The 2nd PSU International Teaching Platform on Tumour Immunology and Immunotherapy

Jointly organized by

Prince of Songkla University, Université Pierre et Marie Curie (Paris 6) and Institut Pasteur

December 15 – 20, 2003

At The Department of Biomedical Sciences

Faculty of Medicine, Prince of Songkla University,

Hat Yai, Songkhla, Thailand

Lecture Course Handout

(December 15-17, 2003)

Hervé Fridman, Université Pierre et Marie Curie

Catherine Fridman, Université Pierre et Marie Curie

Eric Tartour, Université René Descartes

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Programme

The second PSU International Teaching Platform
on *Tumour Immunology and Immunotherapy*
jointly organized by
Prince of Songkla University,
Université Pierre et Marie Curie – Paris 6 and Institut Pasteur
at the Department of Biomedical Sciences, Faculty of Medicine, PSU
December 15-20, 2003

Day 1: Dec. 15, 2003

08.30-10.00	Introduction to the Immune System [CF; Lecture 1] - Innate and adaptive immunity - Dendritic cells and macrophages - T and B lymphocytes - TCR and immunoglobulins
10.00-10.30	*Coffee break*
10.30-12.00	Introduction to the Immune System (contd) [CF; Lecture 1] - MHC-restricted presentation - Cytokines - Th1/Th2 balance
12.00-13.00	*Lunch*
13.00-14.30	Tumour biology & immune surveillance [HF; Lecture 2] - Clinical evidence - Incidence - Relation to age and immunodepression - Experimental models - Tumour antigens
14.30-15.00	*Coffee break*
15.00-16.30	Tumor microenvironment [HF; Lecture 3] - Dendritic cells and T lymphocytes - Cytotoxicity - Macrophages - Cytokines - Th1/Th2 balance - Regulatory T cells Cancer models: prostate, lung, colon, uterus

Day 2: Dec. 16, 2003

08.30-10.00	Cytokines and cancer [ET; Lecture 4] - Physiopathology andimmunotherapy - Gene therapy Cancer models: bladder, kidney, melanoma, hemato
10.00-10.30	*Coffee break*
10.30-12.00	Non-specific cellular therapy [HF; Lecture 5] - Macrophages, NK and LAK Cancer models: ovary, bladder, colon, mesothelioma
12.00-13.00	*Lunch*
13.00-14.30	Specific therapy: monoclonal antibodies [CF; Lecture 6]

Cancer models: breast, lung, lymphoma, LLC

14.30-15.00 *Coffee break*

15.00-16.30 Specific therapy: T lymphocytes [HF; Lecture 7]
- Allografts, TIL, T-cell clones

Day 3: Dec. 17, 2003

08.30-10.00 Vaccination [ET; Lecture 8]
- Mechanisms: dendritic cells, tumour antigens

10.00-10.30 *Coffee break*

10.30-12.00 Vaccination (contd) [ET; Lecture 8]
- Clinical trials
- Immunomonitoring

12.00-13.00 *Lunch*

13.00-14.30 Open discussion with Prof. Fridman

14.30-15.00 *Coffee break*

15.00-16.30 Round table discussion

- *To be determined*

Day 4-6: Dec. 18-20, 2003

Lab course training

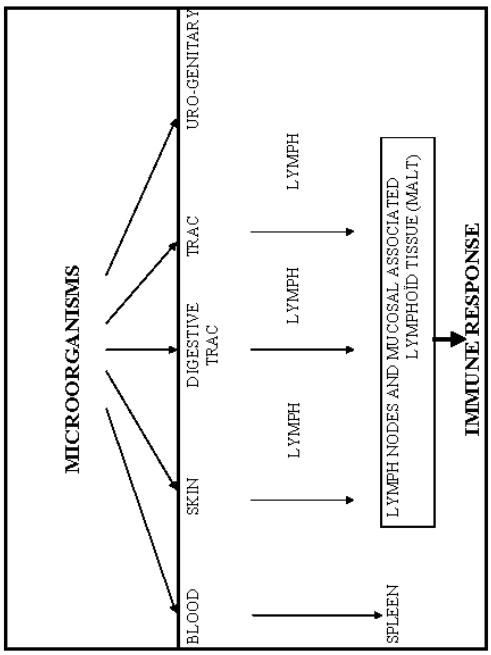
1. Immunomonitoring
   - Intracellular staining of cytokines
   - ELISPOT assay for cytokines
2. Demonstration of detection of tumour antigens by antibodies
3. Exercise session of experimental data in the field of tumour immunology and immunotherapy

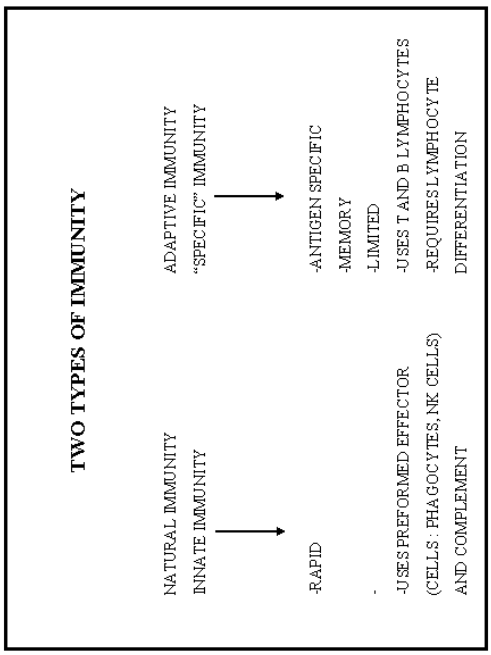
Speakers & Instructors:

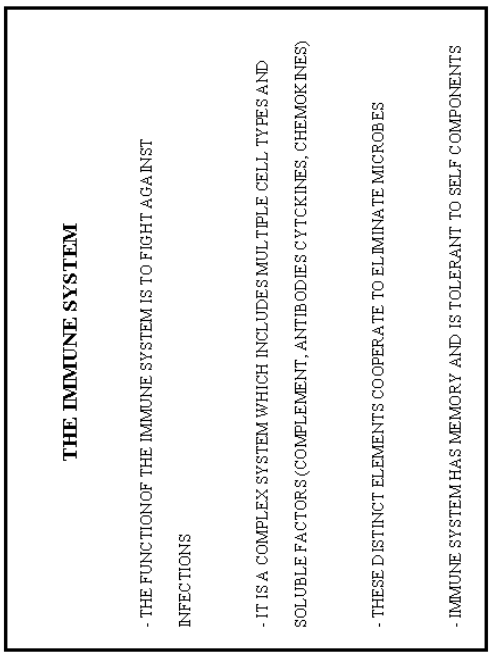
1. Hervé Fridman, M.D., Ph.D.	Prof. of Immunology, Medical School, Université Pierre et Marie Curie – Paris 6
2. Catherine Fridman, Ph.D.	Prof. of Immunology, Université Paris 6
3. Eric Tartour, M.D., Ph.D.	Assistant-Prof. of Immunology, Medical School, Université Renée Descartes – Paris 5
4. Sylvie Garcia, Ph.D.	Investigator, Institut Pasteur
5. Adrien Six, Ph.D.	Assistant-Prof. of Immunology, Institut Pasteur and Université Paris 6
6. Suvina Ratanachaiyavong, M.D., Ph.D.	Department of Biomedical Sciences, Faculty of Medicine, Prince of Songkla University

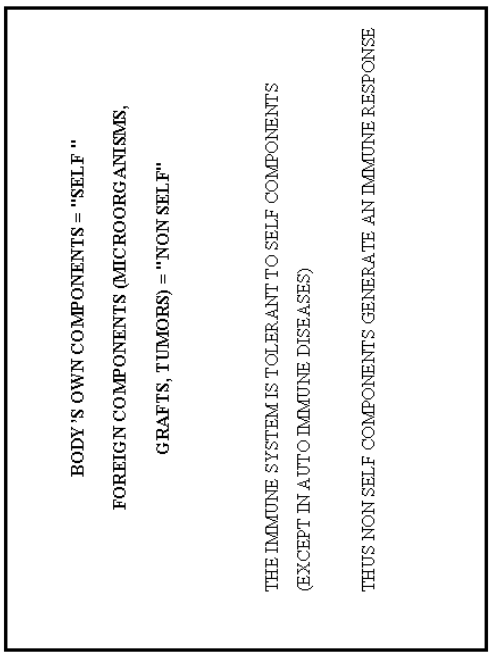
Lecture 1: Introduction to the Immune System [CF]

Introduction







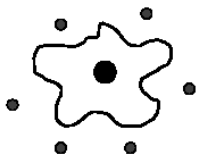


IMMUNE DEFENSES

	BACTERIA	VIRUSES	PARASITES
INNATE IMMUNITY	++	+	±
ADAPTATIVE IMMUNITY	+	++	++

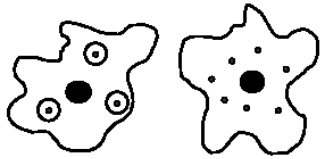
TWO CLASSES OF PATHOGENS

EXTRACELLULAR



BACTERIA :
Streptococcus, Staphylococcus, Neisseria, Salmonella
PARASITES
Plasmodium, Trypanosma, Toxoplasma

INTRACELLULAR



VESICULAR
BACTERIA :
Mycobacteria, Chlamydia, Shigella, Legionella
PARASITES :
Leishmania, Schistosome

CYTOSOLIC
VIRUSES

HUMORAL IMMUNITY (ANTIBODIES, COMPLEMENT) ARE USED TO FIGHT AGAINST EXTRACELLULAR BACTERIA

CELLULAR IMMUNITY IS USED TO FIGHT AGAINST INTRACELLULAR MICROBES (CTL/VIRUSES; TH/INTRACELLULAR BACTERIA)

BOTH TYPES OF IMMUNITY HELP TO FIGHT AGAINST CANCER

INNATE IMMUNITY: THE PRIMARY LINE OF DEFENSE AGAINST INFECTIONS

CELLS

PHAGOCYTES: NEUTROPHILS, MACROPHAGES

DENDRITIC CELLS (pDC and MDC)

EOSINOPHILS
BASOPHILS

NATURAL KILLER CELLS

SOLUBLE FACTORS

COMPLEMENT
CYTOKINES
CHEMOKINES

CELLS FROM INNATE IMMUNITY ARE PRESENT IN AREAS IN CONTACT WITH THE OUTSIDE WORLD: SKIN, MUCOSA, LYMPHOID ORGANS AND BLOOD

ADAPTIVE
IMMUNITY

CELLS — SOLUBLE FACTORS

LYMPHOCYTES B AND T — ANTIBODIES

CYTOKINES
CHEMOKINES

-ANTIGEN SPECIFIC
-MEMORY
-LIMITED
-USES T AND B LYMPHOCYTES
-REQUIRES LYMPHOCYTE DIFFERENTIATION

Immunological memory

→ *used for vaccination*

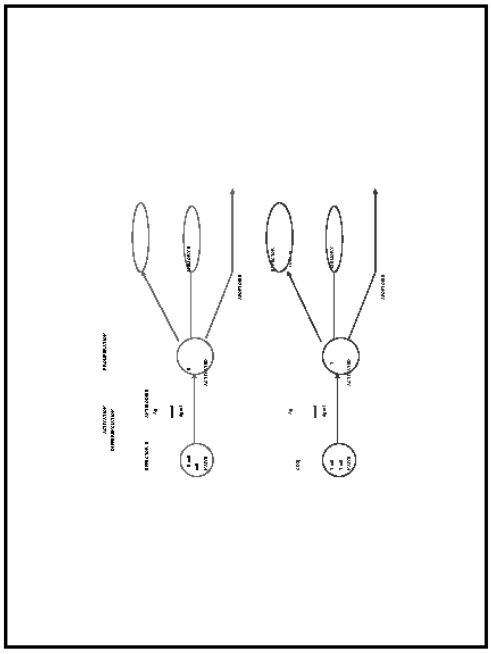
THE LAWS OF LYMPHOCYTE RECOGNITION

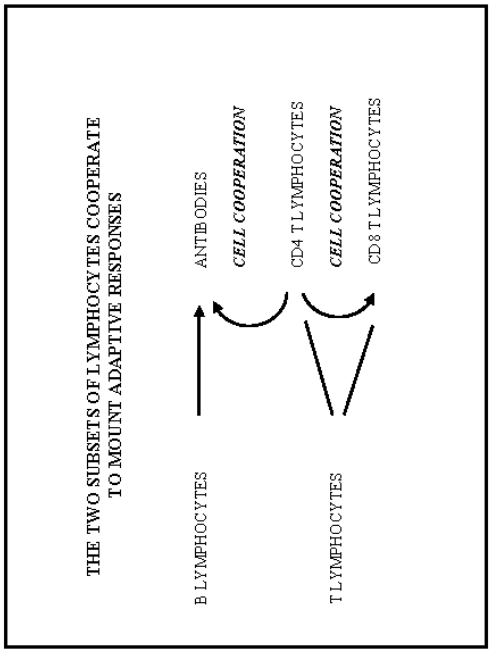
B AND T LYMPHOCYTES RECOGNIZE SPECIFICALLY ANTIGEN THROUGH MEMBRANE RECEPTOR MOLECULES

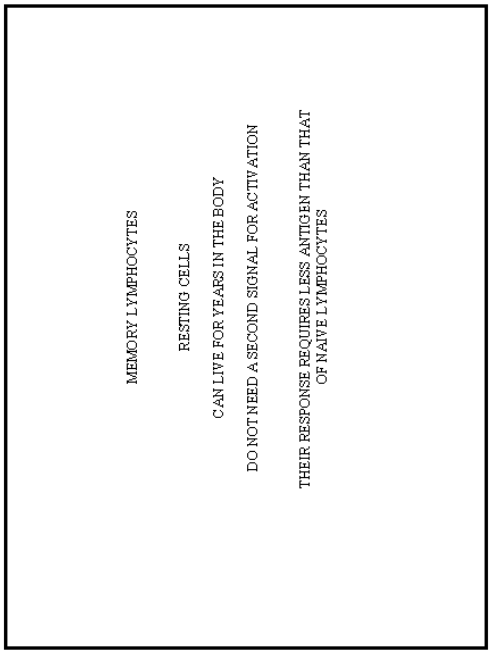
EXPRESSION OF LYMPHOCYTE RECEPTORS IS CLONAL

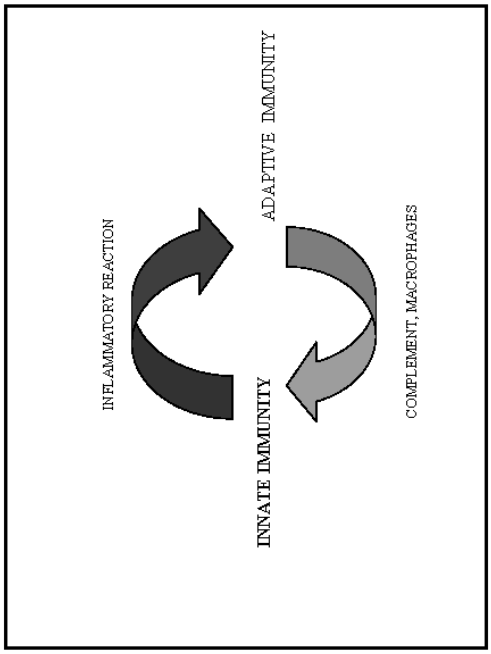
T LYMPHOCYTES RECOGNIZE A PEPTIDE DERIVED FROM ANTIGEN, ASSOCIATED TO SELF COMPONENTS

B LMYPHOCYTES RECOGNIZE THE ANTIGEN ALONE
B LYMPHOCYTES PRODUCE ANTIBODIES OF THE SAME SPECIFICITY THAN THEIR SURFACE RECPETORS




THE TWO SUBSETS OF LYMPHOCYTES COOPERATE
TO MOUNT ADAPTIVE RESPONSES
B LYMPHOCYTES
ANTIBODIES
CELL COOPERATION
CD4 T LYMPHOCYTES
T LYMPHOCYTES
CELL COOPERATION
CD8 T LYMPHOCYTES

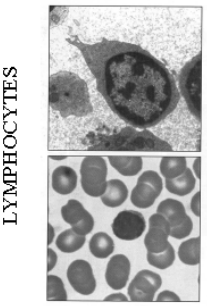

MEMORY LYMPHOCYTES
RESTING CELLS
CAN LIVE FOR YEARS IN THE BODY
DO NOT NEED A SECOND SIGNAL FOR ACTIVATION
THEIR RESPONSE REQUIRES LESS ANTIGEN THAN THAT
OF NAIVE LYMPHOCYTES

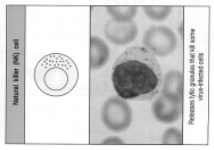

INFLAMMATORY REACTION
ADAPTIVE IMMUNITY
INNATE IMMUNITY
COMPLEMENT, MACROPHAGES

Lymphoid Organs and Cells

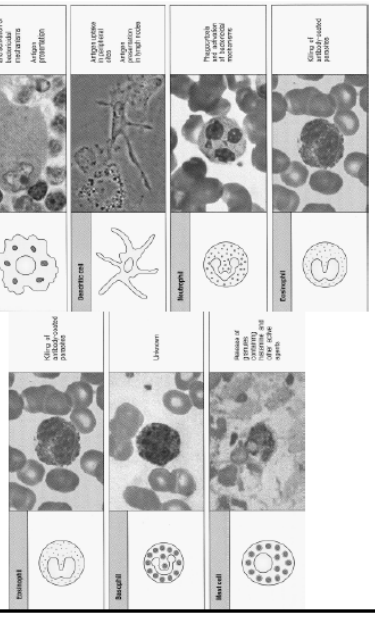
PMM	MACROPHAGES
DO NOT DIVIDE	DO NOT DIVIDE
80% OF BLOOD LEUKOCYTES	DO NOT CIRCULATE
ABSENT IN NORMAL TISSUES	EXIST NORMALLY IN TISSUES (CONNECTIVE TISSUES, LIVER, LUNGS, SPLEEN ...)
SHORT LIFE	LONG LIFE
CONTAIN PRIMARY AND SECONDARY GRANULES	
DEFENSE AGAINST EXTRACELLULAR BACTERIA	DEFENSE AGAINST INTRACELLULAR BACTERIA

LYMPHOCYTES

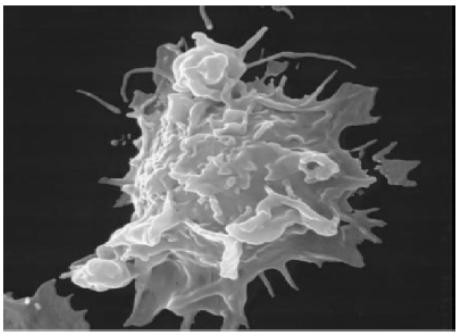


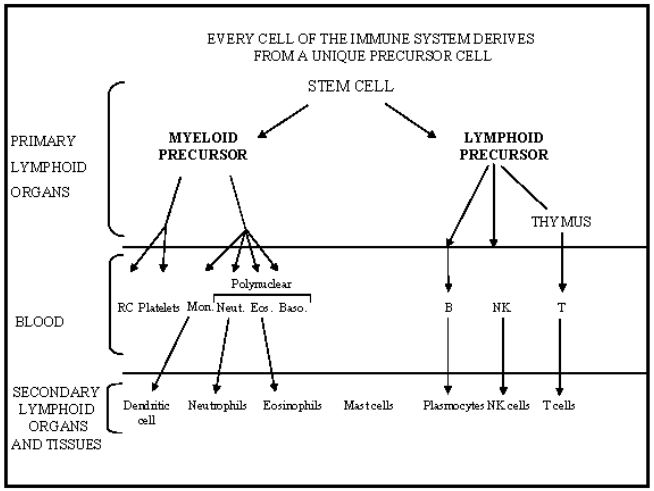


CELLS FROM THE INNATE IMMUNE SYSTEM



DENDRITIC CELL





EVERY DAY THE PRIMARY LYMPHOID ORGANS PRODUCE LYMPHOCYTES WHICH ARE TOLERANT TO SELF COMPONENTS BUT RECOGNIZE NON SELF COMPONENTS

BONE MARROW :
40-60X10^6 CELLS/DAY
15-20X10^6/DAY GO TO THE PERIPHERY

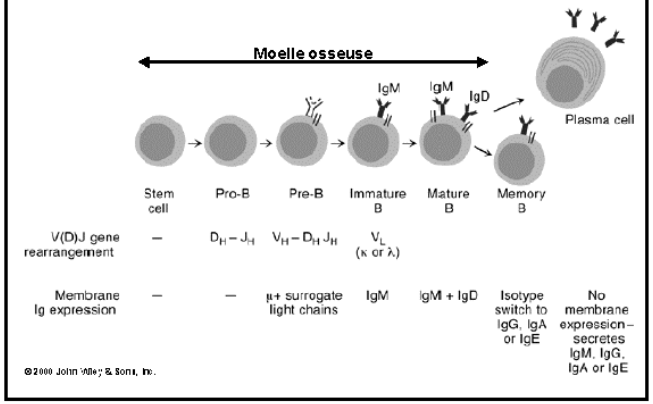
THYMUS :
40-60X10^6/DAY
10^6/DAY GO TO THE PERIPHERY

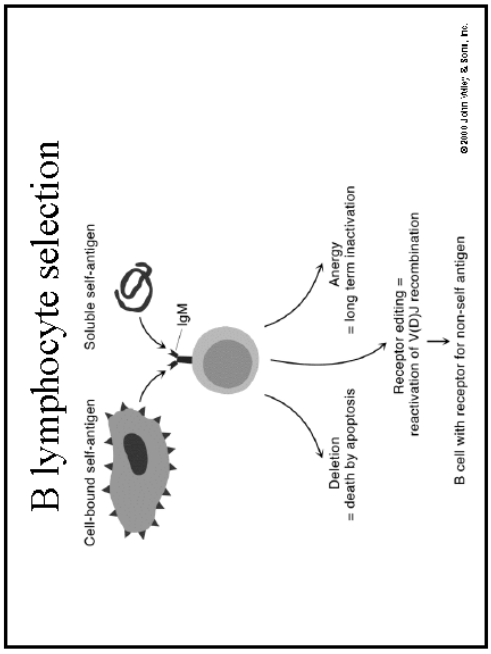
PRIMARY LYMPHOID ORGANS

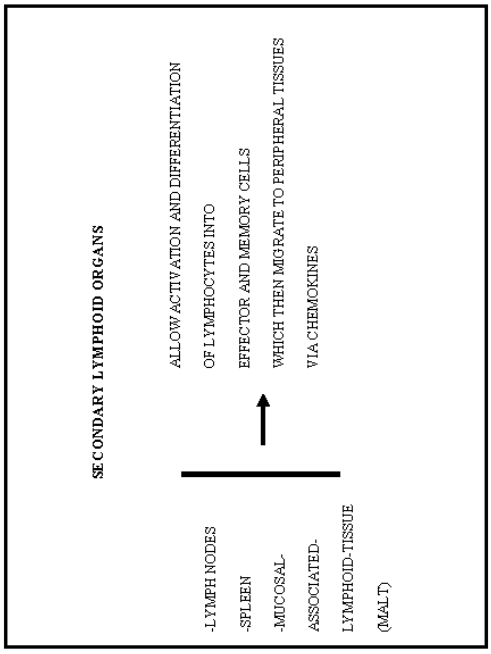
BONE MARROW
FETAL LIVER
THYMUS

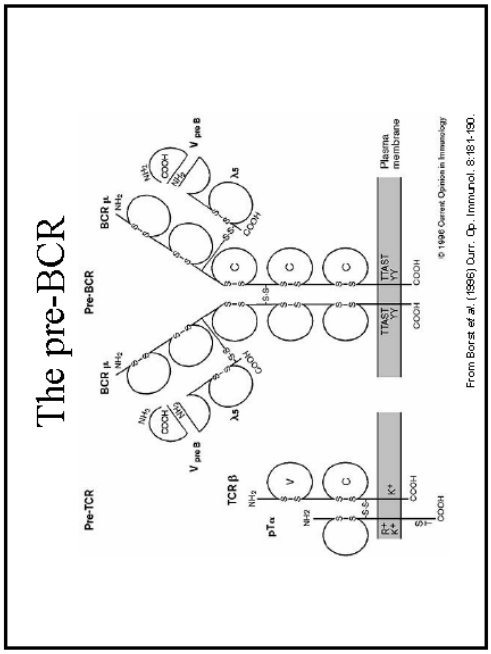
→ ALLOW SELECTION OF LYMPHOCYTES
WHICH ARE TOLERANT TO SELF COMPONENTS
B CELLS COME FROM BONE MARROW AND FETAL LIVER
T CELLS COME FROM THE THYMUS

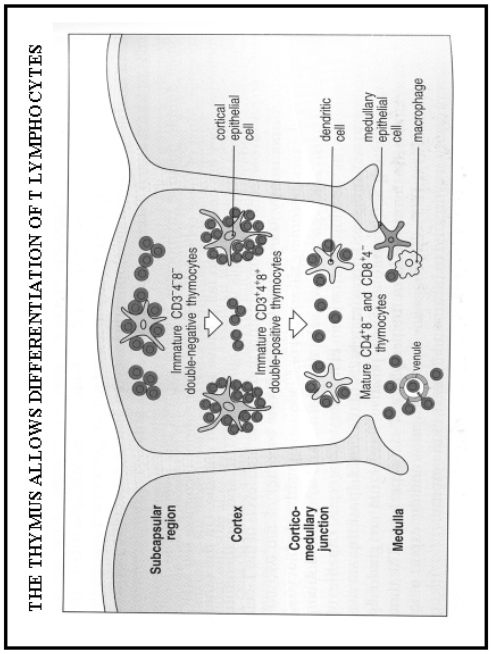
B cell differentiation




B lymphocyte selection
Cell-bound self-antigen
Soluble self-antigen
IgM
Deletion
= death by apoptosis
Anergy
= long term inactivation
Receptor editing =
reactivation of V(D)J recombination
B cell with receptor for non-self antigen
©2000 John Wiley & Sons, Inc.


SECONDARY LYMPHOID ORGANS
-LYMPH NODES
-SPLEEN
-MUCOSAL-
ASSOCIATED-
LYMPHOID-TISSUE
(MALT)
ALLOW ACTIVATION AND DIFFERENTIATION
OF LYMPHOCYTES INTO
EFFECTOR AND MEMORY CELLS
WHICH THEN MIGRATE TO PERIPHERAL TISSUES
VIA CHEMOKINES


The pre-BCR
Pre-TCR
Pre-BCR
From Borst et al. (1996) Curr. Op. Immunol. 8:181-190.
© 1996 Current Opinion in Immunology


THE THYMUS ALLOWS DIFFERENTIATION OF T LYMPHOCYTES
Subcapsular region
Cortex
Cortico-medullary junction
Medulla
Immature CD3-4-8- double-negative thymocytes
Immature CD3+4+8+ double-positive thymocytes
Mature CD4+8- and CD8+4- thymocytes
cortical epithelial cell
dendritic cell
medullary epithelial cell
macrophage
venule

Effector T cells and antibody molecules return to the circulation. They leave the circulation again at the site of infection, where inflammatory mediators have induced changes in the blood vessel endothelium

CD4 T cells activate macrophages to become more cytotoxic, while antibody recruits complement to lyse bacteria directly and to opsonize them, enhancing their uptake by phagocytes

IN THE PERIPHERY, WHEN NAIVE LYMPHOCYTES ENCOUNTER NON SELF COMPONENTS ADAPTIVE IMMUNITY IS ACTIVATED

LYMPHOCYTES DIVIDE, AND DIFFERENTIATE INTO EFFECTOR AND MEMORY CELLS OR DIE

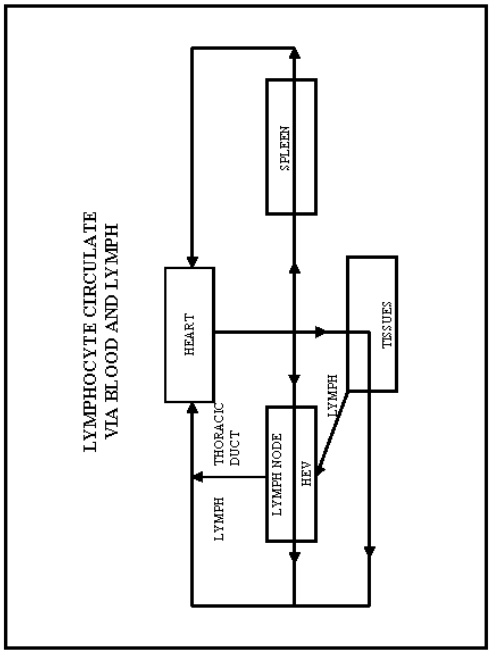
Most pathogens are kept outside of the body by epithelial barriers, such as the epidermis, and are crossed only when there is an injury or tissue damage

After an injury, bacteria cross the epidermis and establish an infection in the underlying tissue

Phagocytic cells in the tissues, such as macrophages and neutrophils, engulf the pathogen. Dendritic cells are also phagocytic, and are activated by binding pathogens to leave the site of infection and migrate to a lymph node

The migrating dendritic cells enter the lymphatic vessels and are collected in a draining lymph node

T cells are activated by antigen presented by the dendritic cells, and in turn activate B cells to secrete antibody


LYMPHOCYTE CIRCULATE
VIA BLOOD AND LYMPH
SPLEEN
HEART
TISSUES
LYMPH
THORACIC
DUCT
LYMPH NODE
HEV
LYMPH

Innate Immunity

INNATE IMMUNITY

- DEVELOP IMMEDIATELY
- LACKS MEMORY
- HIGLY CONSERVED AMONG SPECIES
- IN MAMMALS
  - FIRST LINE OF DEFENSE AGAINST INFECTIONS
  - STIMULATES ADAPTIVE RESPONSE

INFLAMMATION

CALOR

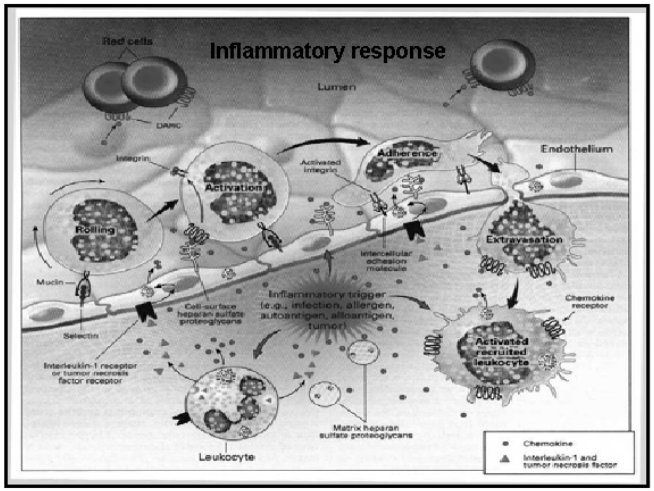
DOLOR

RUBOR

TUMOR

IMMUNE DEFENSES

	BACTERIA	VIRUSES	PARASITES
INNATE IMMUNITY	++	+	±
ADAPTATIVE IMMUNITY	+	++	++



BACTERIA ACTIVATE INNATE IMMUNITY VIA

1 - PRODUCTION OF MICROBIAL PEPTIDES (7MLP)
7TM RECEPTORS

2 - EXPRESSION OF MANNANES
MANNOSE R

3 - EXPRESSION OF "PAMP" (PATHOGEN ASSOCIATED MOLECULAR PATTERN
TOOL-R
- LACK IN MAMMALIAN CELLS
- COSTIMULATORY ACTION

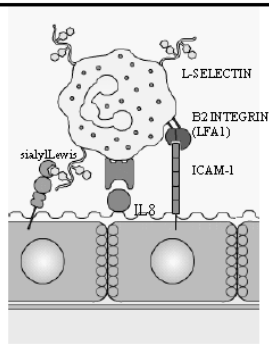
4 - "DANGER" SIGNALS
- PRODUCED B Y HOST OR TUMOR CELLS (NECROSIS = Hsp, DNA, Poly IC)
- CD40L (T LYMPHOCYTES)

5 - COMPLEMENT ACTIVATION
7 TM RECEPTORS
- LEADS TO C5a AND C3a FORMATION (ANAPHYLATOXINS) WHICH INCREASE INFLAMMATORY REACTION

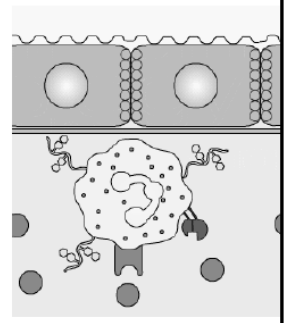
THE INFLAMMATORY REACTION

1-CHIMIOATTRACTANTS INDUCE L-SELECTIN, ICAM-1 AND V-CAM-1 EXPRESSION ON ENDOTHELIAL SURFACE

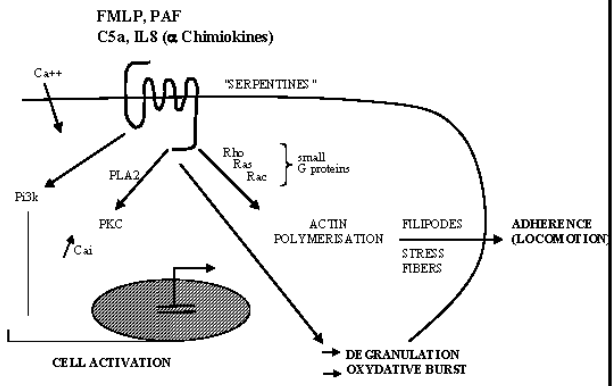
2-THIS ACTIVATES EXTRAVASATION AND MIGRATION TO INFECTED SITE OF NEUTROPHILS, MONOCYTES AND EFFECTOR T CELLS



EXTRAVASATION OF NEUTROPHILS
(from Janeway et al, « Immunobiology », 5th edition Garland ed »)



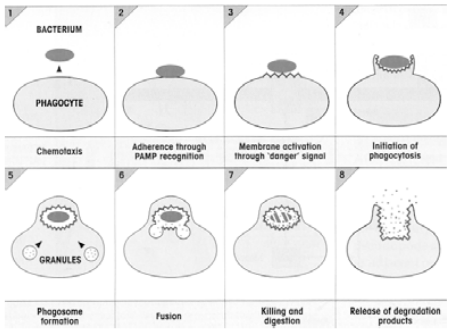
THE INFLAMMATORY REACTION



GRANULES CONTENT OF HUMAN IMN

PRIMARY GRANULES (AZUROPHIL IC/GRANULES)	SECONDARY GRANULES (SPECIFIC GRANULES)
ENZYMES	ENZYMES
MYELOPEROXYDASE	LYSOZYME
LYSOZYME	COLLAGENASE
LACTOFERRIN	ELASTASE
CATHEPSIN B, D	
MICROBICIDAL FACTORS	MICROBICIDAL FACTORS
αDEFENSINS	LL37
BPI (BACERIAL PERMEABILITY INCREASING PROTEIN)	

Engulfed bacteria fuse with PMN granules to form phagosome where bacteria are destroyed



PHAGOSOME ANTIMICROBIAL CONTENT

REACTIVE OXYGEN SPECIES (O2, OH, H2O2)

CHLORE DERIVATIVES (HOC1, CHLORAMINES)

NITRITE DERIVATIVES

LACTOFERRIN

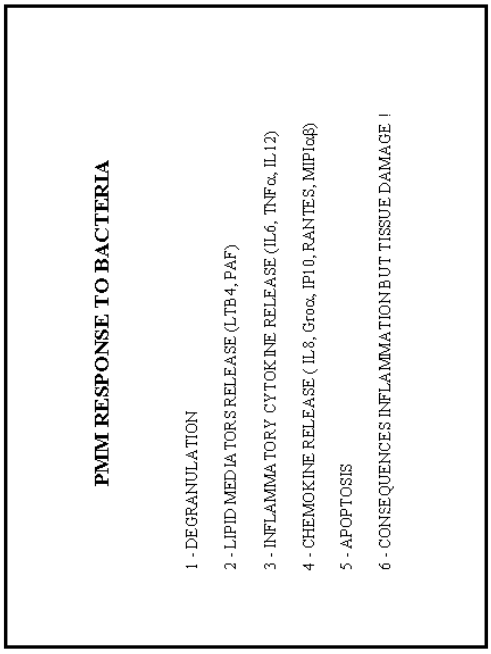
BPI

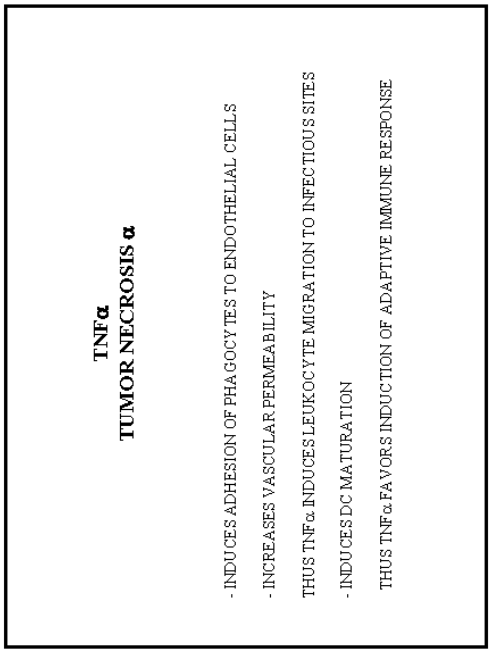
PLA2

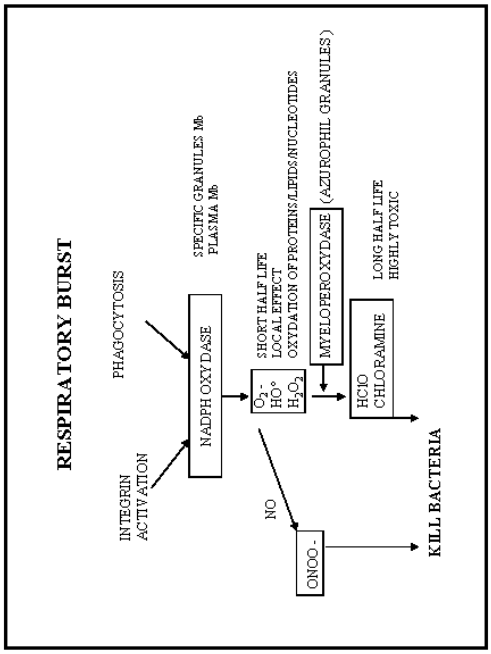
IL37

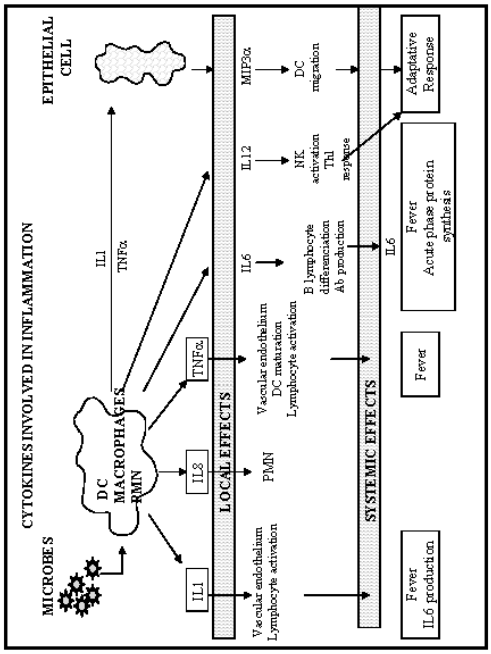
LYSOZYME

DEFENSINS


PMM RESPONSE TO BACTERIA
1 - DEGRANULATION
2 - LIPID MEDIATORS RELEASE (LTB4, PAF)
3 - INFLAMMATORY CYTOKINE RELEASE (IL6, TNFα, IL12)
4 - CHEMOKINE RELEASE (IL8, Groα, IP10, RANTES, MIP1αβ)
5 - APOPTOSIS
6 - CONSEQUENCES INFLAMMATION BUT TISSUE DAMAGE !

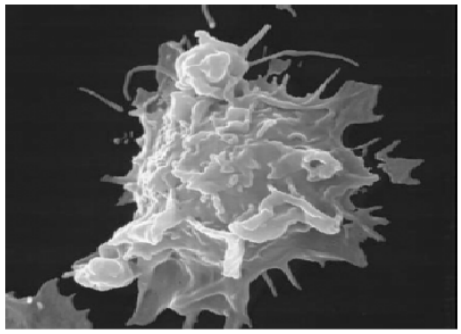

TNFα
TUMOR NECROSIS α
- INDUCES ADHESION OF PHAGOCYTES TO ENDOTHELIAL CELLS
- INCREASES VASCULAR PERMEABILITY
THUS TNFα INDUCES LEUKOCYTE MIGRATION TO INFECTIOUS SITES
- INDUCES DC MATURATION
THUS TNFα FAVORS INDUCTION OF ADAPTIVE IMMUNE RESPONSE


RESPIRATORY BURST
PHAGOCYTOSIS
INTEGRIN ACTIVATION
NADPH OXYDASE
SPECIFIC GRANULES Mb
PLASMA Mb
O_2^- HO° H_2O_2
SHORT HALF LIFE
LOCAL EFFECT
OXYDATION OF PROTEINS/LIPIDS/NUCLEOTIDES
MYELOPEROXYDASE (AZUROPHIL GRANULES)
HClO
CHLORAMINE
LONG HALF LIFE
HIGHLY TOXIC
NO
ONOO-
KILL BACTERIA

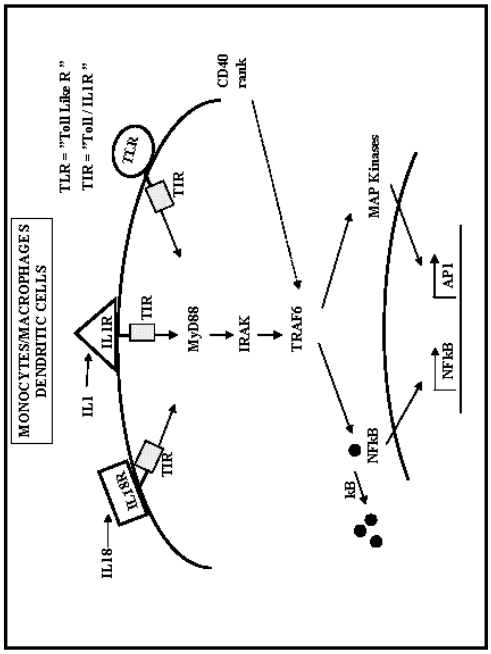

CYTOKINES INVOLVED IN INFLAMMATION
MICROBES
EPITHELIAL CELL
DC
MACROPHAGES
PMN
IL1
IL8
TNFα
IL6
IL12
MIP3α
LOCAL EFFECTS
SYSTEMIC EFFECTS
Vascular endothelium
Lymphocyte activation
Vascular endothelium
DC maturation
Lymphocyte activation
B lymphocyte differenciation
Ab production
NK activation
Th1 response
DC migration
Fever
IL6 production
Fever
Fever
Acute phase protein synthesis
Adaptative Response

HUMAN TOLL RECEPTORS

TLR1	LEUKOCYTES
TLR2 TLR4	MONOCYTES, MYELOID CELLS, DENDRITIC CELLS B LYMPHOCYTES
TLR3	DENDRITIC CELLS
TLR5	MONOCYTES
TLR5	MACROPHAGES EPITHELIAL CELLS
TLR7	MYELOID AND PLASMACYTOID DENDRITIC CELLS
TLR9	PLASMACYTOID DENDRITIC CELLS



DENDRITIC CELL

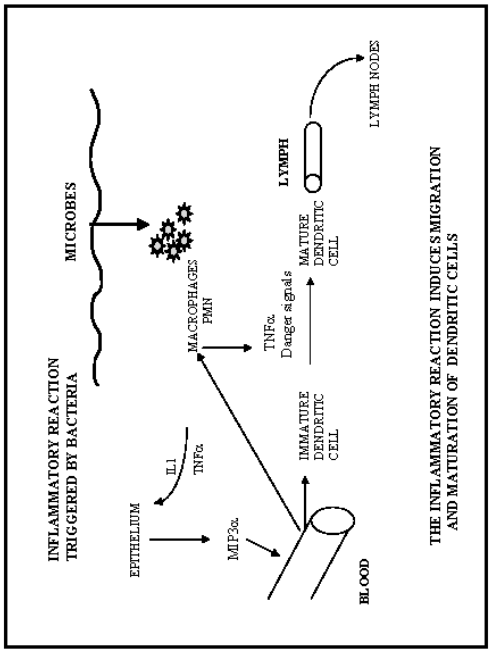


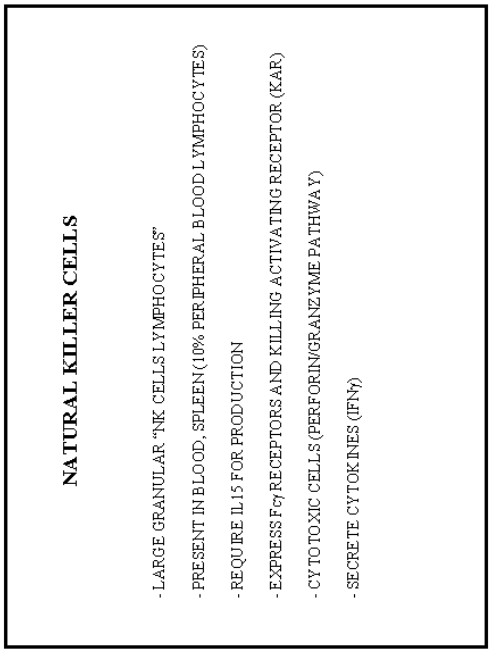
INFLAMMATORY CELLS SECRETE

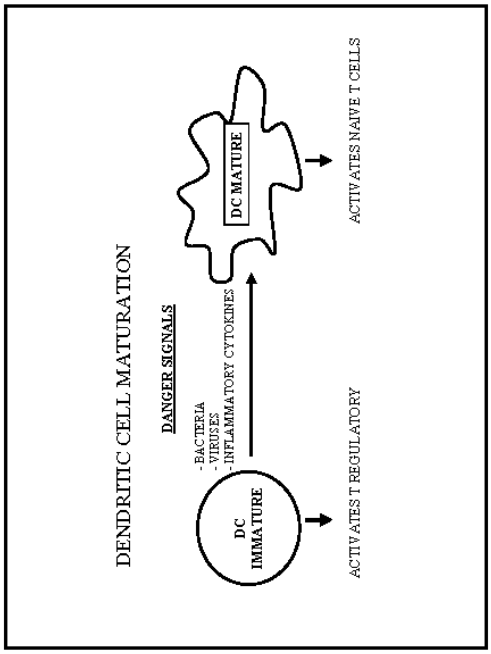
EFFECT ON ADAPTATIVE RESPONSE

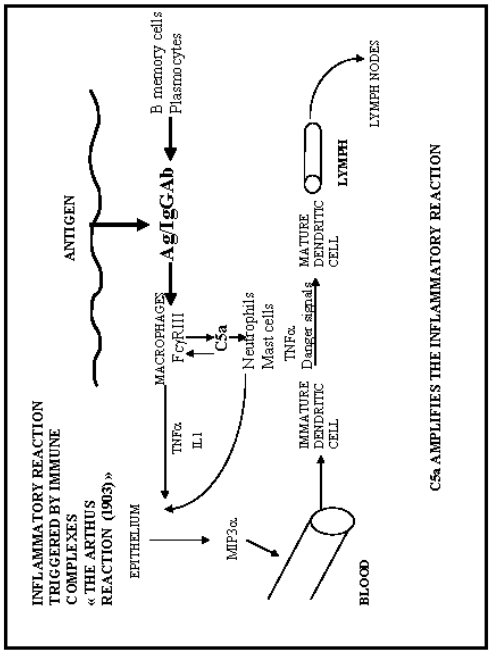
	DEFENSINS	7TMR	ATTRACT T EFFECTOR CELLS ATTRACT DC
NEUTROPHILS	LL37	FMLPR	ATTRACT PMN MONOCYTES T CELLS
MACROPHAGES	TNFα		CD MATURATION
EPITHELIAL CELLS	MIP3α		ATTRACT IMMATURE CD

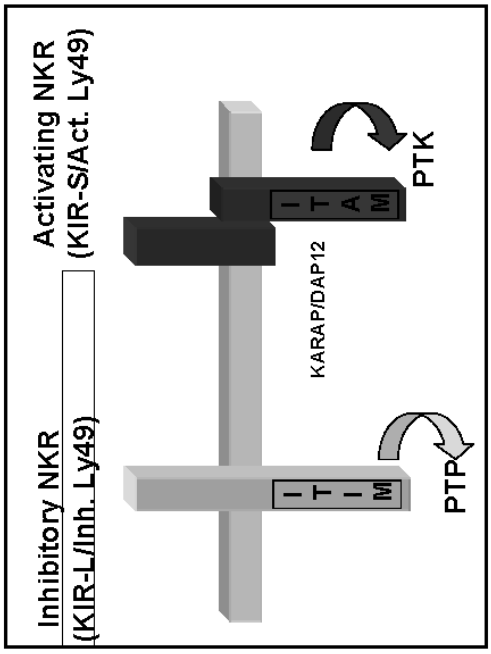
THE INFLAMMATORY REACTION STIMULATES THE ADAPTIVE RESPONSE

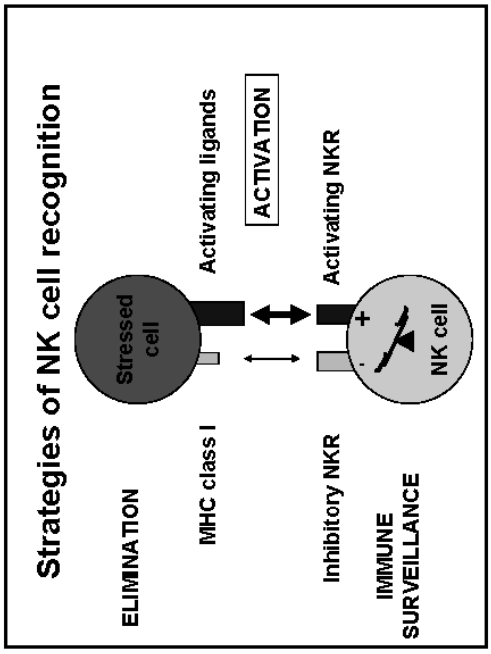

INFLAMMATORY REACTION
TRIGGERED BY BACTERIA
MICROBES
EPITHELIUM
IL1
TNFα
MIP3α
MACROPHAGES
PMN
TNFα
Danger signals
IMMATURE
DENDRITIC
CELL
MATURE
DENDRITIC
CELL
LYMPH
LYMPH NODES
BLOOD
THE INFLAMMATORY REACTION INDUCES MIGRATION
AND MATURATION OF DENDRITIC CELLS

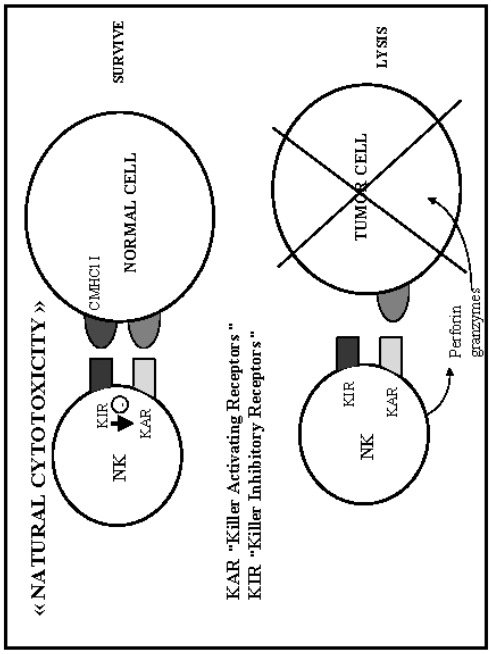

NATURAL KILLER CELLS
- LARGE GRANULAR "NK CELLS LYMPHOCYTES"
- PRESENT IN BLOOD, SPLEEN (10% PERIPHERAL BLOOD LYMPHOCYTES)
- REQUIRE IL15 FOR PRODUCTION
- EXPRESS FcγRECEPTORS AND KILLING ACTIVATING RECEPTOR (KAR)
- CYTOTOXIC CELLS (PERFORIN/GRANZYME PATHWAY)
- SECRETE CYTOKINES (IFNγ)

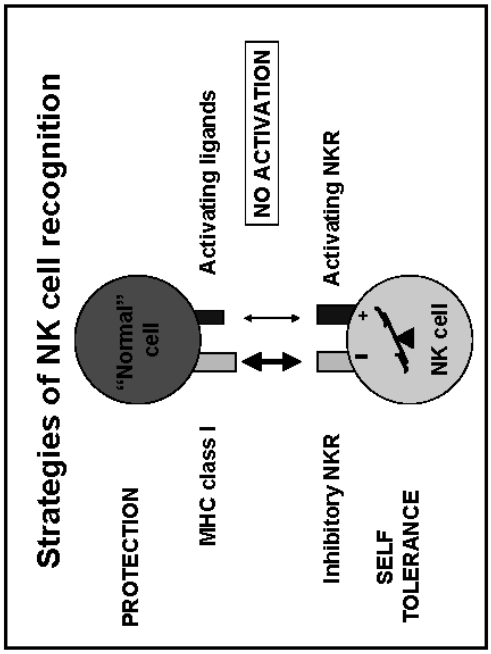

DENDRITIC CELL MATURATION
DANGER SIGNALS
- BACTERIA
- VIRUSES
- INFLAMMATORY CYTOKINES
DC
IMMATURE
DC MATURE
ACTIVATES T REGULATORY
ACTIVATES NAIVE T CELLS

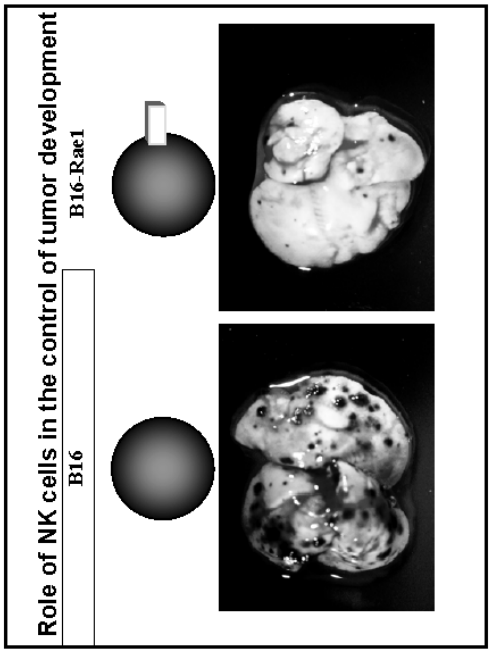

INFLAMMATORY REACTION
TRIGGERED BY IMMUNE
COMPLEXES
« THE ARTHUS
REACTION (1903) »
EPITHELIUM
ANTIGEN
B memory cells
Plasmocytes
Ag/IgG-Ab
MACROPHAGES
FcγRIII
TNFα
IL1
C5a
Neutrophils
Mast cells
MIP3α
TNFα
Danger signals
IMMATURE
DENDRITIC
CELL
MATURE
DENDRITIC
CELL
LYMPH
LYMPH NODES
BLOOD
C5a AMPLIFIES THE INFLAMMATORY REACTION

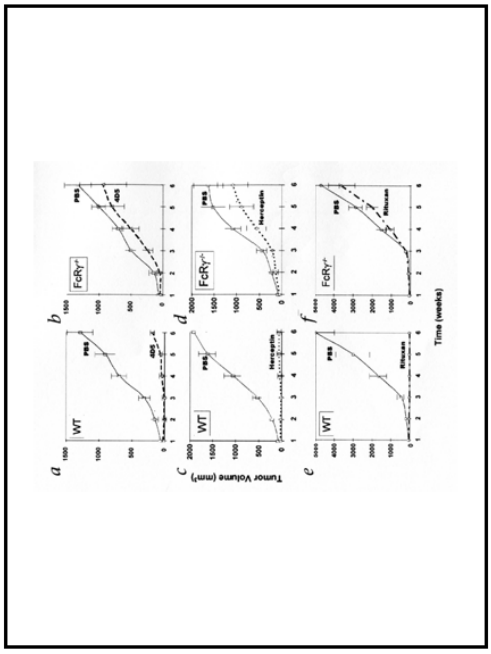

Inhibitory NKR
(KIR-L/Inh. Ly49)
Activating NKR
(KIR-S/Act. Ly49)
KARAP/DAP12
ITIM
ITAM
PTP
PTK

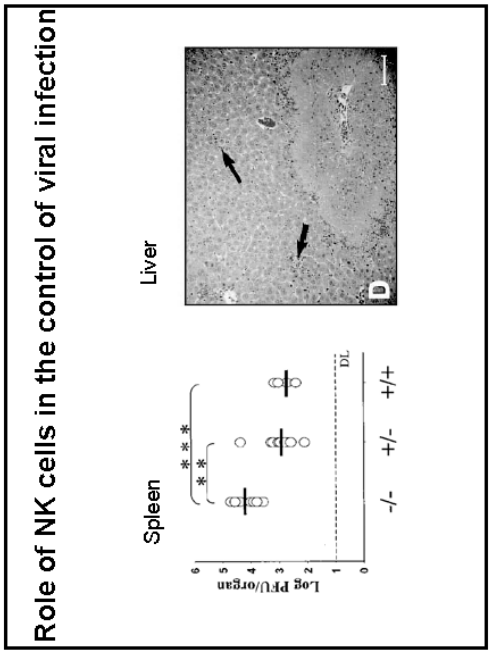

Strategies of NK cell recognition
ELIMINATION
MHC class I
Stressed cell
Activating ligands
ACTIVATION
Inhibitory NKR
Activating NKR
IMMUNE SURVEILLANCE
NK cell

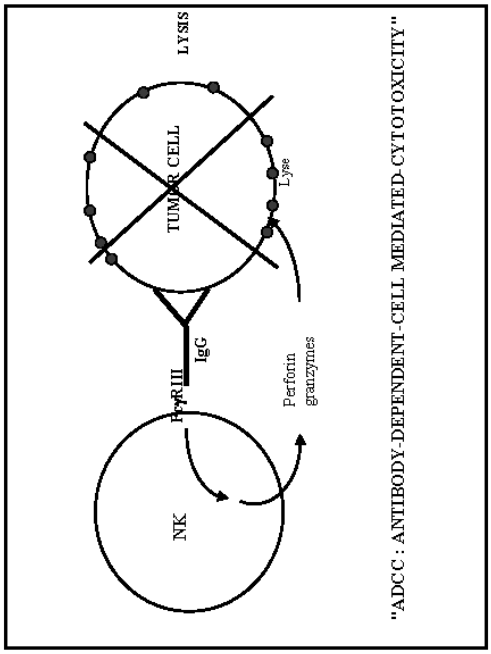

« NATURAL CYTOTOXICITY »
SURVIVE
NORMAL CELL
CMHC I
KIR
KAR
NK
KAR "Killer Activating Receptors"
KIR "Killer Inhibitory Receptors"
LYSIS
TUMOR CELL
Perforin
granzymes


Strategies of NK cell recognition
PROTECTION
MHC class I
"Normal" cell
Activating ligands
NO ACTIVATION
Inhibitory NKR
Activating NKR
SELF TOLERANCE
NK cell


Role of NK cells in the control of tumor development
B16
B16-Rae1


WT
FcRγ-/-
PBS
Tumor Volume (mm³)
Time (weeks)


Role of NK cells in the control of viral infection
Liver
Spleen
Log PFU/organ
DL
-/-
+/-
+/+


"ADCC : ANTIBODY-DEPENDENT-CELL MEDIATED-CYTOTOXICITY"
NK
FcγRIII
IgG
TUMOR CELL
LYSIS
Lyse
Perforin
granzymes

INTERFERONS

- ANTI-VIRAL ACTIVITY
- ACTIVATE NATURAL AND ADAPTATIVE IMMUNE DEFENSES
- THREE TYPES OF INTERFERONS: **α**, **β** and **γ**

The System of Complement

FUNCTIONS OF THE MAJOR COMPLEMENT COMPONENTS

C1 q	BINDS ANTIBODIES
MBL	BINDS MICROORGANISMS
ENZYMES	C1r, C1s, C2b, Bb, D, MASP-1, MASP-2
MEMBRANE-BINDING PROTEINS	C4b, C3b
PEPTIDES INFLAMMATORY	C5a, C3a, C4a
MAC	C5b, C6, C7, C8, C9
COMPLEMENT RECEPTORS	CR1, CR2, CR3, CR4, C1qR, C5aR, C3aR
REGULATORS	C1 INH, C4bp, CR1, MCP, DAF, H, I, P, CD59

(from Janeway et al, « Immunobiology », 5th edition Garland ed »)

THE COMPLEMENT SYSTEM IS A GROUP OF 20 PROTEINS

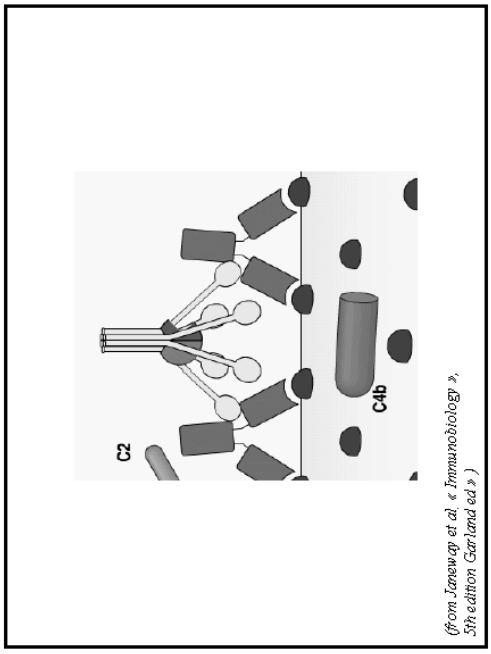
THAT EXIST IN PLASMA AND AS CELL RECEPTORS

WHICH TRIGGER AND REGULATE INNATE AND ADAPTIVE RESPONSES

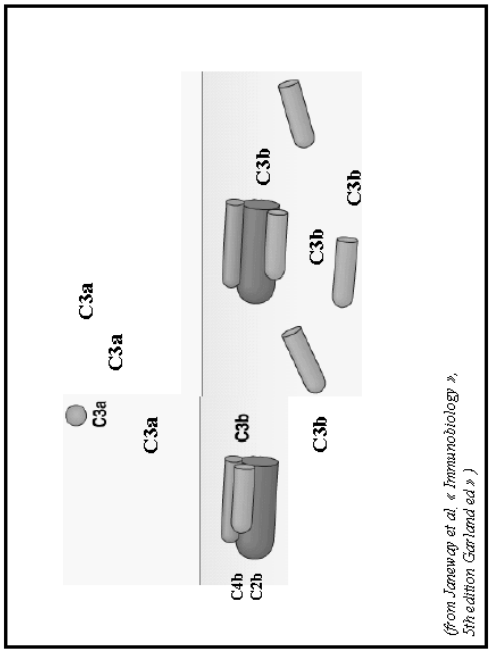
VIA FORMATION OF ENZYMATIC COMPLEXES THAT CLEAVE C3

A SET OF PROTEINS WITH COMMON PROPERTIES

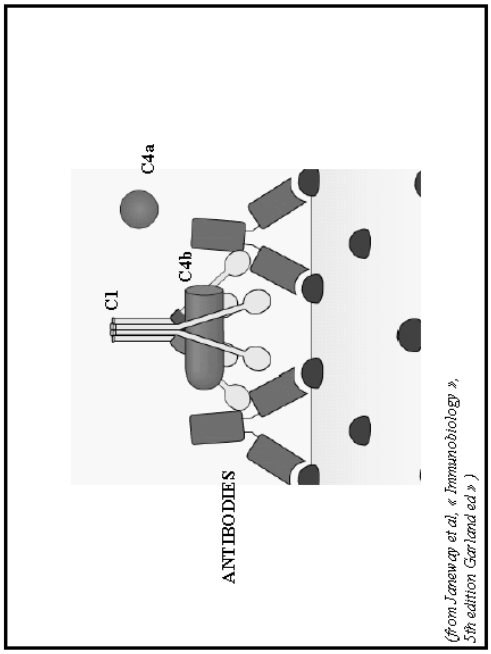
FUNCTIONS / RELATIONSHIPS	PATH			
	ALTERNATE	LECTIN	CLASSICAL	
INITIATION	D	MASP	C1 s	HOMOLOGY
BINDING COVALENT ONTO SURFACES	C3b	C4b	C4b	"
FORMATION CONVERTASES	Bb	C2b	C2b	"
REGULATION	CR1 H	CR1 C4bp	CR1 C4bp	"
OPSONIZATION	C3b	C3b	C3b	IDENTICAL
INITIATION OF LYSIS	C5b	C5b	C5b	"
LOCAL INFLAMMATION	C5a C3a	C5a C3a	C5a C3a	"



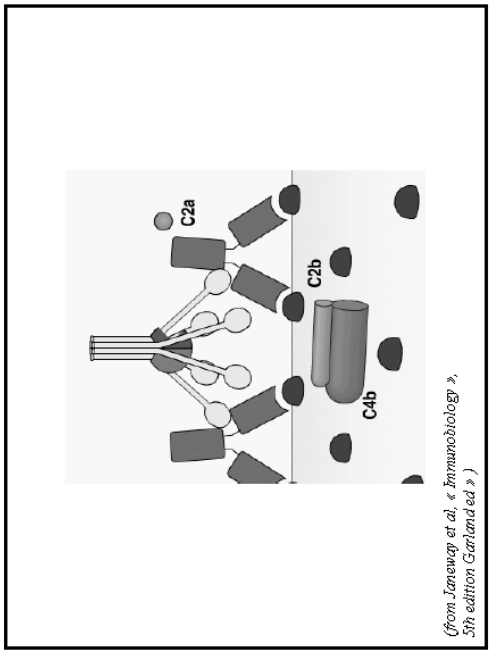
(from Janeway et al. « Immunobiology », 5th edition Garland ed »)



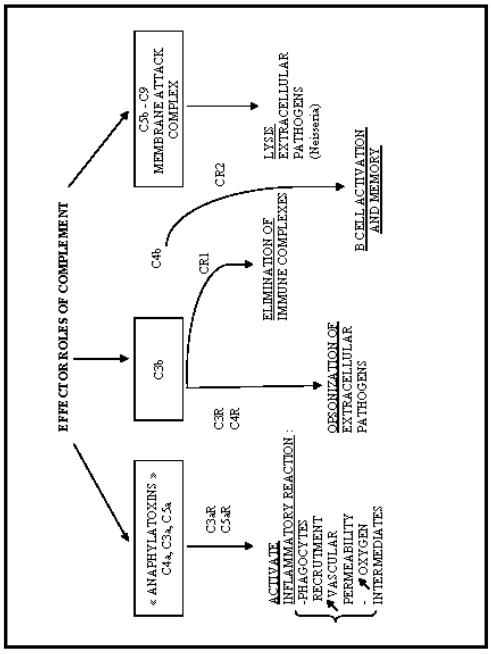
(from Janeway et al. « Immunobiology », 5th edition Garland ed »)

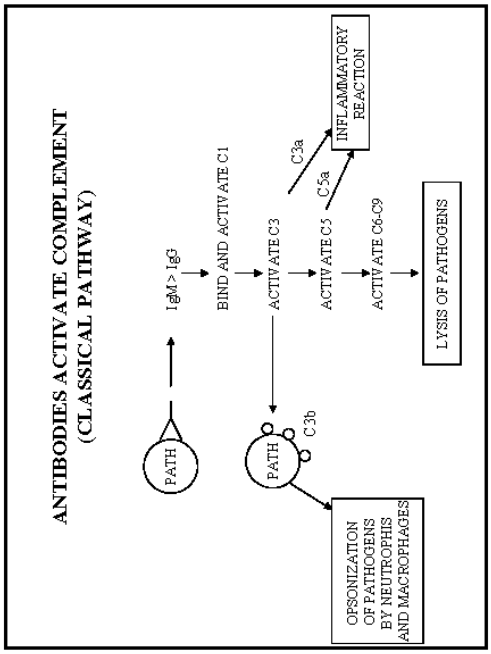


(from Janeway et al. « Immunobiology », 5th edition Garland ed »)



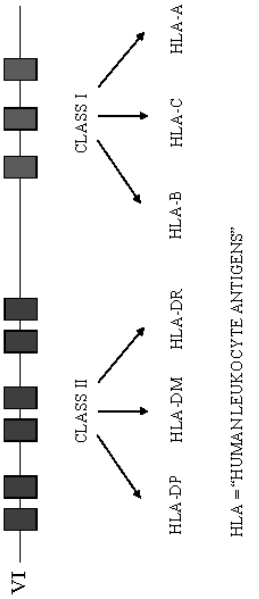
(from Janeway et al. « Immunobiology », 5th edition Garland ed »)


EFFECTOR ROLES OF COMPLEMENT
« ANAPHYLATOXINS »
C4a, C3a, C5a
C3aR
C5aR
ACTIVATE
INFLAMMATORY REACTION :
-PHAGOCYTES
RECRUITMENT
VASCULAR
PERMEABILITY
- OXYGEN
INTERMEDIATES
C3b
C3R
C4R
OPSONIZATION OF
EXTRACELLULAR
PATHOGENS
CR1
ELIMINATION OF
IMMUNE COMPLEXES
C4b
CR2
B CELL ACTIVATION
AND MEMORY
C5b - C9
MEMBRANE ATTACK
COMPLEX
LYSIS
EXTRACELLULAR
PATHOGENS
(Neisseria)


ANTIBODIES ACTIVATE COMPLEMENT
(CLASSICAL PATHWAY)
PATH
IgM > IgG
BIND AND ACTIVATE C1
ACTIVATE C3
C3b
C3a
ACTIVATE C5
C5a
INFLAMMATORY
REACTION
ACTIVATE C6-C9
LYSIS OF PATHOGENS
PATH
OPSONIZATION
OF PATHOGENS
BY NEUTROPHIS
AND MACROPHAGES

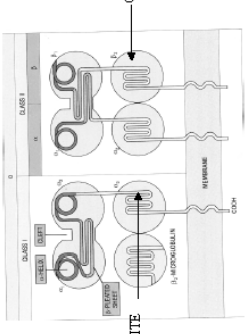
The Major Histocompatibility Complex

HUMAN MAJOR HISTOCOMPATIBILITY COMPLEX



HLA = "HUMAN LEUKOCYTE ANTIGENS"

MHC CLASS I MHC CLASS II



From Essential Immunology, I.M. Roitt et al, 10th edition Blackwell science

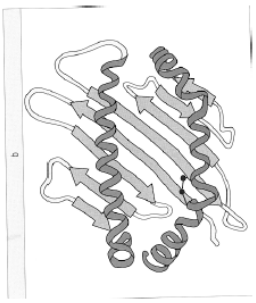
EXPRESSION OF MHC MOLECULES

TISSUE	CLASS I	CLASS II
- ANTIGEN PRESENTING CELLS (APC) :		
B LYMPHOCYTES	+++	+++
MACROPHAGES	+++	++
DENDRITIC CELLS	+++	+++
THYMIC EPITHELIAL CELLS	+	+++
- NEUTROPHILS	+++	-
- T LYMPHOCYTES	+++	-
- OTHER TISSUES	+	-
	+	-
- RED CELLS	-	-

MAJOR HISTOCOMPATIBILITY COMPLEX

HLA : "Human Leukocyte Antigens"
H-2 in mouse
J.Dausset, G.Snell, B.Benacerraf

THE PEPTIDE BINDING GROOVE

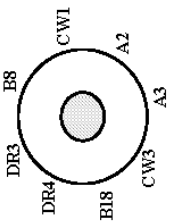


CLASS I PEPTIDES: 9 TO 11 AA
CLASS II PEPTIDES: MORE THAN 13 AA

From Essential Immunology, I.M. Roitt et al, 10th edition Blackwell science

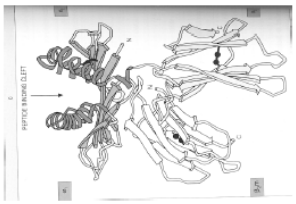
CO-DOMINANCE

MHC ALLELES PROTEINS



FATHER	DR3	B8	CW1	A2	
MOTHER	DR4	B18	CW3	A3	

MHC CLASS I



CD8 BINDING SITE

From Essential Immunology, I.M. Roitt et al, 10th edition Blackwell science

POLYMORPHISM

	CLASS II				CLASS I		
NUMBER OF ALLELES	DPβ	DPα	DRβ	DRα	B	C	A
	89	19	323	2	395	93	195

- MHC MOLECULES BIND SELF AND NON SELF PEPTIDES (ANTI-SELF T CELLS HAVE BEEN ELIMINATED)
- ONE MHC MOLECULE BINDS A RESTRICTED NUMBER OF PEPTIDES

CLASS I PRESENTATION OCCURS IN APC VIA

MHC PROPERTIES

POLYGENIC

POLYMORPHISM

CO-DOMINANCE

→ THIS ALLOWS BINDING OF A LARGE NUMBER OF PEPTIDES ONTO CELLS

TWO CELL COMPARTMENTS

1. CYTOSOL

1. VESICULES
   - ENDOPLASMIC RETICULUM
   - GOLGI APPARATUS
   - ENDOSOMES
   - LYSOSOMES

SECRETORY VESICULES

ANTIGEN PRESENTATION ON MHC CLASS II OCCURS
VIA THE EXOGENEOUS PATHWAY
EXOGENEOUS PROTEIN
ENDOSOMES
ENDOPLASMIC RETICULUM
NUCLEUS
GOLGI APPARATUS
CLASSE II
CMH II
PEPTIDE
CLASS II
MHC II
EXOGENEOUS PROTEIN/
ENDOGENEOUS PROTEIN
PEPTIDE
CLASS I
MHCI
CD8 T CELL ACTIVATION
CD4 T CELL ACTIVATION

MHC/PEPTIDE INTERACTIONS
-CLASS I MOLECULES BIND ENDOGENEOUS AND EXOGENEOUS PEPTIDES
"CROSS PRESENTATION"
- CLASS II MOLECULES BIND EXOGENEOUS PEPTIDES

PEPTIDE LOADING ON MHC CLASS I
CLASS I
TAPASIN
TAP
PEPTIDES
CALRETICULIN
PROTEASOME
(from Janeway et al, « Immunobiology », 5th edition Garland ed »)

MHC CLASS II-PEPTIDE
PATHOGEN
ENDOSOMES
MHC II VESICULES
CLIP
MHC class II
Cytosol
Endoplasmic reticulum
newly synthesized protein
Nucleus
PEPTIDE LOADING ON MHC CLASS II
(from Janeway et al, « Immunobiology », 5th edition Garland ed »)

THE MHC-PEPTIDE COMPLEX IS PRESENTED TO NAIVE T LYMPHOCYTES BY «ANTIGEN-PRESENTING CELLS»

	CHARACTERISTICS of APC		
	DENDRITIC CELL	MACROPHAGE	B LYMPHOCYTE
Entry of antigen	Macropinocytosis Phagocytosis Viral Infection	Phagocytosis	Via Ag receptor
MHC Expression	high in lymphoid tissues (mature DC)	Inducible - / +++	Constitutive Increased by activation
Expression of Cosignal	Constitutive (mature DC)	Inducible - / +++	Inducible - /+++
Ag presented	Peptides Viral Antigens Allergens	Extracellular and Intracellular pathogens	Solubles Toxins Viruses
Localization	Lymphoid Tissue Connective Tissues Epithelia	Lymphoid Tissue Connective Tissue Cavities (peritoneal, pleural...)	Lymphoid Tissue Blood

MHC CLASS I ALLOWS RECOGNITION BY CD8 T CELLS

MHC CLASS II ALLOWS RECOGNITION BY CD4 T CELLS

PATHOGENS
ACUTE VIRAL INFECTIONS

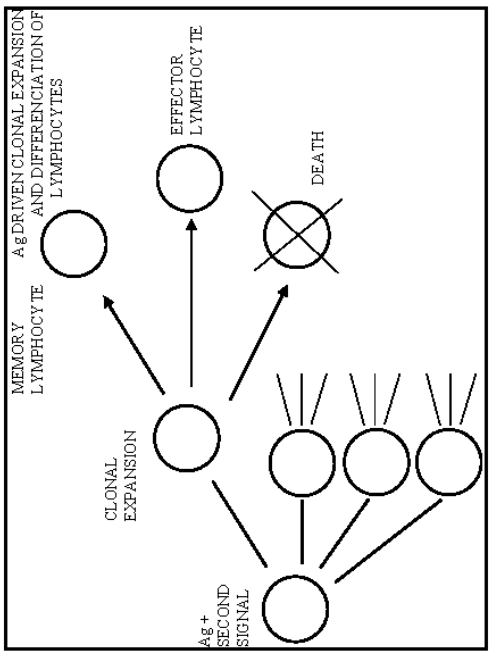
IMMATURE DC
Endocytosis +++
MHC +
COSTIMULATORY MOLECULES +

→ TOLERANCE

MATURE DC
Endocytosis +
MHC +++
COSTIMULATORY MOLECULES +++

→ IMMUNITY

Adaptive Immunity



NAÏVE MATURE T CELLS, CD4 OR CD8, LEAVE THE THYMUS
VIA BLOOD AND REACH THE SECONDARY LYMPHOID ORGANS
WHERE THEY MEET APC IN THE T CELL ZONE

THE NAÏVE T CELLS ARE TOLERANT TO SELF COMPONENTS:
SELF-MHC-SELF PEPTIDE

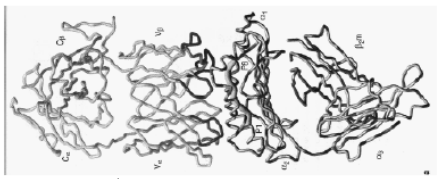
LIKE B CELLS, T CELLS REQUIRE
TWO SIGNALS FOR ACTIVATION

SIGNAL 1(MHC-PEPTIDE)
SIGNAL 2 (B7)
GIVES RESPONSE

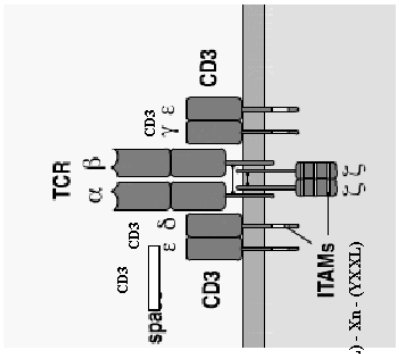
SIGNAL 1(MHC-PEPTIDE)
NO SIGNAL 2
GIVES ANERGY

SIGNAL 1(MHC-PEPTIDE)
SIGNAL 2 NEGATIVE : CTLA4
GIVES ANERGY

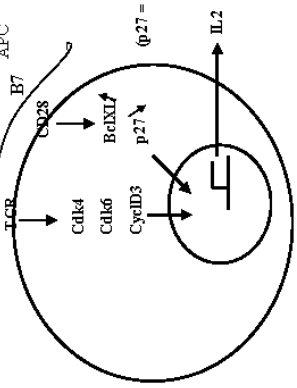
SIGNAL 1-STEP 1
TCR RECOGNIZES THE
CMH-PEPTIDE COMPLEX



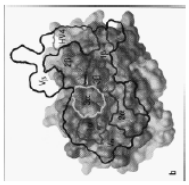
THE TCR IS ASSOCIATED TO SIGNAL TRANSDUCING CHAINS



A SECOND SIGNAL IS REQUIRED FOR ACTIVATION OF NAIVE T CELLS IT LEADS TO IL2 SECRETION

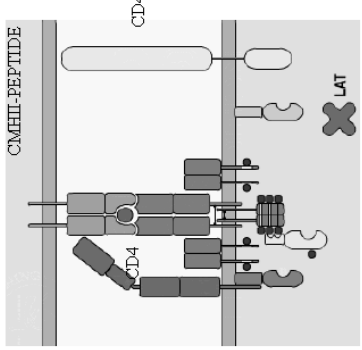


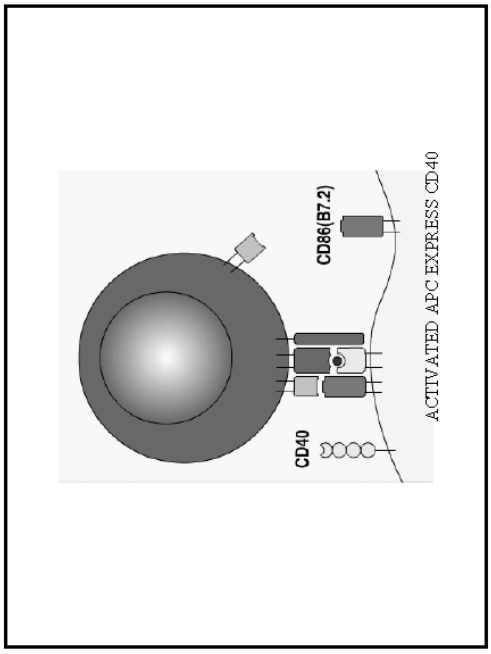
THE HV3 REGIONS OF THE TCR CONTACT THE PEPTIDE AND THE MHC



THUS T CELLS RESPOND TO SELF MHC-PEPTIDE COMPLEX

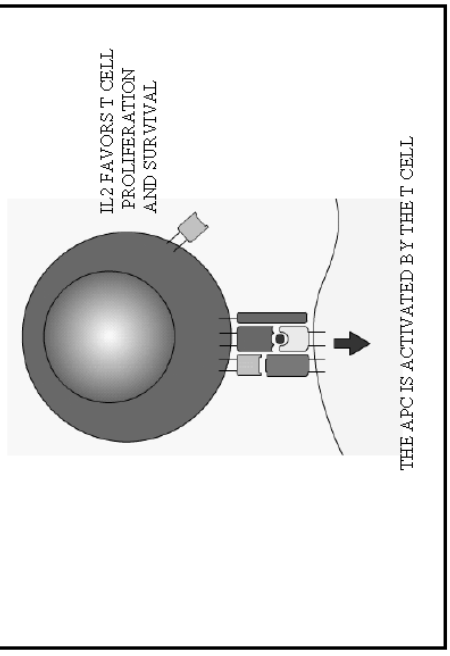
SIGNAL ONE STEP 2 TYROSINE KINASES ARE ACTIVATED WHICH PHOSPHORYLATE LAT

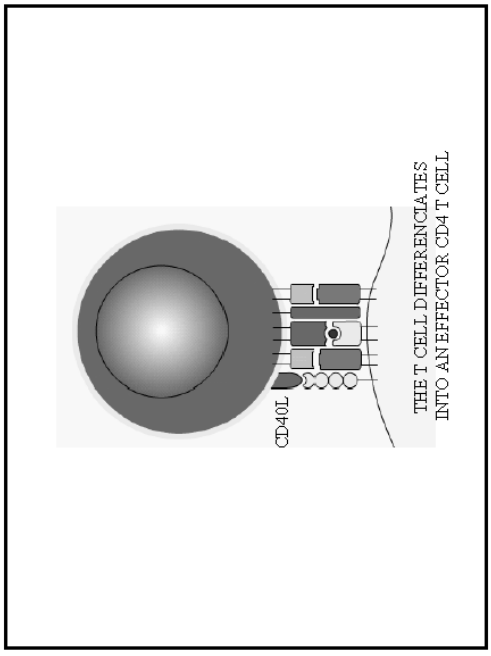




CD4 EFFECTOR T CELLS ARE TH CELLS.

THEY SECRETE CYTOKINES
AND DO NOT REQUIRE CO-SIGNAL TO ACT





EFFECTOR CD4 or CD8 T CELLS AND MEMORY CELLS

LEAVE THE LYMPHOID ORGANS

TO REACH TISSUES, ATTRACTED BY CHEMOKINES

CD4 T LYMPHOCYTES HELP :
1-CD8 T CELLS TO BECOME CYTOTOXIC T CELLS AND
2- B CELLS TO BECOME PLASMOCYTES

B CELLS DIFFERENTIATE INTO PLASMOCYTES TO MAKE ANTIBODIES

CELL COOPERATION VIA CYTOKINES

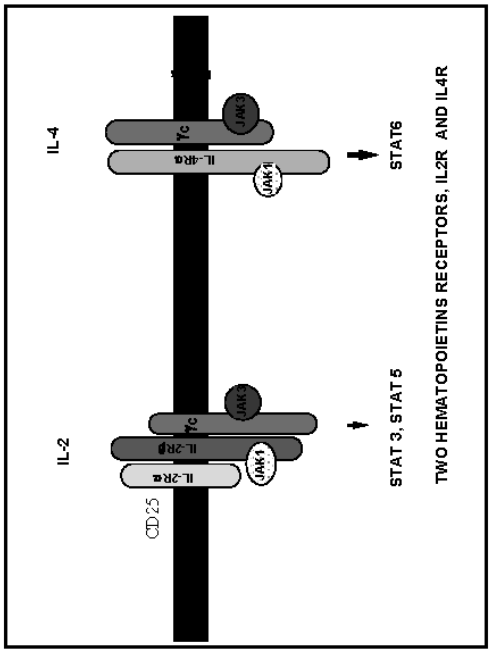
CD4 TH LYMPHOCYTES

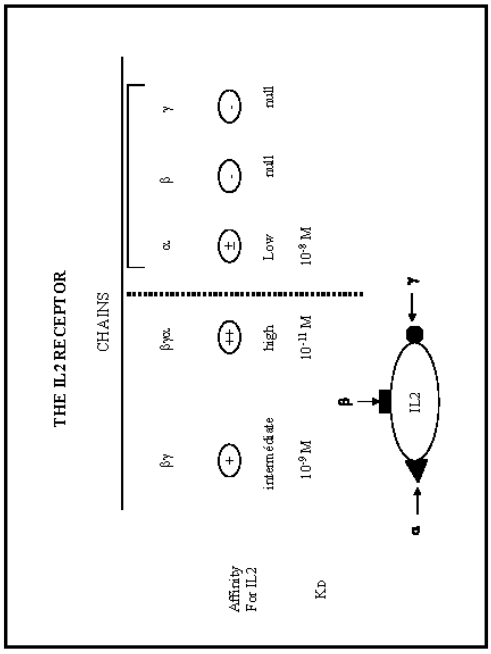
CELL COOPERATION VIA CYTOKINES

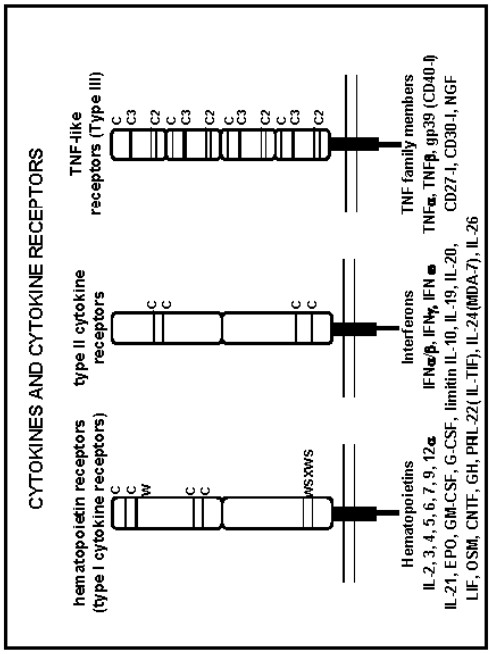
CD8 T LYMPHOCYTES DIFFERENTIATE INTO CYTOTOXIC CELLS

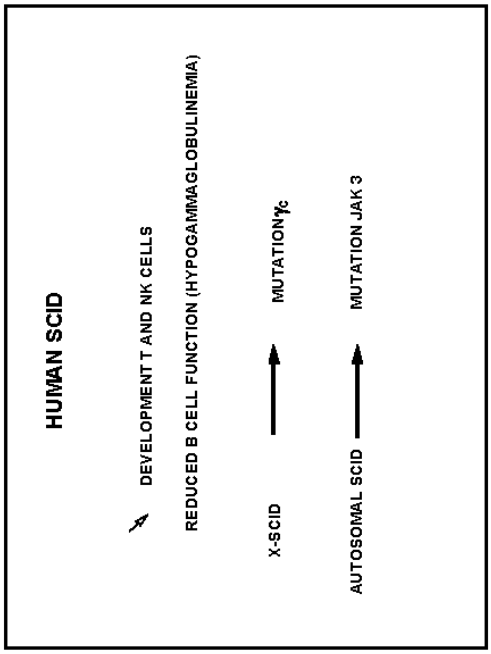
CD8 T CELL DIFFERENTIATION INTO EFFECTOR CELLS REQUIRE CD4 TH CELLS

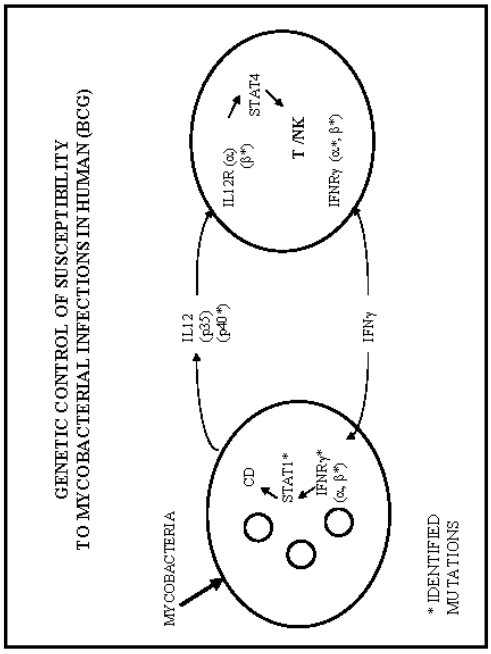
TWO PATHWAYS OF T CELL CYTOTOXICITY

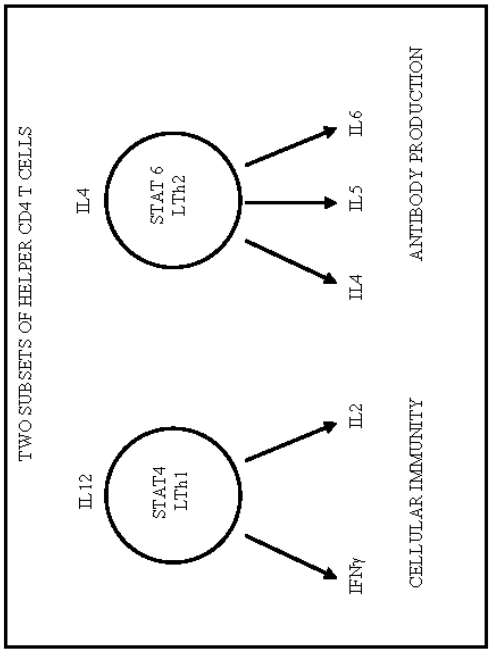

IL-2
IL-4
CD25
IL-2Rα
IL-2Rβ
γc
JAK1
JAK3
STAT 3, STAT 5
STAT6
TWO HEMATOPOIETINS RECEPTORS, IL2R AND IL4R


THE IL2 RECEPTOR
CHAINS
βγ
βγα
α
β
γ
Affinity For IL2
intermediate
high
Low
null
null
Kd
10^{-9} M
10^{-11} M
10^{-8} M
IL2


CYTOKINES AND CYTOKINE RECEPTORS
hematopoietin receptors (type I cytokine receptors)
type II cytokine receptors
TNF-like receptors (Type III)
Hematopoietins
IL-2, 3, 4, 5, 6, 7, 9, 12α
IL-21, EPO, GM-CSF, G-CSF, limitin IL-10, IL-19, IL-20,
LIF, OSM, CNTF, GH, PRL-22(IL-TIF), IL-24(MDA-7), IL-26
Interferons
IFNα/β, IFNγ, IFN ω
TNF family members
TNFα, TNFβ, gp39 (CD40-l)
CD27-l, CD30-l, NGF


HUMAN SCID
DEVELOPMENT T AND NK CELLS
REDUCED B CELL FUNCTION (HYPOGAMMAGLOBULINEMIA)
X-SCID
MUTATION γc
AUTOSOMAL SCID
MUTATION JAK 3





IFNγ GENE DEFICIENT MICE

DEFECT IN MACROPHAGE FUNCTION
Increased susceptibility to intracellular pathogens (Mycobacteria, Leishmania, Listeria)

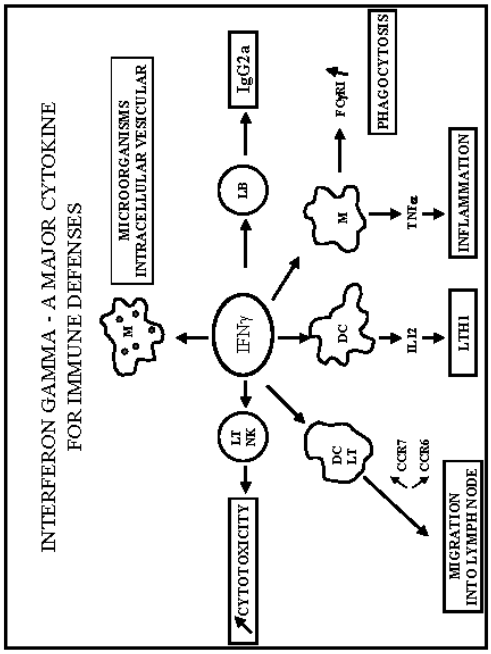
LOSS TH1 RESPONSE, INCREASED TH2

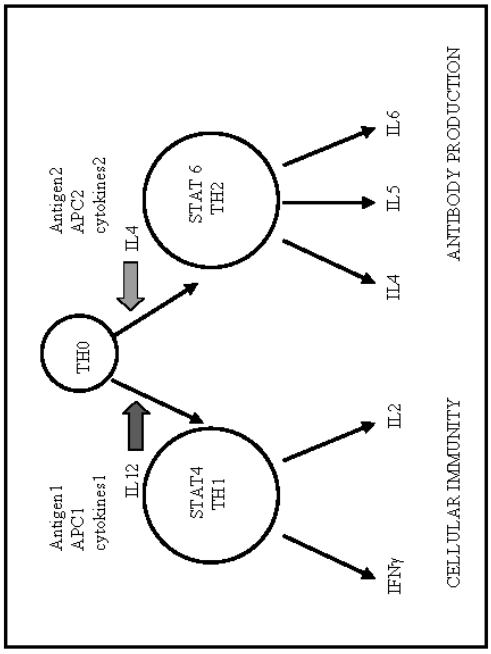
IFNγR GENE DEFICIENT MICE

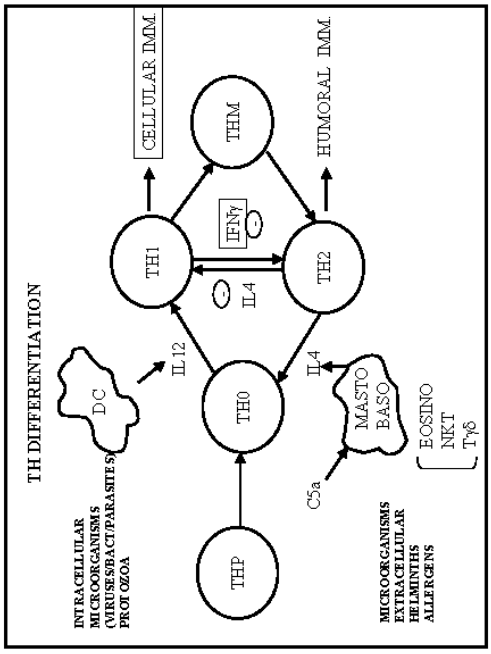
TH1 RESPONSE CAN DEVELOP

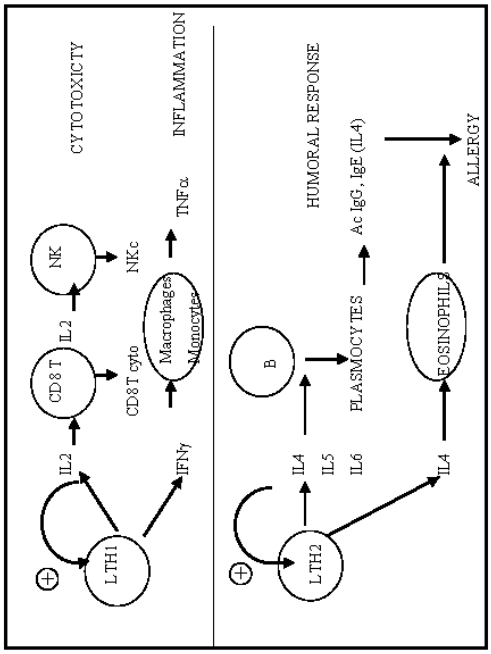
STAT1 GENE DEFICIENT MICE

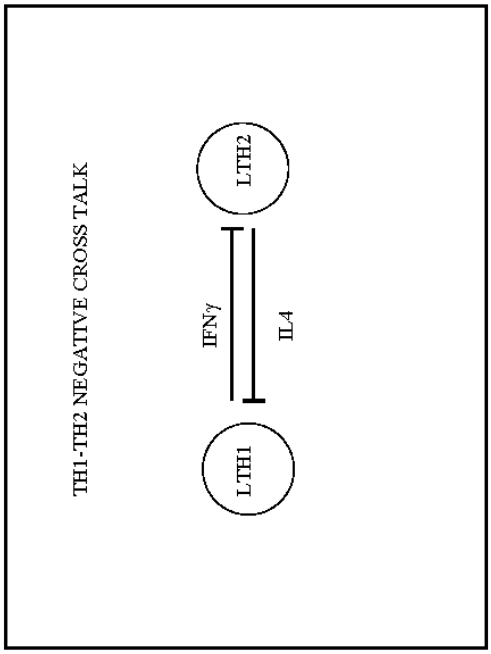
SUSCEPTIBLE TO VIRAL AND BACTERIAL INFECTIONS

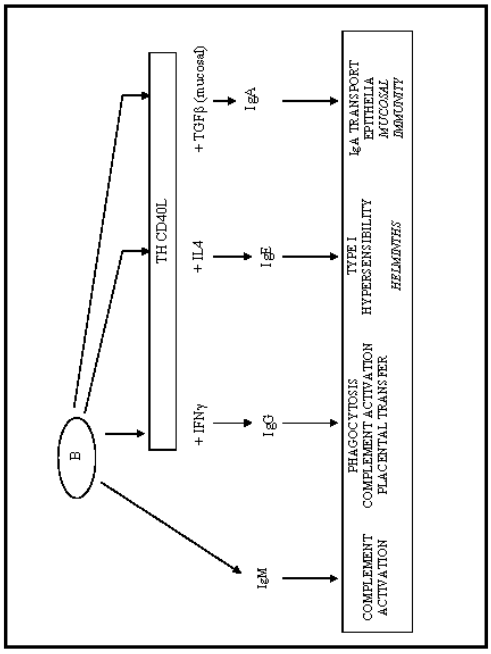


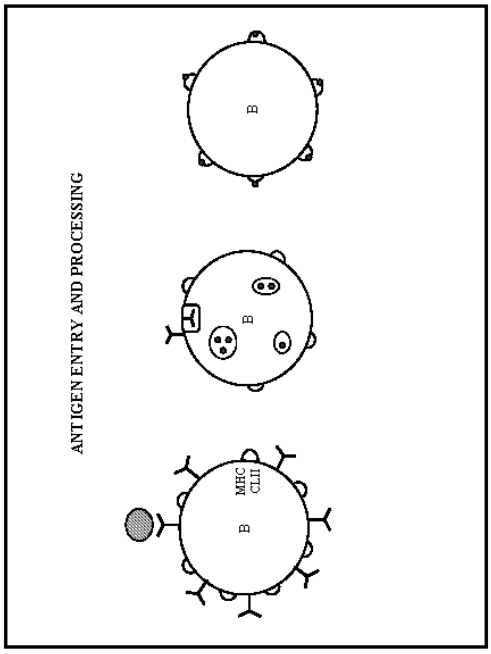

Antigen1
APC1
cytokines1
IL12
TH0
STAT4
TH1
IFNγ
IL2
CELLULAR IMMUNITY
Antigen2
APC2
cytokines2
IL4
STAT 6
TH2
IL4
IL5
IL6
ANTIBODY PRODUCTION

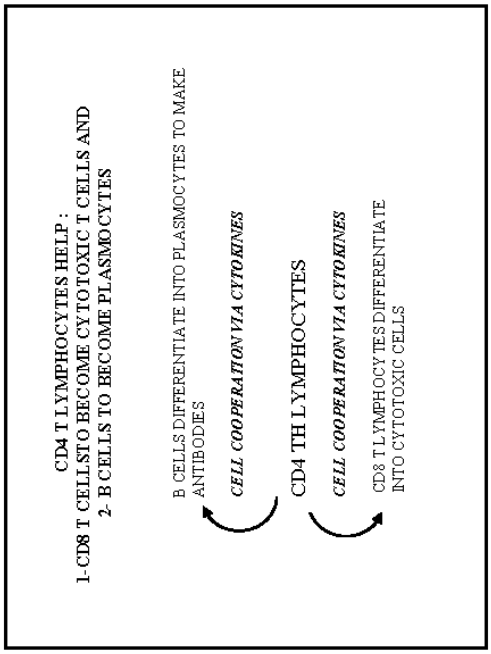

TH DIFFERENTIATION
INTRACELLULAR
MICROORGANISMS
(VIRUSES/BACT/PARASITES)
PROTOZOA
DC
IL12
TH1
CELLULAR IMM.
THM
IFNγ
IL4
TH2
HUMORAL IMM.
TH0
THP
C5a
IL4
MASTO
BASO
EOSINO
NKT
Tγδ
MICROORGANISMS
EXTRACELLULAR
HELMINTHS
ALLERGENS

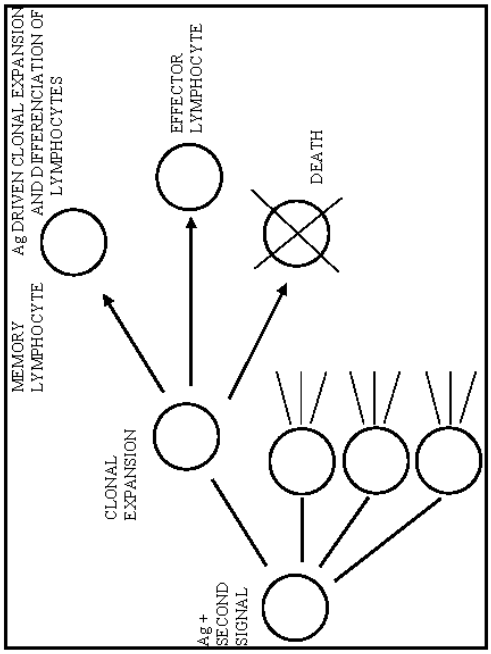

IL2
CD8 T
NK
CD8 T cyto
NKc
Macrophages
Monocytes
TNFα
INFLAMMATION
CYTOTOXICITY
IFNγ
LTH1
B
IL4
IL5
IL6
PLASMOCYTES
Ac IgG, IgE (IL4)
HUMORAL RESPONSE
EOSINOPHILS
ALLERGY
IL4
LTH2

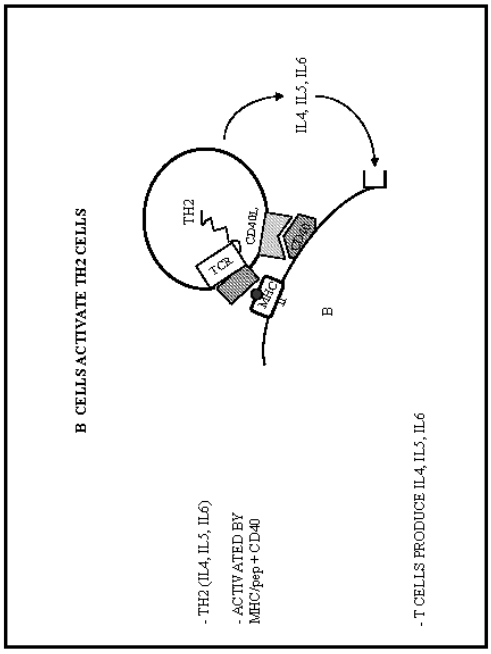

TH1-TH2 NEGATIVE CROSS TALK
LTH1
IFNγ
IL4
LTH2

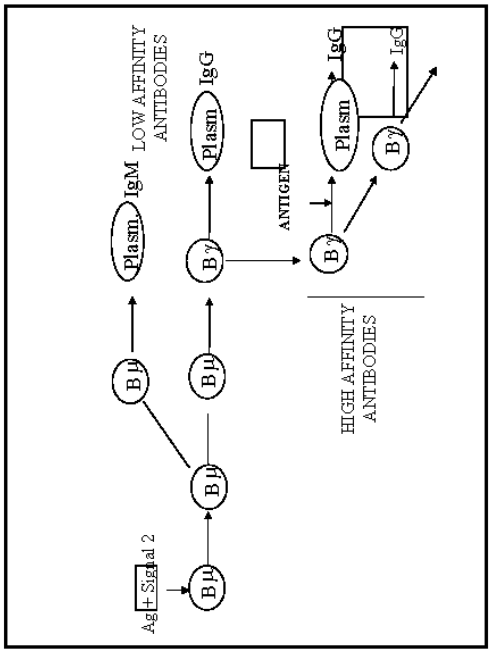

B
TH CD40L
+ IFNγ
+ IL4
+ TGFβ (mucosal)
IgM
IgG
IgE
IgA
COMPLEMENT ACTIVATION
PHAGOCYTOSIS
COMPLEMENT ACTIVATION
PLACENTAL TRANSFER
TYPE I
HYPERSENSIBILITY
HELMINTHES
IgA TRANSPORT
EPITHELIA
MUCOSAL
IMMUNITY

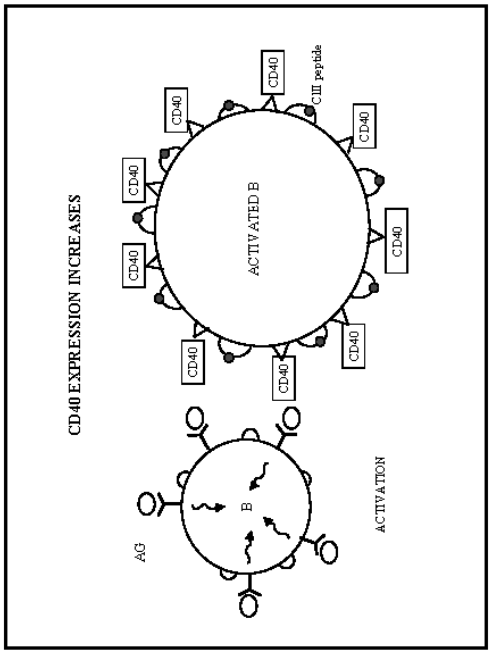

ANTIGEN ENTRY AND PROCESSING
B
MHC CLII
B
B

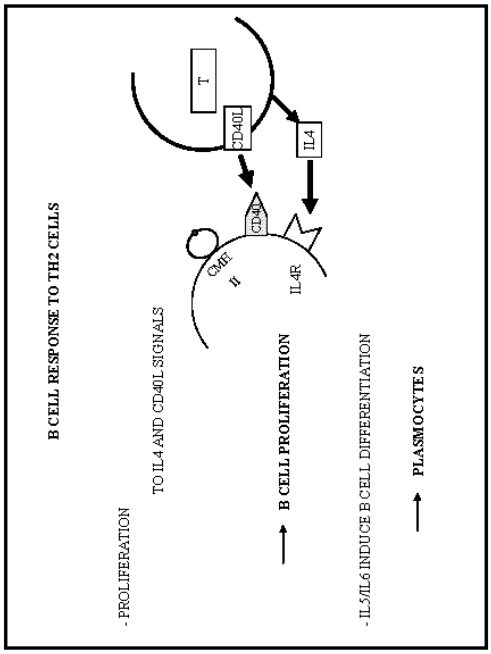

CD4 T LYMPHOCYTES HELP :
1- CD8 T CELLS TO BECOME CYTOTOXIC T CELLS AND
2- B CELLS TO BECOME PLASMOCYTES
B CELLS DIFFERENTIATE INTO PLASMOCYTES TO MAKE ANTIBODIES
CELL COOPERATION VIA CYTOKINES
CD4 TH LYMPHOCYTES
CELL COOPERATION VIA CYTOKINES
CD8 T LYMPHOCYTES DIFFERENTIATE INTO CYTOTOXIC CELLS

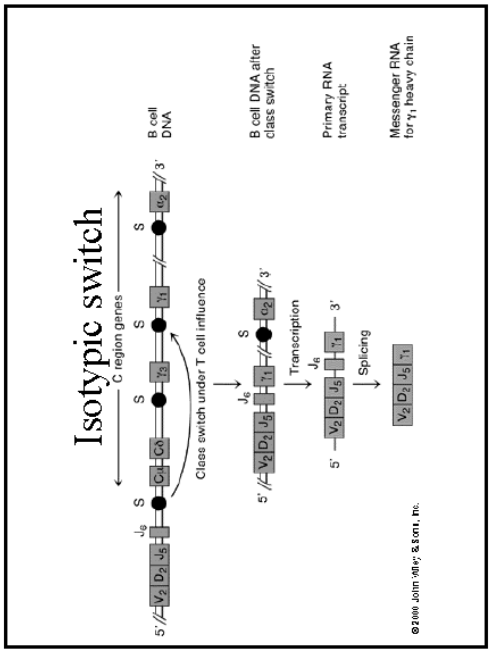

MEMORY LYMPHOCYTE
Ag DRIVEN CLONAL EXPANSION AND DIFFERENCIATION OF LYMPHOCYTES
EFFECTOR LYMPHOCYTE
CLONAL EXPANSION
DEATH
Ag + SECOND SIGNAL

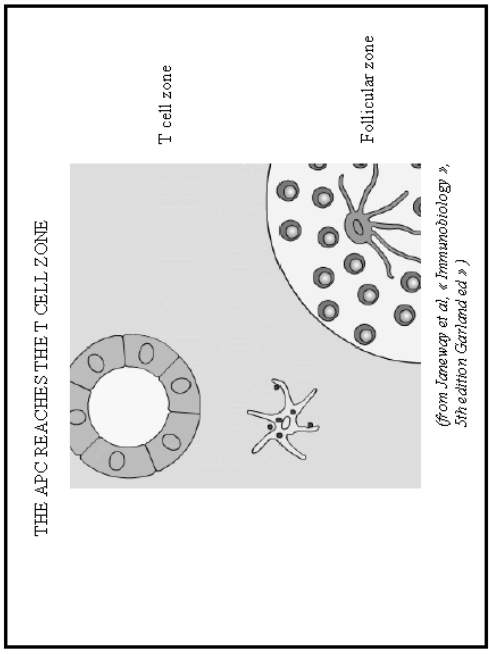

B CELLS ACTIVATE TH2 CELLS
- TH2 (IL4, IL5, IL6)
- ACTIVATED BY MHC/pep+CD40
- T CELLS PRODUCE IL4, IL5, IL6
TH2
TCR
CD40L
IL4, IL5, IL6
MHC
B

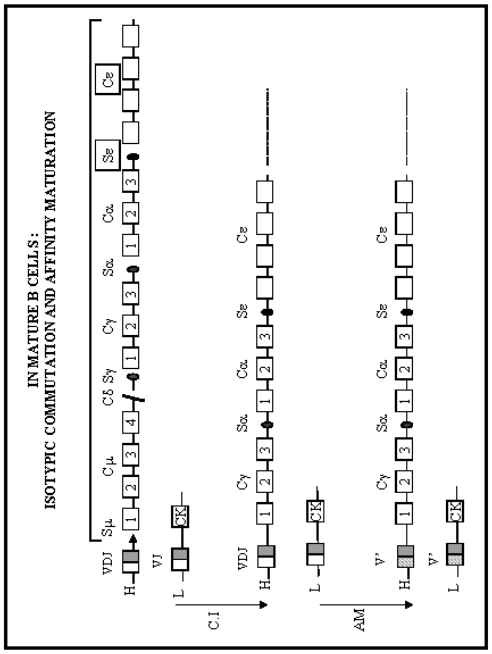

Ag + Signal 2
Bμ
Plasm
IgM
LOW AFFINITY ANTIBODIES
Bγ
Plasm
IgG
ANTIGEN
HIGH AFFINITY ANTIBODIES
Bγ
Plasm
IgG
Bγ
IgG

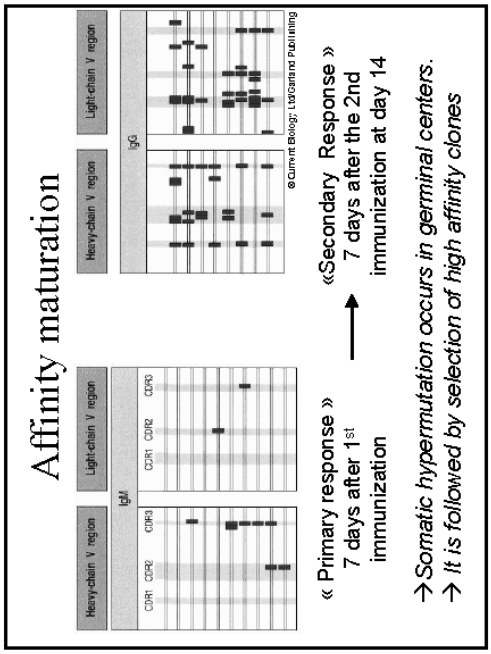

CD40 EXPRESSION INCREASES
AG
B
ACTIVATION
ACTIVATED B
CD40
CIII peptide


B CELL RESPONSE TO TH2 CELLS
- PROLIFERATION
TO IL4 AND CD40L SIGNALS
T
CD40L
IL4
CD40
CMH II
IL4R
B CELL PROLIFERATION
- IL5/IL6 INDUCE B CELL DIFFERENTIATION
PLASMOCYTES


Isotypic switch
C region genes
Class switch under T cell influence
B cell DNA
B cell DNA after class switch
Primary RNA transcript
Messenger RNA for γ1 heavy chain
Transcription
Splicing
©2000 John Wiley & Sons, Inc.


THE APC REACHES THE T CELL ZONE
T cell zone
Follicular zone
(from Janeway et al, « Immunobiology », 5th edition Garland ed.)


IN MATURE B CELLS :
ISOTYPIC COMMUTATION AND AFFINITY MATURATION
VDJ
VJ
CI
AM


Affinity maturation
Heavy-chain V region
Light-chain V region
IgM
IgG
CDR1
CDR2
CDR3
« Primary response »
7 days after 1st immunization
«Secondary Response »
7 days after the 2nd immunization at day 14
→Somatic hypermutation occurs in germinal centers.
→ It is followed by selection of high affinity clones
©Current Biology Ltd/Garland Publishing

B CELLS RESPOND
AND SECRETE
LOW AFFINITY Ab
SOME REACH
THE FOLLICULE

(from Janeway et al, « Immunobiology », 5th edition Garland ed »)

TH1 DEFENSES

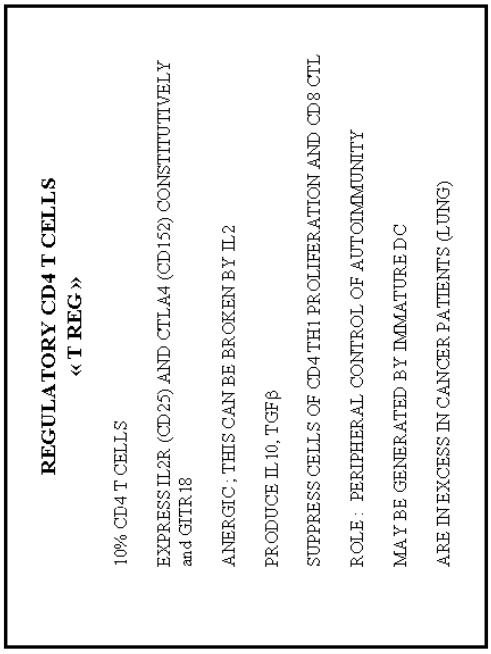
PROTECT AGAINST INTRACELLULAR MICROORGANISMS AND CANCERS

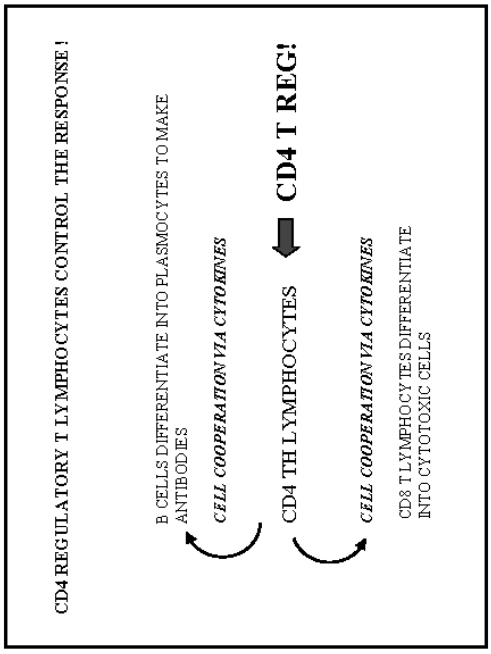
APC ACTIVATE TH

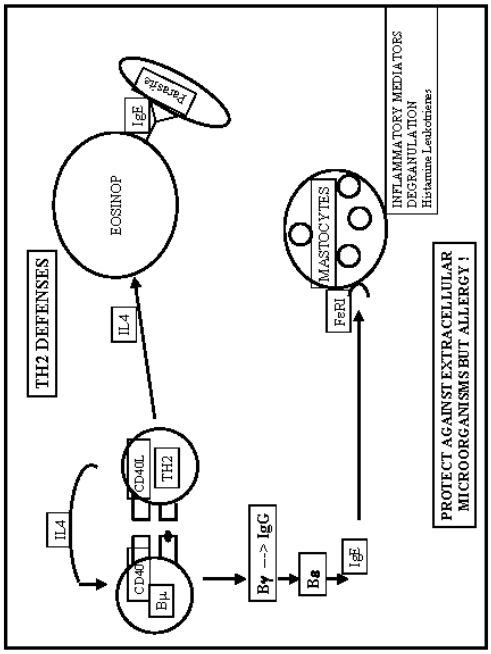
(from Janeway et al, « Immunobiology », 5th edition Garland ed »)

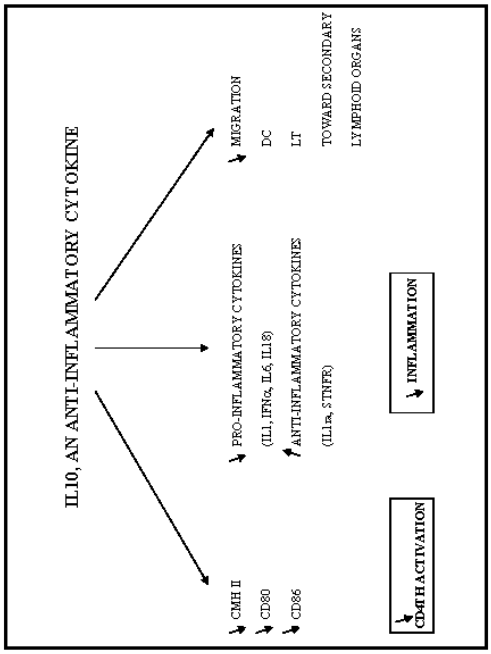
THEY PROLIFERATE, FORM A GERMINAL CENTER
WHERE SOMATIC MUTATION AND ISOTYPE SWITCH CAN OCCUR

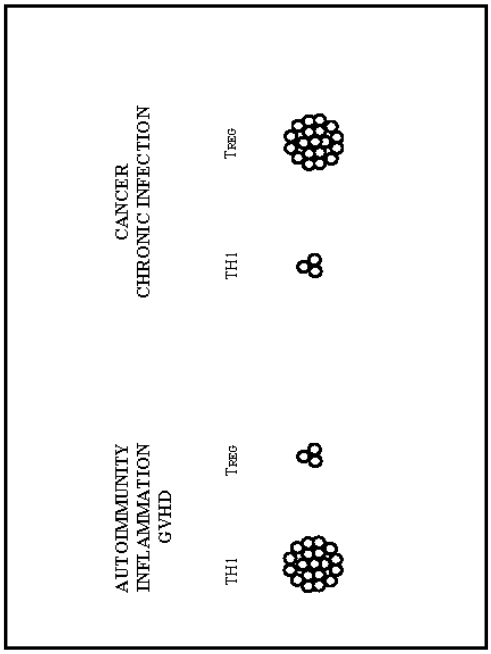
Follicular dendritic cells *(from Janeway et al, « Immunobiology », 5th edition Garland ed »)*


REGULATORY CD4 T CELLS
« T REG »
10% CD4 T CELLS
EXPRESS IL2R (CD25) AND CTLA4 (CD152) CONSTITUTIVELY and GITR18
ANERGIC ; THIS CAN BE BROKEN BY IL2
PRODUCE IL10, TGFβ
SUPPRESS CELLS OF CD4 TH1 PROLIFERATION AND CD8 CTL
ROLE : PERIPHERAL CONTROL OF AUTOIMMUNITY
MAY BE GENERATED BY IMMATURE DC
ARE IN EXCESS IN CANCER PATIENTS (LUNG)


CD4 REGULATORY T LYMPHOCYTES CONTROL THE RESPONSE !
B CELLS DIFFERENTIATE INTO PLASMOCYTES TO MAKE ANTIBODIES
CELL COOPERATION VIA CYTOKINES
CD4 TH LYMPHOCYTES
CD4 T REG!
CELL COOPERATION VIA CYTOKINES
CD8 T LYMPHOCYTES DIFFERENTIATE INTO CYTOTOXIC CELLS


TH2 DEFENSES
IL4
CD40
B
CD40L
TH2
IL4
EOSINOP
IgE
Parasite
By —> IgG
Ba
IgE
MASTOCYTES
FeRI
INFLAMMATORY MEDIATORS
DEGRANULATION
Histamine Leukotrienes
PROTECT AGAINST EXTRACELLULAR MICROORGANISMS BUT ALLERGY !


IL10, AN ANTI-INFLAMMATORY CYTOKINE
CMH II
CD80
CD86
PRO-INFLAMMATORY CYTOKINES
(IL1, IFNα, IL6, IL18)
ANTI-INFLAMMATORY CYTOKINES
(IL1ra, sTNFR)
MIGRATION
DC
LT
TOWARD SECONDARY LYMPHOID ORGANS
CD4TH ACTIVATION
INFLAMMATION


CANCER
CHRONIC INFECTION
TH1
Treg
AUTOIMMUNITY
INFLAMMATION
GVHD
Treg
TH1

Lecture 2: Tumour biology & immune surveillance [HF]

LE CANCER EN FRANCE *

	1980	2000	
INCIDENCE	170 000	278 000	(↗ 63%)
MORTALITE	125 000	150 000	(↗ 20%)

* Rapport de la Commission d'orientation sur le cancer (janvier 2003)

Different Kinds of Cancer

Diseases in which cells grow and spread unrestrained throughout the body

Organs and Tissues

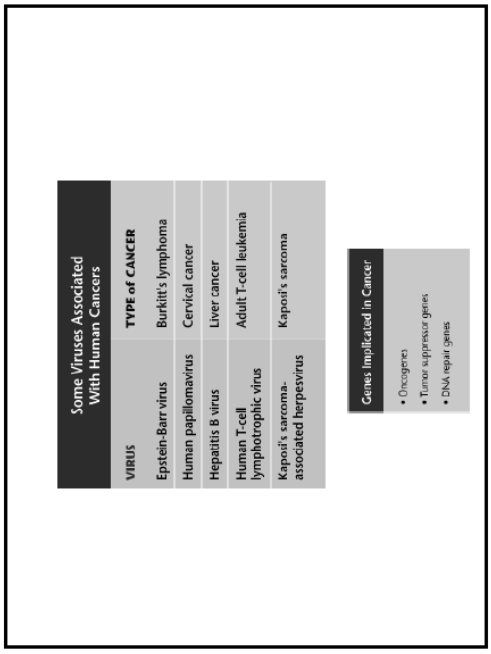
Carcinomas, the most common types of cancer, arise from the cells that cover external and internal body surfaces. (Lung, breast, colon, ...)

Sarcomas are cancers arising from cells found in the supporting tissues of the body (bone, cartilage, fat, connective tissue, and muscle).

Immune System

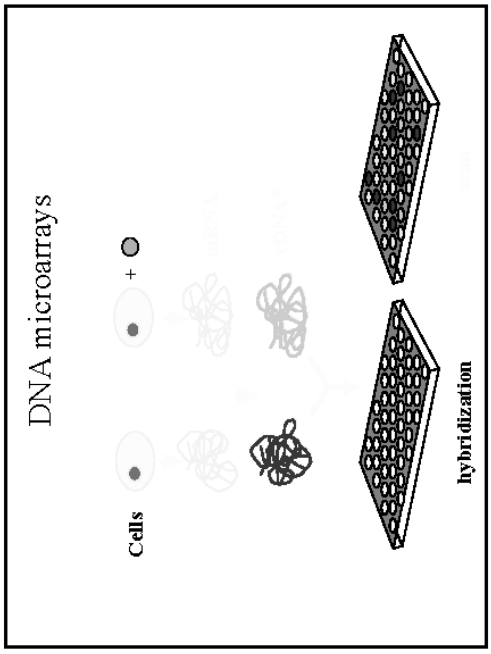
Lymphomas are cancers that arise in the lymph nodes and tissues of the body's immune system.

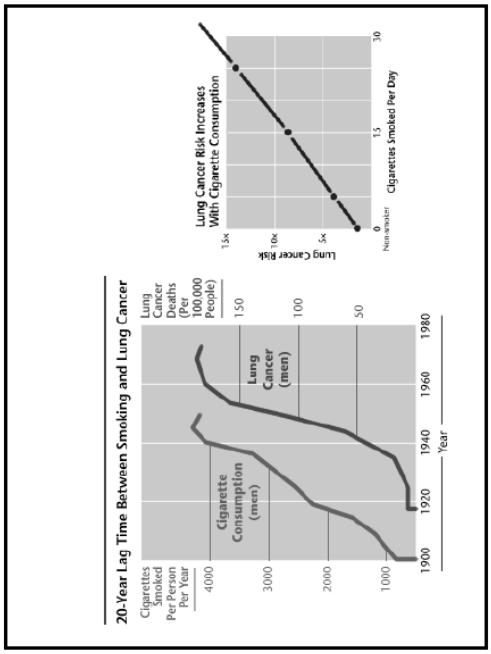
Leukemias are cancers of the immature blood cells that grow in the bone marrow and accumulate in the periphery.

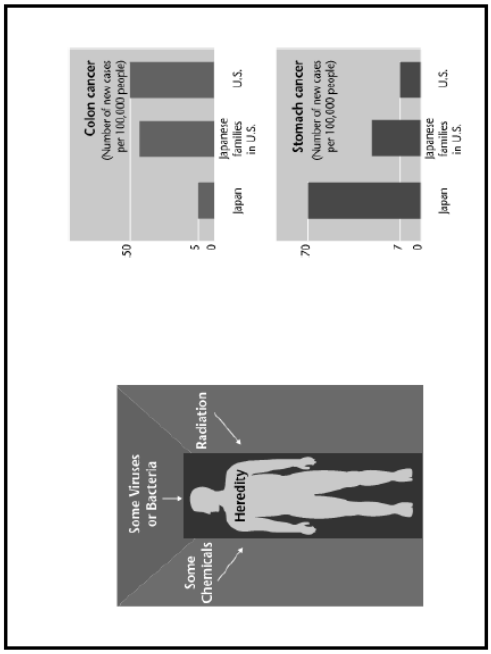


Some Viruses Associated With Human Cancers	
VIRUS	TYPE of CANCER
Epstein-Barr virus	Burkitt's lymphoma
Human papillomavirus	Cervical cancer
Hepatitis B virus	Liver cancer
Human T-cell lymphotrophic virus	Adult T-cell leukemia
Kaposi's sarcoma-associated herpesvirus	Kaposi's sarcoma

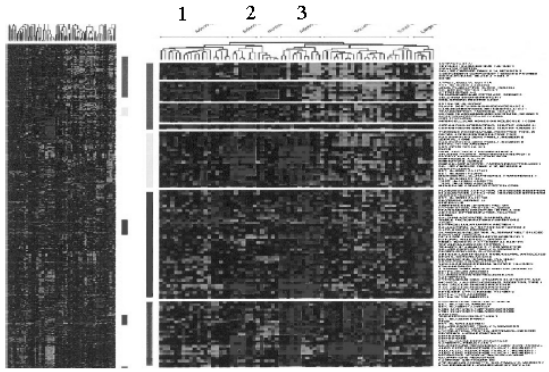
Genes Implicated in Cancer
• Oncogenes
• Tumor suppressor genes
• DNA repair genes







Lung Adenocarcinoma



Diversity of gene expression in adenocarcinoma of the lung.
Proc Natl Acad Sci U S A. 2001 Nov 20;98(24):13784-9.
Garber ME et al.

Lung Adenocarcinoma

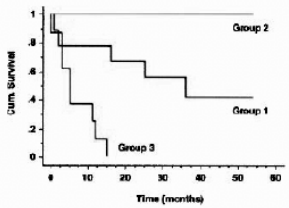
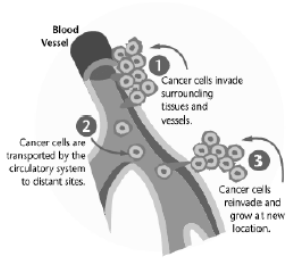


Fig. 4. Kaplan–Meier curves show differences in survival for AC subgroups. AC groups 1–3 were defined by hierarchical clustering (see Fig. 1). Cumulative survival, plotted on the *y* axis, represents percentage of patients living for the indicated times.

Diversity of gene expression in adenocarcinoma of the lung.
Proc Natl Acad Sci U S A. 2001 Nov 20;98(24):13784-9.
Garber ME et al.

Cancers are capable of spreading through the body by two mechanisms:

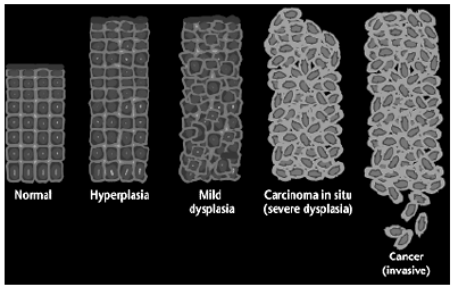
Invasion and Metastasis



Invasion refers to the direct migration and penetration by cancer cells into neighboring tissues.

Metastasis refers to the ability of cancer cells to penetrate into lymphatic and blood vessels, circulate through the bloodstream, and then invade normal tissues elsewhere in the body.

Cancer Progression



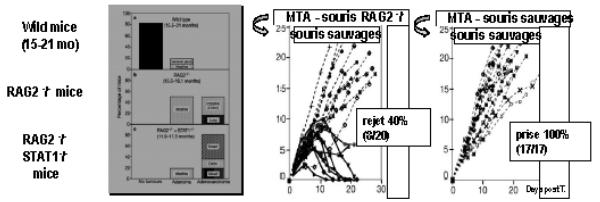
How does the immune system controls (or not) cancer progression?

Anti-tumoral Immunity

A controversial matter

- ✓ Augmentation de l'incidence des tumeurs surtout d'origine virale chez les immunodéprimés
- ✓ Succès modestes mais incontestables de protocoles d'immunothérapie (IL-2, IFNs, TILs,..)
- ✓ Valeur pronostique de l'environnement immunologique des tumeurs
- ✓ Expériences d 'immunisation contre des tumeurs syngéniques => Ag tumoraux
- ✓ Souris invalidées pour l'IFN-γ R, STAT-1 ou la perforine : plus sensibles au MCA

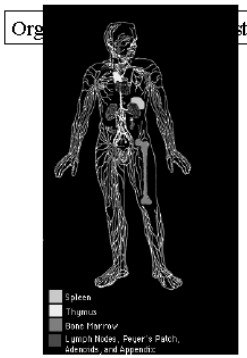
Immune surveillance



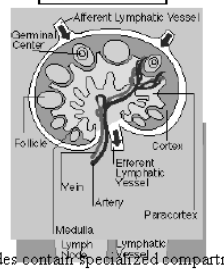
(Shankaran et al.Nature2001)

The Immune System

The immune system is a bodywide network of cells and organs that has evolved to defend the body against pathogens.



Lymph Node



Lymph nodes contain specialized compartments where immune cells congregate, and encounter antigens.

Immune System

Cells of the Immune System		Principle of the Immune System
T Cells	Adaptive Immunity	- Distinct antigenic specificities - Highly specific recognition of foreign antigens - Potent mechanisms for elimination of pathogens bearing such antigens - Capacity to display immunologic memory - Tolerance to self-antigens
B Cells		
NK Cells	Innate Immunity	Consists of molecules and cells that recognize only specific conserved constituents of microorganisms, and whose efficacy is not significantly increased by previous exposure. Inflammatory responses
Eosinophils		
Basophils		
Mast Cells		
Monocytes		
Macrophages		
Dendritic Cells		
PMN		

Tumor killing
CD8
APC
"Danger"
CD4

ANTIGENES DE TUMEURS
A Peptides dérivés d 'antigènes reconnus par des lymphocytes T-CD8
1 Antigènes de différenciation mélanocytaire
- Mart-1 (Melan A)
- Gp100 (pmel-17)
- Tyrosinase
- TRP1 (gp 75)
- TRP2
- MSH- R
2 Cancer-Testis antigen
- Mage 1, Mage 2, Mage 3, Mage 12
- Bage, Gage, Rage
- NY-ESO-1
- N-acetylglucosaminyltransférase V (peptide intronique).

Tumoral micro-environnement
Ac
B
M
DC
CD4
CD8
Tumor Ag
- differentiation
- mutated genes
- tumor testis
- viral proteins
...
CD8
Tumor

Les Lymphocytes T- CD8 et la Réponse anti-tumorale
Un des effecteurs majeurs dans la réponse anti-tumorale
Deux caractéristiques principales
T-CD8
Fas ligand
Fas
Molécules effectrices:
Perforine
Granzymes
Cytokines (IFN-γ, TNF-α/β)
Cellule Tumorale

B peptides dérivés d 'antigènes reconnus par des lymphocytes T-CD4

1 Peptides dérivés d'antigènes non mutés
- **gp100**
- **Mage 1**
- **Mage 3**
- **Tyrosinase**
- **NY-ESO-1**

2 Peptides dérivés d'antigènes mutés
- **Triosephosphate isomerase**
- **CDC-27**
- **LDLR-FUT**

3 Antigènes mutés
- **β catenine**
- **CDK-4**
- **Caspase-8**
- **KIA0205**
- **HLA-A2**

4 Antigènes surexprimés dans les tumeurs
- **G-250**
- **Her-2/neu**
- **p53**
- **Telomerase catalytic protein**
- **ACE**
- **α foeto-proteine (αFP)**

MUC-1

- Large transmembrane glycoprotein which belongs to the mucin family

- 20 Amino-acid sequence which is repeated between 20 and 125 times per Muc-1 molecule

- Found on the apical surface of mucin secreting normal epithelial cells.

- In tumor - Muc 1 is overexpressed on the whole cell surface

  - Muc 1 glycosylation pattern is altered exposing new cancer-specific epitopes to immune system.

IN VIVO IMMUNIZATION EXPERIMENTS USING RECOMBINANT MUC 1 VIRUS

1) IN MICE

Strain of mouse	Immunogen	Tumor challenge	Rejection of tumor challenge
DBA	Vaccine control	P815-Muc1	0/44 (0%)
DBA	Vaccine-Muc 1	P815-Muc1	13/45 (29%)

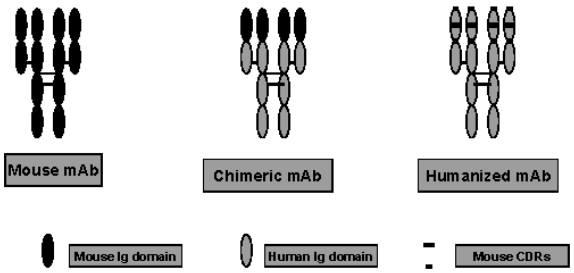
Acres Transgene

2) IN RATS

Vaccination with recombinant vaccinia virus expressing Muc 1 prior to challenge with Muc1 + tumor prevented tumor development in 60-80% of animals

Hareuveni et al 1990

From Mouse to Humanized m Abs



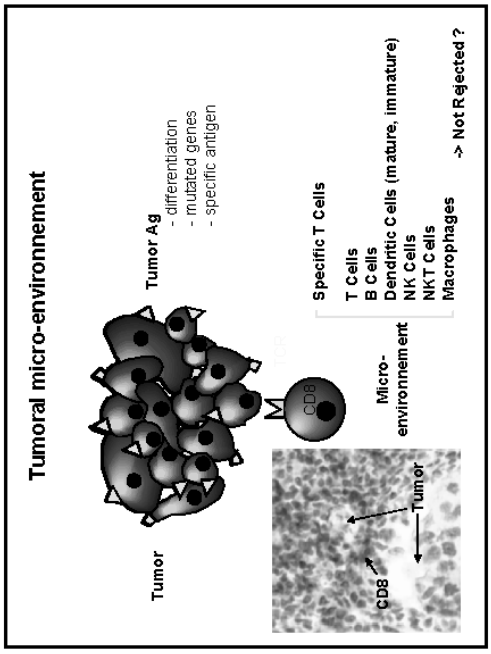
Antibody-based immunological therapies

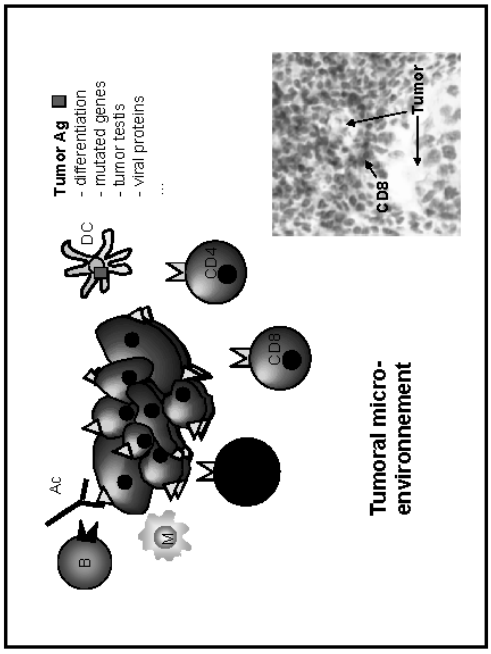
Scott MA and Welt S, Current Opinion in Immunology, 9:717, 1997.

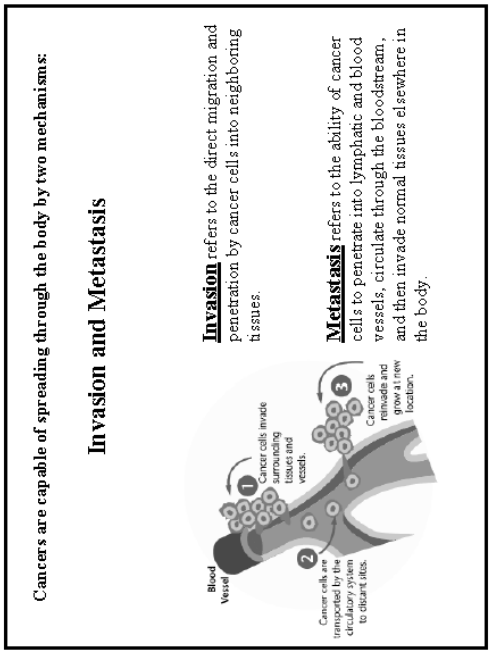
Major tumor associated antigens targeted by monoclonal antibodies in clinical trials.

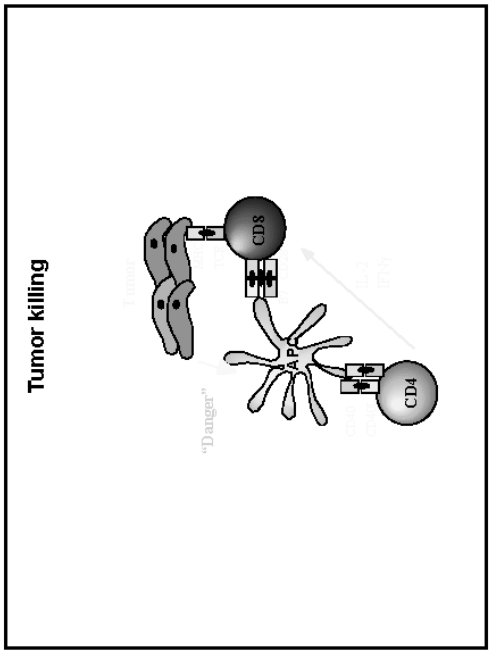
Categories	Examples of antigens	Tumor types	References
Lymphomas/leukemias			
Differentiation antigens	CD5	T cell leukemia/lymphoma	[21]
	CD19, CD20, CD21, CD22, CD37	B cell lymphoma	[4••,5–7,9]
	CD30	Hodgkin's lymphoma	[44]
	CD33, CD45	AML	[10–13]
	CAMPATH-1 (CDw52)	Lymphoid malignancies (T and B cell)	[14]
	HLA-DR	B cell lymphoma	[21]
	Anti-idiotype	B cell lymphoma	[8]
Solid tumors			
Cell surface antigens			
Glycoproteins	CEA, TAG-72, Ep-CAM, MUC1	Epithelial tumors (breast, colon, lung)	[16,22•,32,33]
	Folate binding protein	Ovarian tumors	[43]
	A33	Colorectal carcinoma	[23,24••,25,26]
	G250	Renal carcinoma	[37,38]
Glycolipids	Gangliosides (e.g. GD2, GD3)	Neuroectodermal tumors	[40,41]
Carbohydrates	Le^y	Epithelial tumors (breast, colon, lung)	[27•,36]
	CA-125	Ovarian carcinoma	[21]
Intracellular antigens	Ferritin	Hodgkin's disease, hepatoma	[21]
Growth factor receptors	EGFR	Lung, breast, head and neck tumors	[47]
	p185^{HER2}	Breast, ovarian tumors	[34••,35]
	IL-2 receptor	T and B cell neoplasms	[15]
Stromal/extracellular antigens	FAP-α	Epithelial tumors (colon, breast, lung)	[48]
	Tenascin	Glioblastoma multiforms	[21]
	Metalloproteinases	Epithelial tumors	[3•]

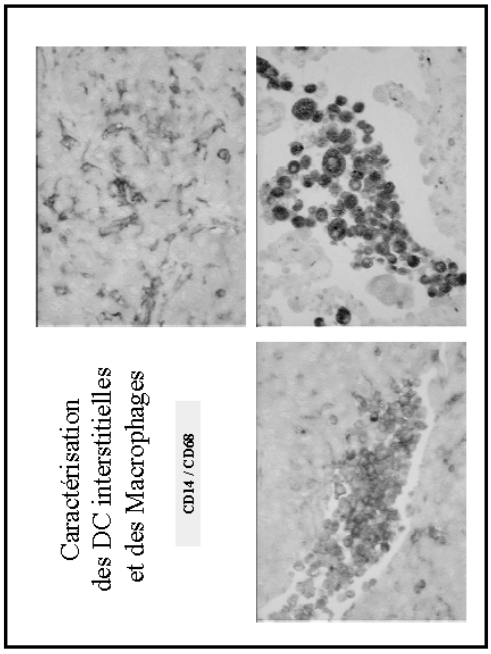
Lecture 3: Tumor microenvironment [HF]

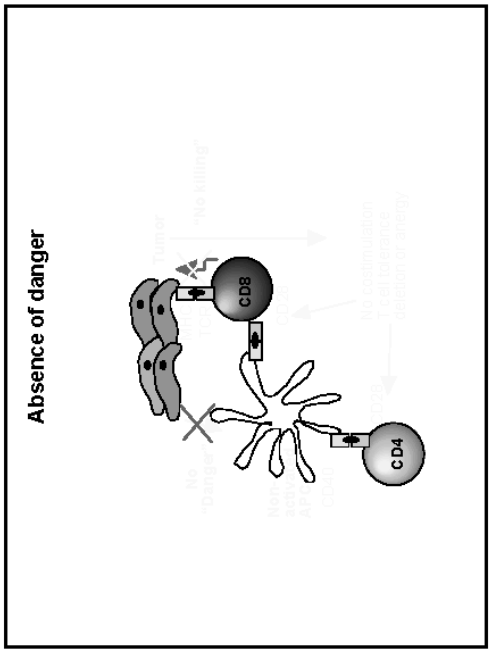


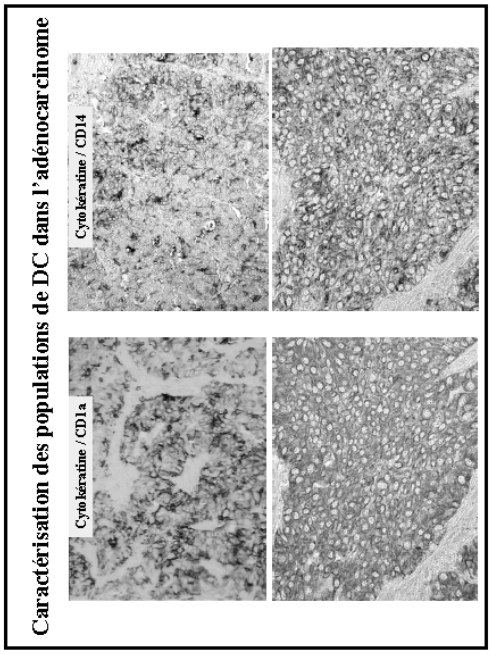


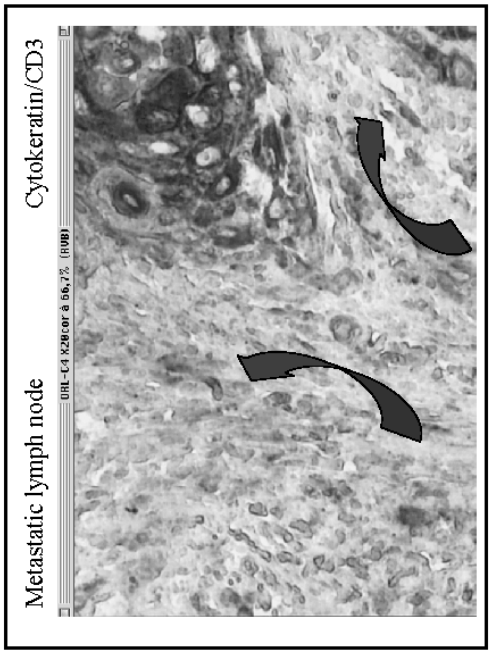





Caractérisation
des DC interstitielles
et des Macrophages
CD14 / CD68


Absence of danger
CD8
CD4


Caractérisation des populations de DC dans l'adénocarcinome
Cytokératine / CD1a
Cytokératine / CD14


Metastatic lymph node
Cytokeratin/CD3

Multivariate prospective analysis :
234 Patients with head and neck cancers

Oral cavity : 112

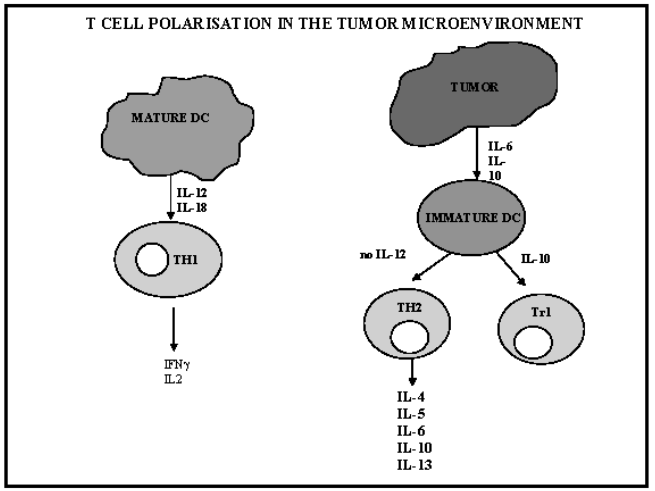
oropharynx : 33

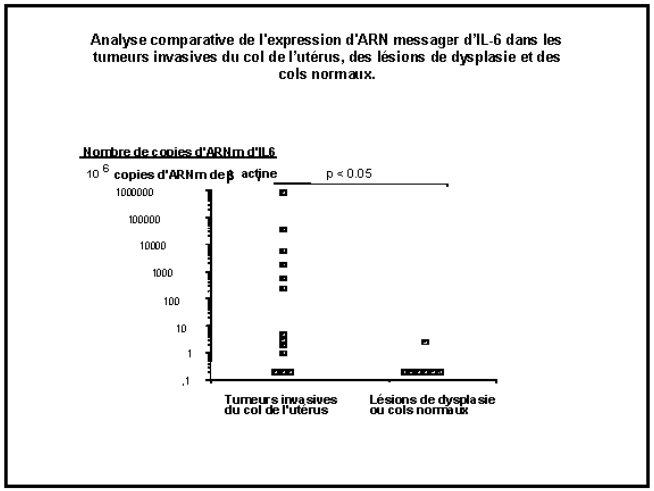
Hypopharynx : 41

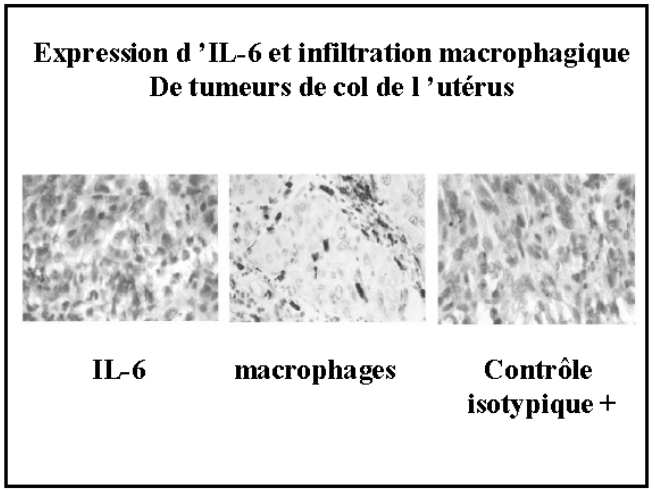
Larynx : 48

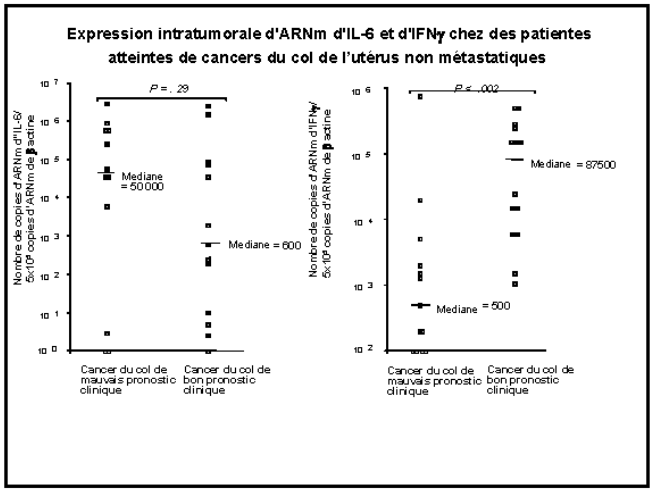
Factors influencing local control, survival and distant metastasis-free interval by the Cox proportional Hazards model.

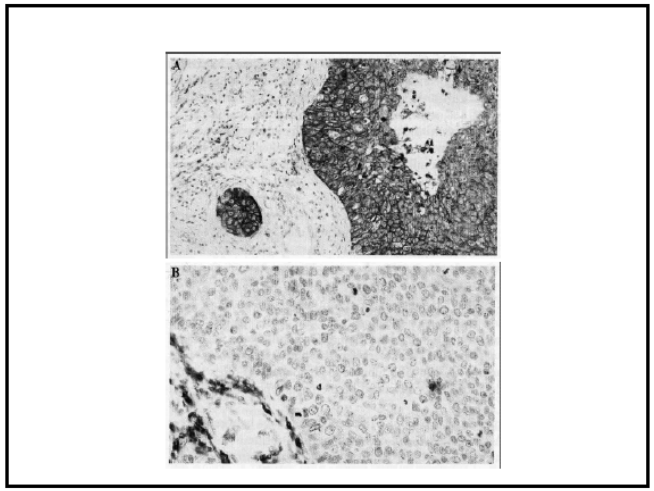
Order of entry in the model	p value	Relative risk (RR) and 95% CI
Survival		RR of death
1. Lymph node involvement	0.0015	
N0N1		1
N2N3		2.3 [1.4 - 3.7]
2. Serum sIL-2R levels	< 0.0001	
< 70		1
≥ 70		3.5 [2.1 - 5.9]
3. Performance status	0.0001	
EZB=0		1
EZB > 0		3.4 [1.9 - 6.1]
4. T stage	< 0.01	
T1 T2		1
T3T4		2 [1.2 - 3.4]
Local control		RR of local failure
1. T stage	0.0001	
T1		1
T2T3T4		7.4 [1.8 - 31.8]
2. Lymph node involvement	0.0006	
N0		1
N1N2N3		2.4 [1.5 - 3.8]
3. Performance status	< 0.045	
EZB = 0		1
EZB > 0		1.9 [1 - 3.4]
Distant metastasis-free interval		RR of metastasis
1. Lymph node involvement	< 0.0001	
N0		1
N1N2N3		4.8 [2.7 – 17.2]
2. Serum sIL-2R levels	0.0002	
< 70		1
≥ 70		5.8 [2.4 – 14.3]











IL-6 AND CANCER

- IL-6 is considered as an autocrine or paracrine growth factor for many tumors (myeloma, lymphoma, renal cell carcinoma, melanoma…)

- High IL-6 serum levels are associated with a poor prognosis and defavorable clinical outcome in various human cancers.

INTERLEUKIN-17 (IL-17 A)

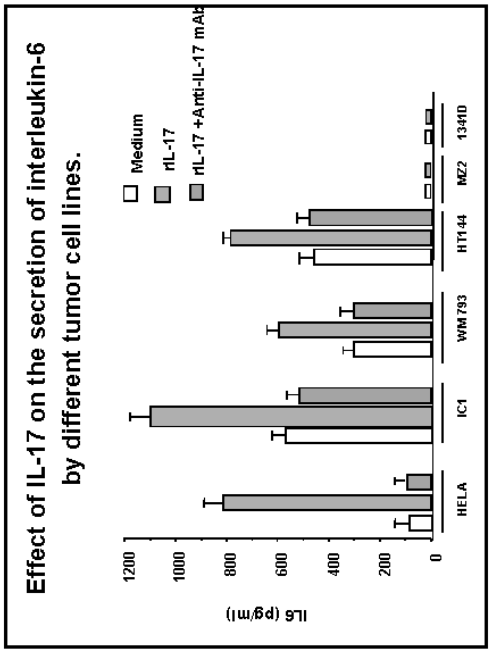
- First member of an emerging cytokine family (IL-17B, IL-17C IL-17 D, IL-17 E, IL-17F) expressed as dimers

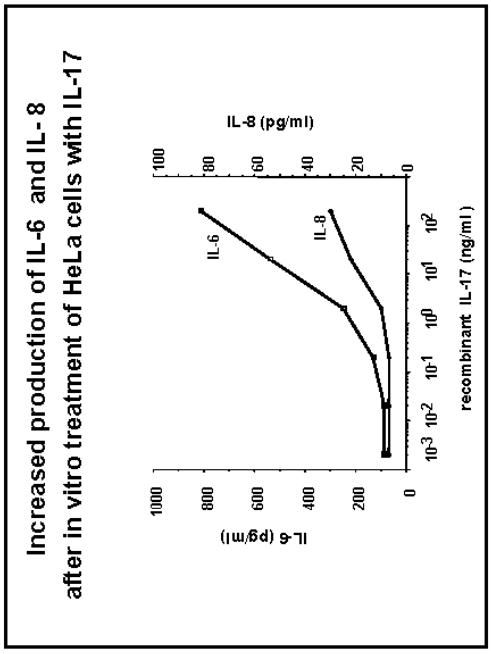
- Produced by activated memory CD4-T cells

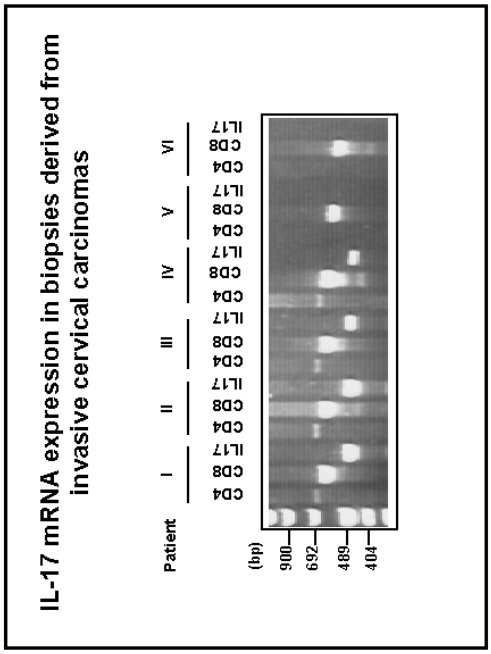
-

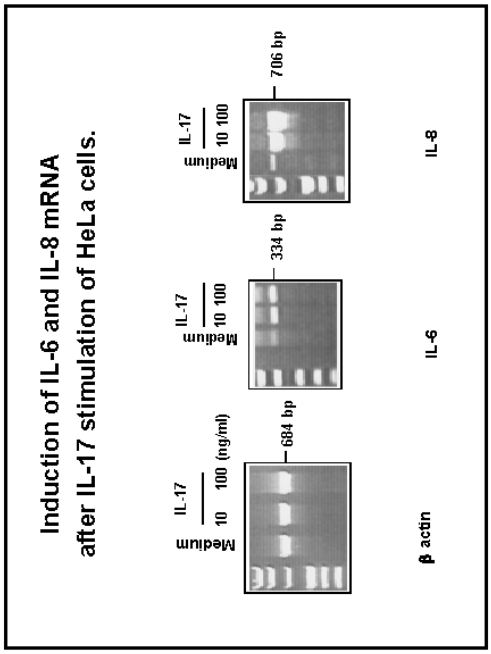
- Pro-inflammatory cytokine which increases the production of chemokines (IL-8, MCP-1, Groα) and hematopoietic growth factor (G-CSF, GM-CSF) thereby promoting the expansion and recruitment of monocytes and neutrophils.

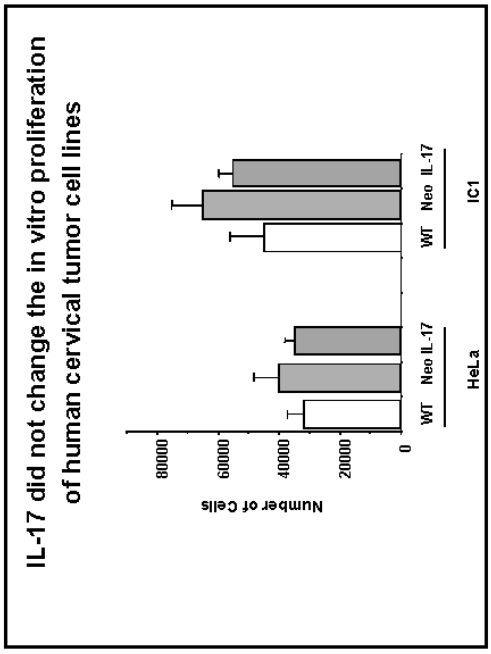
- IL-17 stimulates the production of IL-6 by different epithelial cell lines.

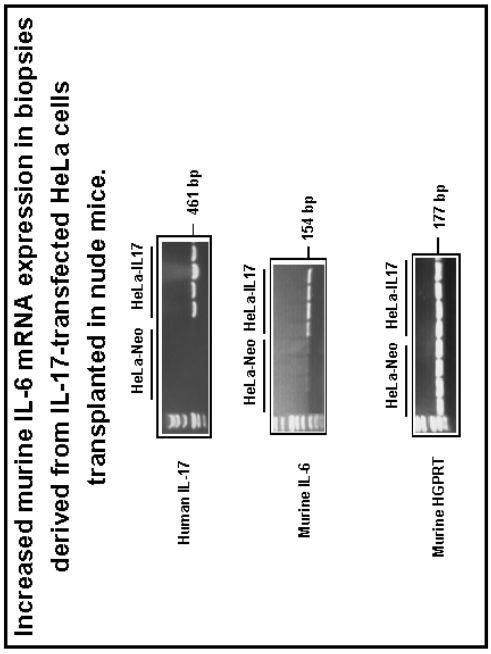

Effect of IL-17 on the secretion of interleukin-6 by different tumor cell lines.
Medium
rIL-17
rIL-17 +Anti-IL-17 mAb
IL6 (pg/ml)
1200
1000
800
600
400
200
0
HELA
IC1
WM793
HT144
MZ2
1341D

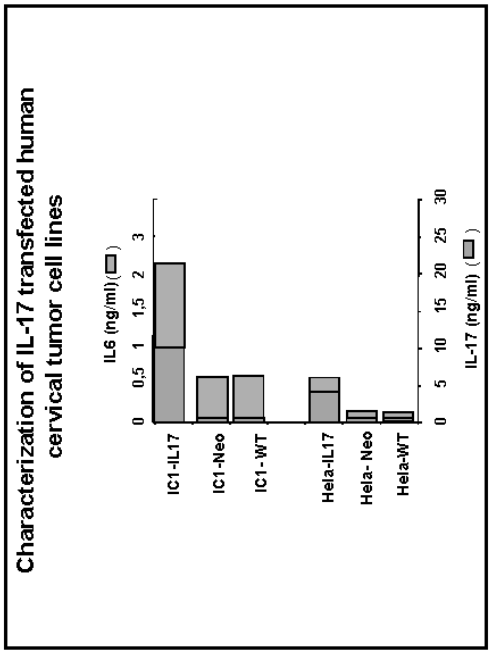

Increased production of IL-6 and IL- 8 after in vitro treatment of HeLa cells with IL-17
IL-8 (pg/ml)
IL-6
IL-8
IL-6 (pg/ml)
recombinant IL-17 (ng/ml)

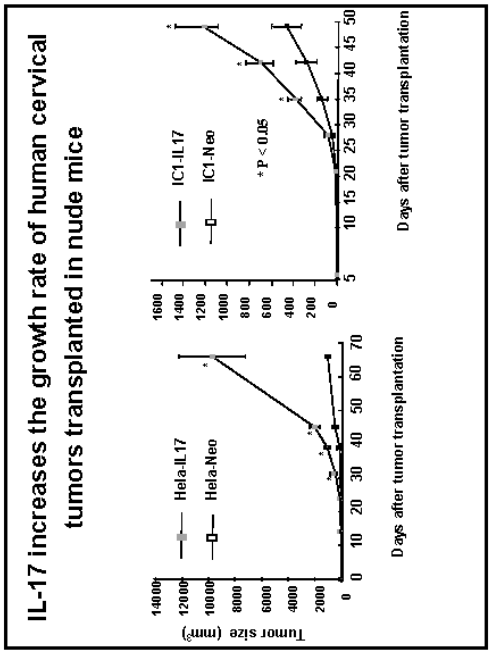

IL-17 mRNA expression in biopsies derived from invasive cervical carcinomas
Patient
I
II
III
IV
V
VI
CD4
CD8
IL17
(bp)
900
692
489
404

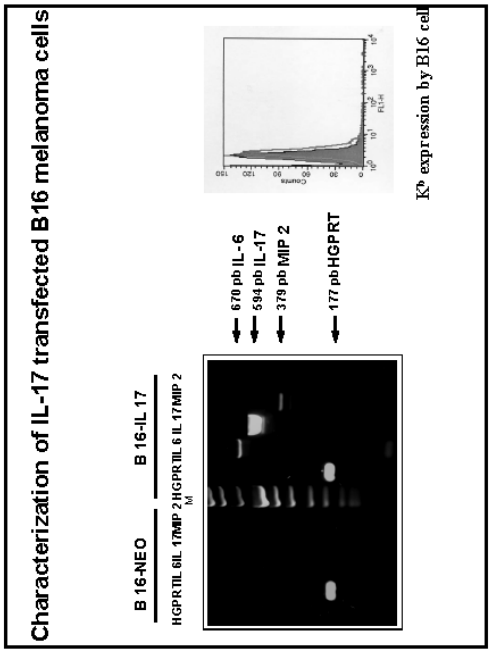

Induction of IL-6 and IL-8 mRNA after IL-17 stimulation of HeLa cells.
Medium
IL-17
10
100 (ng/ml)
684 bp
β actin
334 bp
IL-6
706 bp
IL-8

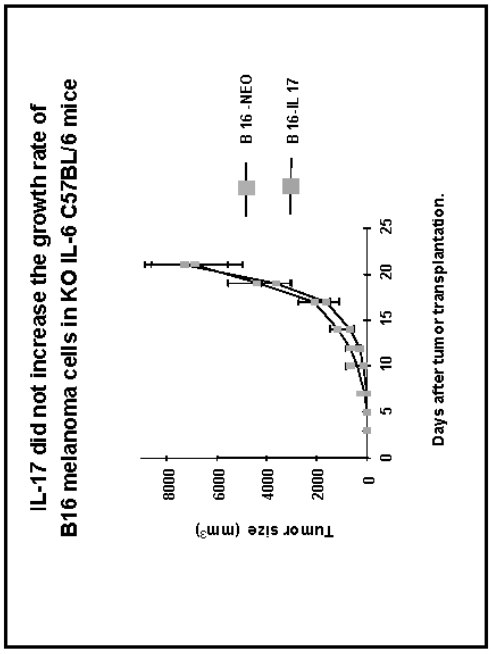

IL-17 did not change the in vitro proliferation of human cervical tumor cell lines
Number of Cells
80000
60000
40000
20000
0
WT
Neo
IL-17
HeLa
WT
Neo
IL-17
IC1

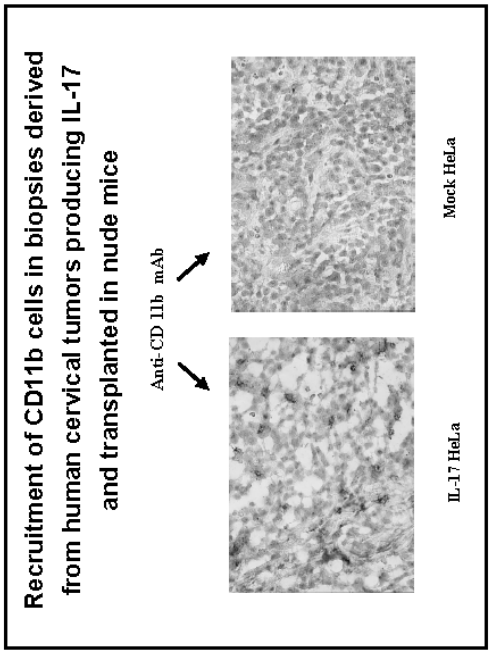

Increased murine IL-6 mRNA expression in biopsies derived from IL-17-transfected HeLa cells transplanted in nude mice.
HeLa-Neo
HeLa-IL17
461 bp
Human IL-17
154 bp
Murine IL-6
177 bp
Murine HGPRT

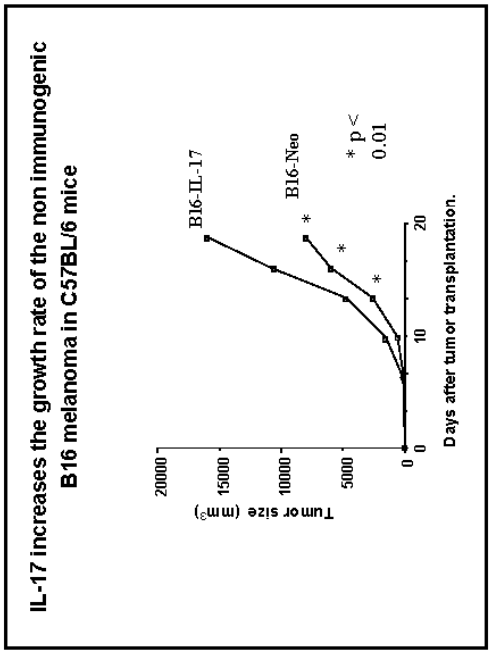

Characterization of IL-17 transfected human cervical tumor cell lines
IL6 (ng/ml)
0
0,5
1
1,5
2
3
IC1-IL17
IC1-Neo
IC1-WT
Hela-IL17
Hela- Neo
Hela-WT
IL-17 (ng/ml)
0
5
10
15
20
25
30

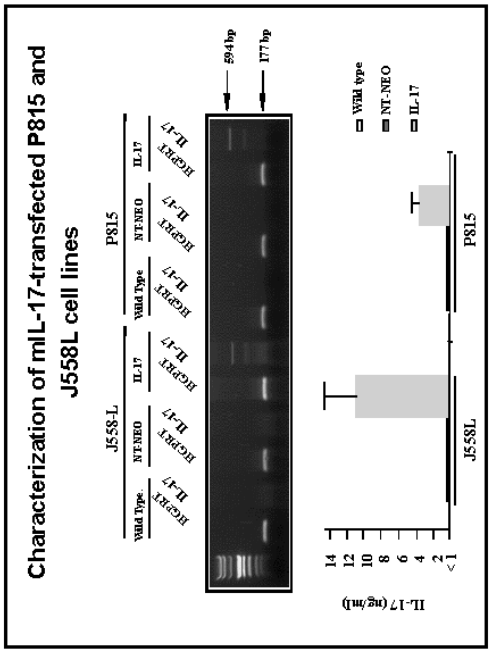

IL-17 increases the growth rate of human cervical tumors transplanted in nude mice
Tumor size (mm²)
Hela-IL17
Hela-Neo
IC1-IL17
IC1-Neo
* P < 0.05
Days after tumor transplantation
Days after tumor transplantation

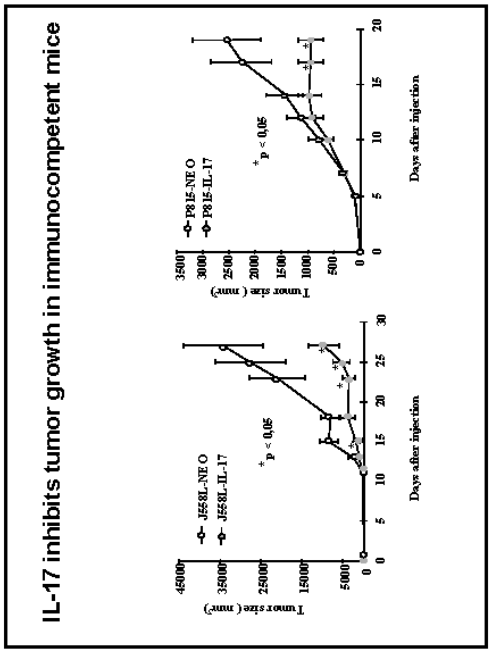

Characterization of IL-17 transfected B16 melanoma cells
B 16-NEO
B 16-IL 17
HGPRTIL 6IL 17MIP 2HGPRTIL 6 IL 17MIP 2
M
670 pb IL-6
594 pb IL-17
379 pb MIP 2
177 pbHGPRT
Kb expression by B16 cell

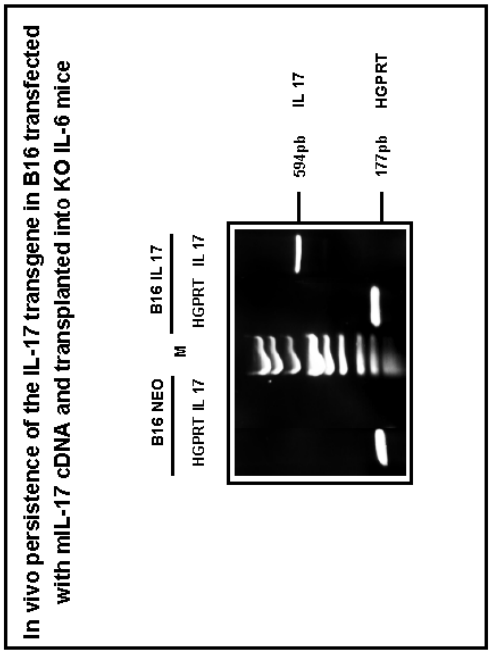

IL-17 did not increase the growth rate of
B16 melanoma cells in KO IL-6 C57BL/6 mice
B 16 -NEO
B 16-IL 17
Tumor size (mm3)
Days after tumor transplantation.

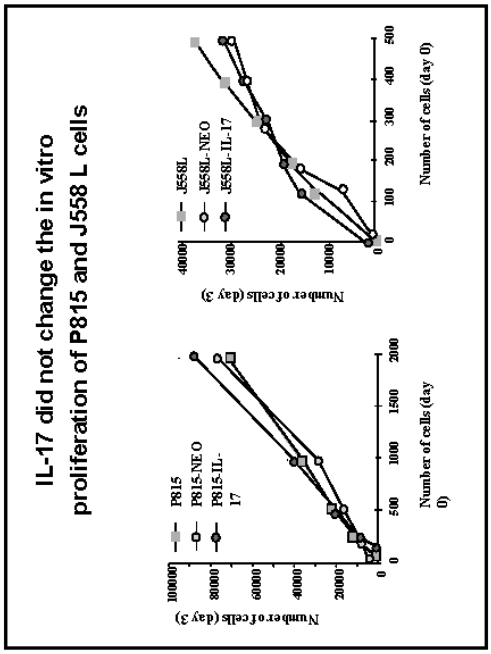

Recruitment of CD11b cells in biopsies derived
from human cervical tumors producing IL-17
and transplanted in nude mice
Anti-CD 11b mAb
IL-17 HeLa
Mock HeLa

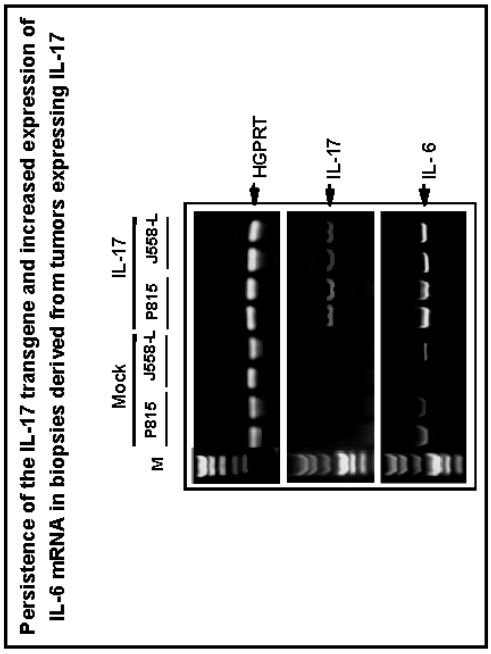

IL-17 increases the growth rate of the non immunogenic
B16 melanoma in C57BL/6 mice
B16-IL-17
B16-Neo
* p < 0.01
Tumor size (mm3)
Days after tumor transplantation.

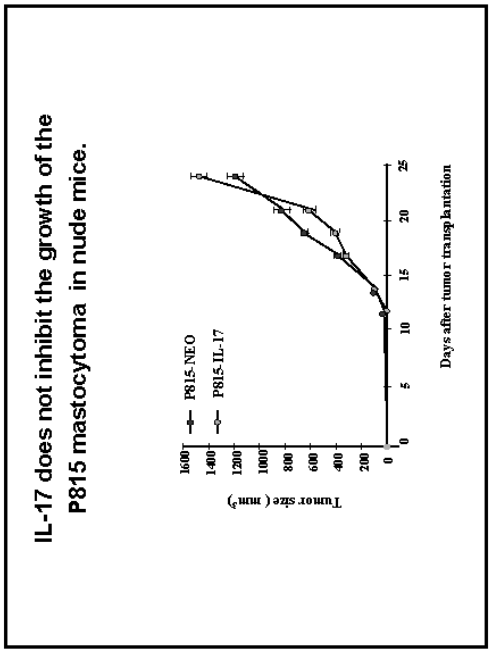

Characterization of mIL-17-transfected P815 and J558L cell lines
J558-L
P815
Wild Type
NT-NEO
IL-17
HGPRT IL-17
594 bp
177 bp
Wild type
NT-NEO
IL-17
IL-17 (ng/ml)
J558L
P815

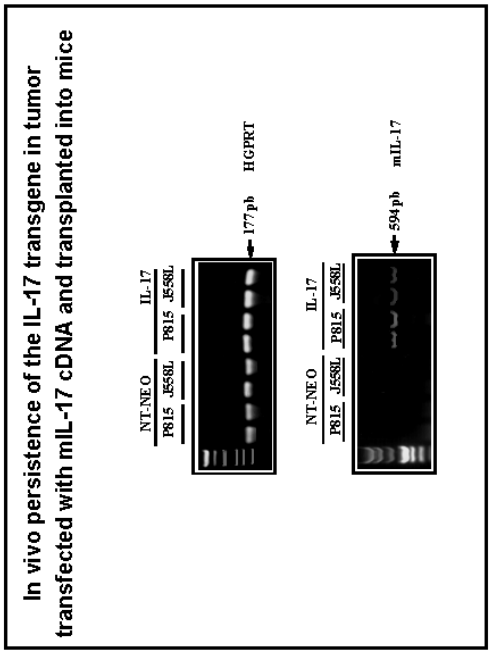

IL-17 inhibits tumor growth in immunocompetent mice
J558L-NEO
J558L-IL-17
P815-NEO
P815-IL-17
Tumor size (mm²)
Days after injection
p < 0,05

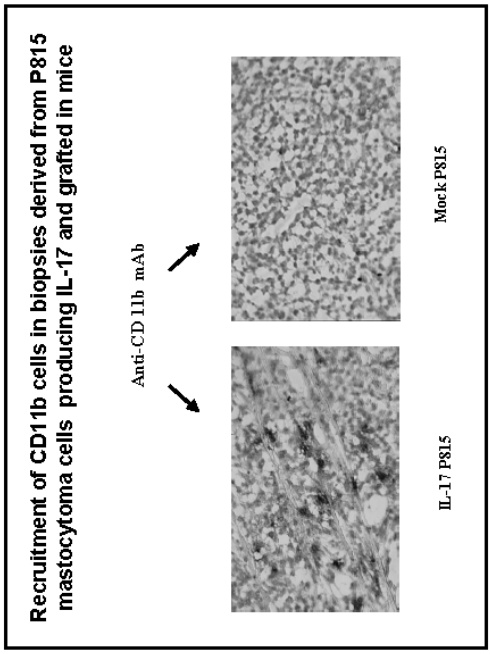

In vivo persistence of the IL-17 transgene in B16 transfected with mIL-17 cDNA and transplanted into KO IL-6 mice
B16 NEO
B16 IL 17
M
HGPRT IL 17
HGPRT IL 17
594pb
IL 17
177pb
HGPRT

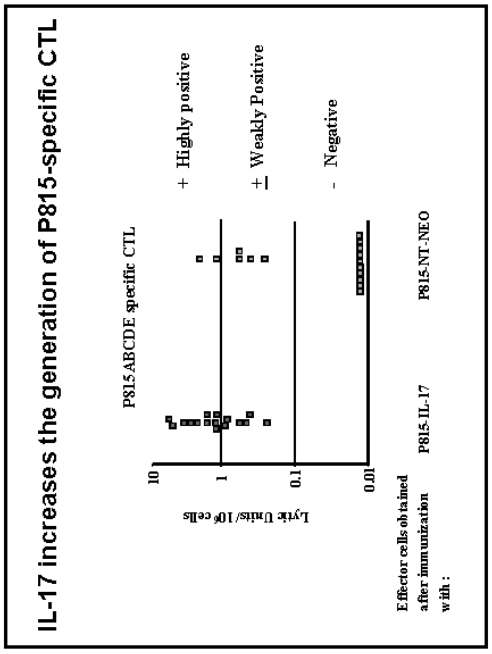

IL-17 did not change the in vitro proliferation of P815 and J558 L cells
P815
P815-NEO
P815-IL-17
J558L
J558L-NEO
J558L-IL-17
Number of cells (day 3)
Number of cells (day 0)

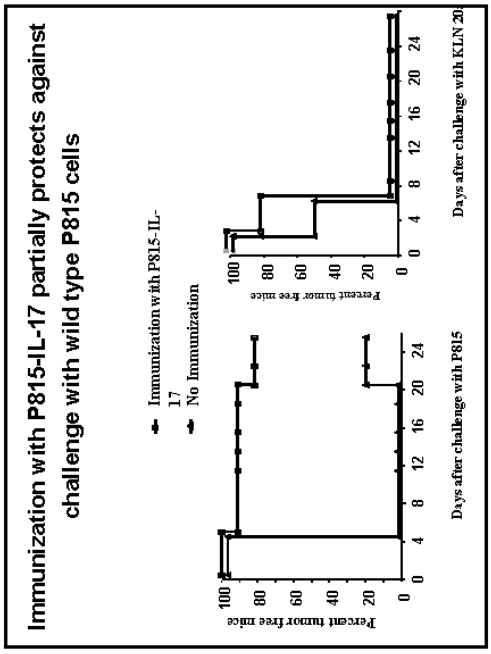

Persistence of the IL-17 transgene and increased expression of IL-6 mRNA in biopsies derived from tumors expressing IL-17
M
Mock
P815
J558-L
IL-17
P815
J558-L
HGPRT
IL-17
IL- 6

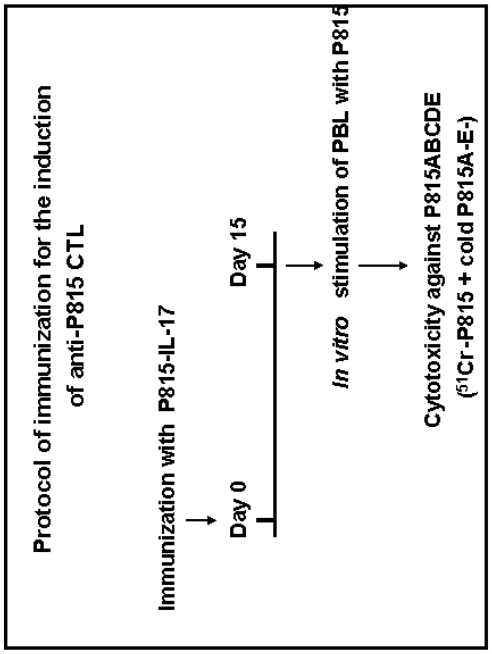

IL-17 does not inhibit the growth of the P815 mastocytoma in nude mice.
P815-NEO
P815-IL-17
Tumor size (mm³)
Days after tumor transplantation

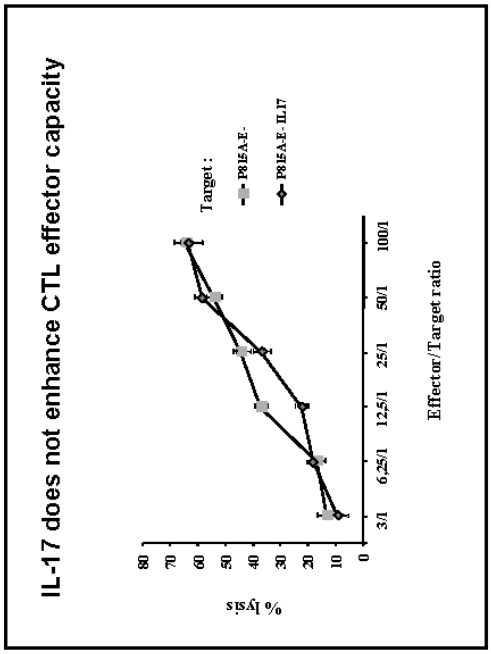

In vivo persistence of the IL-17 transgene in tumor transfected with mIL-17 cDNA and transplanted into mice
NT-NEO
P815
J558L
IL-17
P815
J558L
177 pb
HGPRT
NT-NEO
P815
J558L
IL-17
P815
J558L
594 pb
mIL-17


Recruitment of CD11b cells in biopsies derived from P815 mastocytoma cells producing IL-17 and grafted in mice
Anti-CD11b mAb
IL-17 P815
Mock P815


IL-17 increases the generation of P815-specific CTL
P815 ABCDE specific CTL
+ Highly positive
+/- Weakly Positive
- Negative
Lytic Units/ 10^6 cells
10
1
0.1
0.01
Effector cells obtained after immunization with :
P815-IL-17
P815-NT-NEO


Immunization with P815-IL-17 partially protects against challenge with wild type P815 cells
Immunization with P815-IL-17
No Immunization
Percent tumor free mice
Days after challenge with P815
Days after challenge with KLN 20


Protocol of immunization for the induction of anti-P815 CTL
Immunization with P815-IL-17
Day 0
Day 15
In vitro stimulation of PBL with P815
Cytotoxicity against P815ABCDE
(51Cr-P815 + cold P815A-E-)


IL-17 does not enhance CTL effector capacity
% lysis
Target :
P815A-E-
P815A-E- IL17
Effector/Target ratio
3/1
6,25/1
12,5/1
25/1
50/1
100/1

IL-17 inhibits tumor growth in other murine tumor models

- **Immunization of mice with Meth-A fibrosarcoma cells producing IL-17 protects against a challenge with parent Meth A fibrosarcoma.**

- **This protection is tumor specific and dependent of both CD4 and CD8-T cells.**

Hirahara et al . Oncology 2001

Potential mechanisms which may contribute to the induction of anti-tumor CTL by IL-17

- IL-17 stimulates the production of IL-6 which in some models elicited anti-tumor CTL (Porgador et al 1992, Mule et al 1992)

- IL-17 promotes the maturation of dendritic cells thereby increasing their ability to prime CTL (Antonysamy et al 1999)

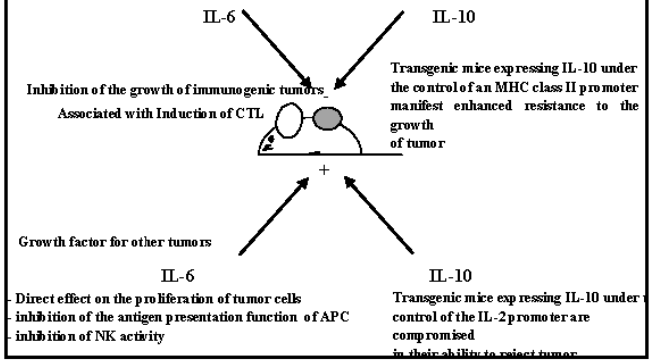
- IL-17 triggers the secretion of IL-12, a cytokine known to induce CTL (Jovanovic et al 1998)

IL-17 a two faces cytokine

- In non immunogenic tumors or in the absence of T lymphocytes ,
IL-17 promotes tumor growth : this effect seems in part mediated by IL-6.

- In contrast, IL-17 inhibits the growth of immunogenic tumors by means of a T cell dependent mechanism.

This ambivalent activity in the control of tumor growth has also been observed for other cytokines



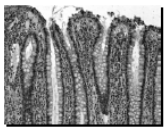
Expression of IL-17 in Sezary syndrome
PMA and Ionomycin
IL-2 and IL-15
Medium
MYLA
Sezary cells produce IL-17
β Actin
Interleukin-17
IL-17 mRNA expression in biopsies derived from Sezary patients

IL-18 : an IFN-γ inducing cytokine
• IL-18 activity : a multifunctional cytokine
Th1 response
IL-2
IFN-γ
Cytotoxic activity
IFN-γ
TNF-α
FasL
perforine
Inflammatory response
IFN-γ, TNF-α, IL-1β
IL-8, MCP-1,
MIP-1
NO, PG
Antigen
Pro IL-18
(24 kDa)
ICE
IL-18
IL-12
IL-12
T CD4+ Th1
T CD8+
NK
Macrophage
APC

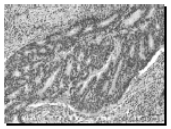
IL-17 and ovarian cancer
11 (64.7%) of 17 ovarian cancers
blood vessels (stained with anti-CD31
mAb
Kato T et al BBRC 2001

IFN-γ et valeur pronostique
Perte d'IFN-γ au site tumoral
Mauvais pronostic
• Cancer du col utérin
(Tartour E. et al. J. Nat. Cancer Inst. 1998)
• Cancer du colon
(Pagès F. et al. Int. J. Cancer 1999)

IL-18 and colon adenocarcinoma



IL-18 is synthezised by epithelial cells of the normal colon mucosa facing the intestinal lumen



• IL-18 expression decreased / abolished in colon adenocarcinoma

IL-18 and colon adenocarcinoma

• Clinical outcome :

- 5/7 patients with markers of IL-18 activity (FasL, IFN-γ) had a cancer strictly confined to the colon.

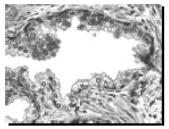
- 7/7 patients with no marker of IL-18 activity (FasL, IFN-γ) presented with distant metastases.

Production of active IL-18 at tumor site may be involved in the host anti-tumor immune defense

IL-18 and prostate cancer

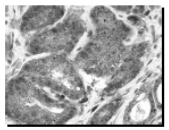
• 36 cases of clinically localized prostate cancer studied

- 27/36 cases with tumor cells producing IL-18
- heterogeneous tumor IL-18 expression (<10 to >66% IL-18+ cells)
- not correlated with the pathological stage or the Gleason score



Normal prostate

basal cells express IL-18

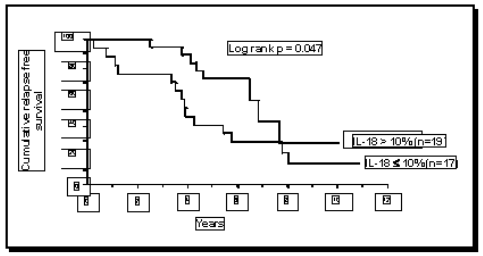


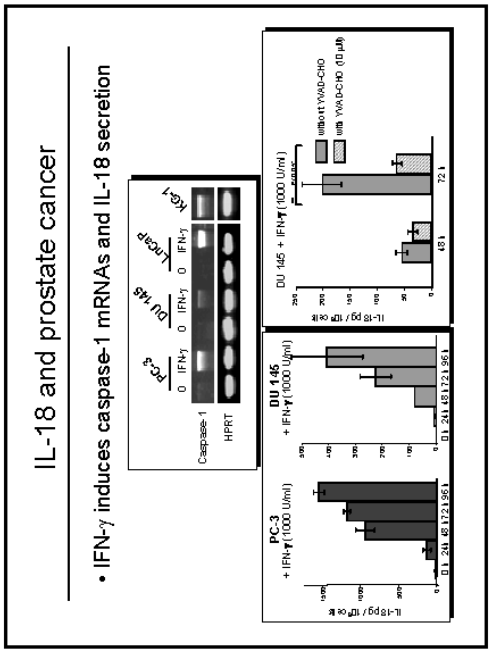
Prostate cancer

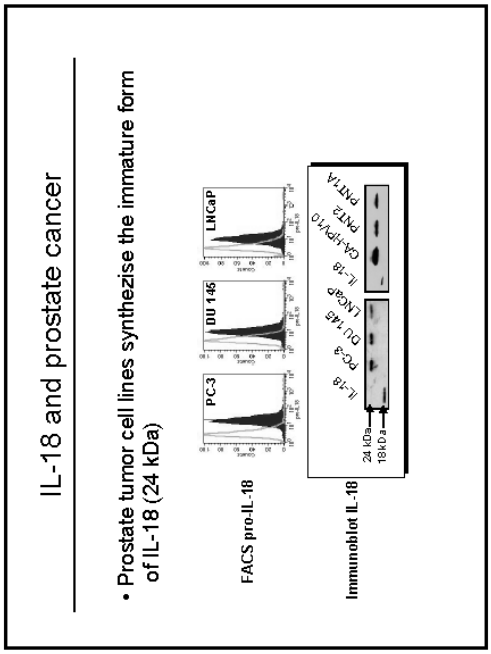
Tumor cells express IL-18

IL-18 and prostate cancer

• median follow-up : 95 months (75-115)
• clinical outcome : 12 disease-free patients
22 relapses (11 bone metastasis)




IL-18 and prostate cancer
• IFN-γ induces caspase-1 mRNAs and IL-18 secretion
PC-3
DU 145
LNCaP
IFN-γ
Caspase-1
HPRT
Ker-1
PC-3
+ IFN-γ (1000 U/ml)
DU 145
+ IFN-γ (1000 U/ml)
without YVAD-CHO
with YVAD-CHO (10 µM)


IL-18 and prostate cancer
• Prostate tumor cell lines synthesize the immature form of IL-18 (24 kDa)
FACS pro-IL-18
PC-3
DU 145
LNCaP
Immunoblot IL-18
24 kDa
18 kDa
IL-18
PC-3
DU 145
LNCaP
IL-18
CA-HPV-10
PANT2
PANT1A

Lecture 4: Cytokines and cancer [ET]

CYTOKINES AND CANCER

1 Pro-tumor activity of cytokines

- Growth or anti-apoptotic factors for tumor cells.

- Inhibitors of T cell activation (TGFβ , IL-10)

- Inhibitors of dendritic cell maturation (IL-6, IL-10...).

2 Anti-tumor activity of cytokines

. Bias in TH1 polarisation (low concentrations of TH1 cytokines, RsIL-2…)

. Clinical use of cytokines in immunotherapy.

3 Dual activity of cytokines in the control of tumor growth.

- IL-17 a model of cytokine with ambivalent activity in oncology.

- Other examples of cytokines with dual activity

- Mechanisms underlying these pleiotropic and antagonist activities.

Cytokines as growth factors for tumor cells

Cytokines stimulate the growth of many tumor cell lines.

- GM-CSF is a growth factor for human ovarian cancer cells

- IL-1 can promote the growth of leukemia cells by inducing GM-CSF

- Cytokines may act as autocrine growth factors or paracrine growth factors.

Interleukin -6

- IL-6 is a growth factor for AIDS Kaposi 's sarcoma derived cells, myeloma, certain T and B cell lymphoma, renal cell carcinoma, hepatocarcinoma, cervical carcinoma and prostate carcinoma cell line.

- Recent studies have shown that IL-6 is a survival factor for myeloma cells by inducing Mcl-1 (Jourdan M. Cell Death Diff 2000)

- Anti IL-6 mAb therapy in myeloma patients : Inhibition of IL-6 activity without real impact on clinical parameters.

- Since IL-6 has been linked with drug resistance mechanisms, association of anti-IL-6 with chemotherapy is being tested.

Cytokines (IL-10, TGFβ) as immunosuppressive factors with the ability to inhibit T cell activation

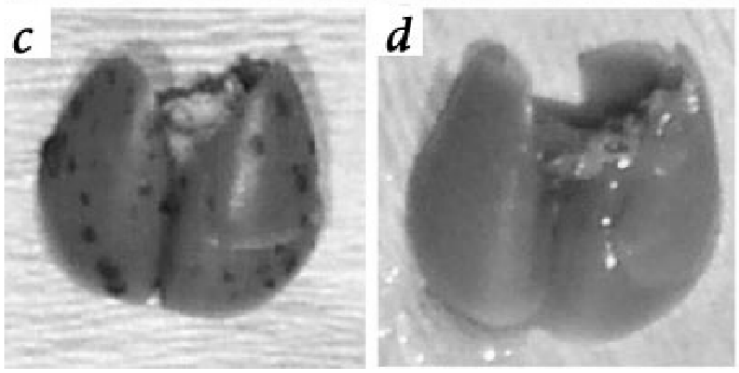
TGFβ

. Drives the balance to TH2 via IL-10 as an intermediate
. Inhibits TH1-type responses directly
. Inhibits T cell activation

IL-10

. Downmodulates the expression of TH1 cytokines
. Regulates expression of TGF-β type II receptor.
. Decreases the expression of MHC molecules
. Inhibits the differentiation of dendritic cells.

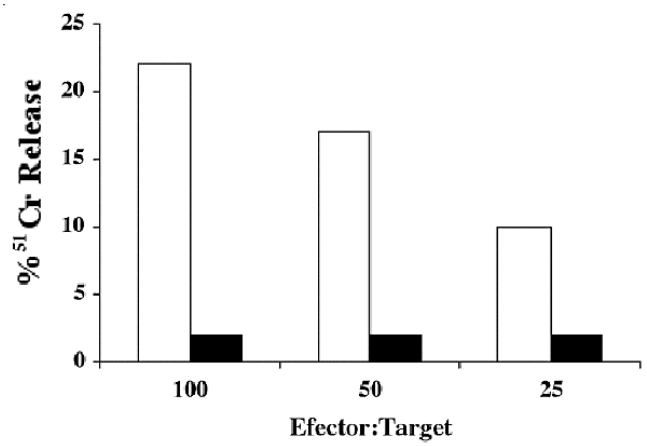
Wild Type Mice **DN TGFRII Mice**



2×10^5
B16-F10 i.v.

Blockade of TGF- signaling in T cells renders mice resistant to tumor challenge.

Gorelik L. Nature Med 2001



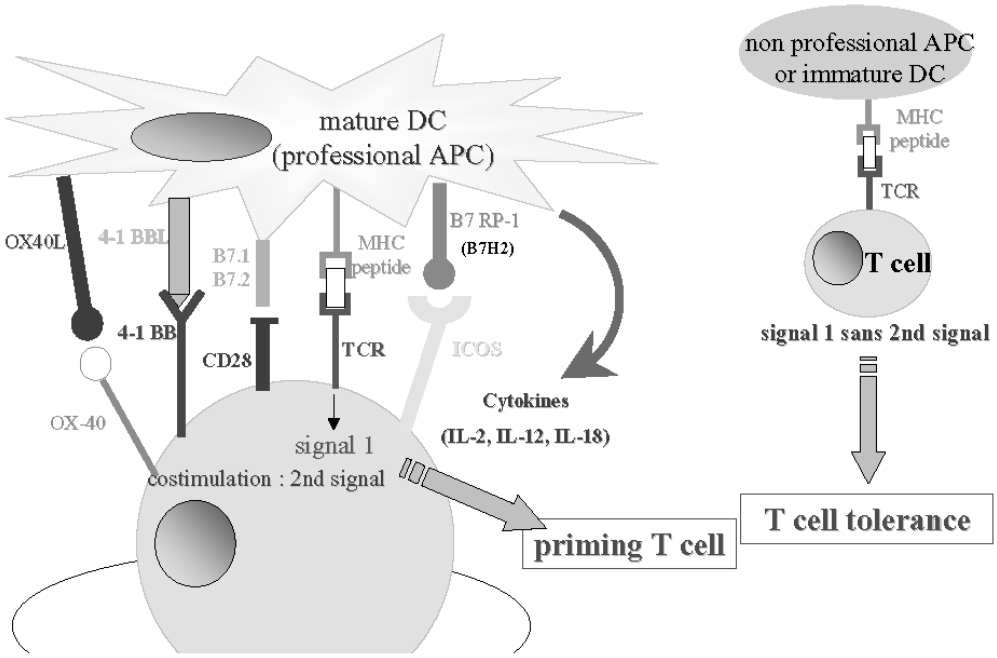
In vivo generation of tumor-specific CD8+ CTLs in the absence of TGFβ- signaling in T cells.

Spleen cells were isolated from transgene-positive (□) or transgene-negative (■) littermate mice challenged with 10^6 live EL-4 cells 10 days prior and were evaluated for their lytic activity by the 51Cr release assay against EL-4 targets.

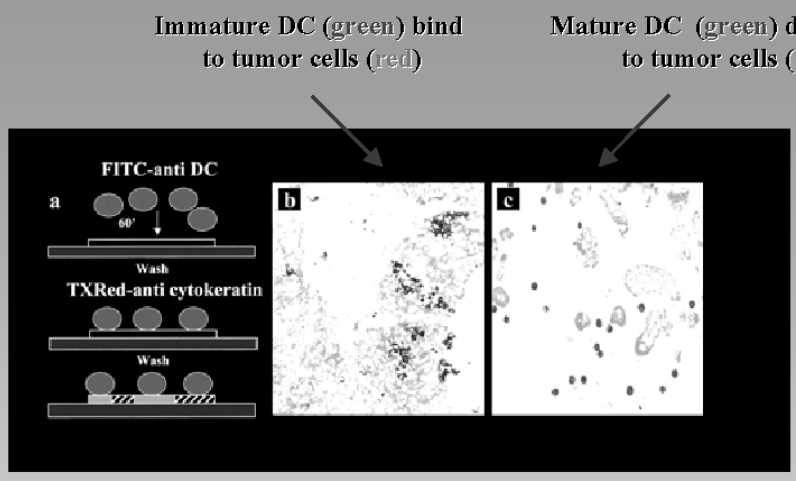
Gorelik L Nature Med 2001

Cytokines inhibit the maturation of dendritic cells during tumor progression

Two signal are required for the optimal activation of T cells



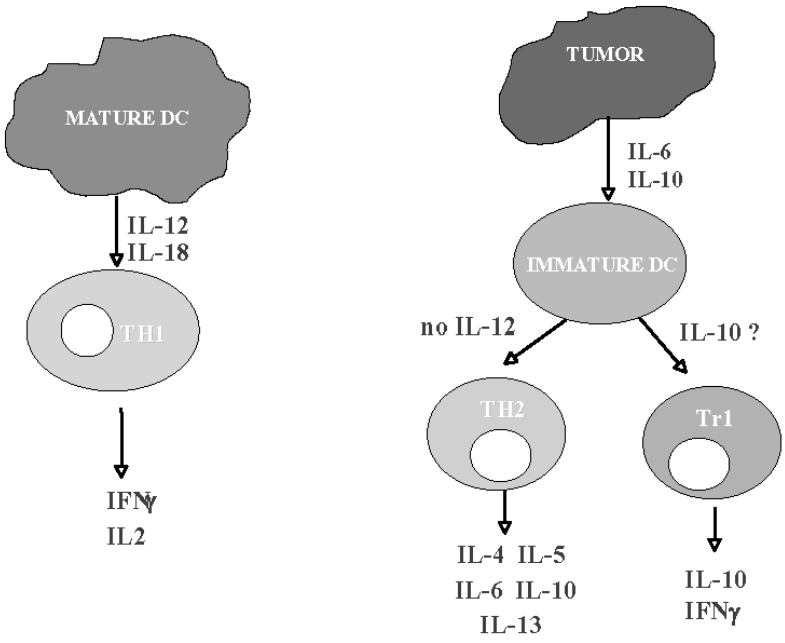
Only immature dendritic cells are found in contact with tumor cells



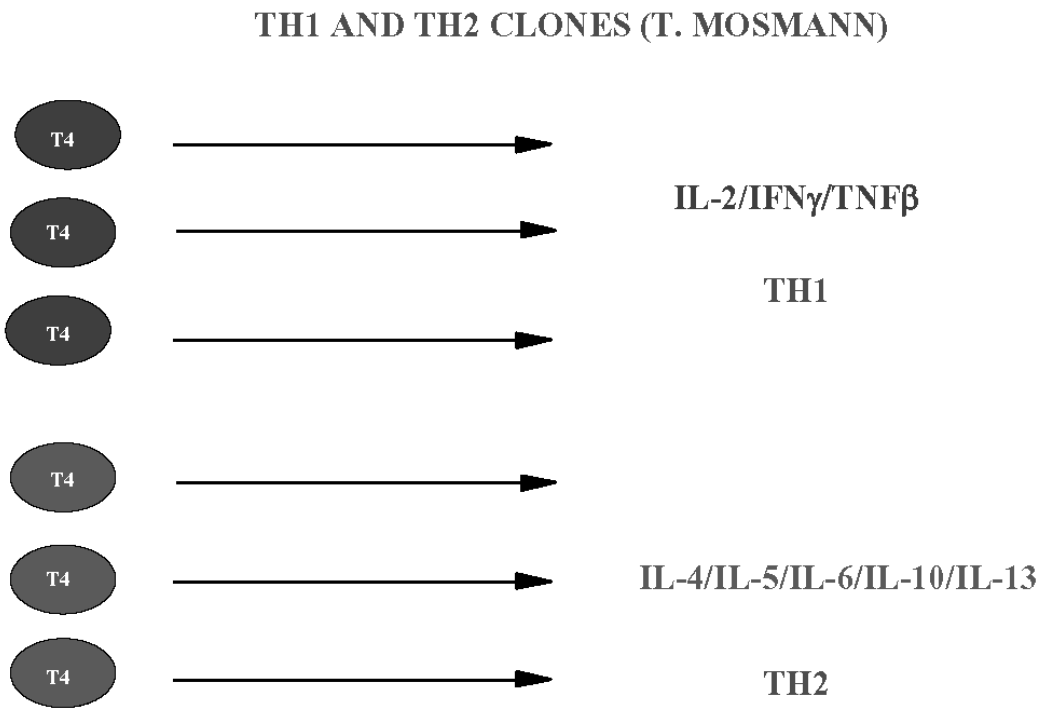
(Bell et al 2000)

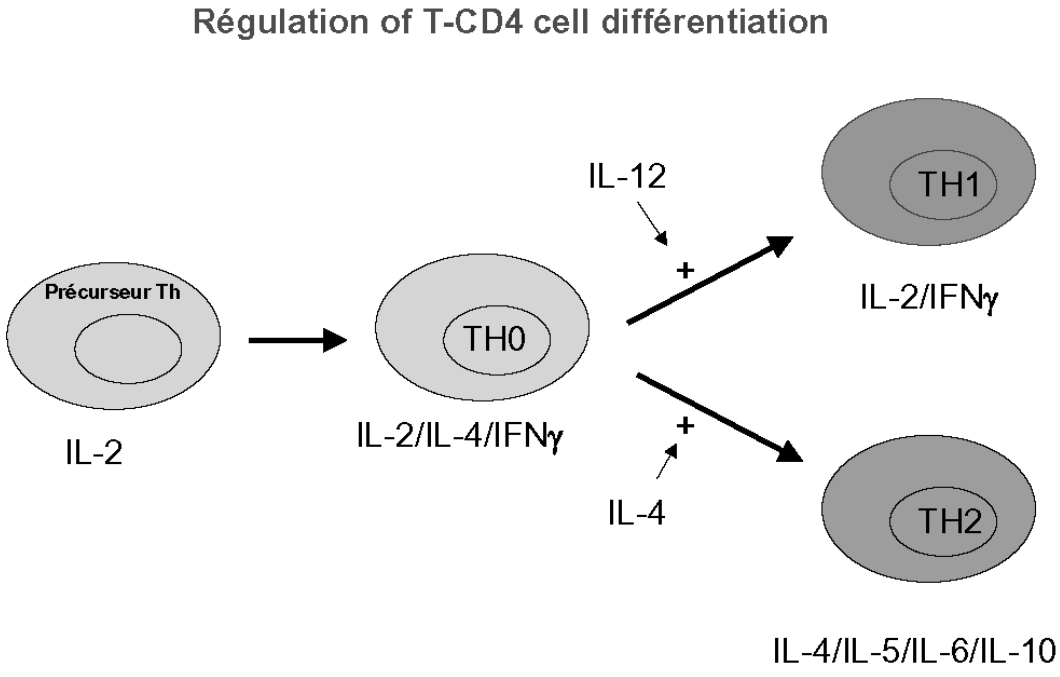
Mature DC were virtually absent in liver tissue from patients with Hepatocellular carcinoma (Chen S et al. 2000)

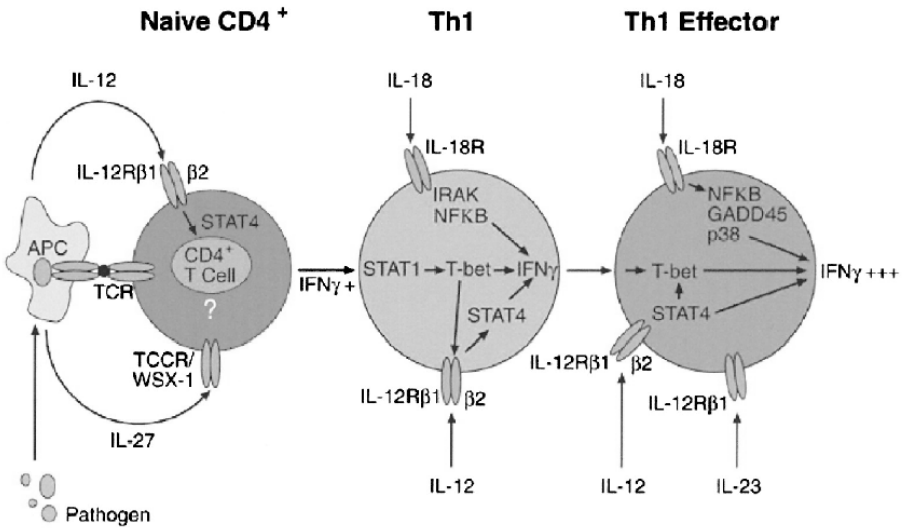
T CELL POLARISATION IN THE TUMOR MICROENVIRONMENT
ROLE OF CYTOKINES AND STATE OF MATURATION OF DENDRITIC CELLS



Anti-tumor activity of TH1 cytokines


TH1 AND TH2 CLONES (T. MOSMANN)
T4
T4
T4
IL-2/IFNγ/TNFβ
TH1
T4
T4
T4
IL-4/IL-5/IL-6/IL-10/IL-13
TH2


Régulation of T-CD4 cell différentiation
Précurseur Th
IL-2
TH0
IL-2/IL-4/IFNγ
IL-12
+
TH1
IL-2/IFNγ
IL-4
+
TH2
IL-4/IL-5/IL-6/IL-10



Checkpoint in TH1 development

- The receptor for IL-18 is induced by IL-12
- T bet induced IL-12 Rβ2 expression

ROLE of TH1 and TH2 cytokines

CD4-TH1
IL-2, IFN γ, TNFβ

→

- Stimulation of T Lymphocytes (Cell mediated immunity)
- Activation of macrophages cytotoxicity
- Favors IgG2a antibody switch.

CD4-TH2
IL-4, IL-5, IL-6, IL-10

→

- Stimulation of B Lymphocytes (humoral immunity)
Favor IgE and IgG1 antibody switch (IgG 4 in human)
- Activation of mastocytes and differentiation of eosinophils (Allergy).

Bias in TH1 polarization in cancer

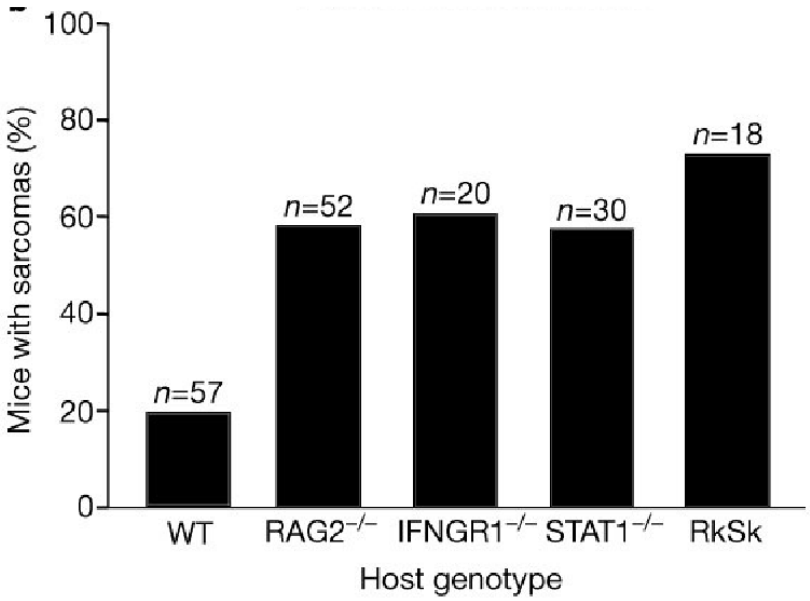
Low TH1 cytokine concentrations in cancer, ↗ RsIL-2

LYMPHOKINE PROFILE OF SPLENIC T CELLS FROM NORMAL AND RENAL CELL CARCINOMA BEARING MICE

Source of Splenic T cells	INTERFERON γ (U/ml)	INTERLEUKIN 2 (Pg/ml)	INTERLEUKINE 4 (Pg/ml)
Normal Mice	3.4	965	< 5
Tumor-Bearing mice	< 1	460	40

Ghosch J. Natl Cancer Inst. 1995

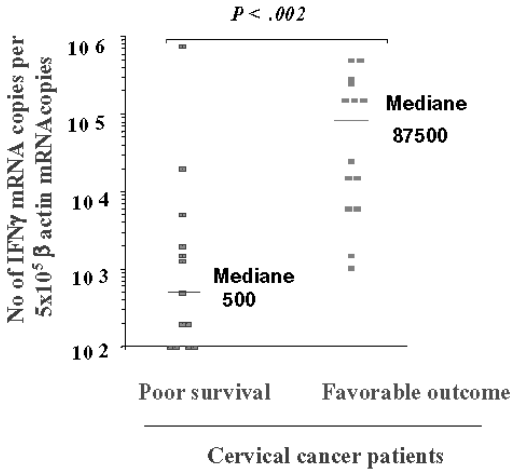
Lymphocyte-deficient mice are highly susceptible to MCA-induced tumour development



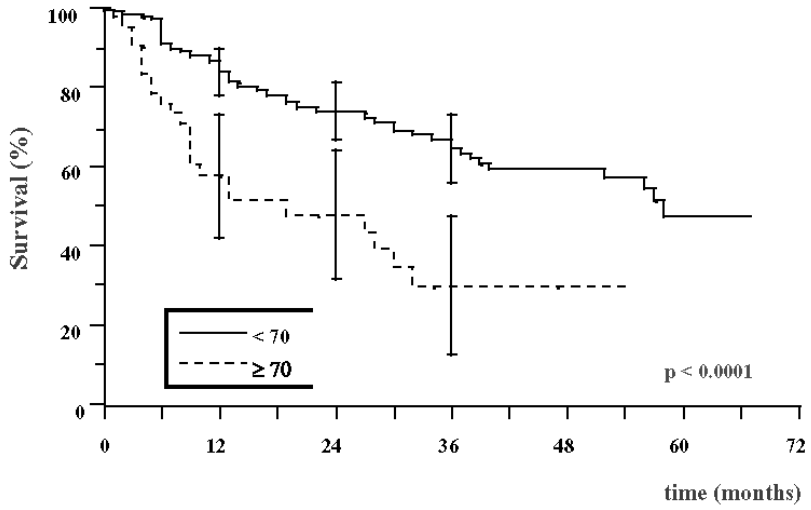
Shankaran et al. Nature 2001

Prognostic value of cytokines in cervical carcinomas

Low levels of IFNγ mRNA in poor prognosis cervical carcinoma patients



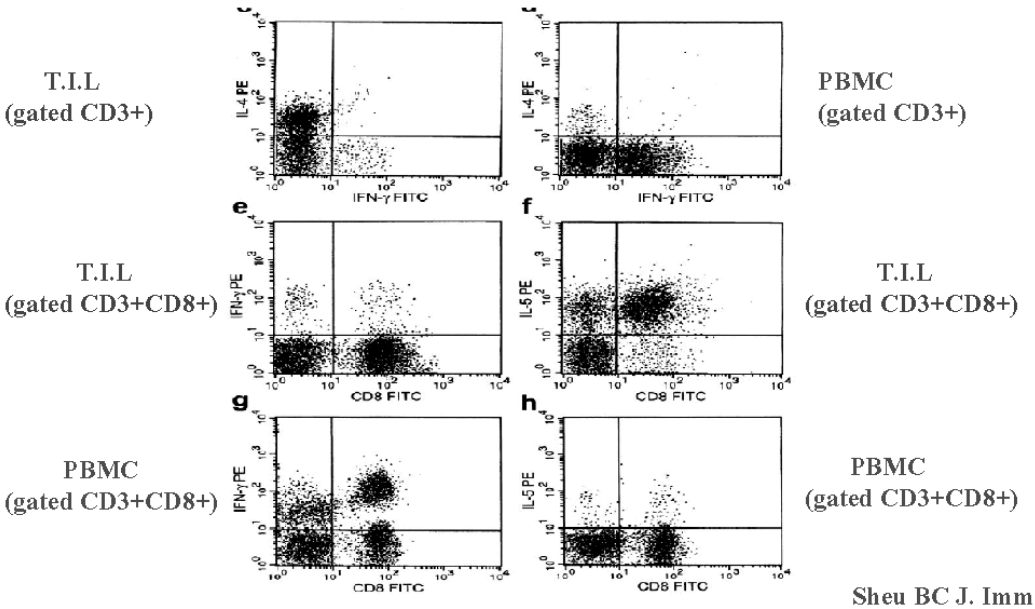
Survival for patients with head and neck squamous cell carcinoma in relation to serum soluble interleukin-2 receptor (sIL-2Rα) levels.



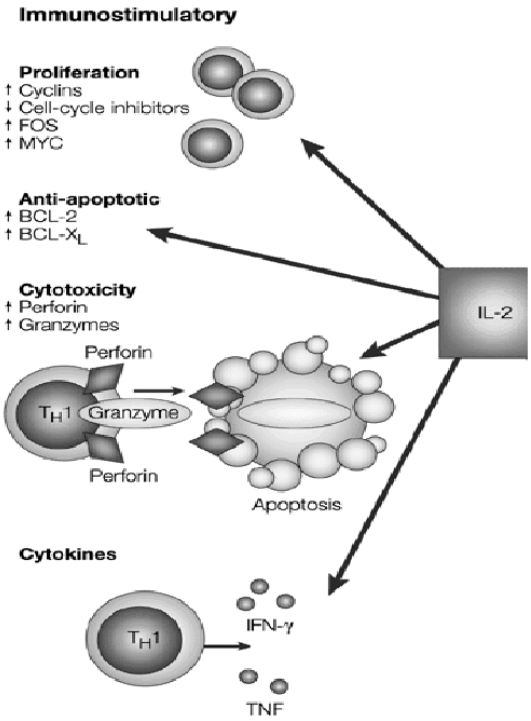
SWITCH IN THE PATTERN OF CYTOKINE FROM TH1 (IL2, IFNγ) to TH2 (IL-4, IL-6, IL-10) GROUPS IN CANCER PATIENTS

- ↘ IL-2 and IFNγ in TIL derived from breast and renal cell carcinomas
- ↗ IL-6 in invasive cervical carcinomas, bladder and renal cell carcinomas
- ↗ IL-10 in melanoma, glioma , bronchogenic and renal carcinomas

Predominant Th2/Tc2 Polarity of Tumor-Infiltrating Lymphocytes in Human Cervical Cancer



Sheu BC J. Immunol 2001



Therapeutic use of cytokines in oncology

IL-2 : Response rate of 10-20% with approximatively 5% complete response in renal cell carcinoma and melanomas.

Initially proposed as high-dose intravenous bolus or intermediate dose-continuous infusion but these schedules give rise to toxicity : capillary leak syndrome (Rosenberg SA N Eng J Med 1987; West WH. N Eng J Med 1987)

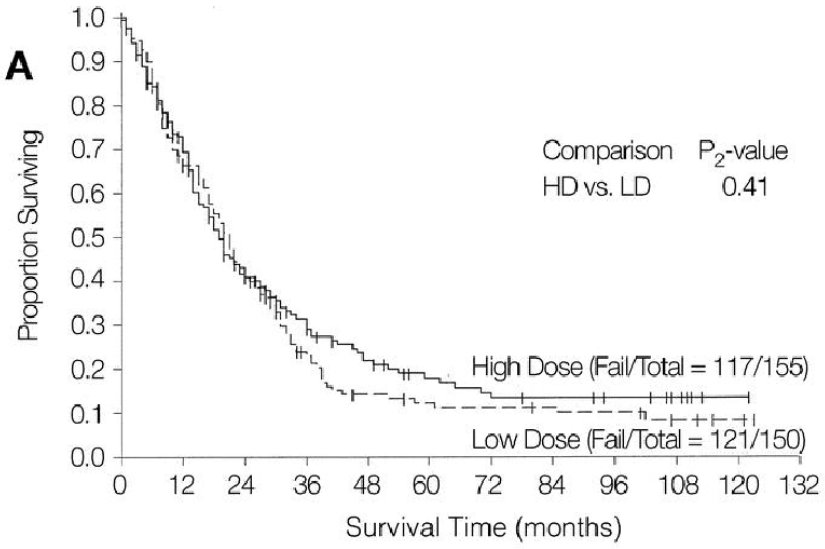
	High-Dose	Low-Dose	Subcutaneous
Total courses (100%)	285	272	181
Thrombocytopenia	9.2	1.5	0
Hyperbilirubinemia	3.2	0.7	0
ALT	3.2	0.7	0.6
Nausea/vomiting	13.4	8.5	3.3
Diarrhea	9.2	3.7	1.7
Peripheral edema	0.4	2.6	0
Creatinine (≥ 8.0)	1.1	2.6	0.6
Oliguria (≤ 80 mL/8 h)	12.0	7.7	1.1
Pulmonary	4.2	1.1	0
Malaise	20.5	9.9	9.4
Infection	2.8	2.6	1.1
Arrhythmia, atrial	4.2	1.5	0
Hypotension	36.4	2.9	0
CNS level of consciousness	2.5	2.6	0
CNS orientation	10.2	3.7	1.7
Death	0	0	0

Toxicities of all patients receiving interleukin-2 therapy for renal cancer
Percentage of courses with grade 3 or 4 toxicity

Yang JC. J Clin Oncol 2003

- To improve tolerance, several investigators proposed subcutaneous administration, which yielded efficacy comparable to that of intravenous administration with the added benefit of less toxicity and outpatient treatment (Buter J . Semin Oncol 1993; Lopez-Hanninen E. J Urol 1996)

- However it persists some controversies about the equivalence of high-dose intravenous IL-2 with low dose regimen



Overall survival of patients randomly assigned to either low-dose (LD) or high dose (HD) intravenous (IV) bolus interleukin-2

Yang JC. J Clin Oncol 2003

Duration (months)					
High-Dose IV IL-2		Low-Dose IV IL-2		Subcutaneous IL-2	
CR	PR	CR	PR	CR	PR
130+	**37**	128+	**24**	**78**+	**28**
121+	**28**	113+	23	**13**	**28**
115+	**24**	**40**+	22		**17**
114+	**23**	20	**21**+		**15**
103+	19	19	**15**		**9**
100+	**17**	3	**13**+		**8**
90+	**17**		11		**2**
52+	16		**11**		
45	15		**8**+		
23	14		7		
19	**14**		**7**		
	14+		**4**		
	13		**4**		
	10				
	9				
	8+				
	8				
	8				
	7				
	6				
	4				
	4				

NOTE. Bold values are for patients concurrently randomly assigned between three arms. + indicates response is ongoing.
Abbreviations: IV, intravenous; IL-2, interleukin-2; CR, complete response; PR, partial response.

Response durations

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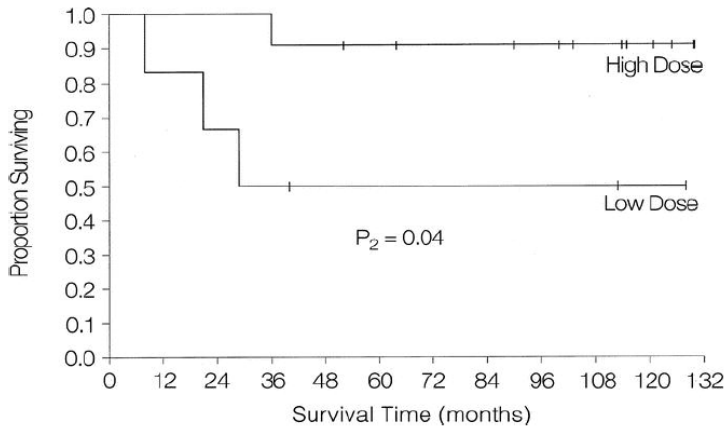
(Atkins J Clin Oncol 1999; McDermott D ProcASCO 2001)



Survival of patients completely responding to high-dose versus low-dose intravenous interleukin-2

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Combination of IL-2 with IFNα or chemotherapy

In a randomized study comparing continuous infusion IL-2 in monotherapy or subcutaneous IFNα monotherapy or a combination of intravenous IL-2/subcutaneous IFNα, the author reported a significant superiority in terms of response for the combination regimen, but this superiority was without survival benefit. (Negrier S. N Eng J Med 1998)

Addition of chemotherapy increased the response rate and toxicity without real impact on survival (Tourani JM J Clin Oncol 1998).

Since IL-2 benefits to only a subgroups of cancer patients many attempts have been pursued to identify predictive factors for response

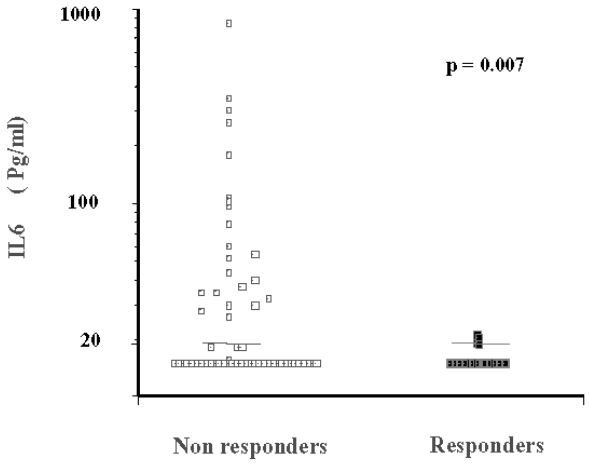
Sites of Metastases Versus Response

Site of Metastases	No. of Patients	Responders No.	Responders %	P_2
SQ and/or cutaneous alone				
Yes	28	15	53.6	.000001*
No	346	43	12.4	
SQ/cutaneous + LN alone				
Yes	23	5	27.7	.38
No	351	53	15.1	
LN alone				
Yes	29	6	20.7	.42
No	345	52	15.1	
Visceral alone				
Yes	69	8	11.6	.32
No	305	50	16.4	
Visceral + SQ/cutaneous alone				
Yes	61	9	14.8	.86
No	313	49	15.7	
Visceral + LN alone				
Yes	56	5	8.9	.14
No	318	53	16.7	
Bone + any other site(s)				
Yes	30	5	16.7	.80
No	344	53	15.4	
Brain + any other site(s)				
Yes	21	1	0.05	.056
No	353	57	16.1	

*Odds ratio, 8.13; 95% confidence interval, 3.33 to 19.81.
Abbreviations: SQ, subcutaneous; LN, lymph node(s).

Phan GQ J Clin Oncol 2001

Melanoma patients with high serum levels of IL-6 before therapy were unlikely to respond to IL-2 therapy.



Posttreatment Factors Versus Response and the Incidence of Long-Term Immunologic Side Effects of IL-2 in Responders

	No. of Patients	Responders		Odds Ratio	95% CI	P_2
		No.	%			
TSH						
Normal	153*	15	9.8	2.25	1.16-4.54	.01
Abnormal	219*	43	19.6			
FT4						
Normal	172*	17	9.9	2.35	1.24-4.60	.0049
Abnormal	200*	41	20.5			
Vitiligo						
Present	84	28	33.3	4.33	2.29-8.14	$< 10^{-6}$
Absent	290	30	10.3			

	Abnormal TSH		Abnormal FT4		Vitiligo	
	No.	%	No.	%	No.	%
Responders	43/58	74.1	41/58	70.7	28/58	48.3
Nonresponders	176/314	56.1	159/314	50.6	56/316	17.7

Abbreviation: CI, confidence interval.

*The total number of assessable patients was 372 because two nonresponders did not have TSH/FT4 levels during follow-up.

Phan GQ J Clin Oncol 2001

IFNα

-Upregulation of MHC class I and II expression

-Potentiation of effector T- and natural killer (NK cells)

-Maturation of dendritic cells

-Antiangiogenic effect

-Direct inhibition of tumor growth.

IFNα

Indications :

Hairy cell leukemia, chronic myelogenous leukemia, Kaposi sarcoma associated with VIH, Follicular lymphoma

- A randomized study showed an improvement in survival in patients with metatstatic renal cell carcinoma treated with IFNa (Lancet 1999)

- Low dose IFNα monotherapy has failed to provide significant clinical improvement in patients at high risk for melanoma (Cascinelli N Lancet 2001; Pehamberger H J Clin Oncol 1998)

Side effects

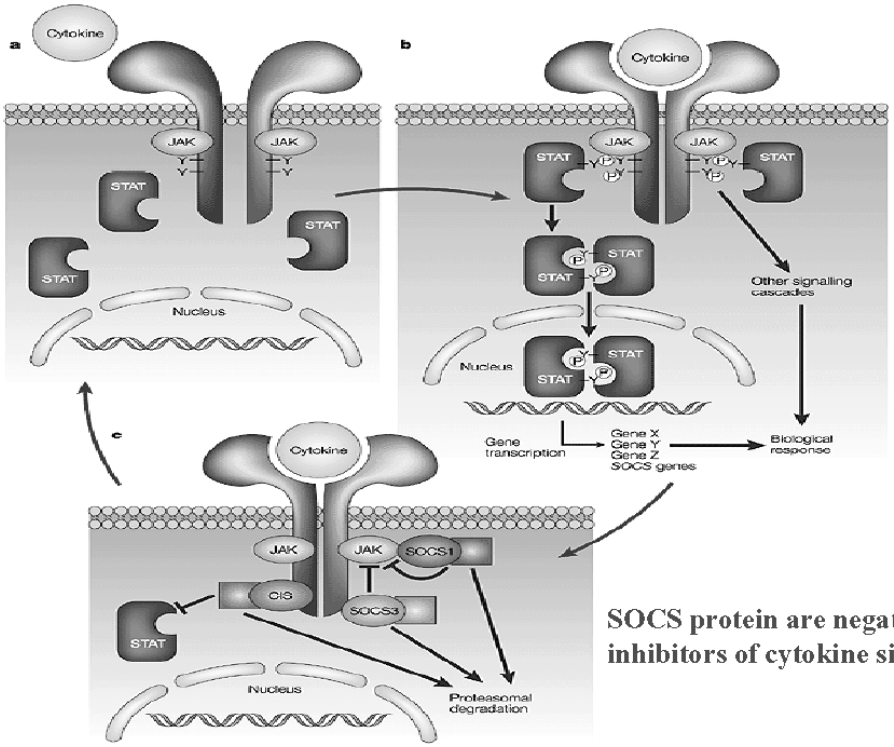
-Flu-like syndrom

-Depression

- Nausea vomiting diarrhea

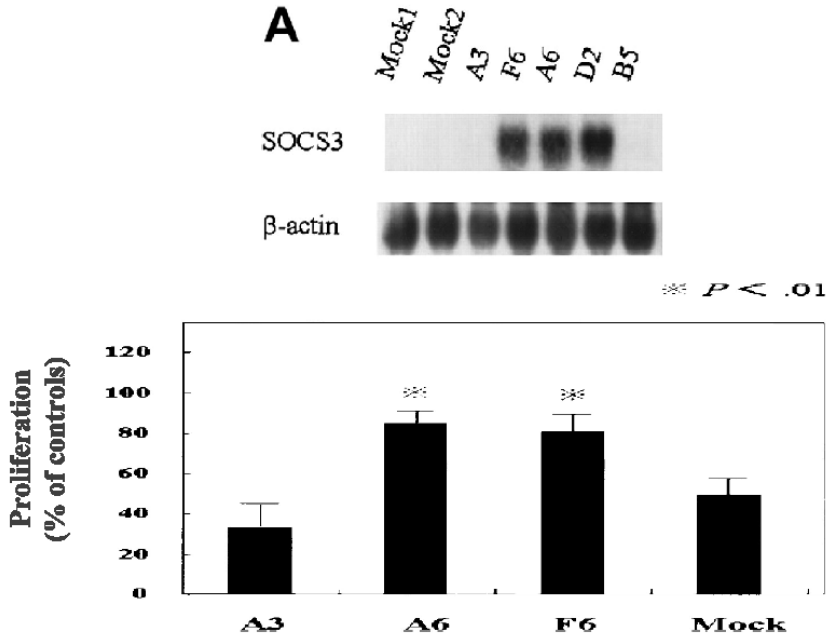
- Increase of ALT

- Retinopathy

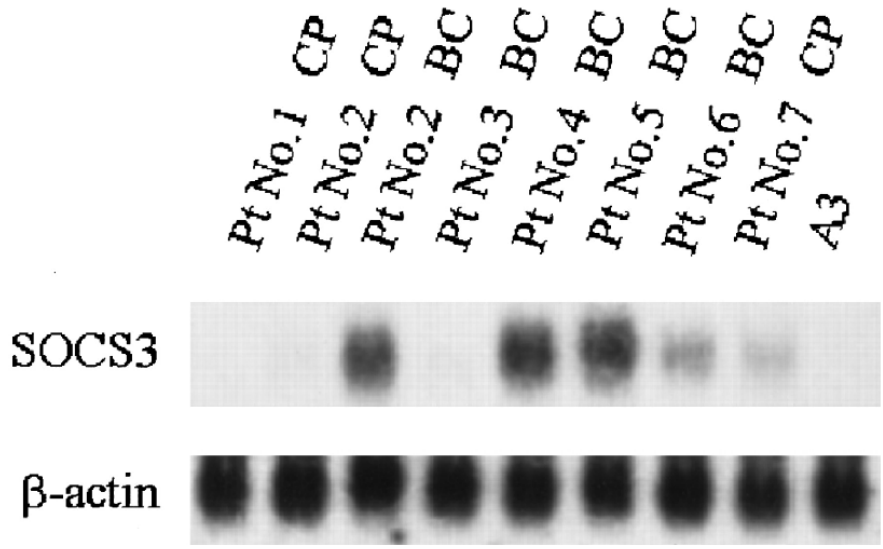


SOCS protein are negative feedback inhibitors of cytokine signal transduction

SOCS 3 confers resistance to IFNα in CML cells



Sakai Blood 2002



Expression of SOCS 3 mRNA in fresh CML cells from chronic phase (CP) or blastic crisis (BC) patients

IL-12

- **Development of Type 1 T-cell response**
- **Enhanced recruitment and activation of NK cells**
- **Antiangiogenic effect**

Toxicity

A phase II trial led to a fatal outcome in two renal carcinoma patients which has markedely delayed clinical testing

Modification of the administration schedule with a pre-dose has reduced the toxicity.

- Clinical results were rather disappointing in solid tumors (melanoma, renal cell carcinoma, ovarian cancer, colon cancer)

- Better results were reported in a small cohort of patients with cutaneous T- cell lymphoma and Sezary syndrome where 56% response rate was observed including tumor reduction (Rook AH 2001)
This was not entirely unexpected since these tumor cells are highly skewed to a TH2 phenotype.

Dual activity of cytokines in the control of tumor growth

INTERLEUKIN-17 (IL-17 A)

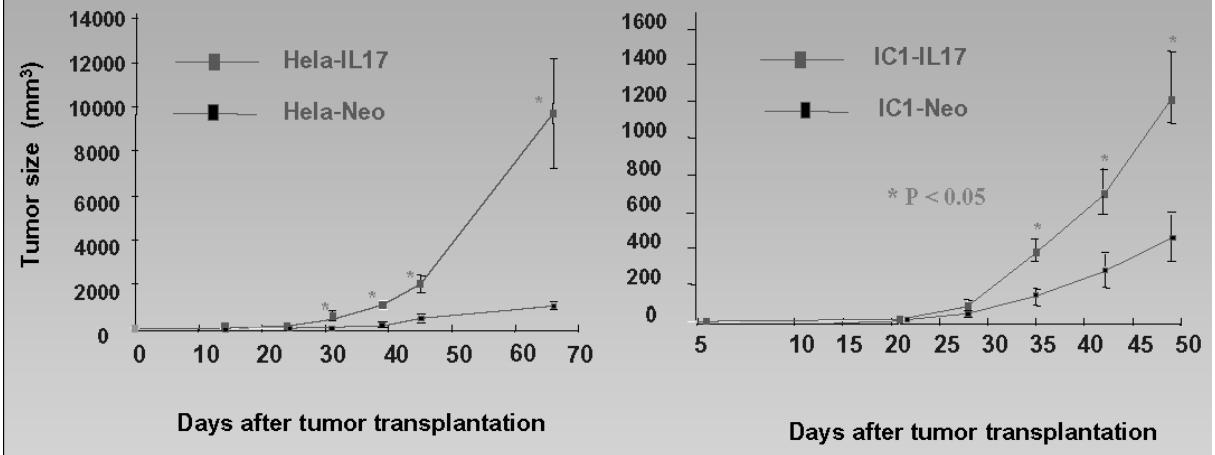
- First member of an emerging cytokine family (IL-17B, IL-17C IL-17 D, IL-17 E, IL-17F) expressed as dimers

- Produced by activated memory CD4-T cells

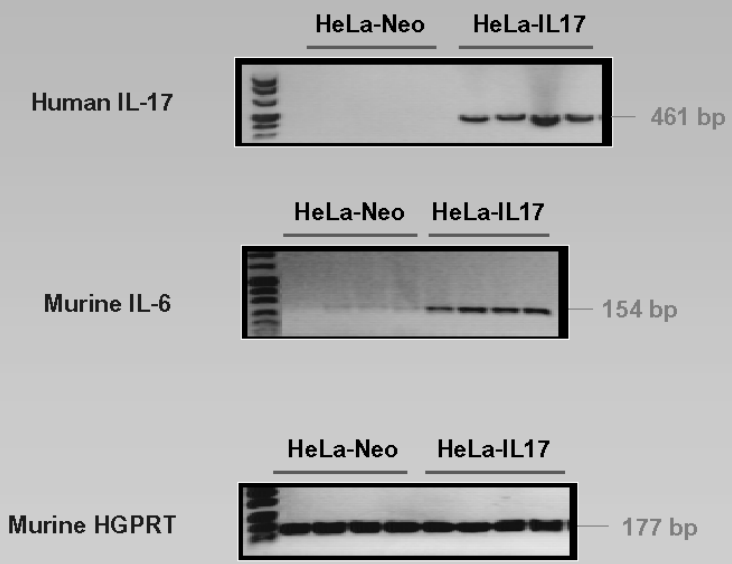
- Pro-inflammatory cytokine which increases the production of chemokines (IL-8, MCP-1, Groα) and hematopoietic growth factor (G-CSF, GM -CSF) thereby promoting the expansion and recruitment of monocytes and neutrophils.

- IL-17 stimulates the production of IL-6 by different epithelial cell lines.

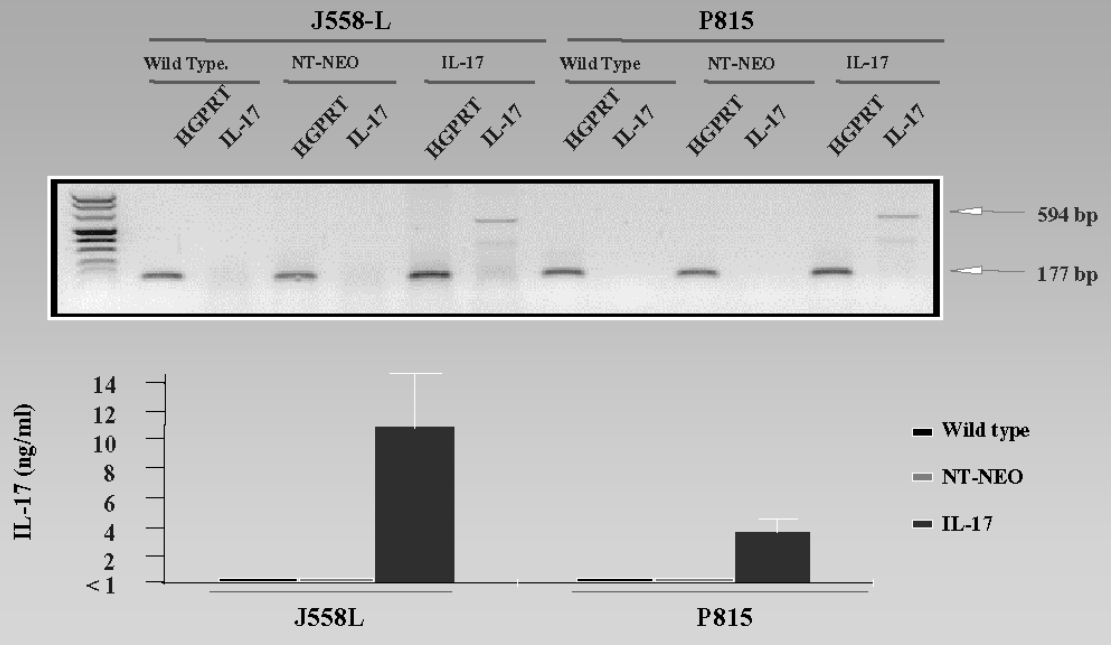
IL-17 increases the growth rate of human cervical tumors transplanted in nude mice



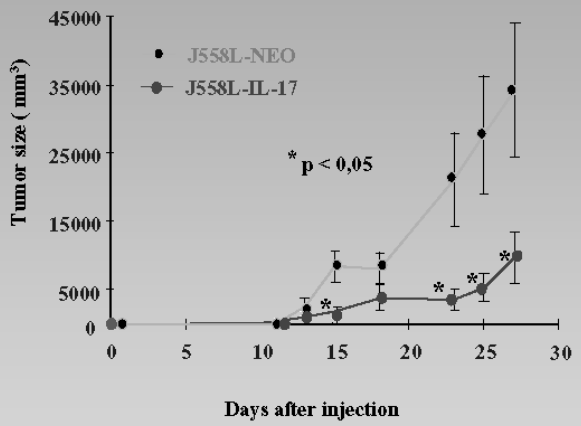
Increased murine IL-6 mRNA expression in biopsies derived from IL-17-transfected HeLa cells transplanted in nude mice.

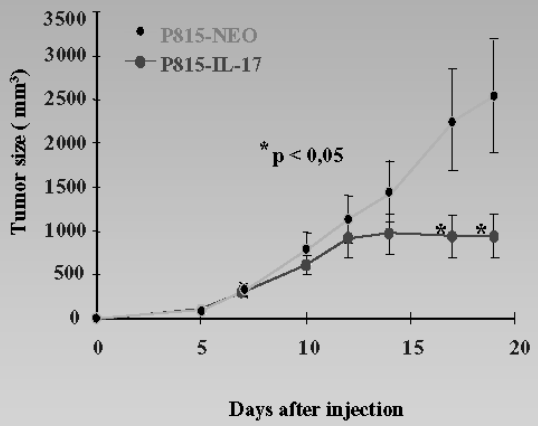


Characterization of mIL-17-transfected P815 and J558L cell lines

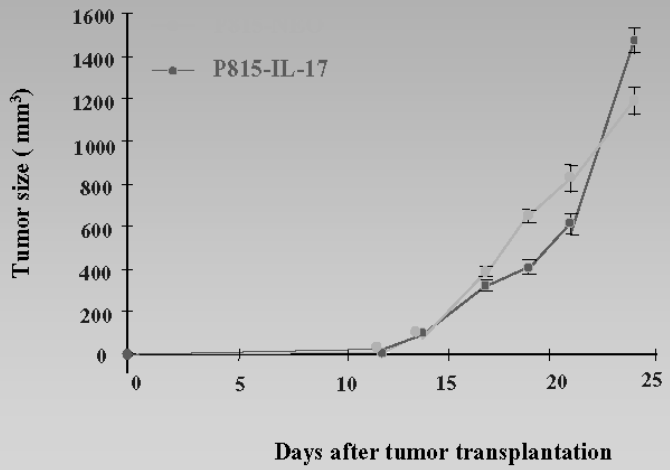


IL-17 inhibits tumor growth in immunocompetent mice

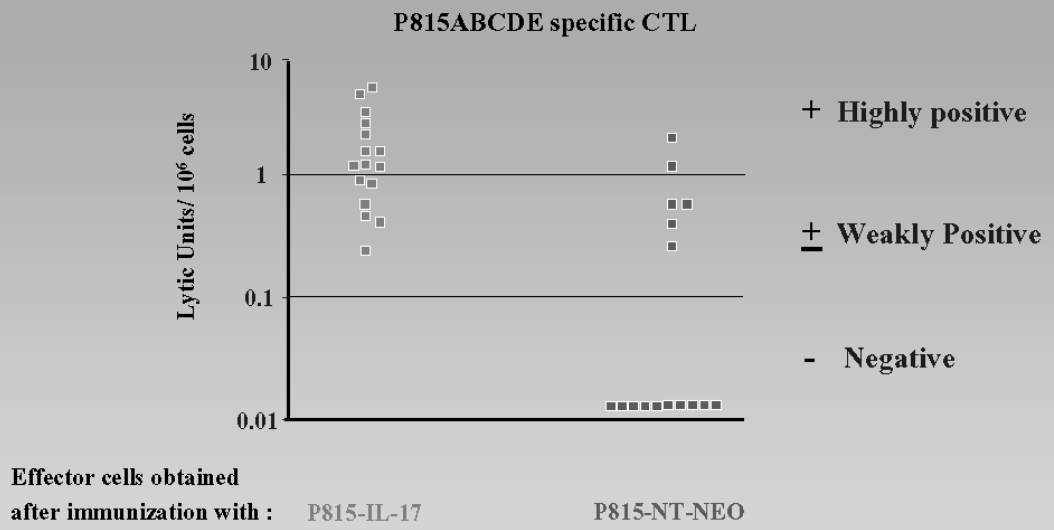




IL-17 does not inhibit the growth of the P815 mastocytoma in nude mice.



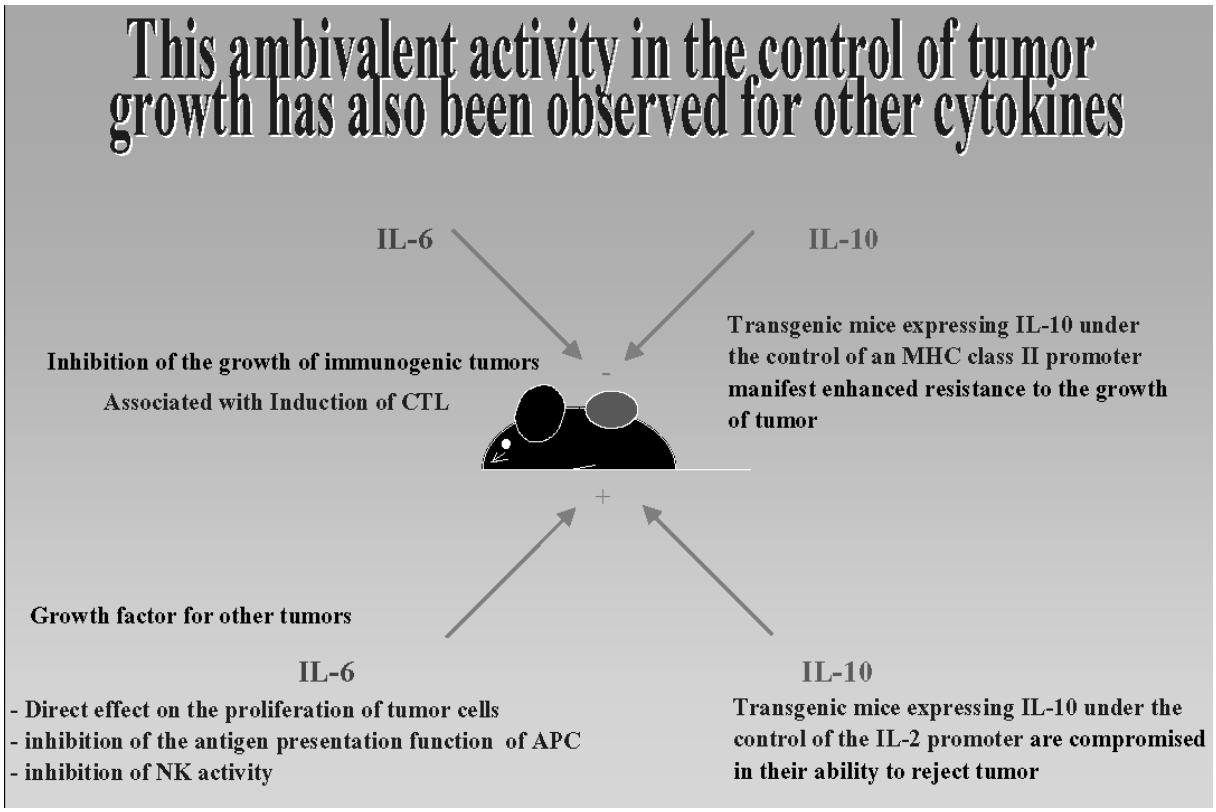
IL-17 increases the generation of P815-specific CTL



IL-17 a two faces cytokine

- In non immunogenic tumors or in the absence of T lymphocytes , IL-17 promotes tumor growth : this effect seems in part mediated by IL-6.

- In contrast, IL-17 inhibits the growth of immunogenic tumors by means of a T cell dependent mechanism.



Factors regulating cytokine activities in cancer patients

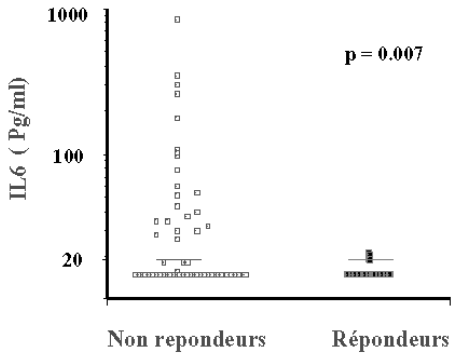
Stage and differentiation of the tumors

- l'IL-6 inhibits in vitro primary melanoma cell proliferation, whereas it rather acts as a growth factor for metastatic melanomas (Kerbel et al. Oncogene 1999)

- TGFβ switch from tumor suppressor to prometastatic factor during breast cancer progression (Tang B J Clin Invest 2003)

- Production of cytokines by tumor cells often reflects a progression of the disease and is associated with loss of sensitivity of tumor cells to the inhibitory activity of cytokines (IL-6, TNFα...).

- Melanoma patients with high serum levels of IL-6 before therapy were unlikely to respond to IL-2 therapy. (Blay et al, Tartour et al…)



Dose and schedule of administration.

IL-2 : well known as an anti-tumor cytokine. However …
At high doses or if it was administered too frequently, the anti-tumor activity of IL-2 is abolished. (Schmidt W PNAS 1995). This paradoxal effect may be mediated by the induction of CTL apoptosis. (Schrikant P J Immunol 2002)

IL-12 : inhibits tumor growth in a dose dependent manner but leads to the development of an antitumor immune response when IL-12 is expressed at the tumor site at the relatively small amount (Tahara H Cancer Res 1994)

How may we improve the clinical use of cytokines in cancer patients.

- Better selection of cancer patients to be included in therapy by cytokines. (stage of tumors, presence and site of metastases)

- Inflammatory syndrome (CRP, IL-6…) : high risk of resistance to immunotherapy.

- Development of pharmaco-immunological study to better design the dose and schedule of administration of cytokines.

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Lecture 4: Cytokines and cancer [ET]: Bibliography

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Cytokines and Cancer

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The relationships between cytokines and cancer are multiple and bidirectional. On the one hand, cytokines may directly influence carcinogenesis and metastasis by modifying the tumor phenotype. On the other hand, during tumor progression, modifications of the cytokine expression in the tumor environment may be induced by the tumor cells, leading to a state of immunosuppression reflected by low cytokine expression in tumor stroma. Cytokines also play a role by stimulating the host immune system to generate anti-tumor specific responses. Finally, the use of cytokines as anti-tumor agents has led to objective clinical responses in about 15–25% of patients with metastatic melanoma or renal cell carcinoma, which presents the basis for the development of promising immunotherapeutic approaches for cancer therapy.

Keywords: Cancer; Cytokine; Immunotherapy; Prognostic marker

A CYTOKINES AND PATHOGENESIS OF CANCER

1 Role of Cytokines in the Control of Tumor Cell Proliferation

(a) Cytokines as Tumor Growth Inhibitors

The ability of cytokines to directly inhibit tumor growth has generated many studies in clinical oncology aimed to use these molecules as anti-tumor agents. Among a vast literature, several examples emerged such as that of TNFα and Interferons which were the first cytokines which demonstrated cytotoxicity towards tumor cell lines [1,2]. IL4 was also shown to inhibit the growth of human colon, renal, lung and breast carcinoma cells in culture [3,4] and IL6 has an

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anti-proliferative effect on the growth of breast and human non small cell lung cancer cell lines [5,6].

The combination of several cytokines may increase their anti-tumor activity. For instance IL1 and IL6 act additively to inhibit the growth of breast cancer cell lines [7]; IL1 and IFNγ exert additive growth inhibitory effects on colon cancer cell lines [8]. When IL4 is combined with IFN or TNF, there is a significant increase of cell growth inhibition of renal cell carcinoma [9]. Finally, in some situation cytokines can counteract the action of other growth factors. For example, on breast cancer cell lines, IL1 and IL6 antagonize estradiol stimulated growth [8].

(b) Cytokines as Tumor Growth Factors

Cytokines may stimulate the growth of some tumor cell lines. IL6 is a growth factor for cervical carcinoma, AIDS Kaposi's sarcoma derived cells, myeloma, renal cell carcinoma, certain T and B cell lymphoma, and prostate carcinoma cell line [10]. GM-CSF is a growth factor for human ovarian cancer cells [11]. *In vitro*, IL1 can promote the growth of leukemia cells by inducing GM-CSF [12].

Since tumor cells release cytokines, these molecules may act as autocrine growth factors. For instance, IL6 functions as an *in vitro* autocrine growth factor of renal cell carcinoma [13] and glioblastoma [14]. On the other hand, IL6 increases the proliferation of ovarian carcinoma cells or plasmocytes via a paracrine mechanism [15,16]. Monocyte-derived cytokines including TNF, IL1 and IL6 can also stimulate the proliferation of ovarian cancer cells [17].

2 Cytokines Modulates the Tumor Phenotype

(a) Role of Cytokines in Tumor Immunogenicity

IL4, TNF or IFN modulate HLA class I and HLA class II expression and increase the surface expression of tumor antigens on different tumor cell lines [9], allowing a better recognition of tumor derived peptides by the immune system.

Conversely, many tumors release IL10, a cytokine which inhibits major histocompatiblity complex class II dependent antigen

presentation, and the activation of type I helper T cells [18]. In addition, incubation of melanoma cells with IL10 results in the inhibition of autologous T cell specific tumor lysis [19]. This could represent an escape mechanism of tumor cells from the immune system.

(b) Role of Cytokines in the Development of Metastasis

Different studies demonstrate that cytokines play a role in the promotion of tumor metastasis. Thus, IL12 inhibits cancer cell motility and invasion by upregulating E cadherin cell surface adhesion molecules [20]. In contrast IL6 promotes tumor metastasis and invasion by increasing the motility and decreasing the adherence of breast carcinoma cell lines [21]. TGFβ increases the metastatic potential of mammary tumor cells and it was shown that breast cancer cell lines become more tumorigenic after transfection with a TGFβ1 expression plasmid [22,23].

3 Cytokines and Tumor Progression

(a) Tumor Progression is Associated with Differences in the Sensitivity of Tumor Cell Lines to Cytokines

Several examples of differential sensitivity of cells of the same lineage to the effect a given cytokine, depending on their malignant or metastatic stage, have been reported. For instance, TNFα and TGFβ are potent inhibitors of the growth of melanocytes and are less effective or even stimulate the growth of melanoma cells. By contrast, IFNα and IFNγ inhibit the proliferation of melanoma cells but not that of melanocytes [24]. Moreover, the growth of melanoma cells obtained from early stage (metastatically incompetent) primary lesions is inhibited by IL6, whereas melanoma cells from more advanced stage (metastatically competent) appear to be resistant to IL6 as well as to other inhibitory factors such as IL1β, TNFα and TGFβ [25].

These findings suggest an alteration in the growth control mechanisms during melanocyte transformation.

A similar phenomenon occurs in other tumors. For example, IL1α and TNFα inhibit the proliferation of normal epithelial cells cultured from human cervix, whereas both cytokines significantly stimulate the proliferation of cervical cell lines immortalized by transfection

with HPV16 or HPV18 [26]. Finally in ovarian tumours, a malignant epithelial phenotype has been associated with acquired resistance to the antiproliferative effect of TGFβ [27].

(b) Switch of Cytokine Pattern During Tumor Progression

The analysis of the expression of mRNA or of the production of TH1 and TH2 cytokines in tumor samples suggest that during tumor progression a switch towards a TH2 phenotype takes place. Indeed IL4, IL5 and IL10 are the dominant cytokine mRNAs found in cutaneous basal cell carcinoma whereas IL2 and IFNγ are identified as the predominant cytokines in seborrheic keratosis, a benign tumor [28]. Conversely, IL10 mRNA expression is detected only in human renal cell carcinoma while non-tumorous kidney tissue are negative for this cytokine [29]. This is in accordance with the inability to detect mRNA for IL2 in freshly explanted renal tumors or in metastases of different tumors [29,30]. An increased expression of IL6 mRNA has also been demonstrated in invasive tumors, as compared to dysplasia or normal tissue [31,32]. Finally a recent report showed that the cytokine profile of peripheral blood lymphocytes from patients with Sezary syndrome (a T cell lymphoma) who achieved a complete remission after IFNα therapy switched from a TH2 towards a TH1 phenotype [33].

4 Tumor-Associated Immunosuppression

The ability of tumor cells to produce immunosuppressive factors including cytokines such as TGFβ or IL10 may explain the state of immune anergy found in most cancer patients. It is well known that TGFβ is the major factor involved in the inhibition of Lymphokine (IL2) activated killer cells [34–35] and that IL10 inhibits cytolysis of tumor cells by specific antitumor CTL [19].

In the vicinity of a tumor, T lymphocytes are often deficient in their response to mitogens or antigens [36,37] and the expression of IL2, IL4 and IFNγ mRNA in biopsies derived from breast cancer or melanoma is very low [38,39]. A specific defect in IL2 and IL4 secretion by TIL has also been reported [40,41]. Since all these defects could be overcome by *in vitro* stimulation with IL2 or mitogen, this

suggest that factors (cytokines?) present in the tumor environment could be responsible for this immunosuppression. Reduced expression of cytokines by tumor infiltrating monocytes has also been observed [42,43].

B CYTOKINES AS CLINICAL MARKERS IN ONCOLOGY

1 Cytokines as Clinical Prognostic Markers

High cytokine serum levels are often associated with poor outcome in patients with cancer. Indeed, the presence of high serum levels of IL6 is an adverse prognostic factor in multiple myeloma, renal cancer, melanoma, glioblastoma, ovarian cancer and lymphoma [44]. In patients with intermediate or high grade non-Hodgkin lymphoma, the presence of detectable serum IL10 at diagnosis correlates with a significantly shorter overall and progression-free survival [45]. High TNF serum levels in post surgery may represent an early marker of relapse [46]. Intense immunostaining for TGFβ1 in human breast cancer is positively associated with rate of disease progression in these patients [47].

2 Cytokine as Predictive Factors for Chemoimmunotherapy

The *in vitro* secretion of cytokines by tumor cell lines was associated with a resistance phenotype to cytokines and to other growth inhibitors. For example, the endogenous expression of TNFα has been correlated with a resistance to the cytotoxic effects of TNFα on some tumor cells [48,49]. In another model, the secretion of IL6 by advanced stage melanoma cell lines was associated with an increased resistance to inhibitory factors such as IL1β, TNF or TGF [50]. This may explain the correlation observed between high pretherapeutic serum IL6 levels and poor responses to IL2 therapy in patients with renal cell carcinoma [51] and melanoma [52] since when administered *in vivo* IL2 is responsible for the secondary release of cytokines which may mediate some of the therapeutic effects of IL2.

In patients with chronic myelogenous leukemia, the levels of circulating TNF were higher in the group of patients who did not respond to IFNα treatment than in the responders [53].

This role of cytokines to predict the response to immunotherapy may be extended to chemotherapy. Indeed, the treatment of IL6 secreting human renal carcinoma with Cisplatin (CDDP) in combination with anti-IL6 antibody or anti-IL6R antibody can overcome their CDDP resistance [54]. The secretion of IL6 protects prostate tumor cells against the cytotoxic effect of CDDP and Etoposide [VP16] and its neutralization sensitizes the tumor cells to drug toxicity [55]. This *in vitro* findings could explain clinical data which showed that higher level of IL6 were found in patients with gynecological tumors unresponsive to chemotherapy as compared to responders [56].

3 Role of Cytokines in the Paraneoplastic Syndrome

The paraneoplastic syndrome represents clinical manifestations such as hypercalcemia, thrombocytosis..., often associated with cancer and probably due to the release of soluble mediators by tumor cells.

An increased production of cytokines may contribute to the disturbances in calcium homeostasis in some malignancies. Indeed antibodies to human IL6 lower the blood calcium in nude mice, carrying human renal cell carcinoma, or other tumors, which produce IL6 and which are associated with hypercalcemia [57,58]. In other models neutralizing antibodies to TNF reduce the plasma calcium level of tumor bearing mice [59].

The administration of anti-IL6 antibodies decreases the thrombocytosis often observed in patients with renal cell carcinoma [60].

Elevated serum levels of IL1β, IL6, TNFα may contribute to the pathogenesis of POEMS syndrome a rare systemic disease characterised by a combination of polyneuropathy, organomegaly, endocrinopathies and skin changes which are considered as paraneoplastic manifestations often found in the course of myeloma and extramedullary plasmocytoma [61].

4 Role of Cytokines in Cachexia

The involvement of cytokines such as TNF, IL1, IL6 and IFNγ has been demonstrated in cachexia. Partial prevention of cachexia was achieved in cancer bearing experimental animals by using specific

antibodies to these different cytokines [62]. *In vitro* it was shown that IL6 reduces adipose lipoprotein lipase activity and this may contribute to the loss of body fat stores associated with some cases of cancer cachexia [63].

C CYTOKINES AS ANTITUMOR AGENTS

1 Interleukin 2

The ability of cytokines to directly inhibit tumor cell growth or to enhance the immune response against tumor cells led different groups to test these agents as anti-tumor molecules. IL2 was the first immunological agent which demonstrated an anti-tumor effect by activating immune effector cells.

(a) IL2 in Animal Tumor Models

IL2 alone, or associated with lymphocytes activated *in vitro* with IL2 (Lymphokine Activated Killer cells: LAK), and reinfused in mice induce the regression of pulmonary or liver micrometastases in a variety of murine tumors [64]. In mice high doses of IL2 alone also eradicated disseminated murine leukemia [65]. It was clearly demonstrated that the immune system is involved in the antitumor effect of IL2 since mice with an impaired immune system do not exhibit any clinical benefit in response to IL2.

(b) Clinical Trials with IL2

Rosenberg *et al.* were the first authors to report clinical responses to IL2 alone or LAK cells plus IL2 in cancer patients [66]. The most significant results were obtained both in melanoma and renal cell carcinoma and to a lesser degree in hematological malignancies. Table I illustrates the major clinical trials developed in melanoma. Fifteen to 25% of patients responded to this mode of treatment [70,75]. In most cases, clinical responses appeared by the end of the first cycle. In some cases, the duration of the response was significant: in the series published by Rosenberg, among 20 patients who achieved a complete response, nine remained free of disease after

TABLE I Response in melanoma patients treated with IL2 alone or TIL + IL2

Authors	*Tumor*	*Mode and schedule of administration*	*Number of patients*	*Response rate*
West [67]	Melanoma	alone (c.i.v)	10	60
Rosenberg [68]	Melanoma	alone (bolus)	16	31
Parkinson [69]	Melanoma	alone (c.i.v)	46	22
Rosenberg [70]	Melanoma	alone (bolus)	41	24
Dorval [71]	Melanoma	alone (c.i.v)	24	33
Legha [72]	Melanoma	alone (c.i.v)	33	22
Rosenberg [70]	Melanoma	+TIL	50	38
Goedegebuure [73]	Melanoma	+TIL	16	19
Rosenberg [74]	Melanoma	+TIL	86	34

13 to 75 months. Because of toxicity, including fever, neurotoxicity, cytopenia and a capillary leak syndrome observed with high dose IL2 i.v regimen, different schedules of administration have been proposed to reduce this toxicity. Indeed subcutaneous or intramuscular low dose administration decrease systemic toxicity but the clinical efficacy of these regimen, as compared to higher dose IL2 remains controversial [76,77].

The use of polyethylene glycol modified IL2 (PEG-IL2), that retains the *in vitro* and *in vivo* activity of IL2 but exhibits a markedly prolonged circulating half life, resulted in a significant decrease of IL2 toxicity in clinical trial [78]. Identification of response predictors to IL2 therapy may enable a better selection of patients who may benefit from IL2 and prevent unnecessary morbidity. The measure of IL6 and CRP levels before treatment has shown a high predictive value for response to immunotherapy in renal cell carcinoma, colorectal carcinoma and melanoma [51,79,80].

2 IFN Alone or Associated with IL2

Objective clinical response rates of 10–15% have been reported after administration of IFNα in renal cell carcinoma or melanoma [81]. The association of IFNα and IL2 improved the response rate as compared to each cytokine administered alone but its impact on the overall survival remains to be established (Table II) [82]. In the same way, in a multicentric study including 425 patients, the association IL2 and IFNα gave better results in terms of responses (18.6%)

TABLE II Clinical trial with IL2 alone or associated with IFN or chemotherapy in melanoma

	Response rate	*Number of patients*	*References*
IL2 + DTIC	22	18	Flaherty [95]
IL2 + DTIC	13	16	Fiedler [96]
IL2 + CDDP	37	27	Demchak [97]
IL2 + cyclophosphamide	20	27	Mitchell [98]
IL2 + CDDP + DTIC	43	18	Flaherty [99]
IL2 + IFN	8	22	Veelken [100]
IL2 + IFN	21	54	Kruit [101]
IL2 + IFN	29	17	Budd [102]
IL2 + IFN	33	39	Rosenberg [103]
IL2 + IFN + CDDP	54	39	Khayat [104]
IL2 + IFN + DTIC + CDDP + BCNU + TAM	57	42	Richards [105]
IL2 + IFN + DTIC + CDDP + VLB	56	30	Legha [106]
IL2 + DTIC + CDDP + TaM	42	38	Atkins [107]
IL2 + IFN + DTIC + CDDP + TAM	55.5	27	Atzpodien [108]
IL2 + IFN + DTIC + carboplatin	35	40	Atzpodien [108]

and of disease free interval than each cytokine alone in renal cell carcinoma [83].

Recently it was shown that intraperitoneal recombinant interferon γ in ovarian cancer with residual disease gave rise to objective clinical responses. Indeed among 98 patients, 23 achieved a complete response and eight additional patients achieved a partial response for a global response rate of 32% [84].

As regards the use of interferons as adjuvant postoperative biological therapy for melanoma, IFNγ failed to show adjuvant benefit in one large cooperative group trial [85], but interferon α2b administered at high dosage for one year improved the continuous relapse free and overall survival of high risk resected melanoma, resulting in the Food Drug Administration (FDA) approval of this agent for adjuvant therapy of patients after surgery for deep primary tumors or regional lymph node metastasis [86].

3 IL12 Alone or Associated with IL2

IL12 has exhibited antitumor activity in a variety of murine cancer models including renal cancer, B16 melanoma, reticulum cell sarcoma and C26 colon carcinoma [87,88]. *In vitro* data suggest that IL2 enhances the expression of IL12 receptors on T and NK cells [89] and IL2 and IL12 act additively in promoting the proliferation, the IFNγ production and the cytotoxic activity of T and/or NK cells [90]. *In vivo* it was shown that IL12, administered in combination with IL2, induced a rapid and complete regression of primary and metastatic renal tumors and displayed greater antitumor activity than that observed with either IL12 or IL2 alone [91]. Clinical trials, with focus on renal cell carcinoma, are presently conducted and their biological and clinical analysis are awaited.

4 Clinical Development of Other Cytokines: IL4, IL6, IL7, TNF

Cytokines such as TNF, IL4, IL6, IL7 have demonstrated antitumor efficacy in murine models.

The toxicity of TNFα in clinical trials led different groups to design TNF muteins which keep antitumor efficacy associated with decreased toxicity, due to their differential binding to the two TNF receptors [92].

As expected from *in vitro* data, the use of IL6 in clinical trials gave rise to objective clinical responses but enhancement of tumor growth was also observed [93,94].

5 Combination of Cytokines with Chemotherapy

In animal tumor models, preliminary results showed that chemotherapy did not impede IL2 immunostimulation [109]. As immunotherapy and chemotherapy may select different tumor escape mechanisms, their association is promising. In a small series of melanoma patients treated with Deticene-CDDP-IL2, 40% partial responses were observed but this was not confirmed by other studies [104,110]. Recently phase II clinical trial in melanoma showed that the association of high dose IL2 plus chemotherapy agents including dacarbazine, carmustine, CDDP and tamoxifen yielded 50% clinical response rate.

However, benefit in terms of survival was not assessed [105,107] (Table II).

Retinoic acid and IFNα act synergistically *in vitro*, to inhibit the growth of different tumor cells. Preliminary clinical results show that the combination of these agents induce objective clinical response in 30% of patients suffering from renal cell carcinoma or cervical carcinoma [111,112].

In a series of 215 renal cell carcinoma patients, Atzpodien *et al.* showed that the association IL2 with IFNα and 5 fluorouracil was superior in term of responses (39%) than each cytokine used alone or in combination [113].

D BLOCKADE OF CYTOKINE ACTIVITY

As previously described, cytokines such as IL6 may behave for some tumor as growth factors.

An example is provided by myeloma which is sensitive to IL6 for its proliferation *in vitro*. In SCID mice, anti-human IL-6R antibody as well anti-human IL-6 antibodies inhibit the growth of human myeloma [114]. In patients with advanced multiple myeloma an excess production of IL6 was demonstrated. A clinical trial with anti-IL-6 monoclonal antibody showed that it can suppress *in vivo* the proliferation of myeloma cells. In rare cases, however, a partial tumor regression of the tumor mass was reported [115].

In Castleman disease, anti-IL6 monoclonal antibodies decrease the paraneoplatic syndrome [116].

In mice, the use of antisense Insulin-growth factor I RNA decreased the tumorigenicity of tumor cells and caused the regression of established brain glioblastomas. It was speculated that blocking of IGF-I expression reversed a phenotype that allows glioma cells to evade the immune system [117].

The growth promoting activity of cytokines may support some immunotherapeutic strategies aiming at eliminating some immune cells to induce anti-tumor immunity. Indeed in mice, the treatment with a monoclonal anti-granulocyte antibody, that counteracts the infiltration of the tumor cell inoculum by non-T cell leukocytes resulted in tumor rejection [118].

The subtle balance between anti-tumor and promoting tumor growth of cytokines also underlines the importance of the dose of cytokine administered. For example the best immunization with tumor cell secreting IL2 was achieved with cell lines producing relatively low IL2 levels. When IL2 expression was raised to high levels, the protection was completely abolished because of an impaired generation of tumor specific cytotoxic T lymphocytes [119].

E FUTURE PROSPECTS: CYTOKINES AND CYTOKINE GENES AS TOOLS AND TARGETS FOR IMMUNOSTIMULATION

1 Development of Methods to Increase Local Cytokine Concentrations in the Tumor Environment

Fearon *et al.* firstly reported that the introduction of the IL2 gene in a murine colon cell line increased the recognition of the tumor by the host immune system and allowed the rejection of wild syngeneic tumor (not transfected with IL2) when hosts were preimmunized with IL2 producing tumors [120]. These observations were reproduced when tumors were transfected with other cytokines such as IL4, IL6, IL7, IL10, IL12, GM-CSF [121].

Other studies demonstrated that a high concentration of certain cytokines at the tumor site, even if they were not secreted by the tumor cells increased the immunogenicity of tumors and induced anti-tumor immunity [122]. Such observations led to clinical trials using autologous fibroblasts transfected with IL2 or IL4 cDNAs mixed with irradiated autologous tumor cells [123,124]. This circumvents the laborious step of *in vitro* culture of tumor cells. Furthermore fibroblast cells may be rapidly obtained and their transfection is easy.

Cytokine gene therapy is not the only option to increase tumor immunogenicity. Slow release delivery systems of cytokine proteins may also be effective and provoke useful biological responses. Indeed Golumbek *et al.* showed that biodegradable microspheres releasing cytokines such as GM-CSF when mixed with tumor cells elicit the same anti-tumor immune response than cytokine transfected tumor cells [125].

Another approach consists of using allogeneic or xenogeneic tumors transfected with cDNA encoding IL2, as vector cells, to produce transient high concentrations of IL2 in the tumor environment. This local IL2 production lasts until allogeneic or xenogeneic tumors are rejected. In a murine mastocytoma and lung carcinoma model it was shown that high dose IL2 produced by allogeneic cells can protect against tumor growth [126]. In contrast to individual lymphokine gene therapy described above, in this method the same cells could be used for almost any individual.

At last an antibody fusion protein which combines the targeting ability of antibody to tumor cells and the immunostimulating properties of a cytokine seems suitable to concentrate cytokine in tumor microenvironment. Indeed two tumor specific antibody-interleukin 2 fusion proteins were reported to inhibit the growth of hepatic and pulmonary metastases in SCID mice reconstituted with human myeloid cells whereas the corresponding antibodies plus recombinant interleukin 2 only reduced the tumor load [127].

2 Cytokines as Adjuvant in Anti-Tumor Vaccination

Molecular characterization of tumor antigens in melanoma provided the first support for the development of original clinical trial aimed to activate specific anti-tumor immune response [128]. Cytokines should enable optimization of strategies to immunize against these tumor antigens.

Mutated oncogenes, such as p53 represent an ideal tumor antigen since it is not present on normal cells. Successful immunization against this mutated protein was developed in a murine model only when IL12 was used as adjuvant [129].

Similarly, fusion proteins which associate a tumor antigen and a cytokine could enhance the immunogenicity of antigen. For example, idiotypes of immunoglobulins expressed in lymphoma when coupled with cytokines such as IL2, IL4, GM-CSF, and administered in mice were efficient to protect mice against the parental lymphoma [130].

Finally, therapeutic trials aimed to induce an immune response to the carcinoembryogenic antigen in colon cancer, the Muc1 antigen in breast cancer, and HPV encoded proteins in cervical cancer are

currently performed using vaccinia vector in which a cDNA encoding cytokine such as IL2 or IL12 were also introduced.

CONCLUSIONS

The interaction between tumor cells and the immune system are complex, and evolutive. Cytokines either produced by the tumor or its environment play a key role in these interactions. They are factors involved in cancer growth and invasion and represent predictive and therapeutic tools to control cancer development. The establishment of animal models and the development of well controlled clinical trials in the field of tumor vaccines and gene therapy with cytokines should allow to define the status of cytokines in the therapeutic arsenal against cancer.

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A Fluorospot assay to detect single T lymphocytes simultaneously producing multiple cytokines

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Abstract

Various subpopulations of T lymphocytes—i.e. Type 1, Type 2, Tr1 T cells—play a major role in the homeostasis of the immune system and in the pathogenesis of many inflammatory and auto-immune diseases. At present, in the absence of specific surface markers, these T cells can only be reliably distinguished on the basis of their cytokine production profile. The Elispot assay detects cytokine-producing cells, but in most cases can detect only one secreted cytokine, which represents a major limitation of this technique.

We have developed a Fluorospot assay to detect single cells that simultaneously produce multiple cytokines. The Fluorospot assay permits the detection of regulatory T cells with an immunosuppressive activity, identified by their coexpression of IL-10 and IFNγ. Polarized type 1 and type 2 specific tetanus toxoid T cells are also directly detected using a dual color Fluorospot. This technique will therefore be useful for detailed analysis of T lymphocytes in various disease states in which an imbalance of T cell subpopulations is suspected, but will also provide a better characterization of polarized specific immune responses.

Keywords: Elispot; Cytokine; T-lymphocytes

1. Introduction

Various subpopulations of T lymphocytes, i.e. Type 1, Type 2, TH3 and Tr1 T cells, which play a major role in homeostasis of the immune system, have been characterized. Although many attempts have been made over the past decade to identify specific cell surface markers, these various types of lymphocytes

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are mainly distinguished, at present, on the basis of their cytokine production profile.

Type 1 T cells are identified by their expression of IL-2, IFNγ and TNFβ, whereas type 2 T cells secrete IL-4, IL-5, IL-10 and IL-13. TH3 cells produce TGFβ and variable amounts of IL-10 and IL-4. Type 0 cells are considered to be precursor cells with a mixed phenotype (Fridman and Tartour, 1997). The hallmark of Tr1 cells is their production of high levels of IL-10 and TGFβ, moderate amounts of IFNγ and IL-5, but little or no IL-2 or IL-4 (Roncarolo et al., 2001). The detection of these T cell subpopulations is crucial to determine and analyze the type of immune response induced by antigen priming. For instance, type 1 T cells provide a helper activity for T cell-mediated immunity, whereas type 2 T cells are important for B cell development and antibody production (Mosmann and Sad, 1996). Tr1 cells are involved in down-regulation of immune responses in vitro and in vivo, partly via production of the immunosuppressive cytokines IL-10 and TGFβ (Roncarolo et al., 2001). Immunization of patients with antigen-pulsed immature dendritic cells has been reported to elicit Tr1-specific T cells with a suppressive activity on effector type 1 T cells (Dhodapkar and Steinman, 2002).

An imbalance in the relative numbers of these various T cell subpopulations may affect the state and progression of several diseases, including infectious, allergic and auto-immune disorders, but also cancers (Yssel and Groux, 2000; Clerici et al., 1998).

The enzyme-linked immunospot (Elispot) assay, based on solid phase immunoenzyme technology, is able to detect single cells producing various immunoreactive substances, such as cytokines. The end result of this test is a set of colored spots, each representing a "footprint" of the original cytokine-producing cells (Czerkinsky et al., 1988). However, in most cases, the Elispot procedure detects only one secreted cytokine, which constitutes a major drawback for the characterization of the various T cell subpopulations. For example, IFNγ can be secreted by type 0, type 1 and Tr1 T cells, and IL-10 is produced by both type 2 and Tr1 cells. To overcome this limitation of the Elispot, we have developed a Fluorospot assay, which is a modification of the Elispot based on the use of multiple fluorescent labeled anti-cytokines detection antibodies. This test permits the detection of single cells producing multiple cytokines.

2. Methods

2.1. Cells

The B-EBV cell line V.1 was a gift from Dr U. Blank (Institut Pasteur, Paris) and the Sezary cells, SeAx, were kindly provided by Dr Keld Kaltoft (University of Aarhus, Denmark).

Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine (Sigma, Saint Quentin Fallavier, France), 5 mM sodium pyruvate, and 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin.

2.2. Cell purification

Mononuclear cells from pleural effusion were enriched by a Ficoll-Hypaque gradient. CD3+CD4+ T cells were then purified by magnetic cell sorting using a MiniMACS device (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of recovered cells ranged between 97% and 99%.

2.3. Elispot

The Elispot assay for the detection of IFNγ was purchased from Diaclone (Besançon, France).

2.4. Fluorospot

Ninety-six-well polyvinylidene difluoride flat-bottom plates (Millipore, Molsheim, France) previously treated with ethanol were coated overnight at 4 °C with 100 μl of mouse monoclonal cytokine-specific antibodies (mAbs) at 10 μg/ml in PBS. All anti-human cytokine mAbs (Anti-IFNγ, Anti-IL-2, Anti-IL-5, Anti-IL-10) used were obtained from Diaclone.

The references for all antibodies and amplification reagents used for this assay have been summarized in Table 1.

The plates were then blocked with 2% milk in PBS for 2 h at room temperature and washed twice with PBS containing 0.05% Tween. Cells in a volume of 100 μl were then added in serial dilutions in duplicate and were incubated for various times ranging from 18 h to 48 h at 37 °C in a humidified atmosphere of 5% $C0_2$ in air. The plates were then

Table 1
List and references for all antibodies and amplification reagents used for the Fluorospot assay

	Fluorospot IFNγ	Fluorospot IL-2	Fluorospot IL-5	Fluorospot IL-10
Capture Ab	Mouse IgG1 anti-IFNγ Clone B-B1	Mouse IgG1 anti-IL-2 Clone B-G5	Rat IgG2a anti-IL-5 Clone BZ 25	Mouse IgG1 anti-IL-10 Clone $B.N_{10}$
Detection Ab	Biotinylated or fluorescein-labeled Mouse anti-IFNγ Clone B-G1	Biotinylated rabbit polyclonal anti-IL-2	Biotinylated goat polyclonal anti-IL-5	Biotinylated mouse IgG2b anti-IL-10 Clone $B.T_{10}$
Amplification step	Anti-fluorescein rabbit IgG labeled with Alexa Fluor 488 or Phycoerythrin-conjugated streptavidin (Dako)	Biotinylated goat anti-rabbit IgG (Southern Biotechnology) and Phycoerythrin-conjugated streptavidin (Dako)	Phycoerythrin-conjugated streptavidin (Dako)	Phycoerythrin-conjugated streptavidin (Dako)

washed and 100 μl of labeled anti-cytokine detection antibodies diluted in PBS containing 1% BSA was added.

For the detection of IFNγ, 100 μl of a fluorescein-labeled mouse monoclonal anti-IFNγ antibody (2 μg/ml) was added for 1.5 h at 37 °C and the signal was amplified with 15 μg/ml of an anti-fluorescein rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR) for 1 h at 37 °C in a volume of 100 μl. This amplification step was essential for detection of a fluorescent signal.

When the phycoerythrin fluorophore was selected to reveal the IFNγ Fluorospot, a biotinylated antibody directed against IFNγ was used as the detection antibody. Phycoerythrin-conjugated streptavidin was then added to the amplification step (Table 1).

For the detection of IL-2, IL-5 or IL-10, 100 μl of biotinylated rabbit polyclonal anti-IL-2 or biotinylated goat polyclonal anti-IL-5 or biotinylated monoclonal anti-IL-10 antibodies (Diaclone) were added for 1.5 h at 37 °C. All these antibodies were used at a concentration of 1.5 μg/ml. An amplification step with 100 μl of biotinylated goat anti-rabbit IgG (500 ng/ml) (Southern Biotechnology, Birmingham AL) for 45 min at 37 °C was added for the detection of IL-2-producing cells. This amplification step for IL-2 increased the signal but satisfactory results could still be obtained when it was omitted. After washing, the reaction was revealed with 15 μg/ml of phycoerythrin-conjugated streptavidin (Dako, Trappes, France) for 1 h at 37 °C.

Spots were read and counted using a stereomicroscope (Carl Zeiss, Le Pecq, France) equipped with a fluorescent reading system.

For dual color Fluorospot assays (IFNγ-IL-2, IFNγ-IL-5, IFNγ-IL-10), two pairs of anti-cytokine antibodies were simultaneously included without any change in the protocol.

To ensure that the reaction was specific and to eliminate cross-reactivity between the different anti-cytokine antibodies, pairs of anti-cytokine antibodies were split and one anti-cytokine antibody was replaced by an irrelevant isotype control antibody or a second anti-cytokine antibody not recognizing the same cytokine as the first reagent used. In all cases, no spot was observed.

During the double IFNγ/IL-2 Fluorospot assay, to avoid a possible cross-reactivity between the biotinylated goat anti-rabbit IgG used for the amplification step of IL-2 and the rabbit IgG anti-fluorescein used to reveal IFNγ, we did not add these reagents simultaneously. We first incubated the cells with the biotinylated goat anti-rabbit IgG for 1.5 h at 37 °C and then performed three washings with PBS containing 0.05% Tween. Finally, the anti-fluorescein rabbit IgG conjugated with Alexa Fluor 488 was added for 1 h at 37 °C.

2.5. Cell activation

Peripheral blood mononuclear cells (PBMC) were activated with either phorbol 12-myristate 13-acetate (PMA; 100 ng/ml) and ionomycin (10 μM) or a combination of 10 μg/ml of anti-CD3 mAb (Beckman Coulter, Villepinte, France) and 1 μg/ml of anti-CD28 mAb (Beckman Coulter) for the indicated incubation times.

For the detection of anti-tetanus toxoid specific T cells, PBMC (10^5 cells/well) were incubated with 20

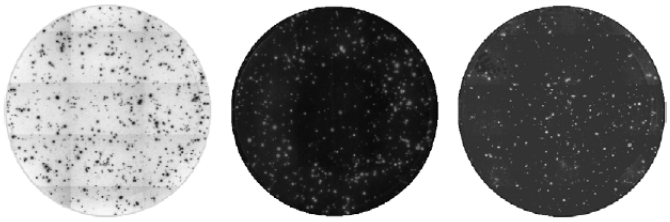

Fig. 1. Illustration of an Elispot and a Fluorospot assay. PBMC (3×10^3) were activated with PMA (100 ng/ml) and ionomycin (10 μM) for 18 h. IFNγ-producing cells were detected with either an alkaline phosphatase Elispot (left) or a Fluorospot assay using Phycoerythrin (middle) or Alexa Fluor 288 (right) conjugated antibodies.

μg/ml of tetanus toxoid (Statens Serum Institute, Copenhagen, Denmark) for 48 h directly in the Fluorospot assay plates.

3. Results

3.1. Development of a Fluorospot assay

During the development of a Fluorospot assay for the detection of cytokine-producing cells, different fluorophores including phycoerythrin (Fig. 1, middle) and Alexa Fluor 488 (Fig. 1, right) were successfully employed. A linear relationship was observed between the number of activated mononuclear cells added to the well and the number of fluorescent spots representing individual cells that secreted IFNγ (Fig. 2). Three different protocols were compared to detect IFNγ-producing cells. The sensitivity of the Fluorospot assay was similar for each fluorophore used (Phycoerythrin or Alexa fluor 488) and comparable to conventional Elispot procedures revealed by alkaline phosphatase (Figs. 1 and 2).

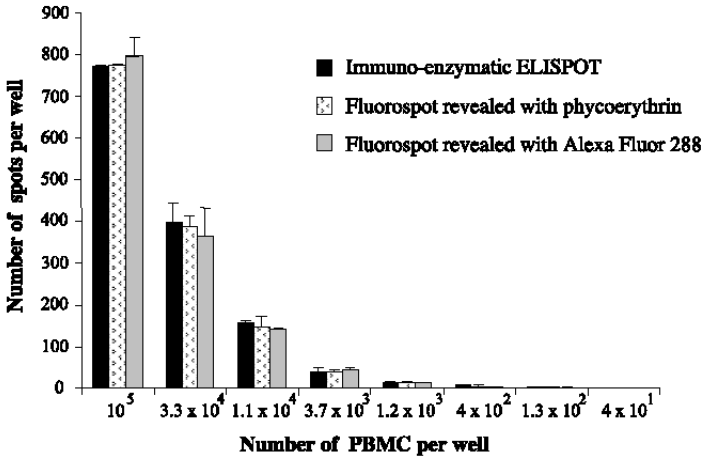

Fig. 2. Analysis of the sensitivity of the Fluorospot assay compared to Elispot. Different concentrations of PBMC were stimulated as in Fig. 1 with PMA and ionomycin. The number of IFNγ-producing cells was compared between Elispot and Fluorospot assays. All experiments were performed in triplicate and non-stimulated cells were introduced as negative controls.

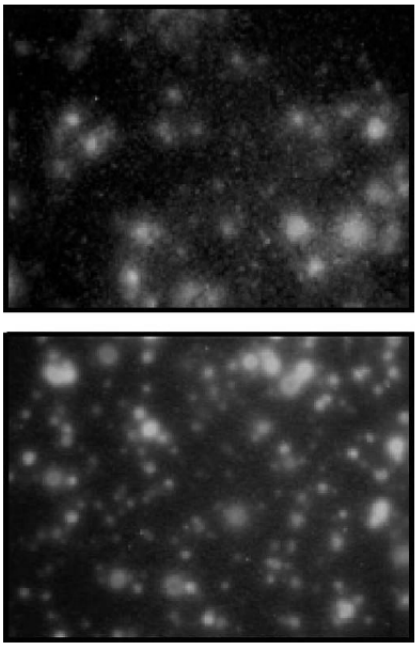

Fig. 3. Dual color Fluorospot for the detection of double cytokine-producing cells. PBMC (5×10^4) were stimulated with PMA and ionomycin on PVDF plates. Double IFNγ and IL-5 (top) or IFNγ and IL-2 (bottom)-producing cells were characterized by a dual color Fluorospot assay. Green spots corresponded to IFNγ secreting cells (top and bottom), whereas red spots were the hallmark of IL-5 (top) or IL-2 (bottom)-producing cells. Yellow spots corresponded to cells coexpressing IFNγ and IL-5 (top) or IFNγ and IL-2 (bottom). No spot was observed when resting cells were used for the dual color Fluorospot.

3.2. Dual color Fluorospot with single cell resolution

A dual color Fluorospot assay was then validated to measure cytokines coexpressed by individual cells. As expected, when cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, green spots corresponding to IFNγ-producing cells (Fig. 3) or red spots representing either IL-5 (Fig. 3, top) or IL-2 (Fig. 3, bottom) secreting cells, were observed. No spot was detected in the absence of cell activation (data not shown). In addition, mixed color (yellow) fluorescent spots representing IFNγ/IL-5 double positive cells (Fig. 3, top) and IFNγ/IL-2 (Fig. 3, bottom) were easily identified. In contrast, we and other groups have encountered difficulties interpreting these mixed colored spots when immunoenzymatic dual color Elispot assays were used (data not shown) (Okamoto et al., 1998; Karulin et al., 2000). Fluorescence, therefore, clearly provides better discrimination and characterization of double cytokine-producing cells than does an enzymatic reaction.

3.3. Accuracy and specificity of the Fluorospot assay

To validate the sensitivity of this dual color Fluorospot, peripheral blood mononuclear cells (PBMC) were stimulated with PMA and ionomycin, and the frequency of IL-2- and IFNγ-producing cells was tested with the single color or double color Fluorospot procedure. The number of cells producing IFNγ and IL-2 in the single color Fluorospot matched the sum of the frequencies of single and double IFNγ/IL-2 secreting cells detected on the dual color assay (with a 5% error) (Table 2). These results indicate that the dual color Fluorospot does not miss single- and double-expressing cells.

Using intracytoplasmic cytokine analysis performed by cytometry in permeabilized cells, the same frequency of double IFNγ/IL-2-producing cells was observed, which reinforces the accuracy of the test (data not shown).

In addition, to confirm that mixed color spots truly correspond to double-expressing cells and not random superimposition of spots derived from different cells, we mixed IFNγ-producing cells that did not secrete IL-2 with IL-2-secreting cells that did not produce IFNγ. No double color Fluorospot was recorded, which supports the correlation between the presence

Table 2
Accuracy of the dual color IL-2 and IFNγ Fluorospot assay

	Number of spots per well
Fluorospot IL-2	48 (47 50)
Fluorospot IFNγ	59 (56 64)
Dual IL-2/IFNγ Fluorospot	IL-2 spots: 46 (44 48)
	IFNγ spots: 58 (56 60)
	Mixed spots: 4 (3 6)

PBMC (10^4) were activated with PMA and ionomycin for 18 h. IFNγ- and/or IL-2-producing cells were detected with single color IL-2 or IFNγ Fluorospot or dual color IL-2 and IFNγ Fluorospot. The results represent the mean (± S.D.) of triplicate wells and are representative of three experiments.

of mixed spots and double cytokine-producing cells (Fig. 4).

3.4. The Fluorospot assay permits the detection of Tr1-like cells and polarized specific T cells

As a potential application of this Fluorospot assay is to detect T cell subpopulations based on their cytokine secretion profile, we purified CD4+CD3+ T cells from pleural effusions derived from patients with breast cancer. When these cells were stimulated with anti-CD3 and anti-CD28 mAbs, single IFNγ- and IL-10-producing cells were clearly identified (Fig. 5, top). We also observed some cells that simultaneously produced IFNγ and IL-10 and which may have been related to Tr1-like cells (Fig. 5, top). No double color spot was detected in the absence of stimulation (data not shown).

When cells were activated with PMA ionomycin, a known inhibitor of IL-10 induction (Schwarz et al., 1995), red spots corresponding to IL-10-secreting cells were no longer observed, which reinforces the specificity of the results (Fig. 5, bottom). Resting cells did not produce IFNγ (data not shown).

Tumor infiltrating T cells producing IL-10 and IFNγ have been previously detected in cancer patients by PCR (Wang et al., 1995; Rabinowich et al., 1996).

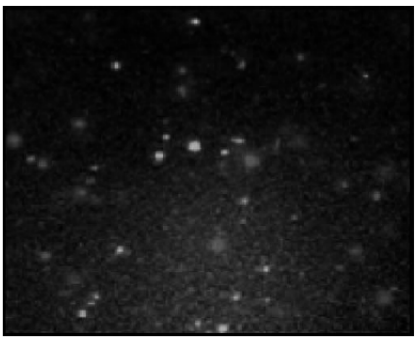

Fig. 4. Specificity of the mixed color spots in the dual color Fluorospot assay. B-EBV.V.1 (5×10^4) and SeAx (10^4) cells were mixed and activated with PMA ionomycin, an inducer of IFNγ without IL-2 in BEBV.V.1 and IL-2 without IFNγ in SeAx cells. IFNγ and IL-2 production was assessed with a dual color Fluorospot. Green and red spots corresponded to IFNγ and IL-2 secreting cells, respectively.

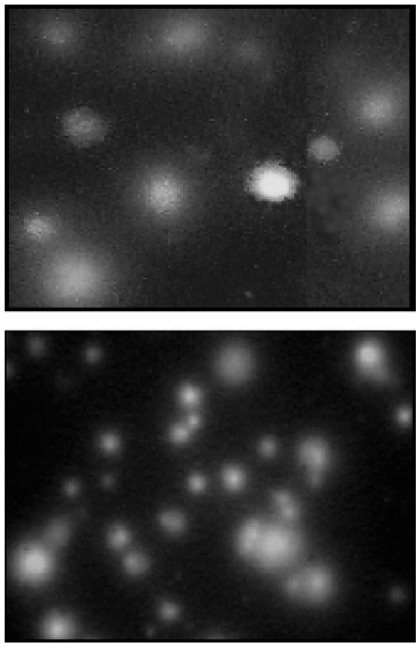

Fig. 5. Detection of Tr1-like cells in pleural effusions. Highly purified CD3+CD4+ T cells from a pleural effusion derived from a breast cancer patient were directly activated in the PVDF plates with anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) (top) or PMA ionomycin (bottom) for 48 h. An IFNγ and IL-10 dual color Fluorospot was used to detect IFNγ- and/or IL-10-producing cells. Green and red spots corresponded to IFNγ and IL-10 secreting cells, respectively. Yellow spots corresponded to cells coexpressing IFNγ and IL-10.

However, in contrast to the Fluorospot assay, coexpression of these cytokines, which is essential for characterization of regulatory T cells with an immunosuppressive activity, could not be analyzed by the previous techniques.

Another advantage of this Fluorospot assay is that it can be used to assess directly polarization of specific immune responses in the same well. Using tetanus toxoid as a model antigen, we directly demonstrated the presence of IFNγ- and IL-5-secreting tetanus toxoid-specific T cells in the blood of healthy patients (Fig. 6). No double IFNγ/IL-5-producing cells were observed in these experiments, which could indicate that these specific T cells were already engaged in a Type 1 or Type 2 differentiation pathway

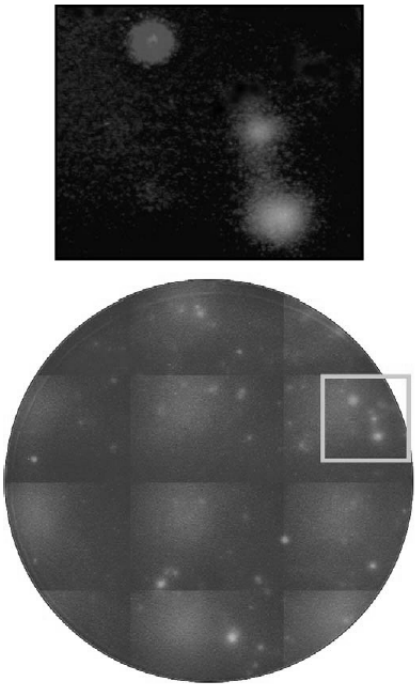

Fig. 6. Detection of polarized specific tetanus toxoid T cells. Bottom: PBMC (10^5 cells/well) were sensitized with tetanus toxoid (20 μg/ml) for 48 h. IFNγ and IL-5 production was assessed with a dual color Fluorospot. Green and red spots corresponded to IFNγ and IL-5 secreting cells, respectively. Top: Greater enlargement of a top right hand quadrant.

(Fig. 6). As a control, no spot was observed when PBMC were not pulsed with antigen (data not shown).

4. Discussion

We have developed a Fluorospot assay for the characterization of single cells secreting multiple cytokines. The sensitivity of the Fluorospot assay is the same as that of the conventional Elispot assay and these two techniques are approximately twice as sensitive as intracytoplasmic staining with FACS analysis for cytokine detection (Helms et al., 2000; Whiteside, 2000).

Unlike intracytoplasmic staining, in which cytokine secretion must be inhibited to increase the signal, the Fluorospot assay directly visualizes the cytokine actually secreted by pharmacologically untreated cells. In addition, detection of a cytokine by cytometry cannot always be equated with secretion of that cytokine, and the assay does not, therefore, measure a cellular function (Whiteside, 2000).

This Fluorospot assay can directly visualize, in the same reaction, the balance between specific type 1 and type 2 T cells. This test will therefore be useful to monitor patients with chronic hepatitis C treated with IFNα-based regimens, in which the initial change in the ratio of type 1 and type 2 HCV-specific CD4 T cells is indicative of treatment outcome (Cramp et al., 2000). A predominant tumor antigen-specific type 2 CD4 T cell response and a frequent decrease of a type 1 specific response has also been reported in cancer patients (Tatsumi et al., 2002).

The use of an immunoenzymatic dual color Elispot assay failed to easily discriminate between double cytokine secreting cells because of difficulties in the interpretation of mixed color spots (Okamoto et al., 1998; Karulin et al., 2000). In this study, we have demonstrated that the Fluorospot assay is better adapted than the Elispot to distinguishing single from double cytokine-producing cells. This property is directly relevant for the analysis of an immune response. For example, during a primary immune response, 80 90% of the population of T cells that express IFNγ fail to coexpress IL-2. Similarly, coexpression of the Type 2 cytokine is rare. However, following the second stimulation, the coexpression pattern more closely resembles a Type 1- and Type 2-like pattern and the incidence of Type 0-like cells is decreased (Bucy et al., 1995).

The ability to detect cells that coexpress cytokines may also have direct applications in the follow-up of HIV and cancer patients.

Various recent studies have shown a correlation between the number of HIV-specific CD4 T cells that secrete both IFNγ and IL-2 and a good clinical outcome in HIV infection, while no difference in the number of specific CD4 T cells secreting only IL-2 or IFNγ was observed between patient groups with different clinical prognosis (Boaz et al., 2002; Sieg et al., 2001).

In melanoma patients immunized with a preparation of heat-killed *Mycobacterium vaccae*, the induction of

IL-2 producing T cells was associated with improved survival (Maraveyas et al., 1999). Surprisingly, the number of T cells secreting IFNγ or coexpressing IL-2 and IFNγ was not associated with a better outcome.

This study has focused on validation of the dual color Fluorospot assay mainly applied to cytokine production by T cells, but the availability of a large range of fluorophores should permit the extension of this technique to multiparameter analysis of molecules secreted by various cell types.

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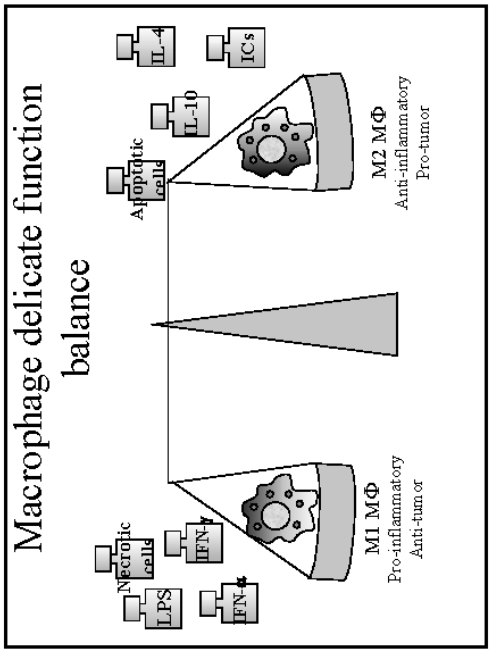
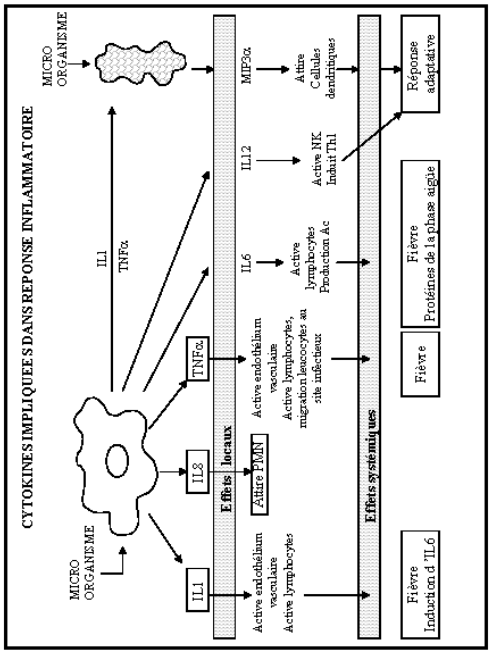
Lecture 5: Non-specific cellular therapy [HF]



Macrophage pro-tumor effector functions

- Phagocytosis: apoptotic cells or via IC
- Soluble secreted factors: cytokines: TGFβ, □EG□, PDGF
- Soluble secreted factors: prostaglandins, Metalloproteases, RONs
- Receptor mediated: Inhibitory Fc receptor cross linkage, scavenger receptors



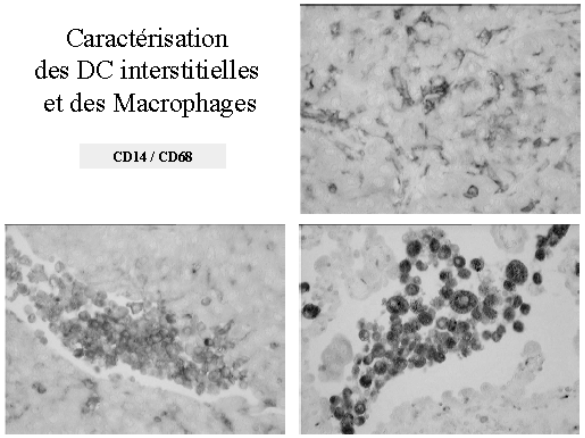
Macrophage anti-tumor effector functions

Phagocytosis: CD3, FcR

- ADCC: FcR (CD16, CD64)
- Soluble secreted factors: cytokines: TNFα, IL1β
- Soluble secreted factors: metabolites and other molecules: RONs, RNIs, prostaglandins, proteases
- Receptor mediated: Fas-FasL apoptosis induction

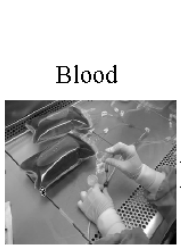
Caractérisation
des DC interstitielles
et des Macrophages

CD14 / CD68

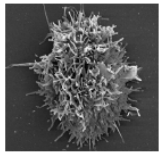


Cell Drugs against cancer

Blood

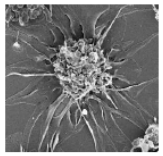


GM-CSF + IFN-γ



Macrophages

GM-CSF + IL-13



Dendritophage™

IDM © 2003

Design of the clinical trial

IDM

• Study design :

17 patients with TaGIII or rec.TaGII superficial bladder cancer

TUR → TaGII/III → 1 month

apheresis (10.10⁹ mononuclear cell-enriched leukocytes)

MAK ™ preparation :
GM-CSF : 500 UI/ml J1-J6
IFN-γ : 250 U/ML J7
2.10⁸ MAK ™ / bag

• Therapeutic schedule :

Aph. D7 D14 D21 D28 D35 D42 M3 M6 M9

MAK n°1 MAK n°2 MAK n°3 MAK n°4 MAK n°5 MAK n°6 MAK n°7 MAK n°8 MAK n°9

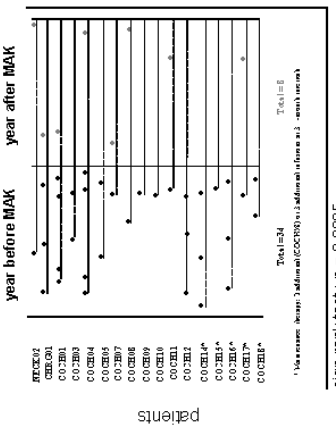
Phase I/II clinical trial of intravesical administration of MAK ™ in patients with superficial bladder cancer

• Rational of this immunotherapeutic approach :

- widespread use of adjuvant BCG-therapy.
- local immunostimulation with an increasing number of macrophages and CD4 T cells.
- Th1 associated cytokines (IFN-γ, IL-12, IL-12, IL-18) detected in urine of BCG-responders.

- *in vitro* and murine models :
IFN-γ activated macrophages are able to kill tumor cells.

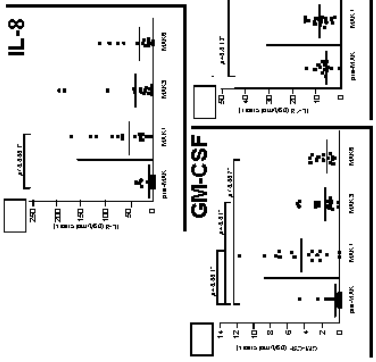
Clinical outcome



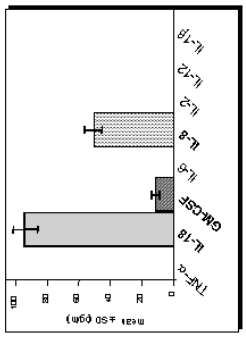
sign rank test : p = 0.0005

(Thiounn N. et al, J. Urol. In press)

Cytokines in urine 2 hours after MAK infusions



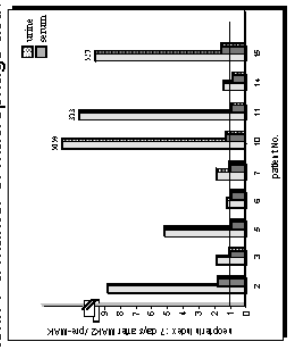
Cytokine production by MAK™ *in vitro* when incubated in urine



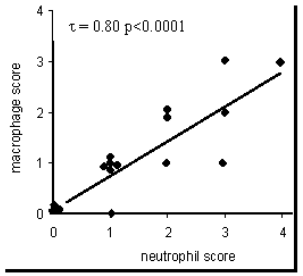
(Pagès F. et al, Clin. Exp. Immunol. 2002)

Neopterin in urine and blood samples

- neopterin : a marker of macrophage activation



Cytological evaluation of urine 2 months after treatment



Conclusion

- Infusion of IFN-γ activated macrophages in the bladder Results inIL-8, IL-18 and GM-CSF production in urine.
- This argues for a local immunostimulation and is associated with a decrease of local tumor recurrences.
- Macrophages in an activation state persist weeks after infusions.

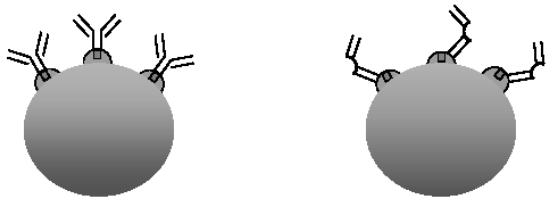
Safety

- Administration of MAK™ or DΦ™ is very well tolerated regardless of the route of injection.
- Out of 605 cell injections carried out by IDM in 89 patients in 5 clinical studies, no serious events related to treatment were reported.
- Only minor events (grade I or II) were observed in a limited number of patients.

Antibodies-based Cell Drugs

MAK armed with conventional mAb

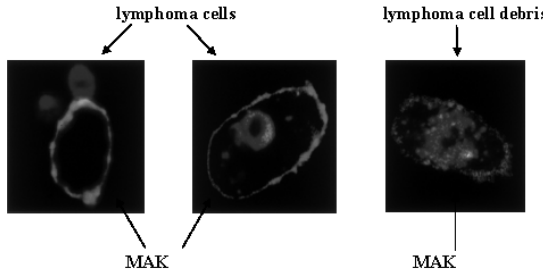
MAK armed with Bab



MEDICAMENTS INNOVANTS AVEC AMM

NOM	CLASSE	INDICATION
GLIVEC	Antityrosine Kinase	LMC
HERCEPTIN (Her2/neu)	Anticorps monoclonal	Cancer du Sein
RITUXIMAB (CD20)	Anticorps monoclonal	Lymphomes
CAMPATH (CD52)	Anticorps monoclonal	LLC

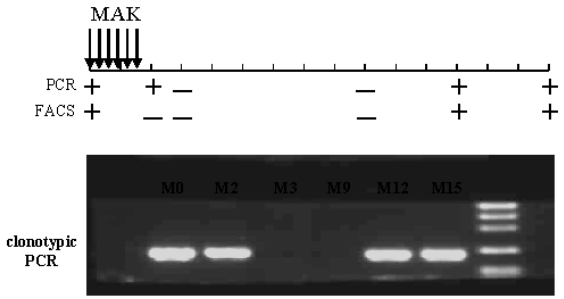
Monocyte-derived Activated Killer cells



IDM © 2003

Fadul et al.

Transient elimination of tumor cells in a CLL patient



IDM © 2003

MAK + @CD20 in CLL. Principal Investigator : L. Sutton

MAK-based clinical trials

Indication	Product	Phase	Pts #	Status
Ovary	OSIDEM™	I/II (IDM)	17	De Gramont *et al., Genycol. Oncol.*, 2002
		III (IDM)	60	on going
Bladder	BEXIDEM™	I/II (IDM)	17	Thiounn *et al., J. Urol*, 2002
		II/III (IDM)	100	in preparation
Mesothelioma		II (Monnet)	19	Monnet *el al., Chest*, 2002
CLL		I/II (Sutton)		on going
NHL		I/II (Favrot)		on going
Glioblastoma		I/II (Wallace)		on going
Ovary		I/II (Prince)		on going

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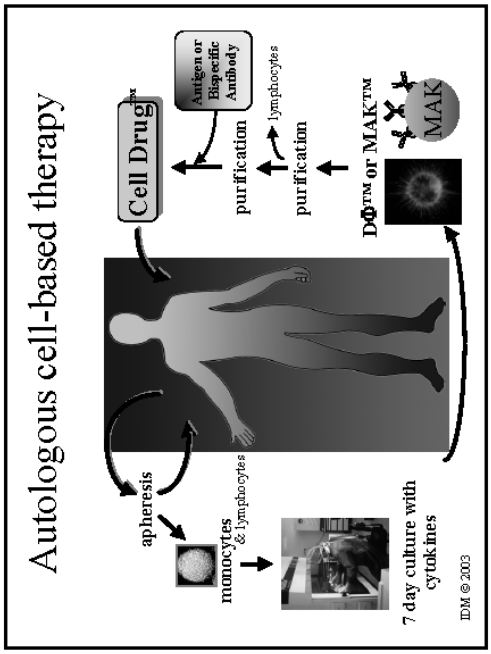
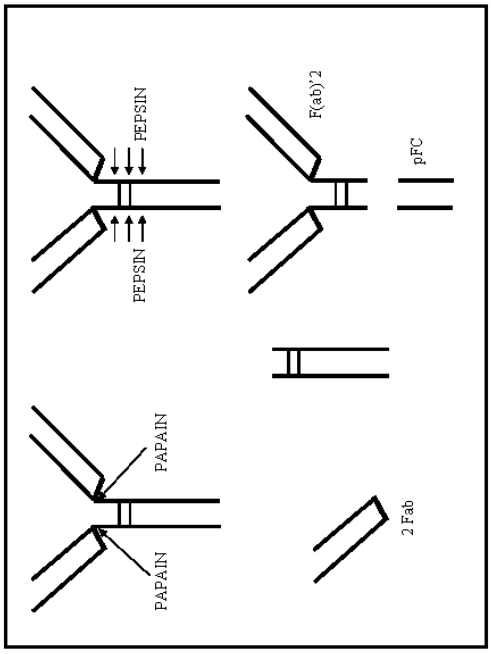
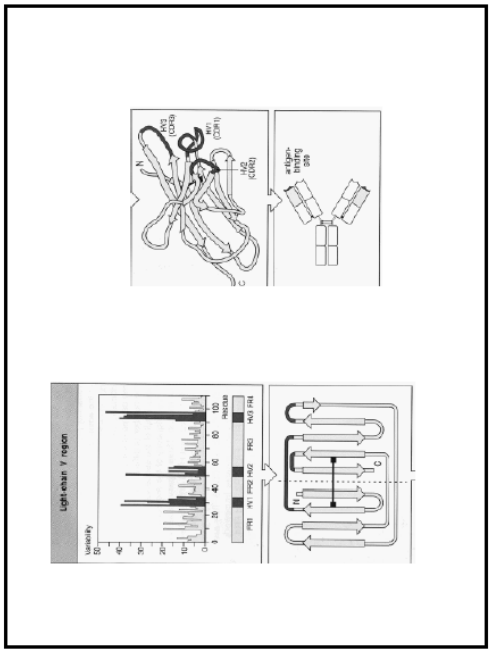
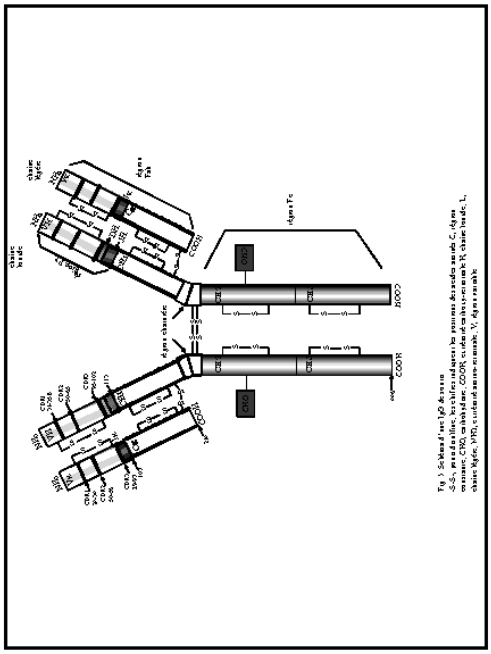
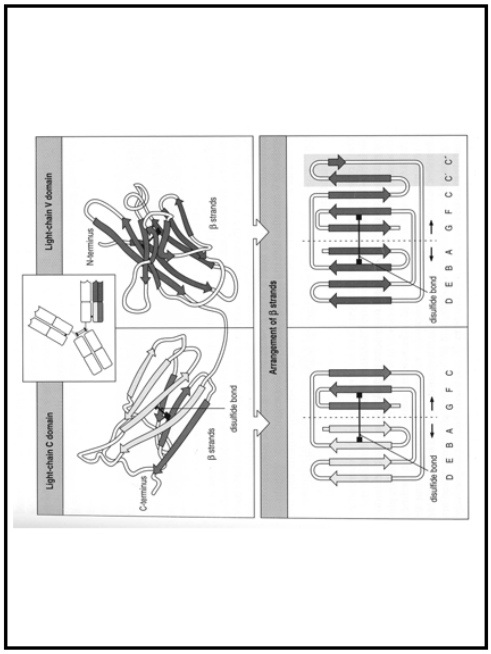

Autologous cell-based therapy
Cell Drug
Antigen or Bispecific Antibody
apheresis
monocytes & lymphocytes
purification
lymphocytes
purification
DC™ or MAK™
MAK
7 day culture with cytokines
IDM © 2003

Lecture 6: Specific therapy: monoclonal antibodies [CF]









Ag/Ab REACTION

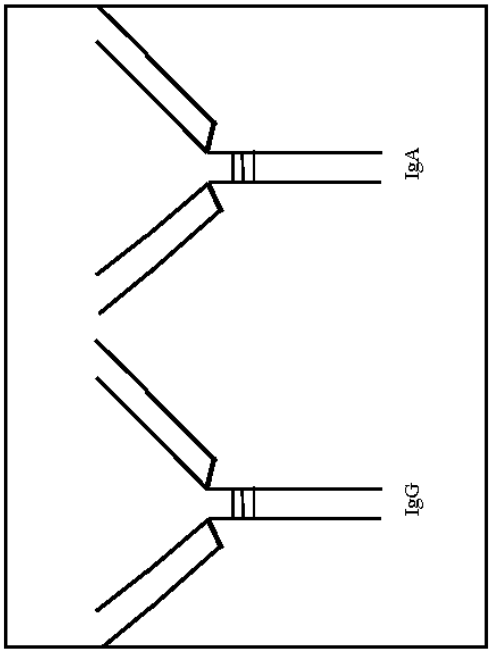
$$Ag + Ac \underset{Koff}{\overset{Kon}{\rightleftharpoons}} AgAc$$

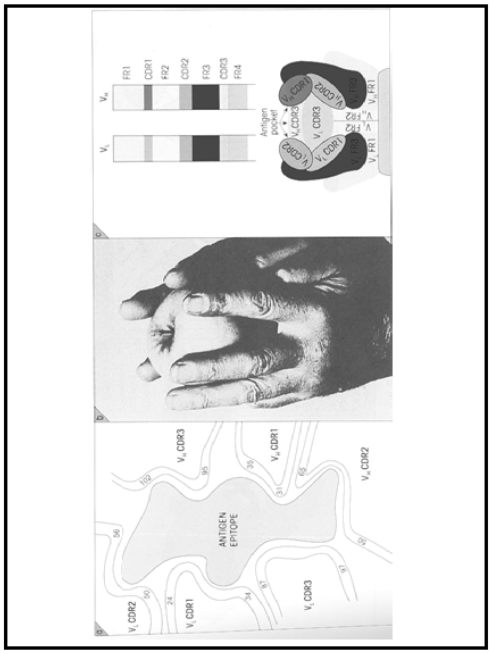
Kon : $m^1 s^{-1}$
Koff : s^{-1}

$$K_A = \frac{[AgAc]}{[Ag][Ac]} = \text{Affinity constant} \quad (K_A : L/M^{-1})$$

$$K_D = \frac{1}{K_A} = \text{Dissociation constant} \quad (K_D : M.L^{-1})$$

K_A = FOR ONE SITE
AVIDITY = FOR WHOLE Ig MOLECULE





CLASS	SUB-CLASS	MW (kDa)	SERUM CONCENTRATION (g/L)
IgG	IgG1	146	9
	IgG2	146	3
	IgG3	165	1
	IgG4	146	0.5
IgM		190x5=950	1.5
IgA	IgA1	160	3
	IgA2	160	0.5
IgD		184	0.03
IgE		186	$5x10^6$

FUNCTIONS OF ANTIBODIES

IgM	PRESENT IN BODY FLUIDS AND TISSUES, DEFENSES AGAINST INFECTION AND CANCER
IgG	PRESENT IN BODY FLUIDS AND TISSUES, DEFENSES AGAINST INFECTION AND CANCER
IgA	PRESENT IN MUCOSAL SURFACES, NEUTRALIZATION OF PATHOGENS
IgE	PRESENT IN TISSUES AND ON VASCULAR ENDOTHELIUM, ALLERGY, DEFENSES AGAINST HELMINTHS

ANTIGENIC DETERMINANTS OR EPITOPES

- RABBITS PRODUCE Ab1, Ab2, Ab3
- 1, 2 AND 3 ARE ANTIGENIC DETERMINANTS OR EPITOPES
- Ab1, Ab2 AND Ab3 DERIVE FROM THREE DISTINCT CLONES OF B CELLS

IgM

ISOTYPES HAVE DISTINCT BIOLOGICAL ROLES

	IgM	IgG	IgA	IgE
COMPLEMENT ACTIVATION	+	+	-	-
NEUTRALISATION OF PATHOGENS	-	+	+	-
OPSONISATION	-	+	±	-
ADCC	-	+	-	-
ACTIVATION OF MAST CELLS	-	-	-	+

MYELOMA CELL LINES
MOUSE MYELOMA MOPC21
P3X63 Ag8
Sp 2/0
NON IgSECRETING
HGPRT NEGATIVE
RAT MYELOMA
EBV-TRANSFORMED HUMAN LYMPHOMA LINES

Ag
MOUSE
SPLEEN CELLS
MYELOMA CELLS (HGPRT-)
MIX AND FUSE (PEG)
HAT (SPLEEN CELLS DIE MYELOMA DON'T SURVIVE)
HYBRIDOMAS
SELECT HYBRIDOMAS THAT MAKE RELEV ANT ANTIBODY
CLONE SELECTED HYBRIDOMA
GROW HYBRIDOMA
PURIFY MAb

POLYCLONAL
ANTISERUM
MONOCLONAL
ANTIBODY
B1
B2
B3
Ab1
Ab2
Ab3
B1
Ab1

HAT MEDIUM
"Hypoxanthine Aminopterin Thymidine"
AMINOPTERIN
BLOCKS NEOSYNTHESIS PATHWAY
HYPOXANTHINE
AND THYMIDINE
FOR SALVAGE PATHWAY IN HYBRIDOMAS

	MOUSE ORIGIN	HUMAN ORIGIN
CHIMERIC MAbs	VARIABLE REGIONS	CONSTANT REGIONS
HUMANIZED MAbs	HYPERVARIABLE REGIONS	FRAME WORK AND CONSTANT REGIONS
HUMAN MAbs IN XENOMOUSE	NONE	WHOLE MAb

MAb PRODUCTION FROM HYBRIDOMAS

IN VITRO	IN VIVO : MOUSE, RAT
- CULTURE FLASKS	- ASCITIS (NUDE MICE OR
- BIOREACTORS	RATS)
- GMP CONDITIONS	

THERAPEUTIC MAbs

AUTOIMMUNITY (PAR)	ANTI TNFα ANTI IL8	TNFα IL8
TRANSPLANTATION	OKT3	CD3 (T cells)
ALLERGY	ANTI-IgE ANTI-FcεRI	IgE FcεRI
GLAUCOMA	ANTI-TGFβ	TGFβ

Rituximab against low-malignancy lymphomas
rechutes/maladie réfractaire
N pts (éval.)
taux de réponse
Maloney (1997) follic./lymphocyt. 34 50% 3 RC, 14 RP
McLaughlin (1998) follic./lymphocyt. 151 50% 9 RC, 67 RP
Piro (1999) follic./lymphocyt. 35 60% 5 RC, 16 RP
Davis (1999) follic./lymphocyt. 28 43% 1 RC, 11 RP
Nguyen (1999) lymphocyt. 15 7% 0 RC, 1 RP
1ère ligne de traitement (faible masse tumorale)
Colombat (Blood 2001) follic. 73% 13 RC, 23 RP
CHOP x follic./lymphocyt. 95% 22 RC, 16 RP

Probability of Event-free Survival
Years after Randomization
CHOP plus rituximab
CHOP
P<0.001
No. at Risk
CHOP plus rituximab 202 177 137 108 63 19
CHOP 197 144 101 72 42 17
Coiffier et al., NEJM2002

Non-conjugated Rituximab (anti-CD20) antibody against « low malignancy » lymphomas

Non-conjugated Rituximab (anti-CD20) against aggressive lymphomas

Monoclonal antibodies against chronic lymphoid leukemia (CLL)

- **Rituximab**
- **Campath-1H**

THERAPEUTIC ANTITUMOR MAbs
MECHANISMS OF ACTION

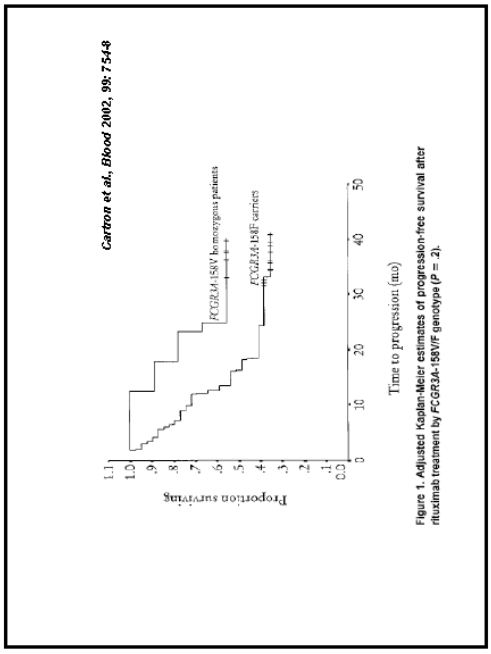
1 - ADCC via NK CELLS

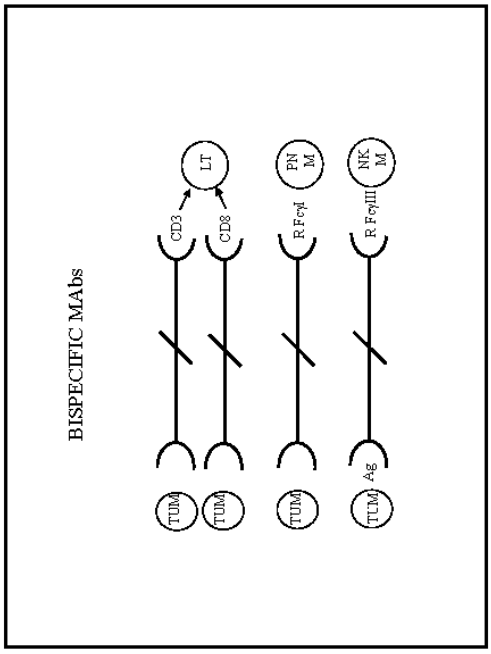
2 - PHAGOCYTOSIS OF TUMOR CELLS VIA MACROPHAGES

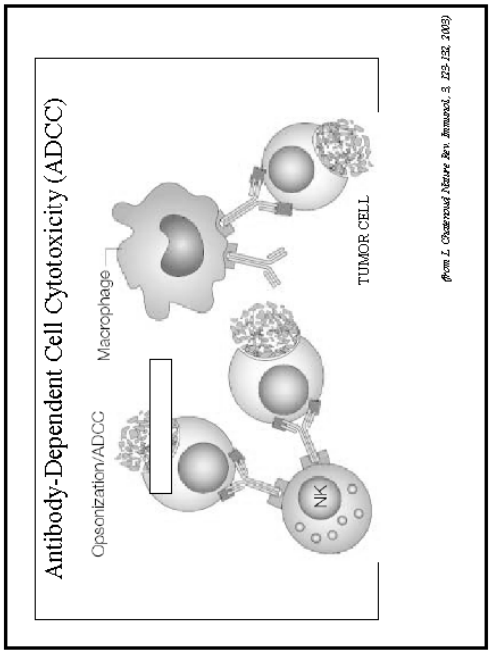
3 - CDC

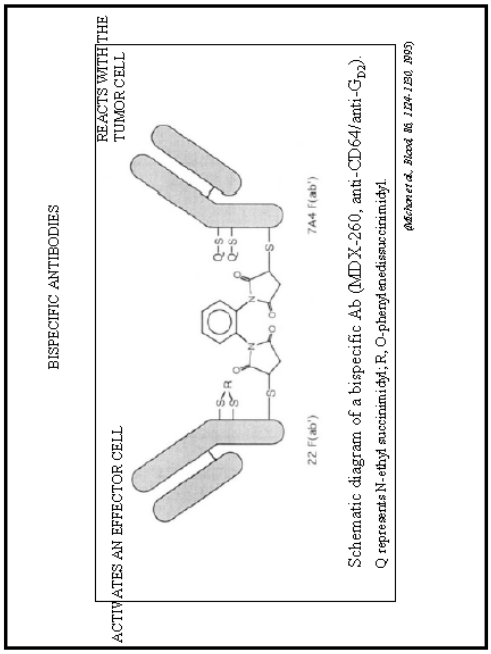
4 - APOPTOSIS

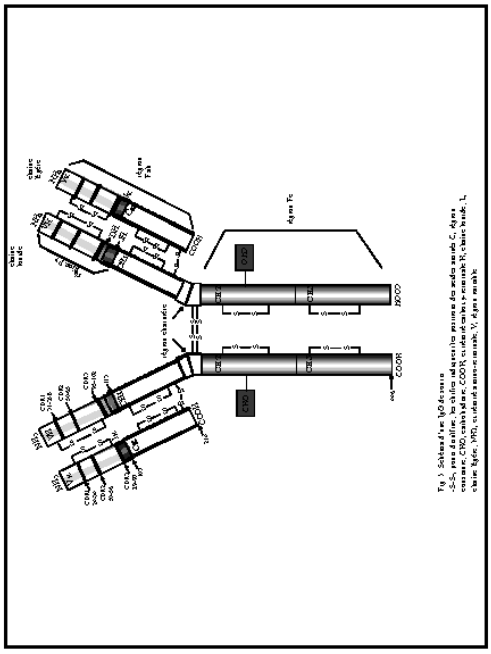
5 - ANTI PROLIFERATIVE EFFECT

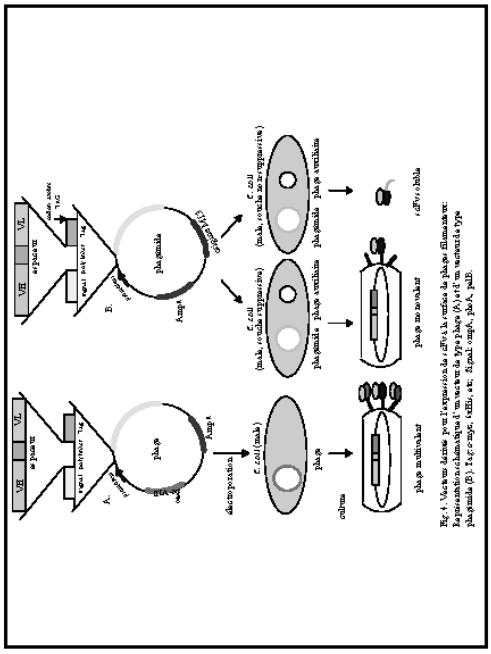

Cartron et al., Blood 2002, 99: 7548
Proportion surviving
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0.0
FCGR3A-158V homozygous patients
FCGR3A-158F carriers
0
10
20
30
40
50
Time to progression (mo)
Figure 1. Adjusted Kaplan-Meier estimates of progression-free survival after rituximab treatment by FCGR3A-158V/F genotype (P = .2).

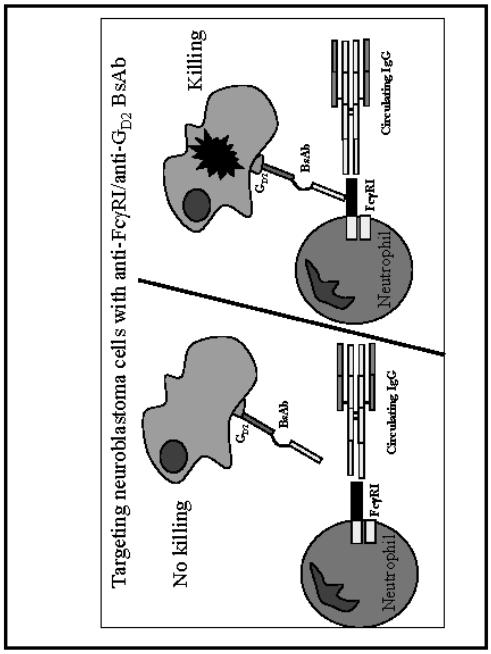

BISPECIFIC MAbs
TUM
CD3
LT
TUM
CD8
TUM
R FcγI
PN
M
TUM
Ag
R FcγIII
NK
M

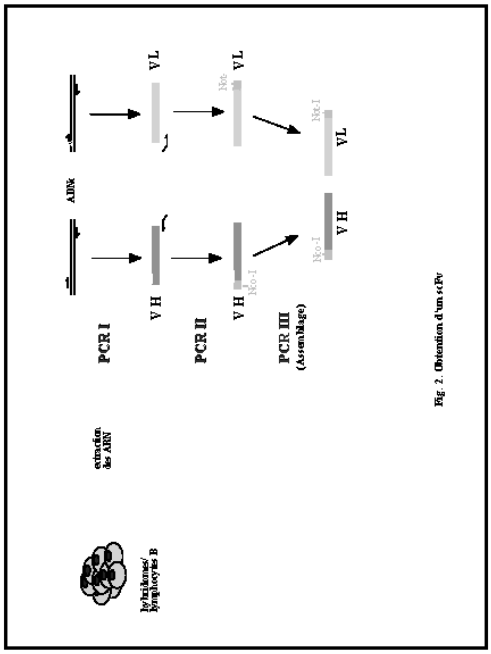

Antibody-Dependent Cell Cytotoxicity (ADCC)
Opsonization/ADCC
Macrophage
NK
TUMOR CELL
(from L. Chasreyeul Nature Rev. Immunol. 3, 125-132, 2003)

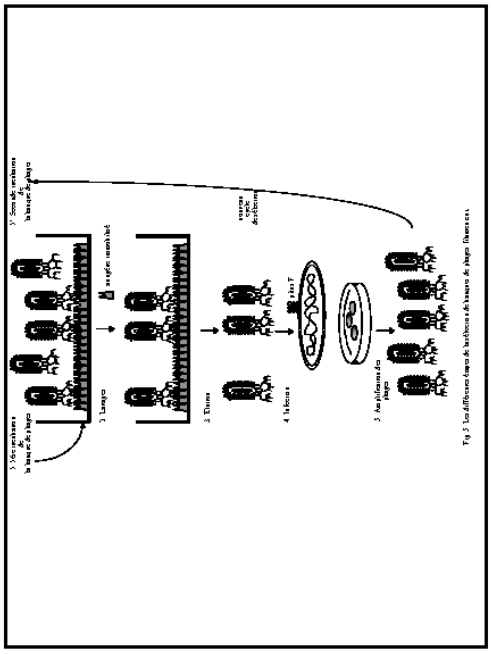

BISPECIFIC ANTIBODIES
ACTIVATES AN EFFECTOR CELL
REACTS WITH THE TUMOR CELL
22 F(ab')
7A4 F(ab')
Schematic diagram of a bispecific Ab (MDX-260, anti-CD64/anti-G$_{D2}$).
Q represents N-ethyl succinimidyl; R, O-phenylenedisuccinimidyl.
(Adams et al. Blood 86, 1254-1261, 1995)

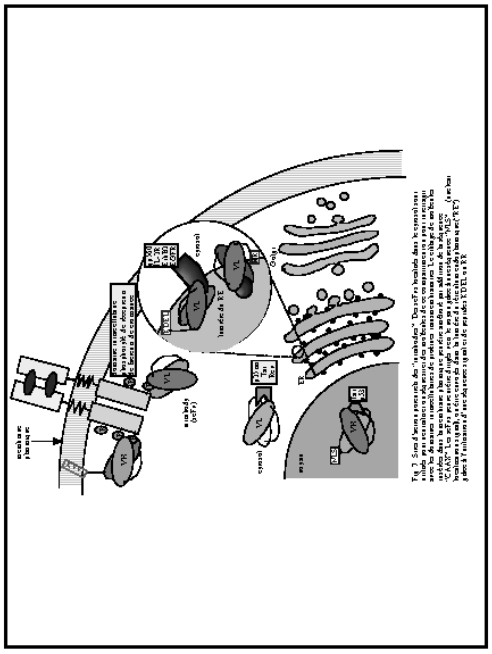


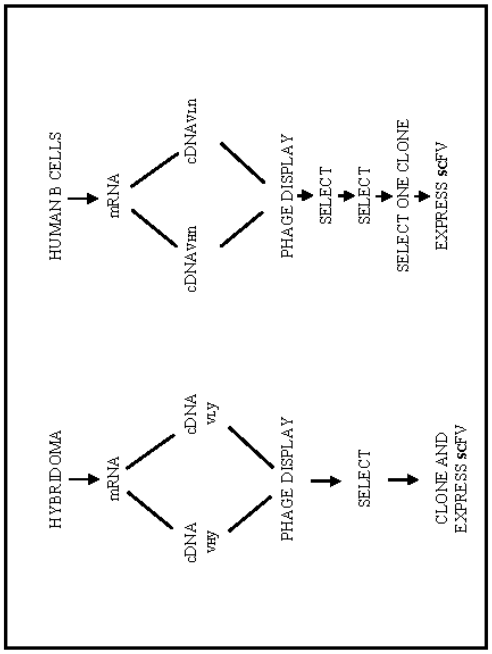


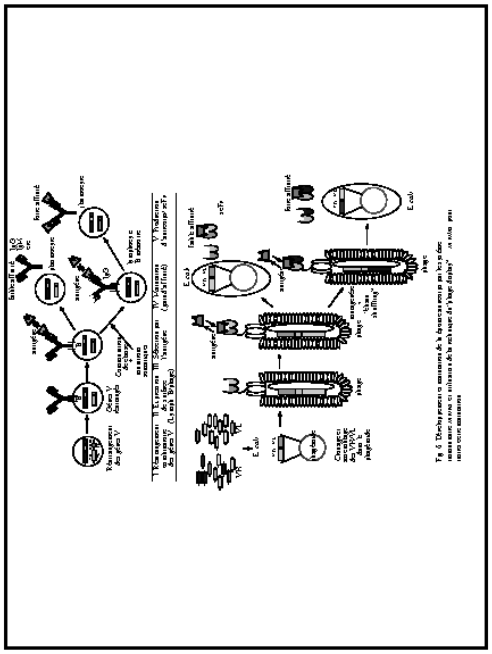

Targeting neuroblastoma cells with anti-FcγRI/anti-G_{D2} BsAb
Killing
No killing
BsAb
Circulating IgG
FcγRI
Neutrophil


PCR I
PCR II
PCR III
(Assemblage)
VL
V H

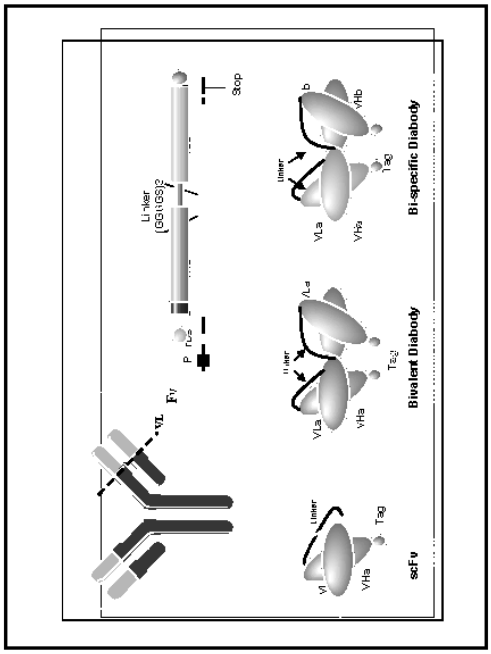





HYBRIDOMA
mRNA
cDNA
VHγ
cDNA
VLγ
PHAGE DISPLAY
SELECT
CLONE AND
EXPRESS scFV
HUMAN B CELLS
mRNA
cDNAVHm
cDNAVLn
PHAGE DISPLAY
SELECT
SELECT
SELECT ONE CLONE
EXPRESS scFV



	ADVANTAGES	PITFALLS
MAb	LONG HALF LIFE	SIZE
	HIGH AFFINITY	NEED Ag FOR IMMUNIZATION
		HYBRIDOMA INSTABILITY
		IMMUNOGENICITY
ScFv	DO NOT NEED IMMUNIZATION	LOW AFFINITY
	CAN BE EXPRESSED	MONOVALENT
	INTRACELLULARLY	SHORT HALF LIFE



Lecture 7: Specific therapy: T lymphocytes [HF]

Greffe hématopoïétique

- Greffon
  - moelle osseuse
  - sang après mobilisation
  - placenta / cordon
- Donneur
  - autologue
  - allogénique
    - géno-id intra-familiale
    - phéno-id (donneur du fichier)
    - haplo-identique intra-familiale
- Greffe à conditionnement
  - myélo-ablatif
  - non myélo-ablatif

Infusion de lymphocytes T lors d'une rechute de LMC après greffe hématopoïétique allogénique

Greffe allogénique de cellules souches hématopoïétiques (CSH)

- Greffon de « CSH »
  - **cellules souches**
  - cellules immunocompétentes: **lymphocytes T, cellules NK,...**
- Reconnaissance allogénique
  - **tissus sains**: **l'effet** Greffon vs Hôte (la GvH)
  - **hématopoïèse résiduelle**: **prévention de** l'effet Hôte vs Greffe (HvG)
  - **cellules d'hémopathies malignes**: l'effet Greffon vs Leucémie (la GvL)

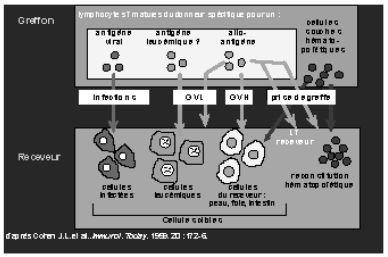
Greffe de CSH allogénique:

- *greffe hématopoïétique*
- *immunothérapie adoptive allogénique*

La GvL

- Barnes (1955): Souris leucémique
  - greffe de moelle syngénique: décès par récidive leucémique
  - greffe de moelle allogénique: pas de récidive mais décès ultérieur par GvH
- Mathé (1965)
  - "guérir la leucémie par la GvH »
- Effet GvL
  - désparité allogénique
  - lymphocytes T matures dans le greffon
  - potentialisé par la survenue d'une GvH
  - rôle des cellules Natural Killer et lymphocytes γδ?

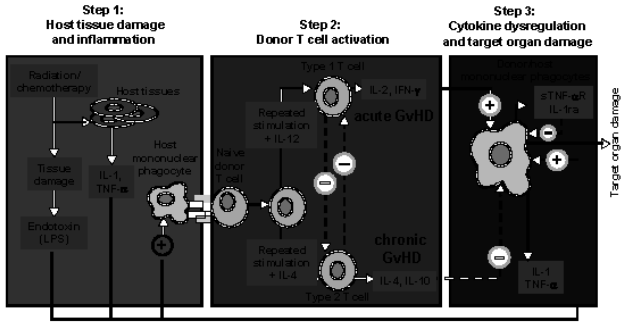
Effets bénéfiques et néfastes des lymphocytes T présents dans le greffon



La maladie du Greffon contre l'Hôte

- Forme aiguë et chronique
- Responsable d'une mortalité (15 à >de 50%) et morbidité (GvH chronique++) importante
- Deux modalités de prévention:
  - immunosuppression après greffe
  - déplétion T du greffon
- Traitement des formes sévères de GvH peu efficace et toxique
- Associée à un puissant effet anti-tumorale

Physiopathologie de la GvH

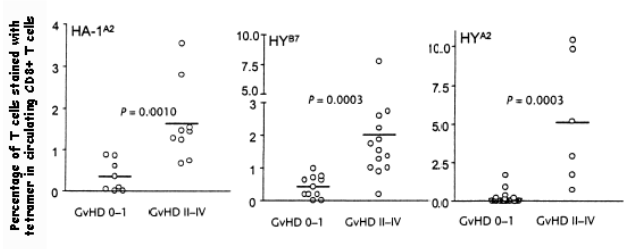


Krenger W and Ferrara JL. J Hematother 1996;5:3–14

GvH/GvL: Cibles reconnues

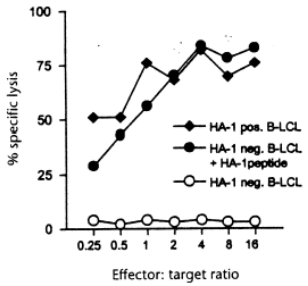
- Ag tumoraux ?
- Ag mineurs d'histocompatibilité
  - peptides endogènes dotés d'une variabilité allélique et présentés par les molécules HLA de classe I ou II
  - ségrégation mendélienne et indépendante du CMH
  - restriction d 'expression au tissu hématopoïétique possible pour certains Ag ?
  - cibles de lymphocytes T du donneur dont la proportion augmente après greffe
- « Ag » majeurs d'histocompatibilité +/- peptides

Corrélation entre la fréquence de CTL circulant anti-HA-1 et anti-HY et la GvH



Mutis et al., Nature Medicine, 1999

Analyse fonctionnelle des CTL anti-Ag mineur



Mutis et al, Nature Medicine, 1999

GvH/GvL: Cibles reconnues

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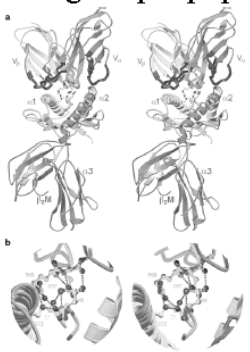
- « Ag » majeurs d'histocompatibilité +/- peptides

Représentation d'un complexe allogénique peptide/MHC - TCR



Complexe BM3.3-pBM1-H-2Kb
Reiser et al, Nature Immunology, 2000

Alloréactivité après greffe hématopoiétique: facteurs de «risques»

- Degré d'histocompatibilité
  - complexe majeur d 'histocompatibilité
  - Ag mineurs d 'histocompatibilites
- Degré de « danger »
  - toxicité du conditionnement et des traitements préalables
  - contexte microbiologique
  - age du receveur (et du donneur?)

Prévention de la GvH

- Immunosuppresseurs
  - cyclosporine, FK 506, Rapamycine, ...
- Déplétion T du greffon
- Déplétion/Anergie des lymphocytes T alloréactifs présent dans le greffon
  - CD25/toxine (Cavazzana-Calvo et al)
  - blocage de la co-stimulation CD28/CD80-CD86 (Guinan et al)
- Cellules mononuclées apoptotiques
- ...

Prévention de la GvL?

Greffe T-déplétée vs non T-déplétée: Etude randomisée

Maraninchi et al, Lancet, 1987

Effet Greffon versus Leucémie dans la Leucémie Myéloïde Chronique

- Rechute après greffe hématopoïétique:
  - greffon allogénique non T-déplété: 10 à 20% de rechute
  - greffon allogénique déplété en lymphocytesT: > 50 % de rechute
  - greffon syngénique: > 70% de rechute
- Infusion de lymphocytes du donneur après rechute:
  - rechute hématologique: > 80% de remission complète

AcMo anti-IL-2R après greffe de moelle allogénique, *Blaise et al, Lancet, 1994*

Etude randomisé, n=101
AcMo de J1 à J28
Recul: 58 mois

Bras AcMo:
- GvH aiguë: incidence identique mais survenue plus tardive
- toxicité: identique
- rechute: 42 % vs 22% (p<.08)
- survie sans rechute: 41% vs 62% (p<.05)

Traitement de la GvH

- Immunosuppresseurs
  - corticoides
  - serum anti-lymphocytaire
  - FK506, mycophenolate mofetil
  - antagonistes de cytokines inflammatoires (Ac, recepteurs solubles)
- Lymphocytes T exprimant un gène suicide
  - thymidine kinase (HS-tk) / Ganciclovir

GvH / GvL : Trois pré-requis

• la présence de cellules immuno-compétentes dans le greffon

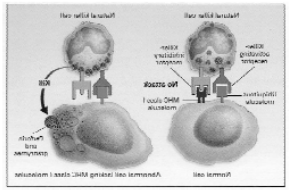
• l'incapacité pour l'hôte de rejeter les cellules administrées

• une histo-incompatibilité entre donneur et receveur

Billingham, 1955

Greffe intra-familiale haplo-identique

- Donneur (presque) toujours disponible
- Greffon important: « megadose »
- Déplétion T du greffon
- Conditionnement très immuno- et myelo-suppresseur
- Réactivité allogénique médiée par les cellules Natural Killer: Incompatibilité KIR donneur anti-receveur
  - effet facilitant sur la prise de greffe
  - effet GvL (dans les LAM++)
  - pas de GvH



Delves, N Engl J Med, 2000

Table 2. Donor-Recipient Pairs With Potential for Donor-Versus-Recipient NK Cell Allorecognition

Transplant No.	Donor	Recipient	Donor KIR Ligand Missing in Recipient
1	A24, B35, Cw4 A24, B62, **Cw3**[*]	A24, B35, Cw4 A24, B35, **Cw4**	Group 2 HLA-C
2	A2, B35, Cw4 A24, **B51**, Cw–	A2, B35, Cw4 A1, **B35**, Cw–	HLA-Bw4
3	A33, B35, Cw4 A3, **B44**, Cw4	A33, B35, Cw4 A2, **B60**, Cw3	HLA-Bw4
4	A32, B44, Cw8 A3, B18, **Cw6**	A32, B44, Cw8 A3, B52, **Cw8**	Group 1 HLA-C

Ruggeri et al, Blood, 1999

Donor Natural Killer Cell Alloreactivity in Mismatched Hematopoietic Transplants

Ruggeri et al, Science, 2002

Table 1. Clinical data and transplantation outcomes in HLA haplotype-mismatched transplants with and without KIR ligand incompatibility in the GVH direction. KIR ligand incompatibility in the GVH direction was defined as absence in recipients of donor class I allele group(s) recognized by KIRs (9–11). Such groups are HLA-C alleles with Asn^{77}-Lys^{80}, Cw2, 4, 5, 6, and related alleles; HLA-C alleles with Ser^{77}-Asn^{80}, Cw1, 3, 7, 8, and related alleles; HLA-Bw4 alleles; and HLA-A3/A11. Twenty-six pairs (11 in ALL and 15 in AML) were mismatched for HLA-C groups, 8 (3 in ALL, and 5 in AML) were mismatched for HLA-Bw4 group; the HLA-A3/A11 mismatch was never found alone but only in conjunction with HLA-C group mismatches (2 pairs).

KIR ligand incompatibility in GVH direction	No	Yes
Number of transplants	58	34
Donors displaying antirecipient NK clones	1/58	34/34*
Disease		
ALL	21	14
AML	37	20
Transplantation outcomes		
Rejection	15.5%	0%*
Acute GVHD, ≥ grade II	13.7%	0%*
Probability of relapse at 5 years		
ALL	90%	85%
AML	75%	0%**

*$P \leq 0.01$; **$P < 0.0008$ (22).

Alloreactive NK cells cause immune-and myeloablation but not GvHD

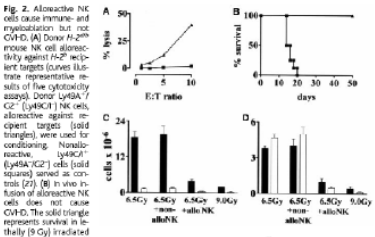
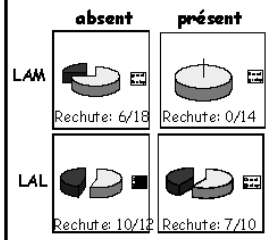


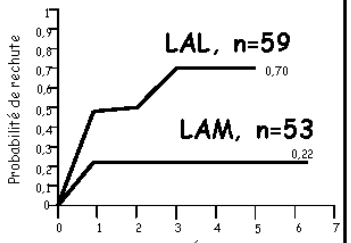
Fig. 2. Alloreactive NK cells cause immune- and myeloablation but not GVHD. (**A**) Donor $H\text{-}2^{bm}$ mouse NK cell alloreactivity against $H\text{-}2^{b}$ recipient targets (curves illustrate representative results of five cytotoxicity assays). Donor $Ly49A^{+}/C2^{-}$ ($Ly49C/I^{+}$) NK cells, alloreactive against recipient targets (solid triangles), were used for conditioning. Nonalloreactive, $Ly49C/I^{+}$ ($Ly49A^{-}/C2^{+}$) cells (solid squares) served as controls (22). (**B**) In vivo infusion of alloreactive NK cells does not cause GVHD. The solid triangle represents survival in lethally (9 Gy) irradiated $H\text{-}2^{b}$ mice given $H\text{-}2^{bm}$ bone marrow plus 16×10^{6} alloreactive $H\text{-}2^{bm}$ NK cells versus survival of mice (solid square) given $H\text{-}2^{b}$ bone marrow plus 10^{6} $H\text{-}2^{b}$ T cells (number of mice in each group = 10). (**C** and **D**) Mice were given nonlethal TBI (6.5 Gy), nonlethal TBI plus 4×10^{6} nonalloreactive NK cells (nonallo NK), nonlethal TBI plus 4×10^{6} alloreactive NK cells (allo NK), or lethal (9 Gy) TBI alone. Alloreactive NK cells ablated bone marrow (**C**) and spleen (**D**) granulocytes (black bars) and T cells (white bars) in nonlethally irradiated mice (mean ± SD of data from a total of nine mice, three each in three independent experiments).

Ruggeri et al, Science 295:2097-2100, 2002

Alloréactivité anti-leucémique médiée par les cellules Natural Killer



Relation entre la présence d 'une alloréactivité NK anti-receveur et rechute



Probabilité de rechute après greffe haplo-identique pour leucémie à haut risque

Aversa et al, Blood Reviews, 2001

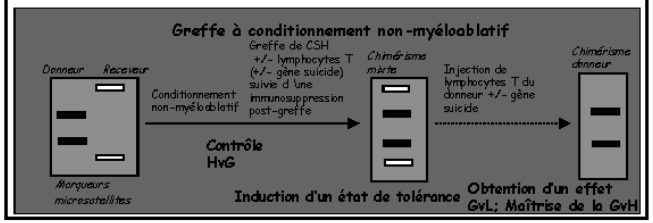
Greffe hématopoïétique allogénique: une immunothérapie efficace mais toxique

Destruction et remplacement d'une moelle « malade »

↓

Processus à plusieurs étapes dont l'objectif est d'obtenir:

- un état de tolérance: *greffe d'organe*
- un chimérisme mixte: *maladies génétiques*
- un rejet de tumeur: *hémopathies malignes, tumeurs solides*
- un rejet de l'immunité autologue: *auto-immunité*



Cancer rénale métastatique: conditionnement non-myéloablatif et greffe hématopoïétique allogénique

- 19 patients, médiane de suivi : 402 jours
- Réponse anti-tumorale : 10/19 (53%)
  - -3 rémissions complètes persistantes,
  - -7 réponses partielles
- 2 décès toxiques, 8 décès par progression de la maladie
- Réponse anti-tumorale :
  - médiane de 129 jours après la greffe
  - lors de l'arrêt de la cyclosporine
  - survenue plus fréquente en présence d'une GvH (80% vs 10% en l'absence de GvH)

Childs et al, N Engl J Med, 2000

Greffe de rein et de moelle après conditionnement non-myéloablatif

- Patiente de 55 ans
- Myélome et insuffisance rénale terminale
- Conditionnement par Endoxan, SAL et irradiation thymique
- Pas de GvH
- Chimérisme mixte
- Arrêt de l'immunosuppression à J73
- Normalisation de la fonction rénale
- Réponse anti-tumorale

Spitzer et al, Transplantation, 1999

Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients

- greffe de CSH T-déplétée
- prophylaxie
- n=39
- 2 à 4 infusion de lymphocytes T polyclonaux anti-EBV (CD4+ et CD8+) exprimant un gène marqueur (NeoR)
- survie in vivo > 18 semaines (> 38 mois dans des lignées T anti-EBV)
- réduction de 2 à 4 log de la charge virale chez les 6 patients présentant une charge virale importante
- pas de toxicité
- pas de lymphome-EBV

- traitement:
  - 2 lymphomes immunoblastiques EBV-induit
  - 2 réponses complètes et prolongés

Rooney CM et al
Blood 1998, 92:1549-55

Lecture 8: Vaccination [ET]

CANCER VACCINES

1) **Arguments supporting the concept of immunosurveillance leading to the development of cancer vaccine to boost the immune system**

2) **Cancer vaccine based on antibody induction**

- Preventive vaccine against virus

- Vaccine against melanoma antigen

3) **Cancer vaccines based on the induction of T lymphocytes (CTL)**

- Peptides (Heteroclitic Immunization, adjuvant…)

- Polyepitopic vaccine : Vectorization by viruses or non replicative vectors

4) Use of Dendritic cells

- Rational

- Clinical results

- Optimization

5 Cancer vaccine and risk of auto-immunity

6 Potential mechanisms leading to tumor escape from immune recognition.

Arguments supporting the concept of immunosurveillance leading to the development of cancer vaccine to boost the immune system

Epidemiology and cancer

- Increase in the frequency of some cancers (Kaposi sarcoma, EBV associated lymphomas, cervix carcinomas...) in immunodepressed patients. :

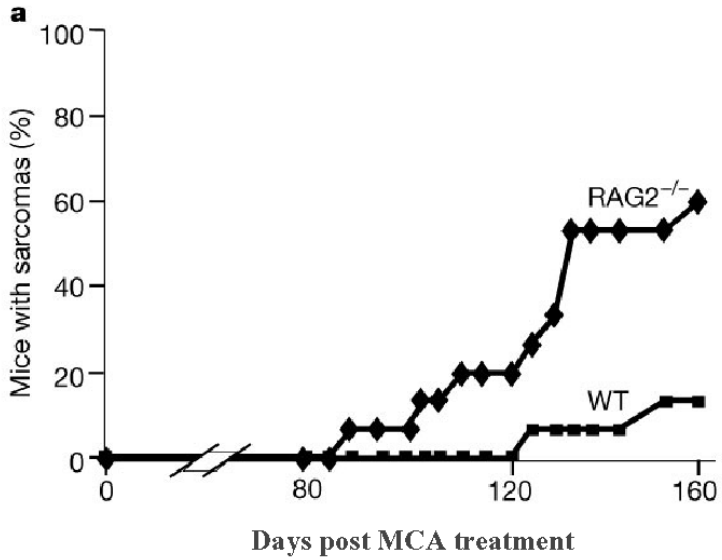
  . Congenital immunodeficiency.

  . Acquired immunodeficiency - AIDS,

  - Immunosuppressors

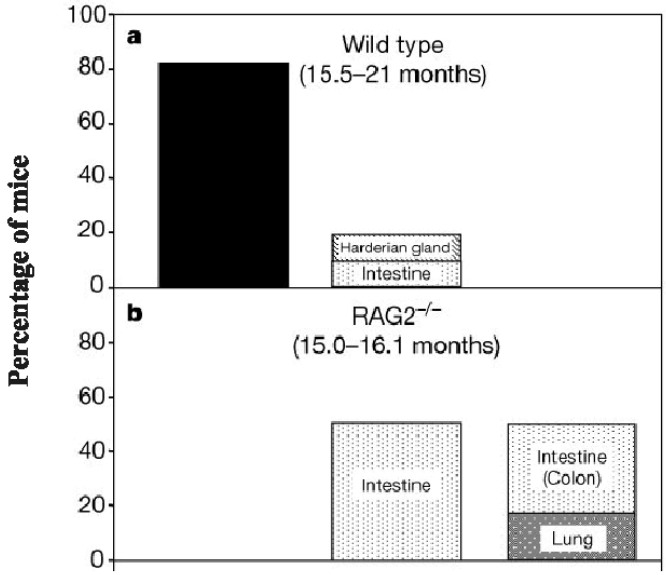
- Graft Versus Leukemia (GVL) during allogeneic bone marrow transplantation.

Lymphocyte-deficient mice are highly susceptible to MCA-induced tumour development

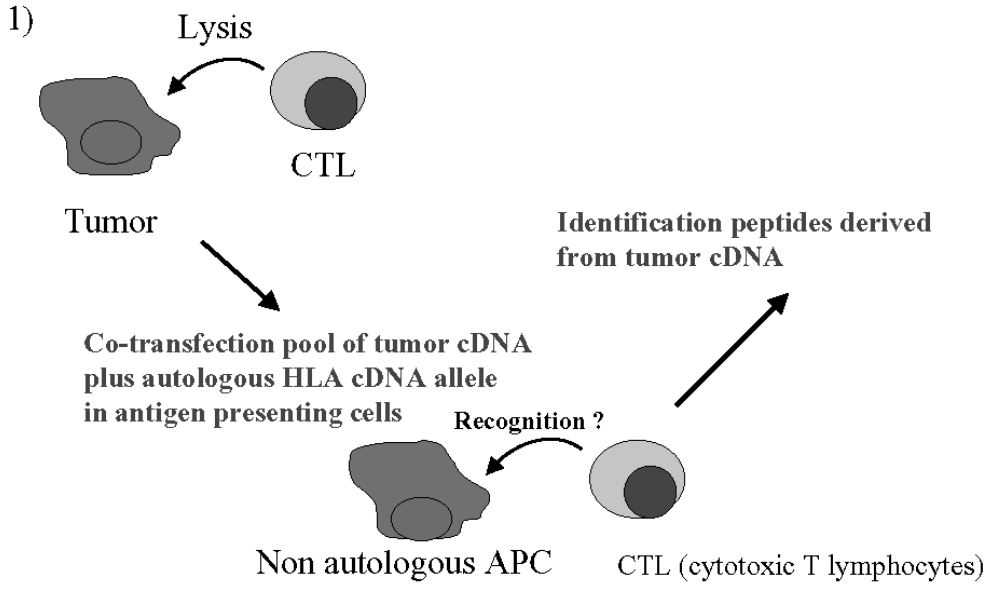


Shankaran et al. Nature 2001

Increased development of spontaneous neoplastic disease in immunodeficient mice.



METHODS FOR THE IDENTIFICATION OF TUMOR ANTIGENS



Technique SEREX

Screening phages or bactéria transfected with a cDNA library derived from tumors with sera of cancer patients.

- Sera are easier to obtain than tumor specific CD4-Tor CD8-T lymphocytes.

- Many antigens were characterized during these last years using this technique.
  .Ex : NY-ESO
  (http://www.licr.org/SEREX.html).

Tumor antigens

A Peptides derived from antigens recognized by CD8-T cells.

1 Melanoma-melanocyte differentiation antigen
- **Mart-1 (Melan A)**
- **Gp100 (pmel-17)**
- **Tyrosinase**
- **TRP1 (gp75)**
- **TRP2**
- **MSH- R**

2 Cancer-Testis antigen
- **Mage 1, Mage 2, Mage 3, Mage 12**
- **Bage, Gage, Rage**
- **NY-ESO-1**
- **N-acetylglucosaminyltransferase V (peptide intronique).**

3 Mutated antigens
- **β catenine**
- **CDK-4**
- **Caspase-8**
- **KIA0205**
- **HLA-A2**

4 Antigènes present in normal tissue but surexpressed in tumors
- **G-250**
- **Her-2/neu**
- **p53**
- **Telomerase catalytic protein**
- **ACE**
- **α foeto-proteine (αFP)**

B peptides dérived from antigens recognized by CD4-T cells

1 Peptides derived from non mutated antigens (cancer testis or Melanoma-melanocyte differentiation antigens)
- **gp100**
- **Mage 1**
- **Mage 3**
- **Tyrosinase**
- **NY-ESO-1**

2 Peptides dérivded from mutated antigens
- **Triosephosphate isomerase**
- **CDC-27**
- **LDLR-FUT**

VIRUS	TUM0RS	Other symptomes associated with viral infections
EBV	- Burkitt Lymphomas - Cavum carcinomas - Hodgkin lymphomas	Infectious mononucleosis Hemophagocytosis syndrome. Immunodeficiency (Purtillo syndrome)
HTLV1	- T -Leukemias	Spasmodic paralysis syndrome
HPV16,18	- Cervix carcinomas	Cervical intraneoplasia laryngeal papillomatosis
HPV1-45	- Bowen disease (In situ carcinoma) - Squamous-cell carcinomas (immunodépressed patiens)	Dyskératosis , Wart
HBV/ HCV	- Hépatocarcinoma	Hépatitis, Cirrhosis
KSHV (HHV8)	- Kaposi Sarcomas	Castleman disease

Natural humoral and cell mediated immunity against cancer

1 Humoral response

- Antibodies against many tumor antigens (p53, HER2/neu, Muc 1, GD2, NY-ESO1, HU…) in the serum of cancer patients.

2 CD4-T cells and CD8-T cell against tumor peptides in the blood or TIL of cancer patients.

Cancer-testis antigen (Mage family. (T Boon)), **Mage A10, NY-ESO**).
Melanoma differentiation antigens : Melan A, Tyrosinase and gp100
Self antigens : Muc 1, HER-2/neu, Proteinase 3, survivin
Virus.

Cancer vaccine based on antibody induction

- **Preventive vaccine against virus**
- **Vaccine against melanoma antigen**

Efficacy Analyses of a Human Papillomavirus Type 16 (HPV-16) L1 Virus-like–Particle Vaccine.

	HPV vaccine	Placebo
Persistent HPV-16 infection	**0/768**	**41/765**
Cervical intraepithelia neoplasia	**0**	**9**

Median follow up : 18 months

Koutsky LA . N Engl J Med 2002

Efficacy Analyses of a Human Papillomavirus Type 16 (HPV-16) L1 Virus-like–Particle Vaccine.

Immunogenicity Analysis

After the third dose (month 7), the geometric mean titer of HPV-16 antibodies was 1510 mMU per milliliter among the 619 women who received HPV-16 vaccine and less than 6 mMU per milliliter among the 631 women who received placebo.

For reference, the geometric mean titer of HPV-16 antibodies was 25.7 mMU per milliliter at enrollment among 337 women who had detectable HPV-16 antibodies on day 0.

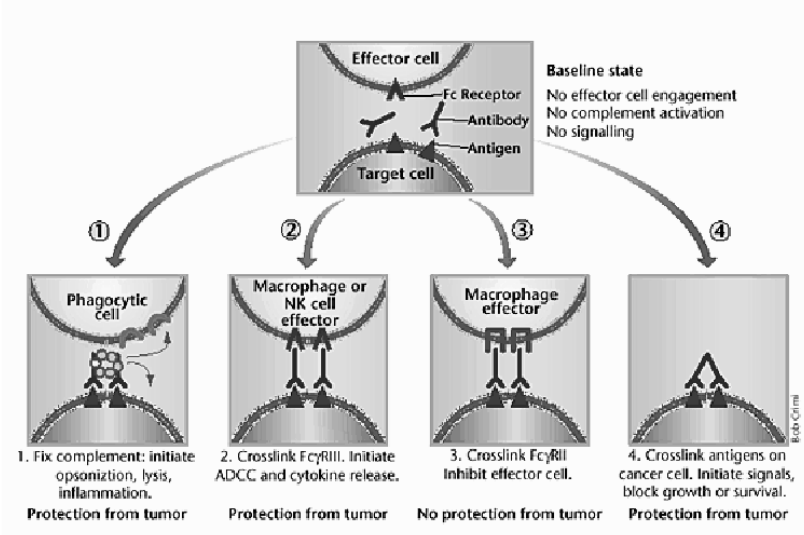
TABLE 4. INCIDENCE OF LIVER CANCER PER 100,000 CHILDREN IN BIRTH COHORTS DETERMINED ACCORDING TO THE DATE OF IMPLEMENTATION OF THE HEPATITIS B VACCINATION PROGRAM.

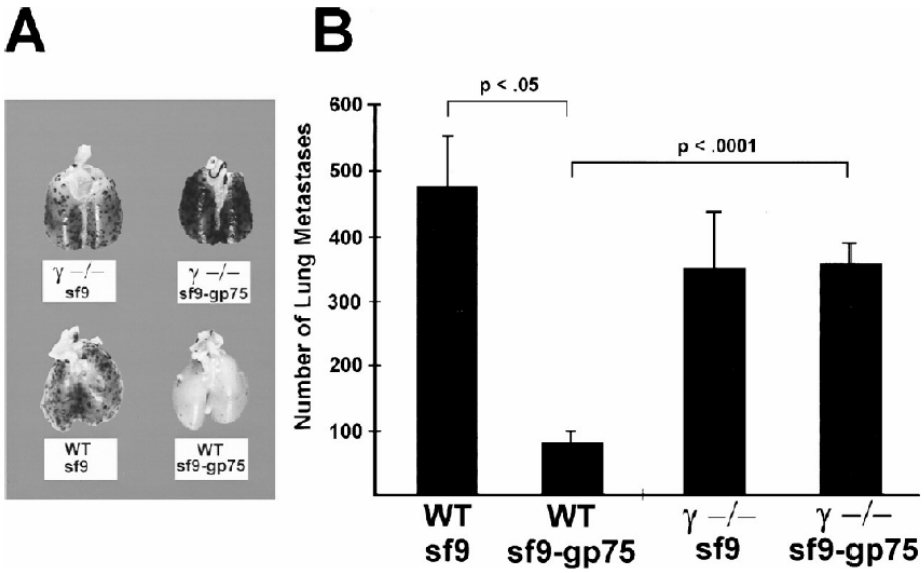
AGE AT DIAGNOSIS (YR)	BEFORE-PROGRAM COHORT (JULY 1974–JUNE 1984)		AFTER-PROGRAM COHORT (JULY 1984–JUNE 1986)	
	POPULATION	NO. OF CANCERS (INCIDENCE)	POPULATION	NO. OF CANCERS (INCIDENCE)
6	3,940,747	18 (0.46)	648,642	0 (0.00)
7	3,938,119	21 (0.53)	647,051	1 (0.15)
8	3,931,983	19 (0.48)	644,892	2 (0.31)
9	3,928,721	24 (0.61)	340,521*	0 (0.00)
Total	15,739,570	82 (0.52)	2,281,106	3 (0.13)†

*This value is based on data for the cohort born from July 1984 to June 1985.

†P<0.001 for the comparisons between birth cohorts.

Chang MH N Engl J Med 1997





Active protection from melanoma metastases requires FcRs.

Clynes R P.N.A.S 1998

Monoclonal antibodies in the treatment of cancers in human

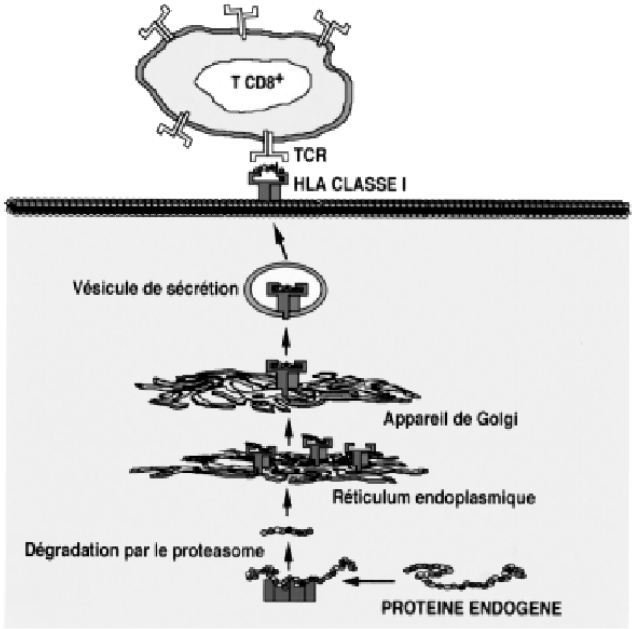
	Target	Indications
Trastuzumab* Herceptin	**HER2/neu**	**Breast Cancer**
Rituximab* (Chimeric Abs)	**CD20**	**Follicular Lymphoma** **B-CLL**
Alemtuzumab* (Campath-1H) Humanized Abs	**CD52** (Malignant B and T cells)	**B-CLL** **Sezary syndrome**

Cancer vaccines based on the induction of T lymphocytes (Cytotoxic T lymphocytes (CTL))

ROLE OF CYTOTOXIC T LYMPHOCYTES IN THE CONTROL OF TUMOR DEVELOPMENT

- Clinical responses after administration of CTL and TIL in adoptive immunotherapy protocols.
- Identification of CTL in biopsies of cancer lesions derived from spontaneous regressing tumors.
- Correlation in murine models between the ability to elicit CTL and anti-tumor clinical responses.

Antigen presentation restricted by HLA class I molecules



In spite of some rare studies in which peptide vaccine were shown to elicit CTL (Feltkamp Eur J Immunol 1995, Cormier JN. Cancer J. Sci Am 1997, Pass HA. Cancer J. Sci Am 1998) **most cancer vaccines based on peptide immunization failed to induce efficient anti-tumor CTL.** (Marchand.Int J. Cancer 1999, Lewis JJ. Int J. Cancer 2000)

To improve the peptide immunization efficiency different groups proposed to :

- Increase the binding of peptide to HLA molecule by introducing mutations in wild type tumor peptides which will enhance the affinity of peptide to HLA molecules. This more stable HLA-peptide complex should lead to better induction of CTL…without changing the recognition of the wild type peptide by the induced CTL.

Peptides derived from Glycoprotéine $B_{498-505}$
from herpes simplex virus.

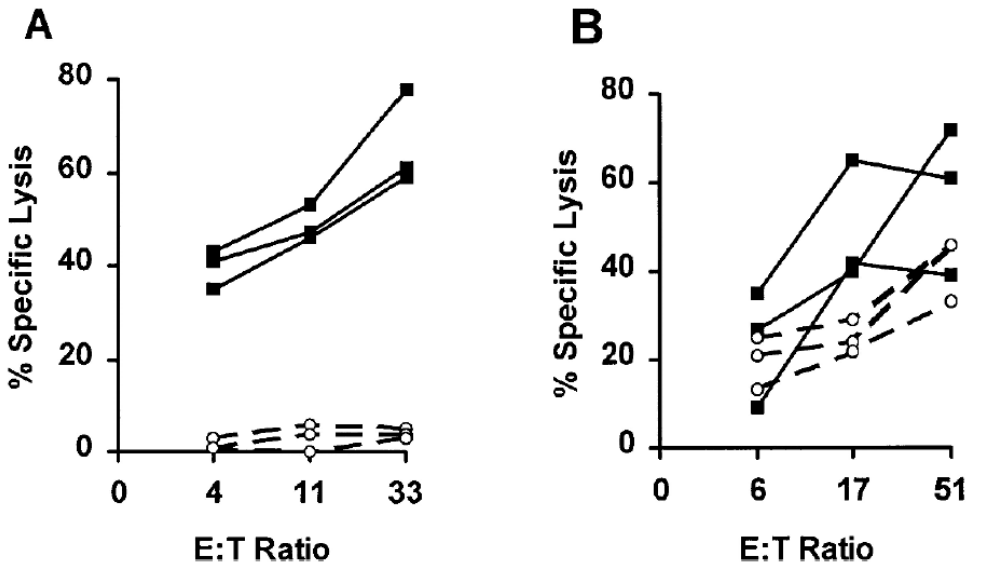
SEI : SEIEFARL (Natural peptide) **SSI** : SSIEFARL

The aminoacid in position 2 of the SEI and SSI peptides bind to the aminoacid 24 of the K^b molecule

B6 mice: H-2K^b
B6 C-H-2^{bm8} mice : H-2K^{bm8} : aminoacid 24 of the K^b molecule is mutated

SEI binds poorly to H-2K^b ; SSI binds strongly to H-2K^b

SEI et SSI bind strongly to the H-2K^{bm8}

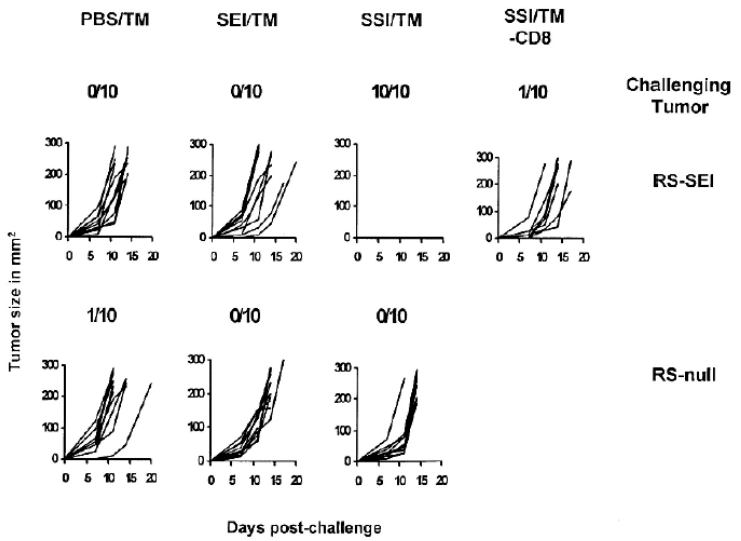


3 mice per groups were immunized with peptide SSI ■ or SEI ◯

A B6 mice : (H-2K^b)

B B6 C-H-2^{bm8} (H-2K^{bm8}) mice

Dyall et al. J. Exp. Med 1998



Dyall et al. J. Exp. Med 1998

In vivo ability of the heteroclitic vaccine SSI to protect mice against a transplantable tumor expressing SEI.

Rosenberg SA. Nature Med 1998.

- $gp100_{209\text{-}217}$ natural peptide which binds to intermediate affinity to HLA-A2

- Mutation of the peptide in AA position 2 (AA2 T ⟶ M) = $gp100_{209\text{-}2M}$

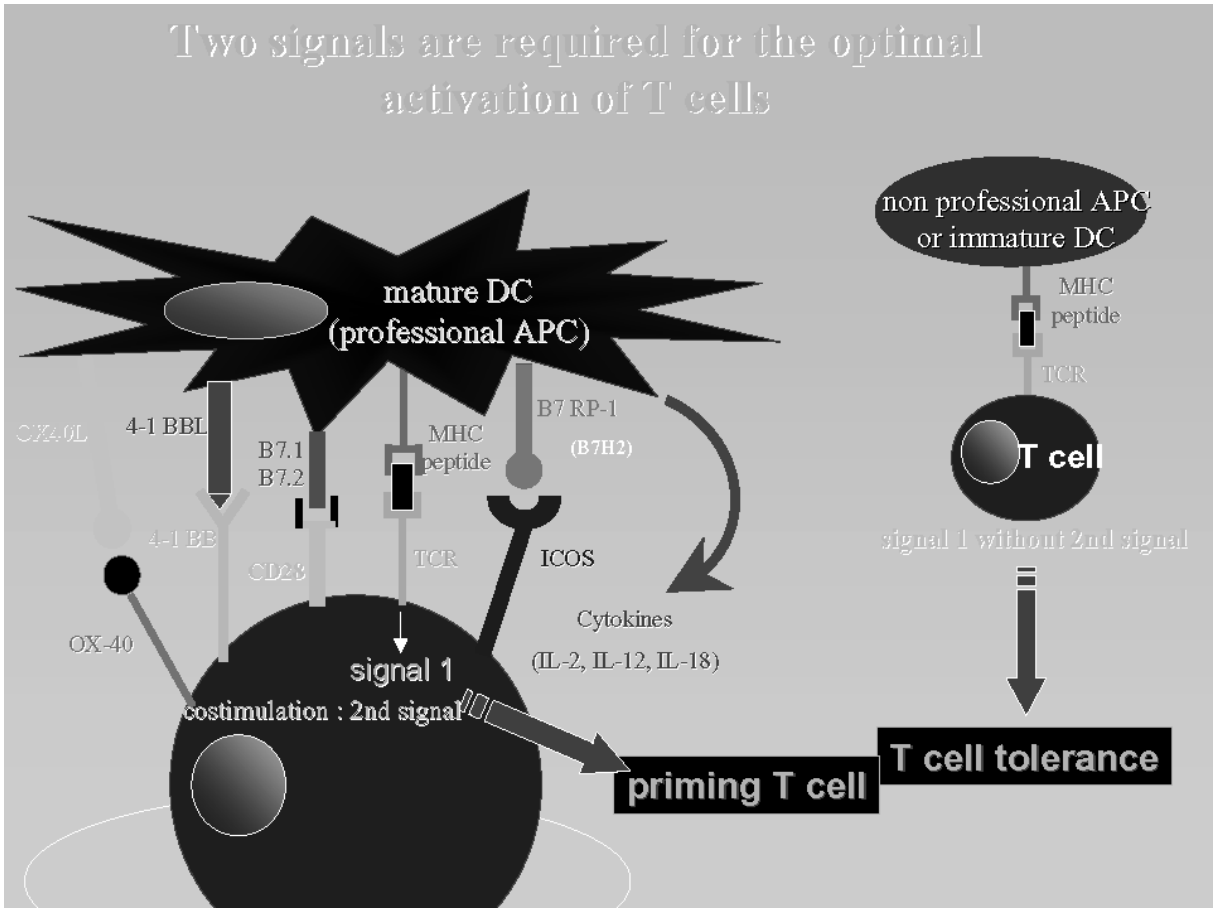
8 melanoma patients were immunized with natural peptide :
$gp100_{209\text{-}217}$ + IFA: 2 T cell response (CD8) (25%) and no clinical response

19 melanoma patients were immunized with modified peptide
$gp100_{209\text{-}2M}$ + IFA + IL-2 : 3 T cell response (CD8) (15%) associated with **clinical response in 42% of patients.**

To improve the peptide immunization efficiency different groups proposed to :

- Increase the binding of peptide to HLA molecule by introducing mutations in wild type tumor peptides which will enhance the affinity of peptide to HLA molecules. This more stable HLA-peptide complex should lead to better induction of CTL…without changing the recognition of the wild type peptide by the induced CTL.

- Peptide will not target antigen presenting cells and in the absence of second signal, this peptide vaccination may lead to tolerance.



ADJUVANT

Use of adjuvants which recruit and/or activate antigen presenting cells.

. GM-CSF

. Anti-CD40

. CpG

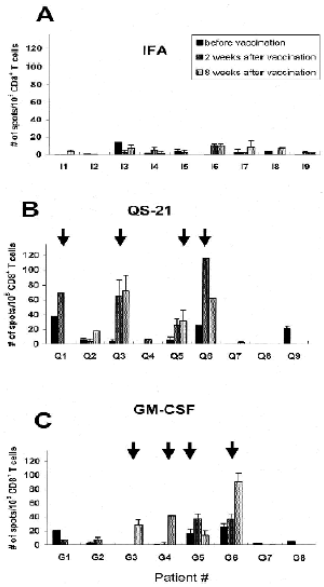
. IFNα

GM-CSF

- In mice, GM-CSF cDNA transfection into tumors increased the immunogenicity of the tumors (Dranoff et al. P.NAS. 1993)

- In human : Immunisation of HLA-A2 melanoma patients with Mart 1, tyrosinase and gp100 peptides during three cycles. The addition of GM-CSF during the 4th cycles increased CTL response against these peptides. (Jager E. Int J. Cancer 1996)

T-cell responses against tyrosinase 368-376(370D) peptide in HLA*A0201+ melanoma patients: randomized trial comparing incomplete Freund's adjuvant, granulocyte macrophage colony-stimulating factor, and QS-21 as immunological adjuvants.



Schaed G Clin Cancer Res 2002

Limits in the development of CD8 peptide based cancer vaccines

- Accumulative evidences also suggest that tumor cells could easi ly escape to immune attacks induced by peptide by means of partial or total loss of the expression of tumor antigen and MHC class I molecules.

- The use of peptides is also restricted to patients with a particular type of HLA and is not applicable to large outbred population.

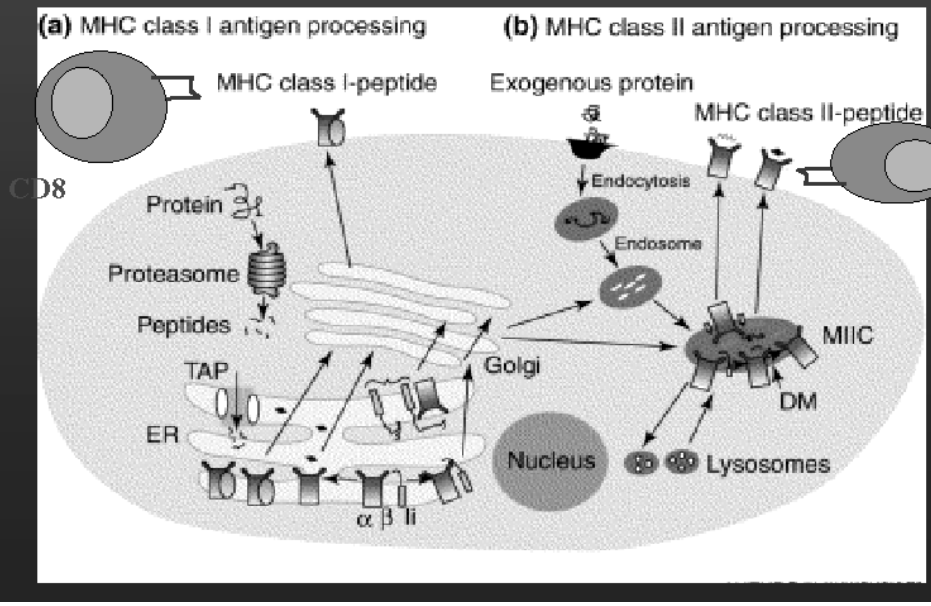
- Polypeptides may allow the activation of CD4 T helper cells which play a critical role in initiating immune response and in the priming and differentiation of CD8+T cells

- In some tumor models, CD4+T cells are the main effector cells responsible for the induction of anti-tumor immunity

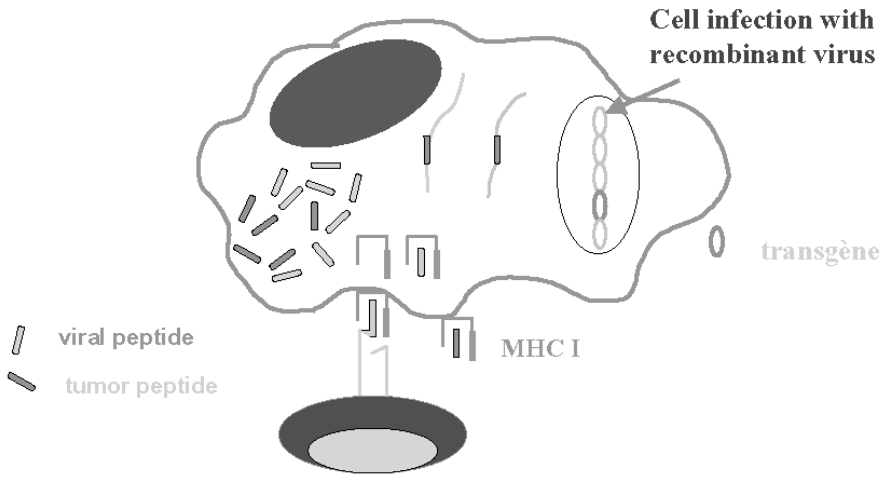
- Unfortunately, recombinant proteins is efficient to elicit humoral responses but failed to induce specific CD8-T lymphocytes.

- Inefficiency of cross presentation may explain this failure.

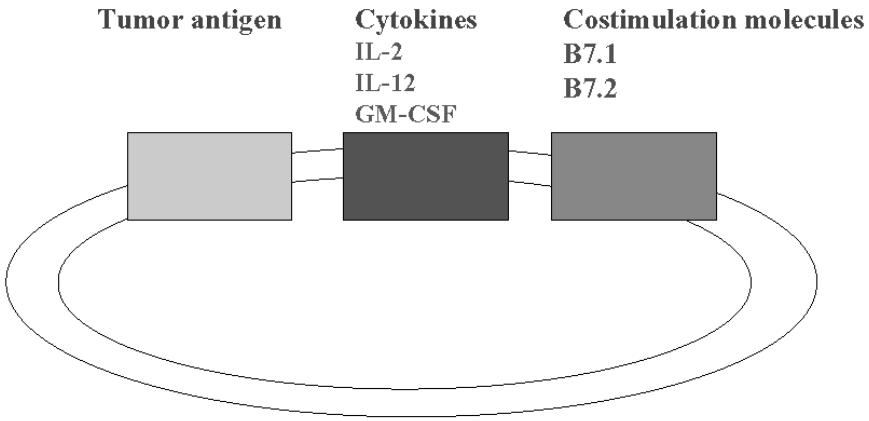
Knowledge in the antigen presentation pathways led to the concept that antigen needs to be vectorized to increase its immunogenicity.



Viruses have the ability to deliver exogenous antigens in the cytosol of the cells



Design of anti-cancer vaccines



Virus : Vaccinia virus, Adenovirus, Retrovirus…

Overview of cancer vaccine protocols based on the use of recombinant viruses.

Vaccinia-E6-E7 HPV16 et 18 **Cervix carcinoma**: .	1 CTL out 3 patients treated	Borysiewicz LK. Lancet 1996
Retrovirus p53 **Non small cell lung carcinoma**	3 local regression out 9 injected tumors	Roth JA. Nature Med 1996
ADV-Mart 1 **mélanoma**	1CR/16 patients 5/23 CTL	Rosenberg JNCI 1998
ADV-gp100 **mélanoma**	No PR no CR 0/16 CTL	Rosenberg JNCI 1998
Vaccine-Muc1 **breast cancer**	2/9 CTL	Scholl S J. Immunother 2000
Alvac-CEA **adénocarcinoma**	No PR no CR CTL 7/9	Marshall JL. J. Clin Oncol 1999

Problems encountered with the use of recombinant virus

- Several safety issues have not been conclusively resolved. For example, reversion of attenuated live vectors to virulent strains by genetic recombination cannot be excluded.

- Even attenuated viral or bacterial strains are associated with health risks for immunodeficient recipients.

- The immune response to virus proteins is a significant limitation to successful immunization in animal and human

Synthetic vectors which target exogenous antigens into the MHC class I pathway

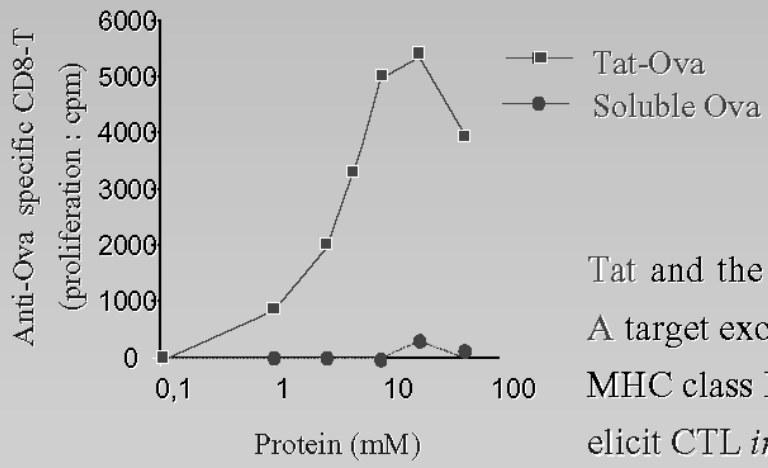
1- Heat Shock Proteins (HSP) GRP94 (gp96), HSP70

2- Toxins
- Adenylate cyclase toxin (CyaA) from *Bordetella pertussis*
- Pertussis toxin from *Bordetella pertussis*
- Lethal Factor from *Bacillus anthracis*
- Shiga toxin from *Shigella dysenteriae*
- Pseudomonas exotoxin A from *Pseudomonas aeruginosa*

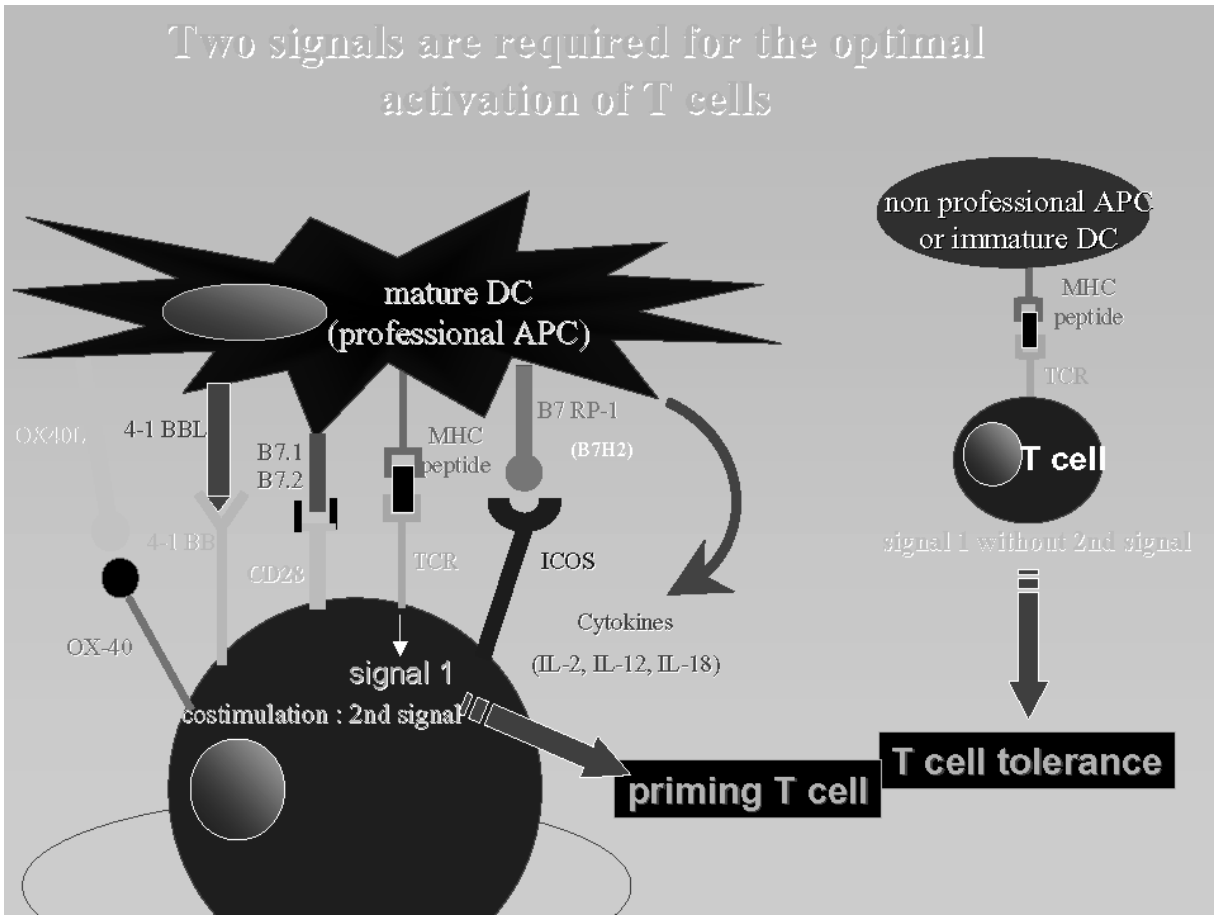
3- Virus Like Particles

4- Other transduction proteins
- Tat (HIV)
- Outer membrane protein A from *Klebsiella*
- Homeodomain from *Antennapedia* (AntpHD)

Targeting exogenous antigens in the MHC class I pathway does not always allow the induction of CTL in mice.



Tat and the Pseudomonas exotoxin A target exogenous antigen into the MHC class I pathway but did not elicit CTL *in vivo*.

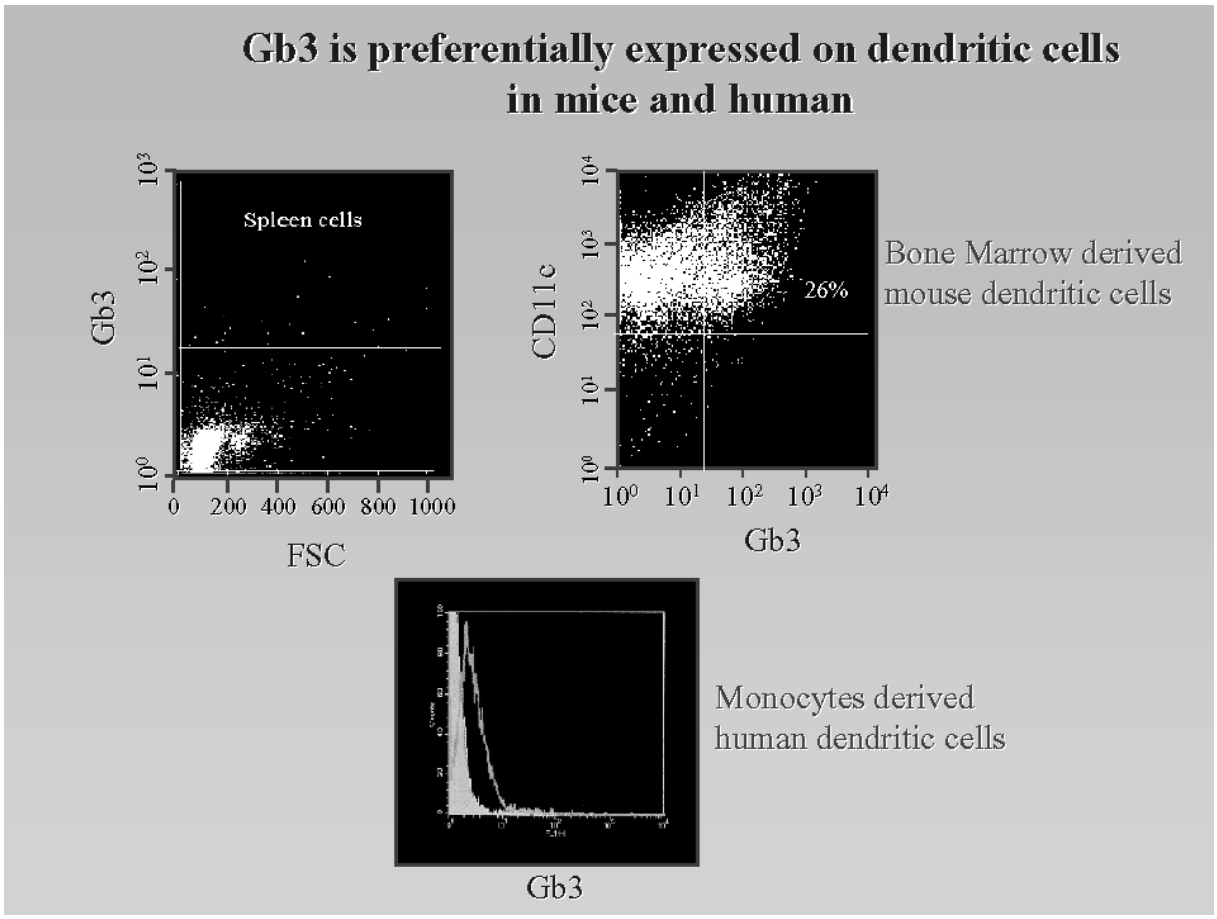


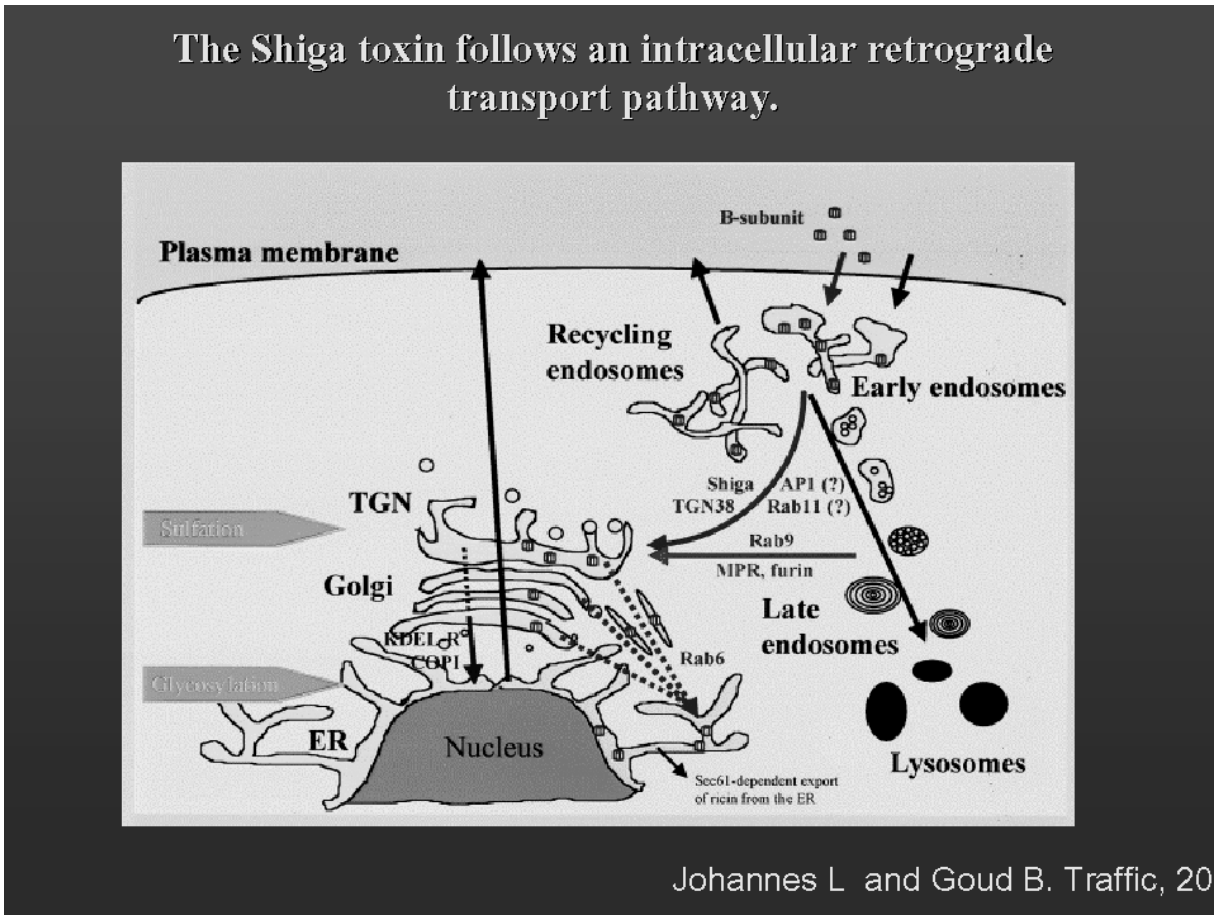
Rationale for the design of a synthetic vector to be developed in cancer vaccines.

- Ability to target exogenous antigen into the MHC class I pathway.

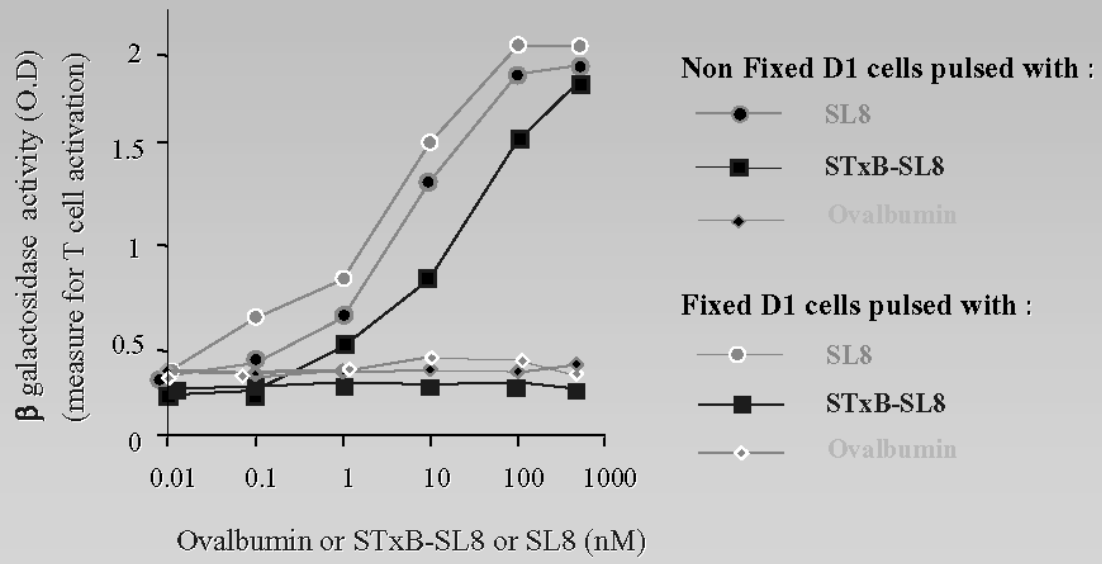
- Preferential delivery of antigen in professionnal antigen presenting cells (dendritic cells).

- Role in the activation and maturation of dendritic cells.

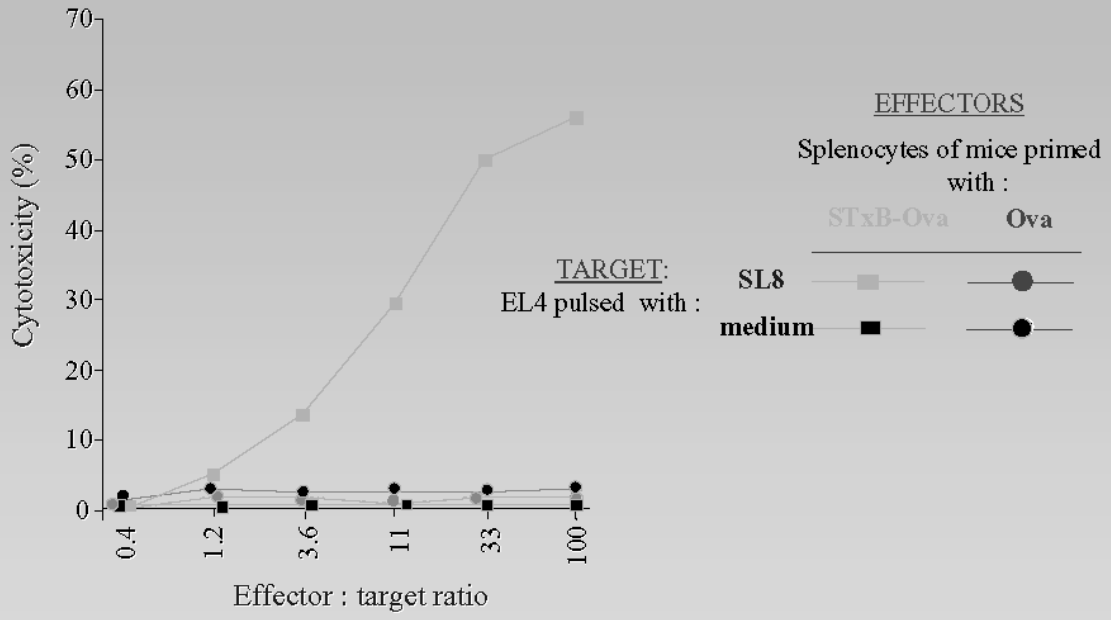

Gb3 is preferentially expressed on dendritic cells in mice and human
Spleen cells
Gb3
FSC
CD11c
26%
Gb3
Bone Marrow derived mouse dendritic cells
Monocytes derived human dendritic cells
Gb3


The Shiga toxin follows an intracellular retrograde transport pathway.
Plasma membrane
B-subunit
Recycling endosomes
Early endosomes
TGN
Shiga
TGN38
AP1 (?)
Rab11 (?)
Rab9
MPR, furin
Golgi
Late endosomes
KDELR
COPI
Rab6
ER
Nucleus
Lysosomes
Sec61-dependent export of ricin from the ER
Johannes L and Goud B. Traffic, 20

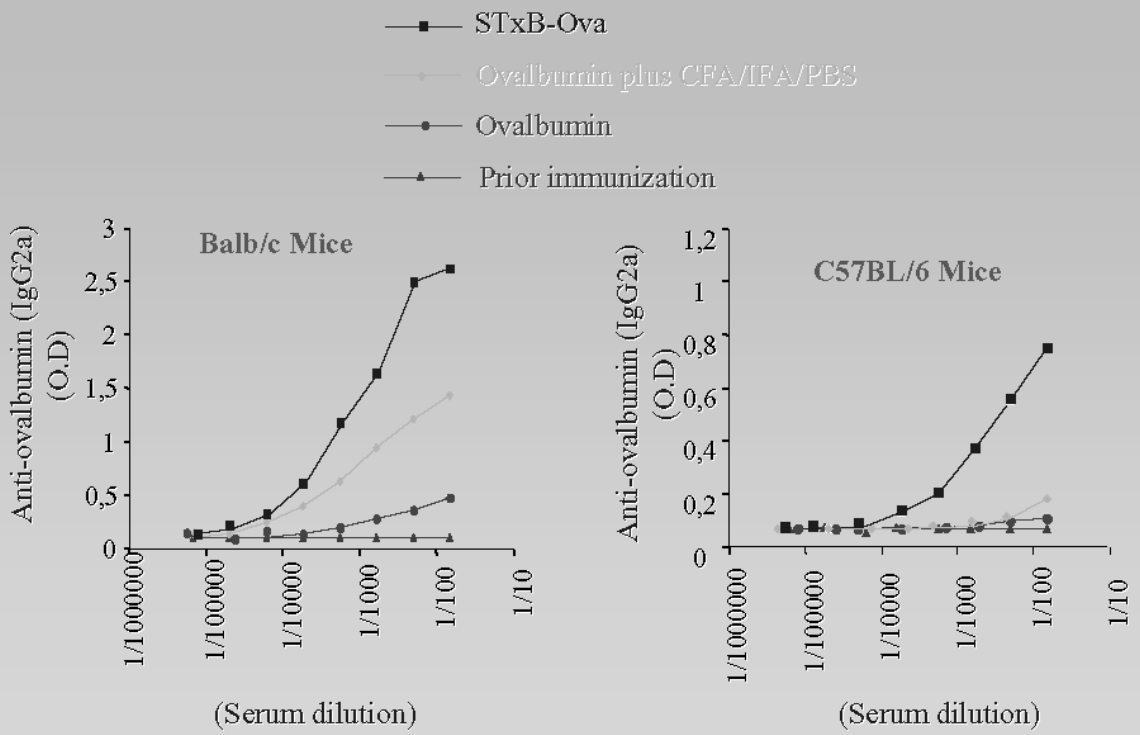
Dendritic cells present CD8 epitopes derived from Shiga-SL8 fusion proteins.



Shiga B subunit coupled to whole protein elicits specific cytotoxic T lymphocytes



STxB increases anti-ovalbumin IgG 2a antibodies



Characteristics of Shiga toxin as a synthetic vector

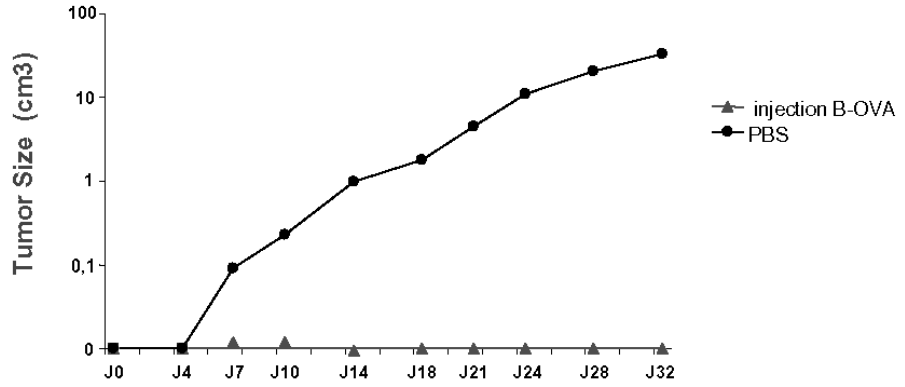
. Targets dendritic cells in mice and human.

. Delivers exogenous antigen into both the classical MHC class I and class II pathway

. Stimulation of CD4 specific T cells with a dominant TH1 polarization

. Induction of CTL against different antigens (P815 A, ovalbumin…) without adjuvant

Vaccination with B-Ova protects against a challenge with Ova-expressing tumor (EG7)



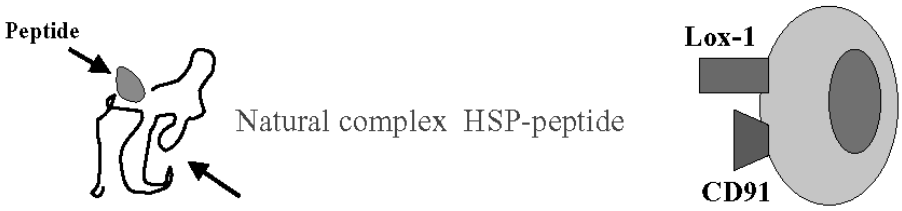
Days after tumor challenge

Comparative features of toxins and related molecules as vectors for cancer vaccines

	Anthrax toxin (Modified Lethal Factor)	Shiga toxin (B subunit)	Bordetella pertussis (Adenylate cyclase)	Kp OmpA
Ability to deliver whole protein	+	+	+	+
Induction of CTL in mice without adjuvant	+	+	+	+
Activation of specific CD4 TH1 cells	+	+	+	?
Preferential targeting of dendritic cells	?	+ Gb3	+ CD11b	+ TLR2
Tumor protection	?	+	+	+

Heat Shock Protein (Gp96/grp94, HSP70, HSP90)

Vector and natural adjuvant of immunity



- HSPs are highly conserved proteins that serve as chaperones for newly synthesized proteins
- HSPs binds preferentially to Antigen Presenting Cells (Lox-1, CD91)
- HSPs facilitate uptake and cross-presentation of the peptides by antigen-presenting cells

HSP = **Antigenic fingerprint of the cells**.

- Vaccination with autologous tumor-derived HSP-peptide complexes has been shown to elicit CTL against peptides complexes with HSP (Srivastava PK. Nat Rev Immunol 2002)

- Vaccination with autologous tumor-derived HSP-peptide complexes also results in both prophylactic and therapeutic antitumor activity in multiple animal tumor models (Tamura Y Science 1997)

Human clinical protocols developed with HSPs

Janetzki S: - gp96 prepared from autologous tumors
- Induction CTL against autologous tumors in 6/12 patients. (**Int J. Cancer 2000**).

Amato R : - gp96 purified from autologous renal carcinomas
- 1CR et 3PR among 16 treated patients (**ASCO 2000**)

Belli F : In five out of 17 patients, T-cell reactivity increased against autologous melanoma after immunization . **J Clin Oncol 2002**

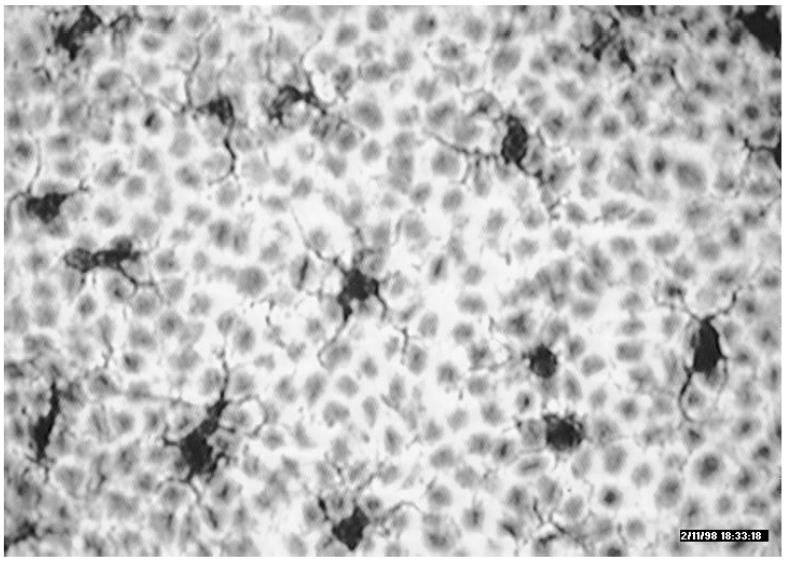
- Two of the 28 patients treated with measurable disease had complete response in multiple, small soft tissue metastases while receiving the vaccine

Limits in the use of HSPs

- Requirement of autologous tumors. Inefficiency of allogeneic HSPs in vaccine protocols.

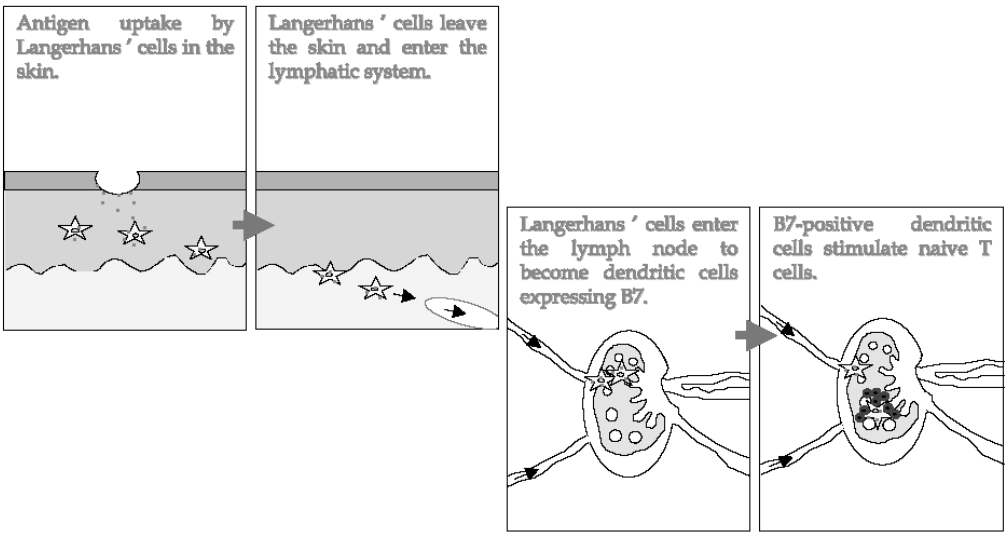
- Purification of HSP is tedious and time consuming.

- Of the 64 patients who had tumor collected, 40% could not receive the initial four weekly injections (ie, one injection per week for 4 weeks), mainly because not enough vaccine could be made or because their melanoma progressed while vaccine was being prepared (Belli F J Clin Oncol 2002)

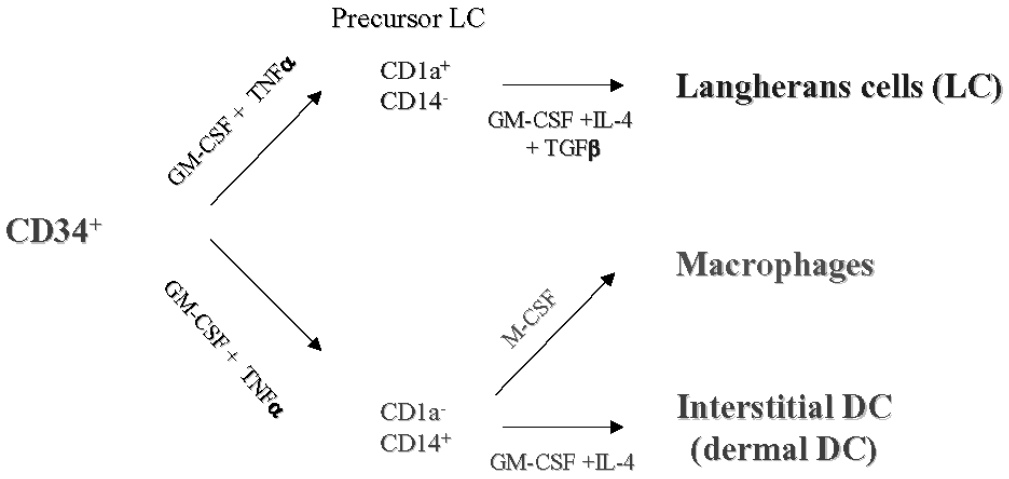


Dendritic cell network in epidermis

Langerhans ' cells can take up antigen in the skin and migrate to lymphoid organs where they present it to T cells.



ORIGIN AND DIFFERENTIATION OF MYELOID DENDRITIC CELLS



Different steps of the immune response involving dendritic cells

- Antigen internalization

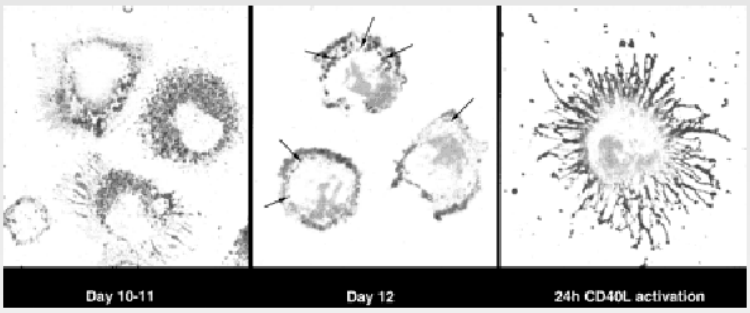
- Migration of dendritic cells to lymph nodes which is associated with their maturation.

- Activation and polarization of T and B lymphocytes in lymph nodes

Analysis by confocal microscopy of the subcellular localization of DC-LAMP during the maturation of CD34+ derived DC.

HLA DR (green)

DC-LAMP (red)



Immature DC | Intermediate | Fully mature DC

Signals required for the maturation of immature human DC

- Ligation of CD40

T cells or anti-CD40 mAb or cells transfected with CD40L (CD154)

- Pathogen derived signals

LPS, double strand RNA, bacteria or bacterial products (SAC, LTA of BG^+, LAM of mycobacteria), immunostimulatory unmethylated CpG oligonucleotides , poly (I:C)

- Cytokines

PGE2, IFNα, TNFα and IL-1β or TNFα alone

- Cell death by necrosis (not apoptosis)

Phenomenon associated with maturation changes of DC

- Increase in the expression of T cell costimulatory molecules like CD86 and CD40

- Increase in the capacity to produce IL-12

- Increase in the capacity to resist to immunosuppression by IL-10

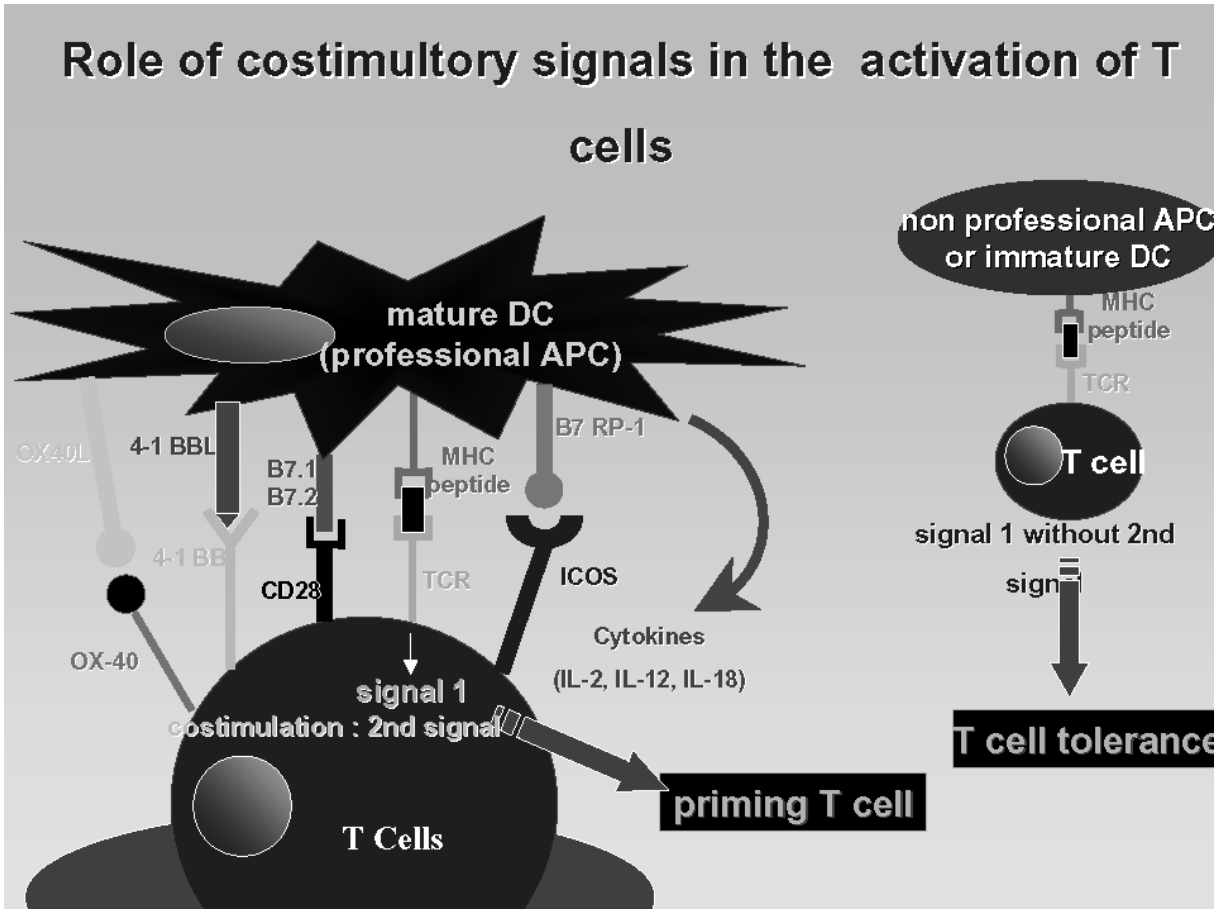
- Development of new repertoire of chemokine receptors, especially CCR7

- Production of DC survival molecules like TRANCE-R (= RANK)

- Redistribution of MHC class II molecules from lysosomes to the cell surface.

- Increase of subunits associated with immunoproteasomes and PA28

- Decreased of phagocytic receptors : $\alpha v \beta 5$ integrin for apoptotic bodies and $Fc\gamma R$ for immune complex.



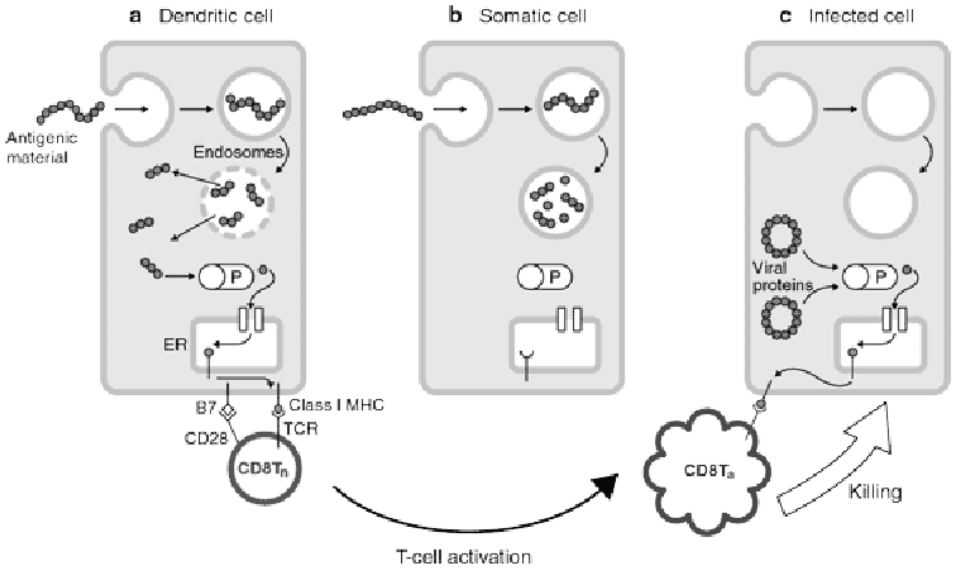
Arguments for using dendritic cells in cancer vaccines

- Cells specialized in the presentation of antigens to T cells.

- Mature dendritic cells produce cytokines favoring TH1 polarization of T cells (IL-2, IL-12, IL-18).

- Dendritic cells have the ability to cross-present antigens.

Ability of dendritic cells to target exogenous antigen in the cytosol



Arguments for using dendritic cells in cancer vaccines

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- Mature dendritic cells produce cytokines favoring TH1 polarization of T cells (IL-2, IL-12, IL-18).

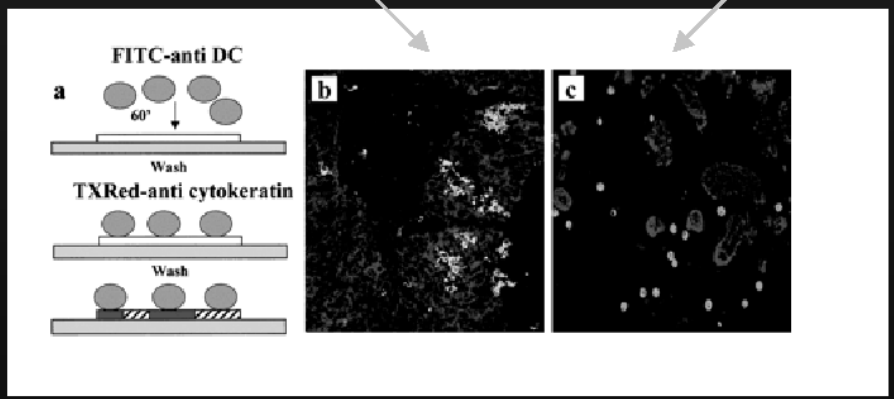
- Dendritic cells have the ability to cross-present antigens.

- Host dendritic cells are blocked in an immature state in tumor microenvironment.

Only immature dendritic cells are found in closed contact with tumors

Immature DC (green) bind to tumor cells (red)

Mature DC (green) don 't bind to tumor cells (red)



(Bell et al 2000)

Mature DC were virtually absent in liver tissue from patients with Hepatocellular carcinoma (Chen S et al. 2000)

Environmental factors and local cytokine production in the tumor microenvironment inhibit DC maturation.

- **PGE2** affects early development of tissue type immature DCs, inducing IL-12 deficient cells with a **TH2 promoting function**.

- **IL-10** inhibits both the ability of DCs to produce IL-12 and their stimulatory capacity . IL -10-exposed DCs have a residual TH2-driving function.
IL-10 treated DC are able to induce **tolerance** in mice and **anergy** in human T cells.

- **IL-6 , M-CSF and VEGF** inhibits the maturation of DC.

Clinical anti-tumor response afer vaccination of mice with dendritic cells pulsed with tumor antigens

TUMORS	METHODS TO LOAD DC	CLINICAL RESPONSES	AUTHORS
- 3 LL (lung carcinoma)	Mut 1 (peptides)	Prevention of tumor growth	Mayordomo JI 1995
- C3-HPV 16	HPV 16 E7 (peptide)	Treatment of established tumor	
- 3T3-p53 fibroblastes transfected with mutated P53	Mutated P53 (peptides)	Treatment of established tumor	Gabrilovich DI 1996
- MCA-205 fibrosarcoma - TS/A (breast carcinoma)	Elution of peptides	Treatment of established tumor	Zitvogel.L. 1996

Clinical anti-tumor response afer vaccination of mice with dendritic cells pulsed with tumor antigens

Tumors	Methods to load DC	Clinical responses	Authors
NFSA-Mart1 (Fibrosarcoma transfected with Mart 1)	Mart 1 (Adénovirus)	**Prevention of tumor growth Treatment of established tumor**	**Ribas A. 1997**
MC-38-Muc 1 (Adénocarcinome transfecté avec Muc 1)	Muc 1 (Adénovirus)	Prévention of tumor growth	**Gong J. 1997**
B16 Melanoma	Tumor lysate	**Treatment of established tumor**	**Shimizu K 2001**
MC38 (Adénocarcinoma)	Fusion tumor-DC	Treatment of established métastases	**Gong J. 1997**

16 MELANOMA METASTATIC PATIENTS

↓

Dendritic cells pulsed ex vivo with peptides or tumor lysates

↓

ADMINISTRATION IN LYMPH NODE

- **No Toxicity**
- **CTL + HSR to tumor antigens in 11/16 patients.**
- **5 Clinical responses (2CR ET 3PR) even in metastatic sites.**

F.O. Nestle . Nature Medecine 1998

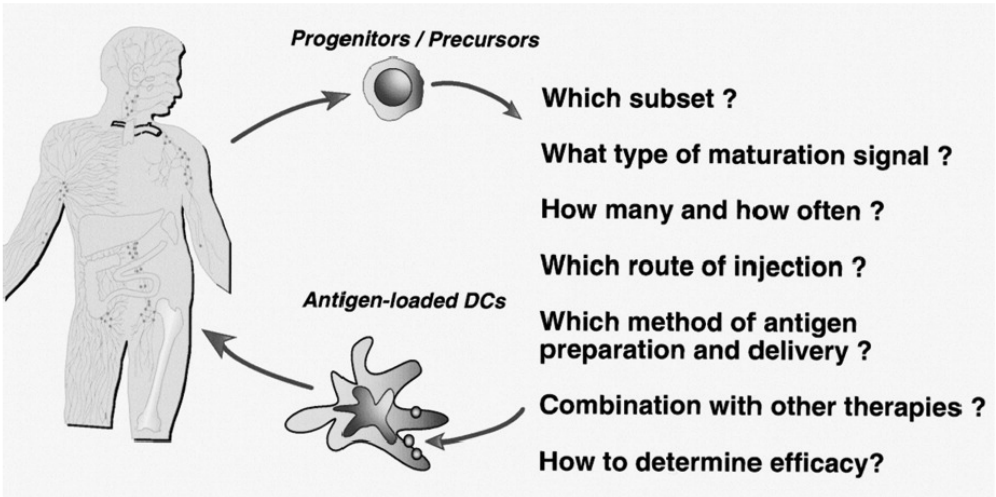
Human Clinical trials with DC

Murphy, Pacific Northwest Cancer foundation
Patients with **prostate cancer** (n = 60)
6 i.v injections (once every six weeks) of $2x10^7$ DCs pulsed with PMSA
25-30% overall response rate

Schuler G., Department of Dermatology, Erlangen, Germany (*J. Exp. Med.*, 1999)
Mature DC pulsed with Mage-3A1 and a recall antigen (TT or tuberculin) in 11 **stage IV melanoma patients**

Expansion Mage 3 -A1 specific CTL in 8/11 patients which declined after the i.v injection.
Regression of individual metastases in 6/11 patients

DC-based Vaccine



Mature or immature DC

- Immature and mature monocytes derived DCs were separately pulsed with a peptide derived from tyrosinase, MelanA/MART-1 or MAGE-1 and a recall antigen. Both DC populations were injected every 2 weeks in different lymph nodes.

- Mature DCs induced increased recall antigen-specific CD4(+) T-cell responses in 7/8 patients, while immature DCs did so in only 3/8 (Jonuleit H et al. Int J Cancer 2001).

- Expansion of peptide-specific IFN-gamma-producing CD8(+) T cells was observed in 5/7 patients vaccinated with mature DCs but in only 1/7 using immature DCs. (Jonuleit H et al. Int J Cancer 2001)

Mature or Immature DCs

The capacity of BmDC to induce an antitumor immune response in vivo correlated to their degree of maturation (Labeur MS et al. J Immunol 1999)

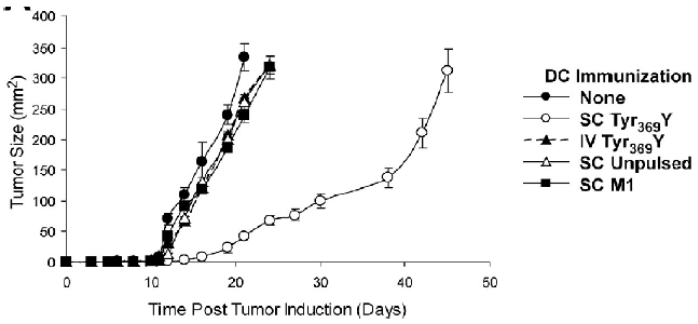
Impaired anti-tumor responses in the absence of CD40/CD154 interactions are the result of a lesion in APC function, namely IL-12 production (Mackey MF et al. J Immunol 1998)

Route of Immunization

DC injected s.c or intradermally migrate to the lymph node. But only 0.4 to 1% of DCs injected s.c reached the draining lymph node (Kupiec-Weglinski JW et al. J Exp Med 1988)(Lappin MB Immunology 1999)

DC administered i.v preferentially migrate to the spleen (< 15% of the number injected) and the lung with no migration or only trace to regional lymph node (Morse MA et al Cancer Res 1999)(Barratt-Boyes SM et al. J Immunol 1997)

In mouse, superiority of s.c injection of DCs over i.v injection in the induction of CTL and anti-tumor immunity against subcutaneously growing tumors (Okada N et al. Br J Cancer 2001; Eggert A et al. Cancer Res 1999; Serody JS et al. J Immunol 2000)

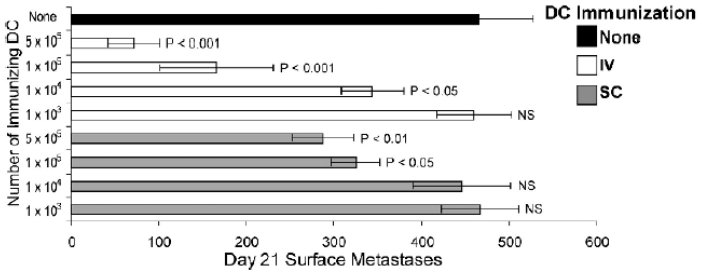


S.C but not i.v immunization with peptide pulsed DCs controls s.c melanoma outgrowth.

Mullins DW. J Exp Med 2003

- In mouse, superiority of s.c injection of DCs over i.v injection in the induction of CTL and anti-tumor immunity against subcutaneously growing tumors (Okada N et al. Br J Cancer 2001; Eggert A et al. Cancer Res 1999; Serody JS et al. J Immunol 2000)

- I.V peptide pulsed activated DC immunization induces memory T cells in the spleen, control metastatic-like lung tumors but not subcutaneous growing tumors (Mullins DW et al. J Exp Med 2003).



Metastatic like lung lesions are controlled by i.v immunization and partially by s.c immunization with peptide pulsed DCs

Mullins DW J Exp Med 2003

- In mouse, superiority of s.c injection of DCs over i.v injection in the induction of CTL and anti-tumor immunity against subcutaneously growing tumors (Okada N et al. Br J Cancer 2001; Eggert A et al. Cancer Res 1999; Serody JS et al. J Immunol 2000)

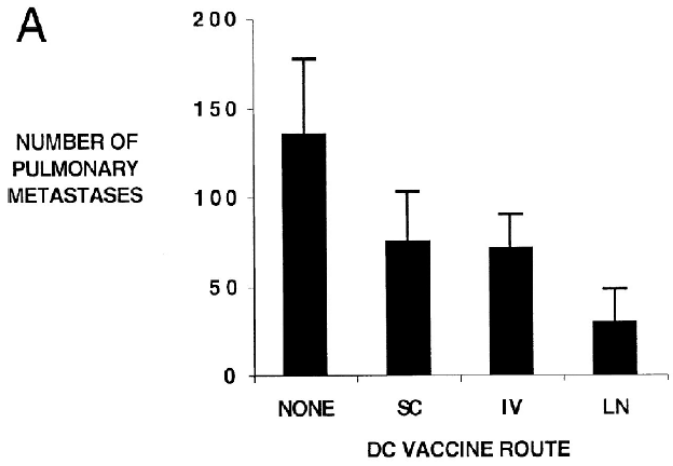
- I.V peptide pulsed activated DC immunization induces memory T cells in the spleen, control metastatic-like lung tumors but not subcutaneous growing tumors (Mullins DW et al. J Exp Med 2003).

- In human, i.v purified DC administration was associated with a significantly higher frequency and titer of Ag-specific Abs (Fong L. J Immunol 2001)

Pulse of DC with antigen coupled to a carrier (KLH) allows an increase in the frequency of antibodies production and IgG isotype (Timmerman JM Blood 2002).

Intranodal immunization

- **Bone marrow-derived, tumor lysate-pulsed DCs administered intranodally generated more potent protective anti-tumor immunity (pulmonary metastases) than s.c or i.v DC immunization. This was associated with greater antigen-specific T- lymphocytes expansion** (Lambert LA. Cancer Res 2001).



Intranodal immunization with TS/A tumor lysate-pulsed DCs is more effective than s.c or i.v immunization

Lambert LA. Cancer Res 2001

Intranodal immunization

- **Bone marrow-derived, tumor lysate-pulsed DCs administered intranodally generated more potent protective anti-tumor immunity (pulmonary metastases) than s.c or i.v DC immunization. This was associated with greater antigen-specific T- lymphocytes expansion (Lambert LA. Cancer Res 2001).**

- **In human, patients were randomly assigned to an intravenous, intranodal or intradermal route of peptide pulsed activated DC immunization.**

All routes of immunization induced comparable increases in tetramer-staining CD8+ T cells.However, the intranodal route induced significantly higher rates for de novo development of CD8+ T cells that respond by cytokine secretion to peptide-pulsed targets (Bedrosian I et al. J Clin Oncol 2003)

Route of Administration and Dose	Tetramer Positivity		Peptide Reactivity		DTH Positivity	
	No. of Patients With Response	No. of Patients Treated	No. of Patients With Response	No. of Patients Treated	No. of Patients With Response	No. of Patients Treated
IV						
5 million dendritic cells	3	4	0	4	0	4
50 million dendritic cells	2	3	0	2	1	3
Total No. of patients	5		0		1	
Total No. of patients		7		6		7
Patients with response, %		71.4		0		14.3
IN						
5 million dendritic cells	3	5	4	5	5	5
50 million dendritic cells	1	2	2	2	2	3
Total No. of patients	4		6		7	
Total No. of patients		7		7		8
Patients with response, %		57.1		85.7		87.5
ID						
5 million dendritic cells	2	3	2	3	2	3
50 million dendritic cells	2	3	0	3	0	3
Total No. of patients	4		2		2	
Total No. of patients		6	6		6	
Patients with response, %		66.7	33.3		33.3	
P, by Fisher's exact test applied to totals		.99	.005		.01	

Abbreviations: DTH, delayed-type hypersensitivity; IV, intravenous; IN, intranodal; ID, intradermal.

Tumor specific peptide reactivity is enhanced after intranodal immunization

Bedrosian I. J Clin Oncol 2003

Route of Immunization and polarization

- KLH-pulsed bone marrow-derived DCs were shown to stimulate a TH-1 cytokine response in the draining lymph node when administered s.c and a TH2 cytokine response when administered i.v (Morikawa Y et al. Immunology 1995)

- Bone marrow-derived, tumor lysate-pulsed DCs administered intranodally generated more potent antigen-specific TH1-type response than when injected s.c or i.v (Lambert LA. Cancer Res 2001)

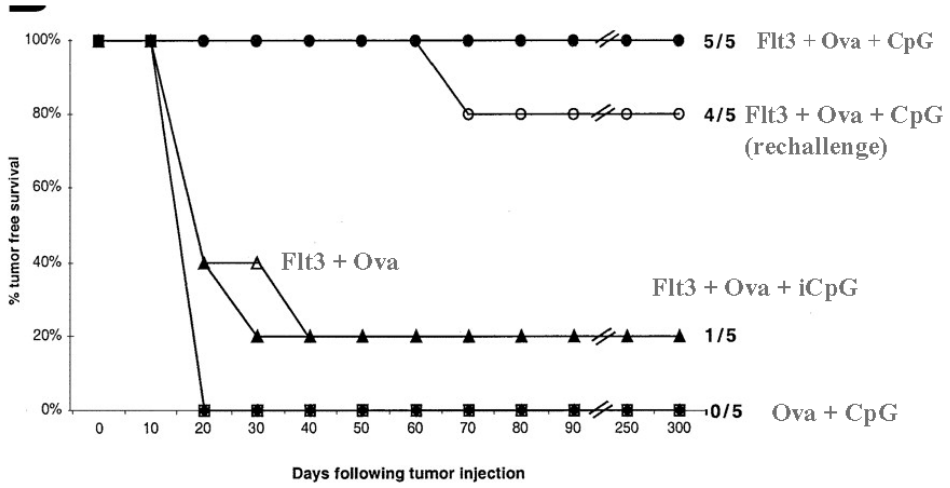
- In human i.d and intralymphatic administrations of purified DC induce TH1 immunity with greater frequency than i.v administration (Fong L et al. J Immunol 2001)

Generation in vivo of mature DC

Flt3 + CpG

Administration of a bone marrow growth factor Flt3 has been shown to expand in vivo the numbers of DCs in lymph nodes, spleen and other tissues (Maraskovsky et al. J Exp Med 1996)
However, a significant proportion of the FL-mobilized DCs are immature and not efficient in inducing Ag-specific T-cell responses.

In mice pretreated with FL, the simultaneous delivery of a tumor antigen and CpG induce potent anti-tumor immunity (Merad M et al. Blood 2002).



Antigen loading and CpG activation of FL-mobilized DCs induce tumor protection

Merad M Blood 2002

Correlation between immune response and clinical response ?

Some studies did not demonstrate any correlation between the induction of immune response and anti-tumor activity.

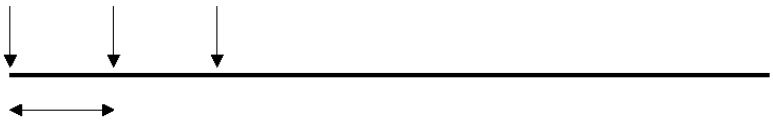
Immunisation of 11 melanoma patients with gp100 modified peptide
$gp100_{209\text{-}2M}$ + IFA : 10 T cell response (CD8) (90%)
Clinical response : No

Immunisation of 19 melanoma patients with gp100 modified peptide
$gp100_{209\text{-}2M}$ + IFA + IL-2 : 3 T cell response (CD8) (15%)
Clinical response : 42%

Rosenberg Nature Med 1998.

Vaccination of metastatic melanoma patients with Mage 3-A1 peptide

Peptide Mage 3

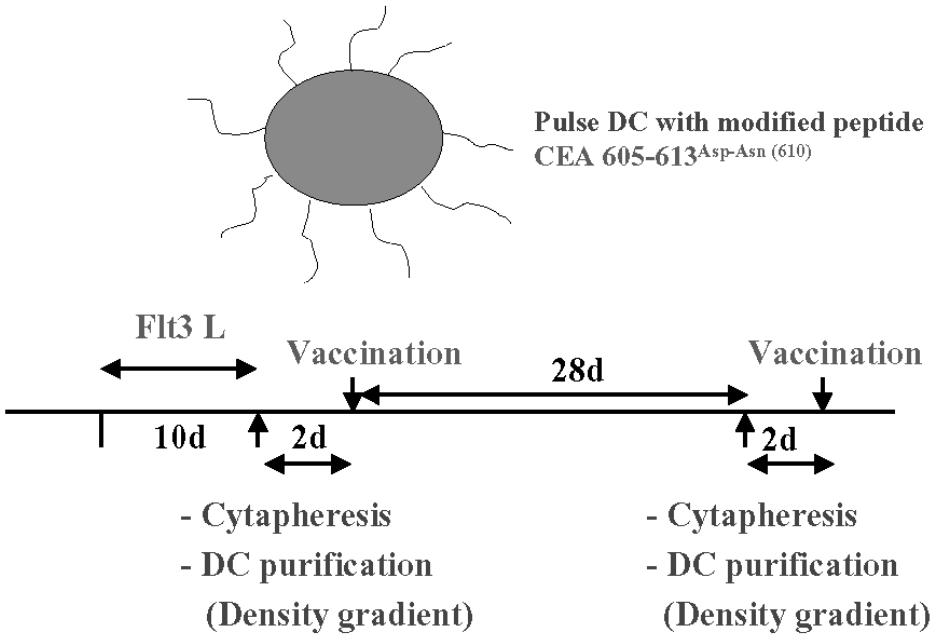


1 month

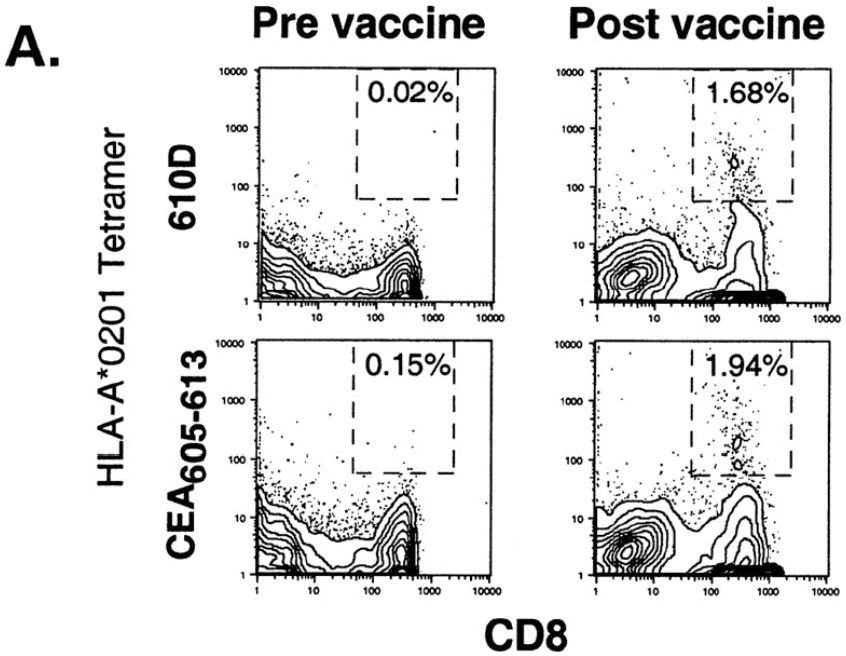
39 patients included.
No toxicity
25 patients received the complete treatment :
4 PR and 3 CR
.
No detection of CTL directed against Mage 3

Marchand et al 1999.

… Whereas other groups reported a correlation between the induction of specific T cells and clinical effects



Phase I : 12 patients
(Colon adenocarcinomas and non small lung carcinomas)

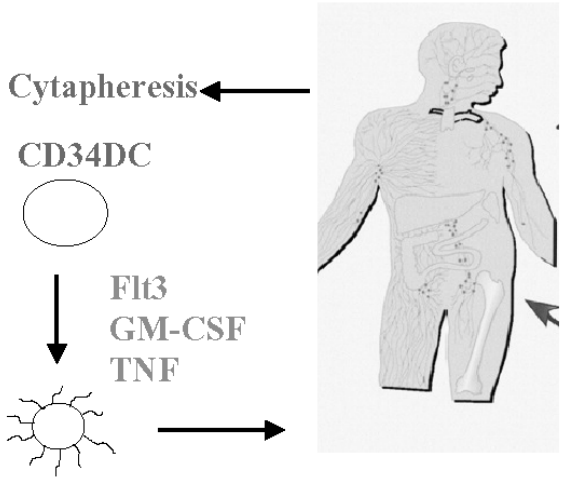


Identification and characterization of antigen-specific CD8 T cells with MHC/peptide tetramers

Correlation between immune and clinical responses* .

Patient	Clinical Response	Tetramer+ Prevaccine%	Tetramer+ Postvaccine%
1	PD	0.08	0.25
2	PD	0.03	0.08
3	**SD**	0.15	**1.11**
4	PD	0.18	0.04
5	**CR-10Mo**	0.4	**1.03**
6	PD	0.10	0.31
7	PD	0.26	0.49
8	**SD**	0.43	**1.05**
9	PD	0.16	0.07
10	PD	0.24	0.5
11	**CR-10Mo**	0.28	**1.03**
12	**MR**	0.12	**1.68**

*** P = 0.002** **Fong L. PNAS 2001**



18 metastatic melanoma pts

Pulse : Tumor peptides :Melan A, tyrosinase,Mage 3, gp100.
Control peptide : Flu-MP
Control protein : KLH

Banchereau Cancer Res 2001

RESULTATS

Cellular response against control antigens :
(CD4 anti-KLH and/or CTL anti Flu-MP) : 16/18 patients.

CTL against ≥ 1 tumor peptide derived from melanoma Ag : 16/18

T-CD8 response	> 2 Mel peptide	< 2 Mel peptide
Tumor progression	1/10	6/7
Regression of at least 1 métastase	7/10	0/7

P =0.015

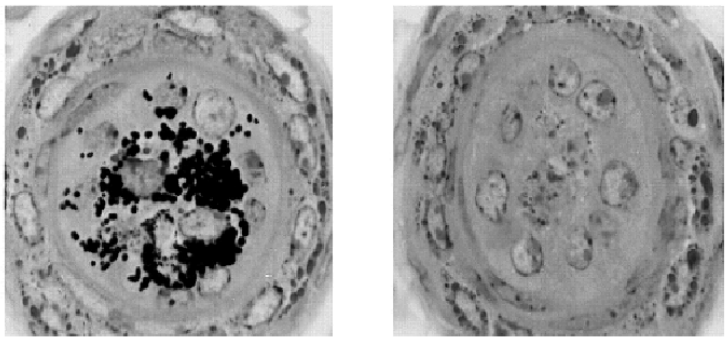
Vitiligo : 2/17 Patients
(responder patients)

AUTO-IMMUNITY AND CANCER

- Anti-tumor CD8-CTL in cancer patients mostly recognize melanoma melanocyte differentiation antigens (Melan A, gp100, TRP1 et 2, Tyrosinase…)

AUTO-IMMUNITE ET CANCER

rVVLacZ rVVmTRP-1



Immunization with rVVTRP-1 induces destruction of cutaneous melanocytes.

0verwijk W et al P.N.A.S.1999

AUTO-IMMUNITY AND CANCER

- **Anti-tumor CD8-CTL in cancer patients mostly recognize melanoma melanocyte differentiation antigens** (Melan A, gp100, TRP1 et 2, Tyrosinase…)

- **Cancer patients** responding to IL-2 therapy **more often develop vitiligo and auto-immune thyroiditis than non responders.** (Rosenberg SA 1996).

- **Autoimmune paraneoplastic syndrome is often associted with anti-tumor clinical response.** (Darnell RB Lancet 1993)

POTENTIAL MECHANISMS LEADING TO TUMOR ESCAPE FROM IMMUNE RECOGNITION.

POTENTIAL MECHANISMS LEADING TO TUMOR ESCAPE FROM IMMUNE RECOGNITION.

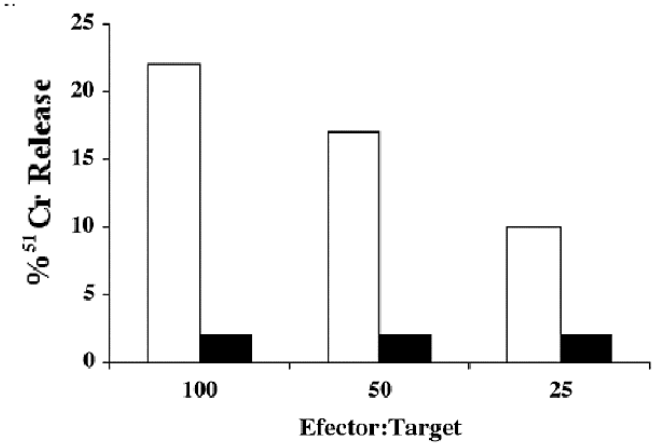
- **Antigen loss or heterogeneity of antigen expression in tumors**

- **Partial or globale Down regulation of MHC class I molecules on tumors (β2 microglobulin loss, peptide transporter defect)**

- **Immunosuppressive factors (IL-10, TGFβ, PGE2…) in the tumor microenvironment.**

- **High expression of molecules associated with tumor resistance to immune attack**

- **Fas ligand expression on tumor cells**

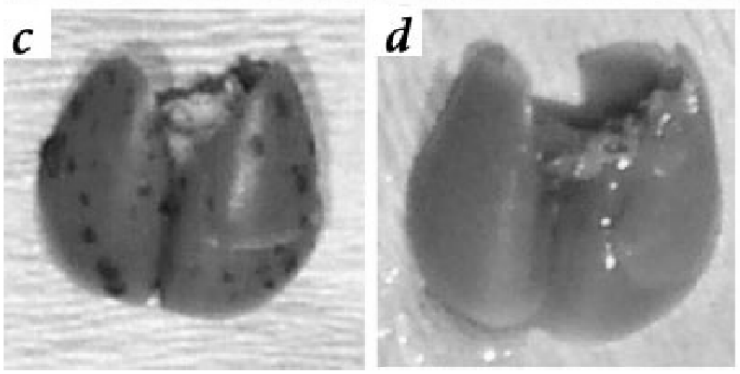


In vivo generation of tumor-specific CD8+ CTLs in the absence of TGFβ- signaling in T cells.

Spleen cells were isolated from transgene-positive (□) or transgene-negative (■) littermate mice challenged with 10^6 live EL-4 cells 10 days prior and were evaluated for their lytic activity by the 51Cr release assay against EL-4 targets.

Gorelik L Nature Med 2001

Wild Type Mice **DN TGFRII Mice**



2×10^5
B16-F10 i.v.

Blockade of TGF- signaling in T cells renders mice resistant to tumor challenge.

Gorelik L. Nature Med 2001

Molecular or functional defects of T lymphocytes infiltrating tumors.

- Defect in the expression of the ζ chain of -CD3

-Defect in the expression of transcription factors belonging to NF-Kappa B family
. p65-Rel A
. c-Rel
. p50

- Defect in the expression of Jak 3

- Low perforine **expression within T cells**

-Expression of KIR by TIL

- Bias in TH2 polarization of TIL

Présence of « suppressive » T lymphocytes

- During cancer progression, increase in the frequency of regulatory T lymphocytes (CD4+CD25+, or Tr1 producing IL-10 or TGFβ or NKT secreting IL-13) able to inhibit the activity of specific anti-tumor lymphocytes.

- In murine models, depletion of CD4+CD25+ T lymphocytes increases the induction of immune response and is associated with tumor regression.

Cancer Vaccine Indications

- **Residual Disease**
- **Adjuvant**
  (After surgery if presence of risk factors)
- **Genetic predisposition to cancers**

PERSPECTIVES

- Molecular identification of tumor antigen associated with the rejection of tumors.

- Better definition of the phenotype of CD8-T cells to be induced after vaccination : avidity Tc1-Tc2, duration of the persistence of the T cell response

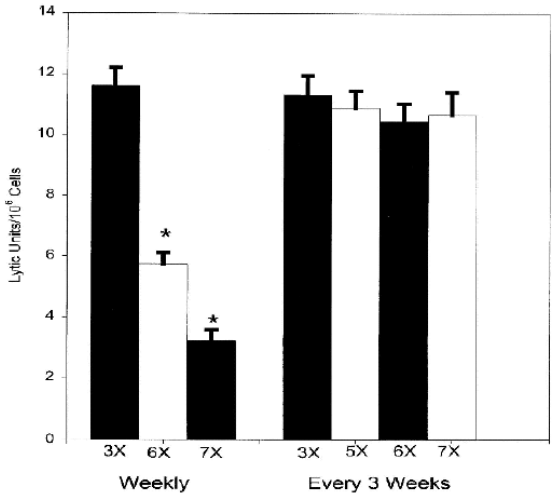
- Better understanding of the factors responsible (chemokines, Adhesion molecules) of the tumor homing of T lymphocytes.

Table 1 Percentage of PR1-specific CTL from PBMC of CML patients

Treatment Group	UPN	Stage[a]	Time from Diagnosis[b]	Treatment Duration[c]	%Ph[d]	Response Type[e]	%PR1-CTL[f]
Interferon	1	CP	15	15	54	MR	1.06
	2	CP	20	18	40	MR	1.02
	3	CP	5	3	75	MR	.51
	4	CP	40	11	38	MR	1.52
	5	CP	18	18	0	CR	.42
	6	CP	10	10	28	PR	.66
	7	CP	9	9	25	PR	.43
	8	CP	10	10	10	PR	.32
	9	CP	14	14	0	CR	1.24
	10	CP	16	16	10	PR	.29
	11	CP	12	12	4	PR	.43
$P = 0.0002$							
	12	CP	42	40	85	MR	< 0.01
	13	CP	12	12	100	None	< 0.01
	14	BC	4	4	100	None	< 0.01
	15	BC	2	2	100	None	< 0.01
	16	CP	5	5	95	None	< 0.01
	17	CP	20	16	100	None	< 0.01
	18	CP	30	24	100	None	< 0.01
	19	CP	15	13	100	None	< 0.01

Molldrem Nature Med 2000

Schedule of DC administration



Weekly administration of peptide-pulsed DCs led to diminishing CTL activity after 6 wk of treatment. This was not found in animals injected with DCs every 3 wk for six treatments

Serody JS J Immunol 2000

Additional Bibliography

ELSEVIER

Vaccine 20 (2002) A32–A39

Vaccine

www.elsevier.com/locate/vaccine

Synthetic and natural non-live vectors: rationale for their clinical development in cancer vaccine protocols

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Abstract

Different arguments suggest that cytotoxic CD8 T lymphocytes (CTL) play a key role in the protection against tumors and in the establishment of anti-tumor immunity. Unfortunately, administration of soluble proteins alone generally does not induce CD8+ T cells presumably because antigen derived peptides are not introduced into the major histocompatibility complex (MHC) class I antigen presentation pathway. Attenuated recombinant live vectors such as viruses or bacteria which have the ability to deliver antigen into the cytosol of cells have been shown to induce cytotoxic T cell response. However, there are safety concerns associated with these approaches especially in immunodeficient patients. Synthetic vectors such as heat shock proteins, virus like particles (VLP) and liposomes could deliver exogenous protein into the cytosol of cells associated with the induction of CTL and tumor immunity. We and other groups have sucessfully exploited the original intracellular traffic of toxins to use them as vectors for tumor antigens.

Keywords: Synthetic vector; Cancer vaccine; Toxin

1. Introduction

CD8+ T lymphocytes (CTL) have been shown to be important mediators of anti-tumor immunity in various animal models. Several observations also suggest a role of CTL in the control of tumor growth in humans. In biopsies from human regressive melanoma an in situ amplification of CTL with anti-tumor activity was demonstrated suggesting that these cells may contribute to the host dependent elimination of tumor cells [1,2]. Recently, a correlation was reported between the efficiency of a cancer vaccine based on dendritic cell therapy and its ability to elicit tumor specific CTL [3,4]. The stimulation of specific CTL therefore represent one major goal in the design of cancer vaccines [5].

CTL recognize antigens as short peptides of 8–10 amino acids associated with major histocompatibility complex (MHC) class I molecules on the surface of cells. The majority of these peptides are derived from the cytosolic degradation of endogenous cellular protein. In contrast, exogenous soluble antigens enter the endosomal pathway and after being degraded in endosomal compartments, the peptides generated associate with MHC class II molecules; then the complex is presented to CD4− T lymphocytes. These observations may explain why in most cases soluble peptides or proteins fail to prime CTL in vivo. To circumvent this difficulty, various vectors have been developed to target exogenous antigens into the MHC class I restricted pathway.

Attenuated recombinant live vectors such as viruses or bacteria and naked plasmid DNA encoding large antigenic proteins have been shown to induce cytotoxic T cell responses [6–8]. However, there are safety concerns associated with these approaches. Reversion of the attenuated live vector to a virulent strain by genetic recombination cannot be excluded. Some viral vectors may cause chromosomal deletions and other rearrangements when they integrate into the genome of human cells [9]. Attenuated organisms also pose a particular risk to immunodeficient recipients [10]. Regarding DNA vaccines, the consequence of stable integration of exogenous DNA into the host genome is not well established. This explains the development of alternative strategies of induction of CTL based on the use of synthetic vectors.

2. Synthetic and non-live natural vectors with the ability to target exogenous peptides into the cytosol and induce CTL

A series of antigen delivery systems that efficiently introduce exogenous molecules into the MHC class I pathway have been described.

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2.1. Heat shock protein and other endoplasmic reticulum (ER) chaperones: natural host vector

Heat shock proteins are molecules induced by heat shock, glucose deprivation and other stress but most HSP are present in abundant levels under normal conditions. HSP70 and HSP90 act as chaperones for peptides in the cytosol, whereas GRP94 (gp96) like other ER chaperones molecules such as calreticulin associate with ER lumenal peptides [11]. HSP complexed with antigenic peptides have been shown to efficiently deliver peptides into the MHC class I processing pathway (cross presentation) [12]. Similarly, murine dendritic cells sensitized with calreticulin associated with an ovalbumin derived peptide efficiently processed and presented this peptide to specific class I restricted T cell hybridomas [13].

Vaccination of mice with HSP was performed with either natural cancer-derived HSP thought to be linked with a large array of tumor peptides or with HSP reconstituted with specific CTL epitopes in the presence or absence of a linker. In both cases a CTL response was elicited against the bound peptides [14–16].

2.2. Toxins

Cell biology of bacterial toxins has shown that the entry and intracellular transport of these molecules is often unconventional and most often allows for the targeting of associated antigens into the cytosol. To face the problem of toxicity, genetic engineering of the wild type toxin was performed. In fact, toxins are often composed of a catalytic A subunit which mediates the toxicity and a B subunit which binds to the receptor and allows for the intracellular traffic of the toxin. In some cases, peptide was directly coupled to the B subunit which has been shown to act as vector in absence of the A chain. We have fused the B subunit of the Shiga toxin derived from *Shigella dysenteriae* to different peptide epitopes and have shown that it could deliver these peptide into the MHC class I intracellular pathway [17,18]. When these Shiga B fusion proteins were injected in mice, CTL were produced that recognized the peptide associated to the B subunit [18]. In other cases, the catalytic domain of a toxin subunit was modified and/or substituted by antigenic peptides. The anthrax toxin derived from *Bacillus anthracis*, is composed of three proteins that act in binary combination to elicit toxic effects, edema and death. Lethal factor (LF) and edema factor (EF) are intracellularly acting proteins, both of which require protective antigen (PA) for their translocation into the cytosol of eukaryotic cells. The amino-terminal domain of LF (LFn) has been found to contain the information necessary for binding to PA and mediating translocation. This domain alone lacks the lethal potentiality which depends on the putatively enzymatic carboxyl-terminal moiety. Different studies have shown that fusion proteins consisting of LFn fused to peptide, when mixed with PA, allow for MHC class I restricted presentation of the associated peptides and stimulate specific CTL in mice [19].

Bordetella pertussis secretes two toxins, the pertussis toxin (PT) composed of an enzymatically active A subunit (S1) associated with a B oligomer (subunit S2-S5) and the invasive adenylate cyclase toxin (Cya A), a 1706 amino-acid long protein containing a 400-residue amino-terminal domain that corresponds to the calmodulin-activated catalytic region. Introduction of mutations in the catalytic region of these molecules led to the production of detoxified toxins. CTL epitopes were fused to the N terminus of the S1 subunit of PT or introduced into the permissive site of the catalytic domain of CyaA. These inactivated toxins were efficient in delivering exogenous peptides into the MHC class I pathway and eliciting CTL against the CD8 epitopes linked to these vectors [20–22].

2.3. Other protein transduction molecules

- The homeodomain of the Antennapedia molecule (AntpHD), a transcription factor of *Drosophila*, spontaneously crosses cellular membranes without being targeted in the lysosome. Antigenic peptides fused to the homeodomain of Antennapedia are processed and presented by MHC class I molecules. Immunization of mice with these fusion proteins could prime CTL. However, the priming required the presence of SDS [23].
- Outer membrane protein A from *Klebsiella pneumoniae* (kpOmpA) form stable complexes with hydrophobic peptides such as melanoma derived peptides and their administration in mice allows for the generation of CTL [24].

3. Mechanisms underlying the delivery of exogenous antigens into the MHC class I pathway

3.1. Delivery of exogenous antigen into the classical MHC class I pathway

As a general rule cytosolic proteins are processed by the multi-enzymatic complex proteasome; the peptides released are then transported by the TAP proteins to the endoplasmic reticulum (ER) where they associate with nascent class I molecules before transport of the complex to the cell surface. The availability of inhibitors of each step of this process enable to follow the pathway of intracellular protein traffic.

- ATX from *B. anthracis* consists of a monomeric cell binding protective antigen (PA) that can form a heptamer once activated by a cell surface protease. Heptameric PA can then bind the enzymatic toxin monomers, LF and/or the EF. After binding to specific surface receptors, the protein complex is endocytosed and trafficked to the endosome, where the bound LF or EF is translocated to the cytosol following endosomal acidification. The recombinant LF associated with peptides appeared then to follow

the normal step of processing by proteasome and further association and the endoplasmic reticulum with MHC class I molecules [25].

- The detoxified Cya A and the B subunit of the Shiga toxin also target exogenous antigens in a classical MHC class I restricted presentation pathway, since the presentation of CD8 epitope is inhibited by both lactacystin, an inhibitor of the proteasome, and brefeldin A which disrupts the Golgi apparatus. It requires the presence of TAP molecules. However, the mechanism of membrane translocation of the fusion protein to the cytosol has not yet been identified [18,26].

3.2. Delivery of exogenous antigen into unconventional MHC class I pathways

- MHC class I restricted presentation mediated by recombinant pertussis toxin (PT) was inhibited by BFA but not prevented by proteasome inhibitor and also occurred in antigen presenting cells from TAP deficient mice [20]. It is hypothesized that PT can deliver epitopes to class I molecules directly by retrograde transport to the ER, thus bypassing the proteasome and TAP dependent endogenous pathway for presentation [27].
- TAP and proteasome independent processing also seems to occur in the case of recombinant pseudomonas exotoxin A (see further) which is endocytosed following binding to the α2 macroglobulin receptor. Antigen presentation with detoxified PexA was also BFA-and lactacystin insensitive and not inhibited by LLnL (calpain inhibitor) which blocks the activity of some ER resident proteases and NH_4Cl which raised the pH of lysosomes [28,29]. These results suggest that epitope generation occurs in compartments (early endosome?) that contain mature or recycling class I molecules that are relatively close of the cell surface [30]. In support of this hypothesis, it was recently found that a small fraction of class I molecules enter acidic endosomes, the pH of which could allow for peptide exchange, before transport to the cell surface and that this could apparently account for non-classical class I presentation of a viral antigen [31].

We don't know if the repertoire of peptides generated by this non-classical MHC class I pathway is similar to the one obtained with endogenous protein or vectors which deliver their immunogenic peptides in the conventional MHC class I pathway. The use of these vectors may allow for the presentation of peptides different from those naturally presented by tumor cells.

3.3. Phagocytosis

The groups of Harding and Song [32] and Kovacsovics-Bankowski and Rock [33] showed that exogenous antigens coupled to different types of particles (beads made of latex, iron or silica) could be targeted into the MHC class I pathway. This effect was due to the internalization of the particles by phagocytosis. A transfer of the protein or peptide from the phagosome to the cytosol was hypothesized to explain the MHC class I presentation of peptides derived from exogenous antigen [33]. Virus like particles (VLP) (see further) also likely enter the cytosol from endosomes and target the classical MHC class I pathway [34].

4. Targeting exogenous antigens into the MHC class I pathway does not always allow for the induction of CTL in vivo

When the Tat protein derived from HIV was conjugated to different enzymes, it was shown that the conjugated proteins crossed the cellular membrane and maintained enzymatic activity suggesting that transport outside the lysosomal compartment occurred. Identification of the short cationic peptide derived from HIV-1 Tat which mediates this translocation has led to the conjugation of proteins such as Ova to this peptide. When antigen presenting cells were exposed in vitro to such protein conjugates, they processed and presented the peptides in association with MHC class I molecules. However, the fusion proteins could not prime CTL in vivo [35].

Similarly, exogenous fusion proteins consisting of the binding and translocating domains of pseudomonas exotoxin A (PE) fused with CTL epitopes are internalized, processed, targeted to and presented by MHC class I molecules but they failed to induce CTL [28,36]. These two observations suggest that in addition to the delivery of antigen into the MHC class I pathway, other factors are required to prime the cellular immune response in vivo.

5. The in vivo efficiency of synthetic vectors is in part determined by their ability to target and activate DC

For efficient T cell priming, two signals have to be delivered to T cells by APC: the first is represented by the recognition of MHC–peptide complex and the second signal requires the expression of costimulatory molecules which are essentially displayed on the membrane of professional antigen presenting cells such as mature dendritic cells and to a lesser extent B lymphocytes. Administration of antigen without any cell specificity will be mainly loaded on non-activated antigen presenting cells and will elicit T cell tolerance [37,38].

These considerations may explain the failure of the Tat peptide or pseudomonas exotoxin A to prime CTL in vivo, since these vectors translocate exogenous peptides in many cell types without any specificity for professional antigen presenting cells.

In contrast, efficient synthetic and natural non-live vectors seem to preferentially target dendritic cells and other antigen

presenting cells (B lymphocytes). For example, CyaA binds to CD11b whose cellular distribution mostly includes dendritic cells, macrophages and neutrophils [39]. The B subunit of the Shiga toxin interacts with the glycolipid Gb3 which is mainly express on dendritic cells, B lymphocytes and some epithelial cells [18,40]. Outer membrane protein A from *K. pneumoniae* specifically bound to professional antigen presenting cells through toll-like receptor 2 [41].

Only professional APCs like dendritic cells, macrophages and B cells are able to bind gp96 molecules [42].

Some of these vectors such as virus like particles, HSP, KpOmpA and PT have also been shown to induce the maturation of DC and to increase the costimulatory capacity of other antigen presenting cells [42–46].

6. Synthetic vectors able to prime CTL without adjuvant

Usually CD4 help is required to allow the priming and differentiation of CTL secondary to the triggering of maturation of dendritic cells. It was therefore not surprising that vectors with the ability to activate dendritic cells such as HSPs, KpOmpA, PT or VLPs could prime specific CD8+ CTL in the absence of CD4+ T cell help or adjuvant [11,20,42,47]. Interestingly, the B subunit of the Shiga toxin and the Cya A derived from *B. pertussis* have not been shown to directly activate DC but were able to stimulate an epitope specific CTL response in mice without any adjuvant [18,48]. Their in vivo ability to induce some DC maturation factors such as TNFα may explain the relative efficiency of these vectors [49,50].

7. Advantages of selecting synthetic vectors with the ability to enhance the immunogenicity of whole protein compared to the vectorization of peptide alone

7.1. Limits and pitfalls of the vectorization of peptide for cancer vaccines

For most tumor antigens, immunodominant and protective peptides have not been identified. In rare cases in which some peptides derived from tumors have been shown to be naturally recognized by T cells, these immunodominant peptides may vary from individual to individual, which may explain that even in subjects with the same HLA Class I haplotype, the detectable CTL response to a given antigen will not necessarily be similar [51]. In addition, peptide vaccination is restricted to patients with a particular type of HLA and is not applicable to large outbred populations.

A vaccine formulation that delivers only peptides derived from tumor antigens will also very likely fail to induce a significant degree of protection against tumors because of the heterogeneity of antigen expression and immunoselection mechanisms which could only be circumvented by anti-tumor polyclonal T cell activation [52–54].

As shown in Table 1 some inactivated toxins (anthrax toxin, CyaA, Shiga toxin) and bacteria derived protein (KpOmpA) were efficient vectors for transferring polypeptides or whole proteins into the MHC class I pathway and eliciting a CTL response [25,41,55].

7.2. Delivery of exogenous antigens in both the MHC classes I and II pathways resulting in the activation of a CD4 T cell response and a humoral response

Helper T-CD4+ T cell responses play a critical role in initiating the immune response and a key role in the priming and differentiation of CD8+ T cells mediated in part through the maturation of APC via their interaction with CD40 [56]. This activation of CD4+ T cells may be essential to support the efficacy of synthetic vectors which do not directly induce the activation of dendritic cells.

CD4+ T cell responses will also help with the induction of the humoral immune response which has been associated with tumor protection in melanomas, lymphomas and breast cancers [57–59].

Different synthetic vectors have been shown to vehicule polypeptides and to activate CD4 T cell help and humoral responses.

- HSP110 is a molecular chaperone highly efficient in binding to large protein substrates. When non-covalently complexed with the intracellular domain of human epidermal growth factor receptor protein 2 (ICD-HER-2/neu), it elicited both CD4 and CD8 T cell responses against peptides derived from ICD-HER-2/neu and significantly enhanced ICD-HER-2/neu specific antibody responses related to those seen with recombinant ICD-HER-2/neu [60].
- Some capsid proteins from different viruses like parvovirus, papillomavirus, and hepatitis B virus can self-assemble into VLP. When fused to other proteins, they keep their properties to form pseudo viral particles [34]. Different investigators have shown that CD4 T cell responses and MHC class I restricted CTL were efficiently primed when antigens were fused or combined with these particles [47,61,62]. Due to the highly repetitive structure of such VLPs, they have also been shown to be very immunogenic for B lymphocytes and induce strong and long-lasting IgG responses in the absence of adjuvants in both animal models and human clinical trials [63,64].
- Liposomes encapsulate peptides or proteins and target liposomal antigen both to the MHC classes I and II pathway, which may explain the ability of liposomes to stimulate both antibody and CTL responses [65]. The main concerns about the use of liposomes is that a consensus to optimize the formulation of liposomes has not yet been established.

Table 1
Main characteristics of synthetic and non-live natural vectors

Vectors	Vectorization of whole protein	Maturation of DC	CTL induction in mice	Humoral response in mice	Anti-tumor clinical responses
HSPs	+	+	+	+	+
VLPs	+	±	+	+[a]	+
Liposomes	+	−	+	+	+
Toxins					
Cya A (*B. pertussis*)	+	?	+	?	+
Lethal factor (anthrax)	+	?	+	?	?[b]
Shiga B subunit	+	−	+	+	?
Pertussis toxin	?	±	+	?	?
Protein transduction molecules					
AntpHD	?	?	+[c]	?	?
KpOmpA	+	+	+	+	+

[a] Both in mice and human.
[b] Viral protection.
[c] In the presence of SDS.

8. Assessing anti-tumor clinical responses using synthetic or natural nonviral vectors

8.1. HSP

HSPs purified from tumors and naturally complexed with an array of tumor associated peptides have been shown to act as an anti-cancer vaccine in both preventive and therapeutic clinical settings [66]. HSPs linked ex vivo to antigen in the form of recombinant fusion proteins also protected mice bearing tumors expressing the vectorized antigen [67].

The success of HSP vaccines in preclinical animal studies has led to the clinical development of phase I/II trials of tumor derived HSP preparations, specifically using GRP94 as autologous tumor vaccine with encouraging results [68,69].

8.2. VLP

After injection of chimeric virus like particles consisting of the L1 major capsid protein from HPV, plus the entire E7 and the L2 minor capsid protein derived from HPV, mice were protected against a challenge with tumor expressing HPV16 E7 protein [70]. These VLP-E7 particles were also efficient even when administered 2 weeks after the tumor graft [71].

In a clinical trial, patients with genital warts exhibited a protein capsid specific immune response after one injection with HPV-6 L1 VLPs and the majority of the patients had complete regression of their genital warts within the 20-week observation period [72].

8.3. Toxin and bacterial products

Using an ovalbumin expressing mouse tumor model, Fayolle et al. showed that recombinant Cya A molecules carrying a CD8+ Ova epitope protected mice against a lethal dose graft of tumor cells and also caused the regression of established tumors [73].

KpOmpA coupled to ovalbumin elicited therapeutic immunity to ovalbumin expressing tumors [41]. When associated with melanoma derived peptides, it inhibits the growth of both induced or spontaneous melanomas [24].

8.4. Liposomes

Zhou et al. demonstrated that previous vaccination of mice with ovalbumin entrapped in liposomes significantly prolonged the survival of mice challenged with tumor expressing the ovalbumin antigen compared to mice immunized with ovalbumin alone [74]. Immunization with liposomal formulation of weak self-antigen like idiotypes derived from lymphoma also induced protection when mice were challenged with lymphoma [75].

9. Concerns about the immunogenicity of synthetic and natural non-live vectors

Live vectors such as viruses are very immunogenic, which may explain the decrease of their efficiency after the first immunization. To face this problem, different groups have developed prime-boost protocols that use more than one type of virus, but this increases the safety concerns [76].

The same difficulties also seem to occur with virus like particles since mice with preexisting neutralizing antibodies against VLPs and vaccinated with HPV16 E7 VLP were not protected from HPV16 E7-positive TC-1 tumor challenge compared to the protection observed in mice lacking these antibodies [77].

As expected, no cellular or antibody responses were detected against natural HSP, which represent a great advantage compared to other live or synthetic vectors.

Regarding the intrinsic immunogenicity of inactivated toxin and bacterial products, it has been reported that preimmunization with CyaA, anthrax toxin and KpOmpA does not compromise subsequent CTL and immune response against the associated peptides [24,55,78].

10. Conclusion

Different studies have now clearly shown that non-live vectors could be used to enhance the immunogenicity of tumor antigens without evidence of toxicity in murine models. Preliminary data also suggest that anti-tumor immunity may also be elicited after in vivo administration of these vectors as cancer vaccines. Most preclinical experiments have been performed with tumors expressing foreign antigens and the development of tumor models targeting self-differentiation antigens commonly expressed by human tumors will allow for the assessment of the versatility and effectiveness of these new vectors compared to classical live vectors.

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CANCER VACCINES: BETWEEN THE IDEA AND THE REALITY

Olivera J. Finn

Whether vaccines are designed to prepare the immune system for the encounter with a pathogen or with cancer, certain common challenges need to be faced, such as what antigen and what adjuvant to use, what type of immune response to generate and how to make it long lasting. Cancer, additionally, presents several unique hurdles. Cancer vaccines must overcome immune suppression exerted by the tumour, by previous therapy or by the effects of advanced age of the patient. If used for cancer prevention, vaccines must elicit effective long-term memory without the potential of causing autoimmunity. This article addresses the common and the unique challenges to cancer vaccines and the progress that has been made in meeting them. Considering how refractory cancer has been to standard therapy, efforts to achieve immune control of this disease are well justified.

Between the idea
And the reality
Between the motion
And the act
Falls the Shadow.

T. S. Eliot

Edward Jenner's landmark publication in 1798 (REF 1) that describes a vaccine against small pox, is considered to be the official beginning of the science of immunology. Immunology has since then made many contributions to scientific enterprise and to many different scientific disciplines, including genetics, molecular biology and cellular biology. The most important contribution of immunology to improving the quality of human life is the development of vaccines.

Twenty-six infectious diseases are preventable through vaccination, at present. In spite of two centuries of vaccine development, however, there are still several parasitic, bacterial and viral diseases, such as Chagas, malaria, tuberculosis and hepatitis C, that have so far eluded protection through vaccines. Modern times have also brought new diseases, such as HIV and cancer. The successes from the past and an ever-increasing level in our understanding of basic immune mechanisms and the ability to manipulate them, predict future victories[2].

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Challenges facing all vaccines

In addition to taking on the challenge to design better vaccines against infectious diseases, immunologists are exploring the possibility of using vaccines against other ailments that involve the immune system. Most notable efforts are directed to developing vaccines for cancer and certain autoimmune diseases. Vaccines that are designed to prepare the immune system for encounter with either infectious pathogens or with cancer or mediators of autoimmunity, all face certain common challenges that are reviewed here.

Choosing the right antigen. Traditionally, successful vaccines have consisted of live attenuated pathogens. Although effective at the population level, these vaccines have a small, but significant, risk of activation that can cause disease or other harmful side effects. On the basis of the successes of attenuated pathogen vaccines and owing to the initial lack of defined tumour antigens, the first cancer vaccines were composed of whole tumour cells that were previously irradiated or otherwise inactivated[3]. In mouse models, this immunization strategy was successful, producing tumour-specific immune responses and rejection of a tumour challenge. These early vaccines used either tumour-cell lines that had accumulated many mutations through numerous passages *in vivo* or *in vitro*

and were, therefore, highly immunogenic, or carcinogen-induced tumours with unique mutations that function as highly stimulatory antigens. As this work expanded to spontaneous tumours that better mimicked human tumours, whole tumour cells proved to be non-immunogenic or weakly immunogenic. Along with these experiments, immunologists were deciphering the exact requirements for antigen specific T-cell activation. They discovered that, in addition to receiving a signal through the T-cell receptor (TCR), naive T cells required additional co-stimulatory signals. This prompted the use of vaccines that were composed of gene-modified tumour cells that expressed various co-stimulatory molecules and/or cytokines, which made them markedly more immunogenic in animal models. Successful animal studies encouraged several clinical trials of cancer vaccines on the basis of gene-modified autologous or allogeneic human tumour cells[4,5].

Just as vaccines that are based on whole pathogens are associated with risks of reactivation and development of disease, whole tumour-cell vaccines present significant health risks. The most serious is the potential for causing autoimmunity. Immature dendritic cells (DCs) that reside in tissues take up and process dying cells and self antigens, but in the absence of strong activating signals, such as those given by pathogens, no immune response to these antigens is generated. To elicit strong immunity, the tumour-cell vaccine must include substances that activate DCs. In the case of whole tumour cells, however, it should be expected that in addition to presenting tumour-specific antigens, activated DCs would prime immunity to many other antigens (autoantigens) that are otherwise subject to peripheral tolerance. This is not a hypothetical case — evidence for autoimmune reactions following vaccination has accumulated from work in animal models, as well as clinical trials[6–9].

The use of whole tumour cells or complex mixtures of tumour-derived material undermines one unique advantage that immunotherapy has over other forms of therapy — that is, specificity. The immune response can recognize epitopes that are expressed by tumour cells and target those cells for destruction without harming normal cells. To take advantage of specificity, the past two decades in tumour immunology have been characterized by considerable effort into the discovery of tumour antigens. Many such antigens were discovered and cancer vaccines based on these antigens have been shown in pre-clinical studies to elicit tumour-specific immunity and establish long-term memory without autoimmunity[10–15]. For breast cancer, for example, vaccines composed of epitopes that are derived from mucin 1 (REF 16), HER2/NEU[17], melanoma-associated antigen 3 (MAGE3) or other members of the MAGE gene family[18], mammaglobin[19] or carcinoembryonic antigen (CEA)[20] have been extensively studied and shown to be immunogenic without causing autoimmunity. Several other antigens under investigation at present will soon be added to the panel of breast-tumour antigens, such as cyclin B1 (REF 21), or one of many cancer-germ-cell antigens that are specifically found in breast tumours[22]. Similarly, there are a large number of antigens available for melanoma vaccines. Extensive studies have been carried out with them in animal models and in clinical trials[23]. In addition to being well explored and understood, many of these antigens are SHARED TUMOUR ANTIGENS. Vaccines that are composed of these antigens can be developed for use in a large number of patients.

Recently, however, in spite of the availability of well-defined tumour antigens, development in the cancer-vaccine field has focused again on the use of whole tumour cells or whole-cell lysates as antigens. The reason being that these complex mixtures will contain UNIQUE TUMOUR ANTIGENS that are expressed only by an individual tumour that, by analogy to unique antigens of mouse carcinogen-induced tumours might be more immunogenic and promote a better anti-tumour immune response[24]. Experiments carried out in mice transgenic for shared tumour antigens have shown that these antigens can elicit equally strong antitumour immunity and tumour rejection[12,25–27]. Furthermore, it has been shown in animal models and in some clinical trials, that a vaccine based on a shared antigen, which elicits an antitumour response, can elicit responses to other antigens on that tumour through a process known as EPITOPE SPREADING[17,28,29] or 'provoked immunity'[14].

The more disturbing reason that might be driving the field away from vaccines that are based on defined tumour antigens is dissatisfaction with the results that have been achieved in the clinic so far. Before we underestimate the potential of defined tumour antigen-based vaccines and go back to undefined tumour mixtures that have the potential for autoimmunity, it must be remembered that antigen-based vaccines have been successful in animal models in which they have been tested almost exclusively in tumour prevention. These vaccines have not yet been given a chance to replicate that success in humans, because they are being tested exclusively as therapeutic agents in advanced disease and often after the failure of standard therapy.

Choosing the right adjuvant. ADJUVANTS are crucial components of all cancer vaccines whether they are composed of whole cells, defined proteins or peptides. Even though, at present, there are only two adjuvants worldwide that are approved for clinical use — aluminum-based salts (alum) and a squalene–oil–water emulsion (MF59) — many other substances that increase the immunogenicity of vaccines have been tested and proven to be effective in animal models and humans. Many new adjuvants are molecules of known function and, therefore, the mechanisms of their adjuvant action are better understood. Adjuvants can activate antigen-presenting cells (APCs) to stimulate T cells more efficiently, activate natural killer (NK) cells or other cells of the innate system to produce cytokines or promote the survival of antigen-specific T cells.

SHARED TUMOUR ANTIGENS
Molecules that are expressed by many tumours and not normal tissues, or expressed by normal tissue in a quantitatively and qualitatively different form.

UNIQUE TUMOUR ANTIGENS
Products of random mutations or gene rearrangements, often induced by physical or chemical carcinogens, and therefore expressed uniquely by individual tumours.

EPITOPE SPREADING
A term originally applied to responses to autoantigens that tend to become more diverse as the response persists. This phenomenon is also known as determinant spreading or antigen spreading. In the setting of a vaccine, it refers to responses that are generated to antigens other than those contained in the vaccine.

ADJUVANT
An agent mixed with an antigen that enhances the immune response to that antigen after immunization.

Cytokines, such as interleukin-2 (IL-2), granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-12, IL-4 and several others, have been used as adjuvants in cancer vaccines[30]. Bacterial products have also been used for many years as effective adjuvants. The two best known are lipopolysaccharide (LPS) from Gram-negative bacteria and monophosphoryl lipid A (MPL) from *Salmonella*. More recently, bacterial DNA was found to have strong immunostimulatory activity owing to the presence of unmethylated CpG dinucleotides[31,32]. These and other bacterial products are bound by many different receptors that are expressed by DCs, macrophages and perhaps NK cells and other cells of the innate system. This induces their maturation, activation and production of pro-inflammatory cytokines. Many of these receptors belong to the family of Toll-like receptors that are located either on the surface of, or inside, cells that recognize invading pathogens[33]. Bacterial products are particularly good at activating cytotoxic T lymphocytes (CTLs), and because of that, they have been of interest to tumour immunologists[34].

Recognition that different antigen-processing pathways control the presentation of antigenic peptides by either MHC class I molecules to $CD8^+$ T cells (endogenous pathway) or MHC class II molecules to $CD4^+$ T cells (exogenous pathway) led to the development of a class of adjuvants that could deliver antigens to a desired processing pathway. Vaccines that are composed of all types of antigen, other than nucleic acids, use mainly the exogenous pathway for the delivery of antigen to APCs. This, in turn, favours the stimulation of $CD4^+$ T cells and the production of antibody. Antigen is required to end up in the cytoplasm for processing by the proteasome and delivery to the endoplasmic reticulum (ER) for binding to MHC class I molecules[35]. Two classes of adjuvants effectively deliver antigens to the cytoplasm: microparticles, such as poly (D,L-lactic-co-glycolic acid) (PLGA) microspheres[36] and virus-like particles[37], as well as immunostimulatory complexes (ISCOMs) — a mixture of Quil A and cholesterol that forms micelles[38]. The particulate nature of the vaccine formulations that are imposed by these adjuvants promotes efficient delivery of antigen to APCs for presentation by both MHC class I and class II molecules. Heat-shock proteins might also belong to this category of adjuvants. They efficiently deliver antigen to the MHC class I pathway and in the process activate APCs[39].

Generating the right type of immune response. Metastatic cancer is a systemic disease that is expected to be monitored by systemic immunity. Many primary tumours, however, originate at mucosal sites in which they are first encountered by the mucosal immune system. Increasing attention is being paid to antigens, adjuvants and routes of administration of vaccines that can effectively stimulate mucosal, as well as systemic immunity[40]. To understand immune responses against tumours at mucosal sites, a better understanding of the immune effector mechanisms that are responsible for protecting the mucosa are required. The mucosal immune system has evolved to keep the balance between a swift reaction against pathogens and no response to food or other environmental antigens and non-pathogenic bacterial flora. Mucosal vaccines need to maintain this well-regulated balance at the same time as strengthening the protective response. Our understanding of the specific characteristics and behaviour of cells of the immune system at mucosal surfaces is still not complete, but information is beginning to emerge with regard to the migration of lymphocytes and APCs to those sites and the induction of immunity versus tolerance[41–44].

None of the cancer vaccines tested so far have been specifically designed to elicit mucosal immunity. One explanation for this obvious omission is that the aim of therapeutic vaccines is to eliminate residual disease, which might be considered as a role for systemic immunity. However, questions are beginning to arise about the potential of a particular immune response to be equally effective against tumours in different sites such as the lung, pancreas, liver or bone marrow. Most experiments carried out with animal models available at present, and especially with transplantable tumours that grow in subcutaneous sites, do not shed light on this subject. Another reason to consider whether a particular vaccine should be applied towards stimulating mucosal rather than systemic immunity is that therapeutic cancer vaccines are expected to boost an already existing, albeit weak, immune response rather than prime new responses. If the existing response was primed against a tumour that originated at a mucosal site — for example, colon cancer, cervical cancer, squamous cell carcinoma of the head and neck (SCCHN), lung adenocarcinoma and bladder cancer — this response might be more effectively boosted by a mucosal rather than systemic route of immunization. Understanding the role of mucosal immunity in cancer is going to be more important in the future for designing preventive cancer vaccines. If, for example, a vaccine is to be used for the prevention of polyps as a means of preventing colon cancer, this vaccine will have to stimulate the type of immunity that can recognize and react against tumour antigens when they are first expressed by the colon epithelium.

While mucosal immunity has not been given appropriate attention by tumour immunologists, the role of T HELPER 1 (T_H1)- versus T_H2-type responses in antitumour immunity and the ability of cancer vaccines to elicit one or the other has been the focus of many studies. Ever since these two types of $CD4^+$ T cell were described[45], their role in many different diseases has been well studied. With few exceptions, most effective antitumour immune responses in animal models have depended on the efficient generation of T_H1-cell immunity that promotes CTL responses. The importance of T_H1-cell immunity for tumour regression is also strengthened by the observation that progressive disease is characterized by an antitumour T-cell response that is skewed to T_H2 cells[46].

T HELPER 1/2 CELLS
T_H1/T_H2 cells. Two subsets of activated $CD4^+$ T cells that can be distinguished by the cytokines they produce. T_H1 cells produce interferon-γ, lymphotoxin and tumour-necrosis factor, and enhance cell-mediated immunity. T_H2 cells produce interleukin-4 (IL-4), IL-5 and IL-13, and support humoral immunity.

Even though in present animal models intentional skewing of the immune response to the T_H1 type leads to tumour rejection, whereas a response skewed to the T_H2 type seems ineffective, in the long run it might be a mistake to focus cancer-vaccine design on the generation of T_H1-cell immunity. T_H2-mediated immunity is characterized mainly by the production of antibodies that have been ineffective against tumour challenge in most animal models. However, in patients, passively administered antibodies that are specific for antigens expressed by tumour cells have shown antitumour effects in B-cell lymphomas[47], breast cancer[48] and colon cancer[49]. Designing vaccines that promote T_H2-type responses to generate such antibodies *in vivo* would seem to have numerous advantages over the passive administration of antibody. This is already being done, with some success, using vaccines against IDIOTYPES expressed by B-cell lymphomas[50]. Vaccine-elicited antibodies can mediate direct effects against tumour cells by fixing complement or facilitating ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY (ADCC). A more important function of tumour-specific antibodies is opsonization of tumour cells to promote their uptake by APCs. Several cancer-vaccine trials have aimed to elicit tumour-specific antibodies and have succeeded. However, owing to advanced stages of disease, the antitumour effects of such antibodies have not been significant[51].

Designing a vaccine that will skew a response to one type (for example, T_H1) or one effector mechanism (for example, CTL) might be an acceptable strategy for present therapeutic vaccinations in which immediate effects are sought. This strategy is unlikely to be beneficial for cancer prevention or in treating early disease in which many mechanisms are required to synergize to create as large a pool as possible of effector cells to guarantee a large pool of memory cells. Until recently, most cancer vaccines were based almost exclusively on MHC class I-restricted peptides[52]. These vaccines did generate some CTL activity, but the frequency and duration of these responses was uniformly low. The requirement for simultaneous activation by a cancer vaccine of many components of the immune system cannot be overestimated.

Elicitation of long-term memory. Immune memory is an important protective mechanism that some vaccines can elicit and others cannot. The nature of immune memory and the requirements for its generation and maintenance have only recently begun to be elucidated[53,54]. The main problem that has hampered this field of investigation has been the relative paucity of specific markers that could separate memory T cells from other T cells. Chemokine receptors have recently been used successfully to distinguish between functional subsets of T cells including memory cells[55]. These, and additional markers, such as mucin-like glycoproteins[56], are starting to be reported. They will help in the evaluation of the role of tumour antigens, adjuvants and routes of injection not only with regard to the complexity and intensity of the immune response they elicit, but also for the type of memory response that is generated.

There is a consensus that a strong primary immune response is required to give rise to a large pool of memory cells. What affects the longevity of memory T cells, however, is not fully understood and there is much controversy with regard to the role of antigen in this process[54,57]. For therapeutic cancer vaccines, these questions are of great importance. The immune system of a cancer patient is exposed to the tumour antigens over a relatively long period of time and the vaccine based on some of these antigens is expected to boost immunity in their presence. It is not known whether the tumour-specific T cells that are present in the patient before vaccination are effector cells or a mixture of effector cells and memory cells. Several papers have claimed the existence of tumour-specific memory cells[58–60]. However, because of the inability to separate clearly effector cells from memory cells and the chronic presence of tumour antigen, it is not clear to what subset of T cells tumour-specific cells in cancer patients belong and how they are affected by vaccination. It is also not clear whether long-term memory can ever be achieved in chronic diseases such as cancer. Certain requirements, especially the need for activation of T_H cells and innate immunity, are coming to light in the setting of chronic viral diseases[61] and to a more limited extent in cancer[62,63]. As reported recently, during the generation of T-cell memory there is a progression from naive cells that become effector cells when antigen is introduced, to effector memory cells when antigen becomes limited, to central memory cells after the clearance of antigen[64]. Although prophylactic cancer vaccines in healthy young adults would be expected to activate this entire differentiation pathway, it is less clear how a therapeutic vaccine might do that in the presence of chronic antigen and many existing cell populations specific for that antigen.

Additional challenges facing cancer vaccines

Aging immune system. Patients with cancer in whom cancer vaccines are presently being tested are, almost without exception, of advanced age (65–80 years), many decades after the thymus has stopped producing naive T cells. Therefore, the generation of an effector-cell population in response to a vaccine depends on the recognition of the vaccine antigen by one or more memory cells in the T-cell repertoire of the patient. Among the T cells that respond to the vaccine there might or might not be the 'best fit' ones that would have been selected from a large pool of naive clones earlier in life. In mouse models, it can be clearly shown that young mice make stronger primary responses than old mice. Generation of the primary response and the conversion to memory is compromised with age[65,66]. This is due to age-associated changes in the function of many components of the immune system[67–69]. At present, there is an important discrepancy between preclinical studies in mouse models and clinical trials of cancer vaccines. Few studies, if any, use old mice. Those that do, report an age-related increase in susceptibility to cancer due to changing patterns of T-cell subsets[70], as well as difficulty in the induction of effective antitumour immune responses[71].

IDIOTYPE
The unique portion of either a T-cell receptor or an immunoglobulin molecule, defined by the hypervariable regions and involved in antigen recognition.

ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY (ADCC). Killing of antibody-coated target cells by cells expressing Fc receptors (FcRs) that recognize the constant region of the bound antibody. Most ADCC is mediated by natural killer cells that express the FcR CD16 or FcγRIII on their cell surface.

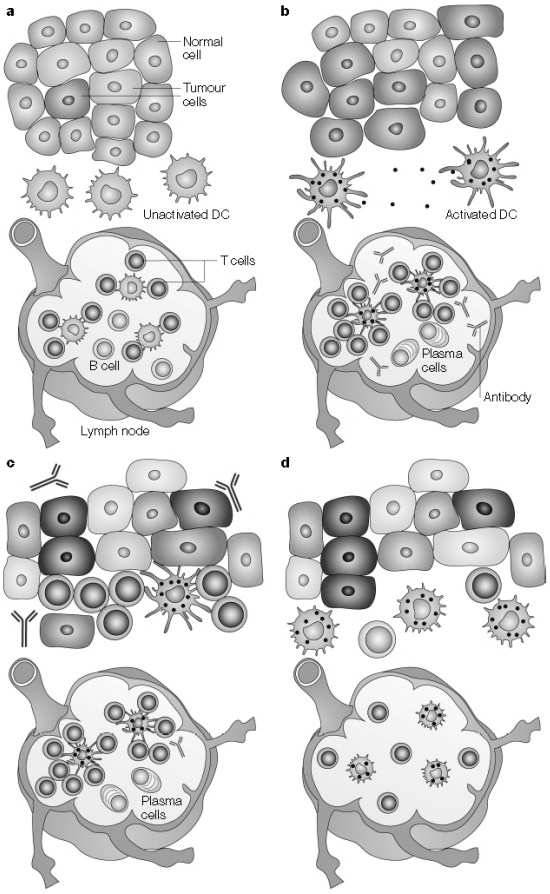

Figure 1 | **A probable model of the evolution and fate of antitumour immune responses that develop coincidently with tumour growth. a** | Tumours develop over a long period of time through a process of accumulation of many mutations. While the tumour is small and does not present a significant danger to the integrity of the organ of origin, the immune system remains ignorant of its presence. Dendritic cells (DCs) in the surrounding tissue are not activated and as a result T and B cells in the lymph node remain in a resting state. **b** | When the tumour becomes larger, heterogeneous and ultimately malignant, damage to the normal tissue and products made by the tumour cells alert the immune system mainly through the activation of resident DCs. Activated DCs that have taken up products derived from damaged normal tissues and tumours, traffic to the draining lymph node in which they begin to present these products as antigens to naive T and B cells. The extent of DC activation determines the extent of lymphocyte stimulation. This in turn is regulated by many factors that determine the immune competence of the patient, including age. **c** | Tumour-specific T cells, antibodies and activated DCs reach the tumour site and attempt to destroy the tumour. They are only partially successful owing to an already large tumour size and marked tumour heterogeneity that allows the tumour to evade many immune effector mechanisms. **d** | The tumour that has evaded the initial immune response continues to grow, disseminate and actively suppress local, as well as systemic, immunity illustrated by the presence at the tumour site of DCs, T and B cells that are not activated and do not exert their respective functions.

In recognition of the fact that therapeutic vaccines for cancer will be given mostly to older individuals, increasing attention should be given to designing vaccines that can overcome at least some age-related problems. For example, engagement of the co-stimulatory molecule 4-1BB (CD137) was shown to amplify T-cell responses in aged mice[72] and, although not yet tested, engagement of other co-stimulatory molecules or inactivation of negative regulators, such as cytotoxic T lymphocyte antigen 4 (CTLA4)[73], might have similar effects. Furthermore, although many adjuvants might work well in young mice, only some might enhance immune responses in aged individuals. CpG–DNA seems to be especially good at enhancing cellular and humoral immunity and promoting T_H1-type responses in old mice[74].

Age-associated immune deficiency indicates that paediatric cancer patients might be better candidates than adult patients for therapeutic cancer vaccines. Few such trials have been carried out. Results from one DC-based vaccine trial conducted on children aged between 3 and 17 years with relapsed neuroblastomas, sarcomas and renal cancers, are unfortunately only slightly more encouraging than results from clinical trials in aged patients[75]. This shows that even in a young patient, there is an influence of previous therapy and/or the advanced stage of the tumour on the immune system, and indicates that successful vaccination strategies would require vaccination not only at an early age, but also in early disease and in the absence of immunosuppressive standard therapy.

Tumour-induced immunosuppression and immune evasion: By the time a tumour is diagnosed, there have been many interactions between the tumour and the immune system (FIG. 1). A tumour might have been growing slowly without much destruction of the surrounding normal tissue and so might not have been detected by the immune system. During that time, tumour cells acquire additional mutations, some of which facilitate growth and invasion. As the tumour becomes larger and begins to cause tissue destruction, in addition to defense processes, such as wound repair and clotting mechanisms, the adaptive immune system is also alerted owing to the activation of DCs. These cells pick up tumour and tissue debris and 'ferry' it to the draining lymph nodes for presentation to T cells. The presence of tumour-specific cellular and humoral responses in cancer patients indicates that the immune system has 'seen' the tumour. The loss of expression of various tumour antigens or MHC molecules by tumour cells indicates that the immune system has tried to get rid of the tumour. Progressive tumour growth, however, indicates that the tumour has ultimately evaded immune defenses. This process of immunosurveillance, which changes the tumour but does not result in complete tumour rejection, is known as 'cancer immunoediting'[76].

Many ways in which tumours influence the immune system have been described and functional defects have been documented in many immune effector mechanisms. The maturation and function of

DCs is inhibited in cancer patients[77,78]. Marked defects are also seen in T-cell activation and function, which was first reported in mice with tumours[79] and later found in patients with many types of tumour[80]. These effects can be mediated by IL-10, transforming growth factor-β (TGF-β) and other cytokines that tumours produce[81–83], or by other less well defined soluble factors[84] or cell-surface molecules[85] expressed by tumour cells.

Suppression of adaptive antitumour immunity can also be mediated by 'improper activation' of innate immunity. It has been reported that the activation of macrophages and polymorphonuclear cells in response to the tumour induces a state of oxidative stress in cancer patients that markedly suppresses the function of T cells[86]. Activation of NKT cells that might result in the production of high levels of IL-13 has also been reported to suppress tumour immunity[87]. There is an ongoing effort to understand these immunosuppressive mechanisms at the molecular level to allow therapeutic intervention. There are encouraging reports that at least some of these defects have been reversible through vaccination in a small number of patients[88,89]. These studies will now have to extend to understanding the role in tumour immunity of the recently described regulatory T cells[90]. A subpopulation of $CD4^+CD25^+$ T cells has been shown to suppress autoimmunity[91] and therefore might be specifically expanded in response to the increased presentation of autoantigens during tumour growth. The limited number of studies that have been carried out with tumours in mice indicate a potential benefit from depleting these cells[92].

A complete understanding of the immune system of patients with tumours is important, especially when trying to manipulate it with therapeutic cancer vaccines. Many of the immunosuppressive mechanisms are common to different tumour types and devising a treatment regiment to reverse immunosuppression before therapeutic vaccination might produce better results.

Therapeutic cancer vaccines

Because many primary tumours can be surgically removed and there is often a long period of time before the tumour recurs at metastatic sites, cancer vaccines have been proposed as therapy that are designed to elicit and/or boost antitumour immunity in patients with minimal residual disease, thereby preventing or prolonging the time to recurrence. Few vaccines have been tested in that optimal clinical setting. Most phase I and II studies have been carried out, so far, in late stage disease and in the presence of a relatively large tumour burden after the failure of standard therapies. Even under the best of circumstances, the success of therapeutic vaccines will depend on the ability of the immune system to overcome tumour-induced, therapy-induced or age-induced immunosuppression. An additional factor that influences the effectiveness of therapeutic vaccines will be the outgrowth of tumour cells that, for one reason or another, can evade the immune response (FIG. 2).

DELAYED-TYPE HYPERSENSITIVITY (DTH). A cellular immune response to antigen injected into the skin that develops over 24–72 hours with the infiltration of T cells and monocytes, and depends on the production of T helper 1-specific cytokines.

The therapeutic vaccine effort that has accumulated the most clinical results has been the development of vaccines for melanoma patients. It started with the use of cell lysates from allogeneic tumour-cell lines in combination with adjuvants[93,94] or protein products that are shed into the supernatants of such cell lines[95,96]. Hundreds of patients with advanced stage III or IV melanoma, many with metastatic disease having failed chemotherapy, have participated in these studies. In the case of one of these vaccines, Melacine (Corixa Corporation, Seattle, Washington, USA), phase I and II trials in stage IV patients showed a 10–20% response rate (clearing of some metastatic sites) and in another 10–20% of patients disease was stabilized (no progression for various periods of time of tumours that were growing at the start of the vaccine protocol). In a multi-centre phase III study, Melacine was compared with a four-drug chemotherapy regimen and the response rates and survival were the same[97]. The advantage of Melacine over chemotherapy was that it was non-toxic and therefore allowed a better quality of life compared with chemotherapy. For that reason, Melacine is now available on prescription to patients in Canada and is awaiting approval in the United States. A similar vaccine preparation, Canvaxin, was evaluated in ~1,000 stage IV melanoma patients and compared with an equal number of patients who were treated with surgery and chemotherapy during the same time period, but did not receive the vaccine. This single-institution study showed a small, but statistically significant, increase in the overall survival in the vaccinated group[94]. The vaccine is now being tested in a multi-centre phase III randomized trial.

More recent versions of cancer vaccines that are based on autologous tumours and their various products include modified tumour cells[98,99] and tumour-derived heat-shock proteins[100]. The latest report from a phase I trial in 35 patients with non-small-cell lung cancer vaccinated with irradiated autologous tumour cells that are engineered to secrete GM-CSF, shows post-vaccine infiltration of metastatic sites with macrophages, granulocytes and lymphocytes, as well as DELAYED-TYPE HYPERSENSITIVITY (DTH) responses against unmodified tumour cells in most patients. Correlation of these events with the clinical outcome is less clear, with only five patients showing stabilization of disease[101]. Similarly, the latest report on the autologous tumour-derived heat-shock protein gp96 vaccine in 39 patients with resected stage IV melanoma indicates that 11 patients had increases in melanoma-specific T-cell reactivity, of which two patients had a complete response (disappearance of all detectable tumours) and three patients had stable disease[100].

DC-based vaccines[102] are the newest development in cancer vaccine design. DCs can be loaded with autologous or allogeneic tumours[103], apoptotic bodies[104], tumour lysates[105], tumour RNA[106,107] and tumour DNA[108,109]. Most of these preparations have shown to be immunogenic and have the potential for

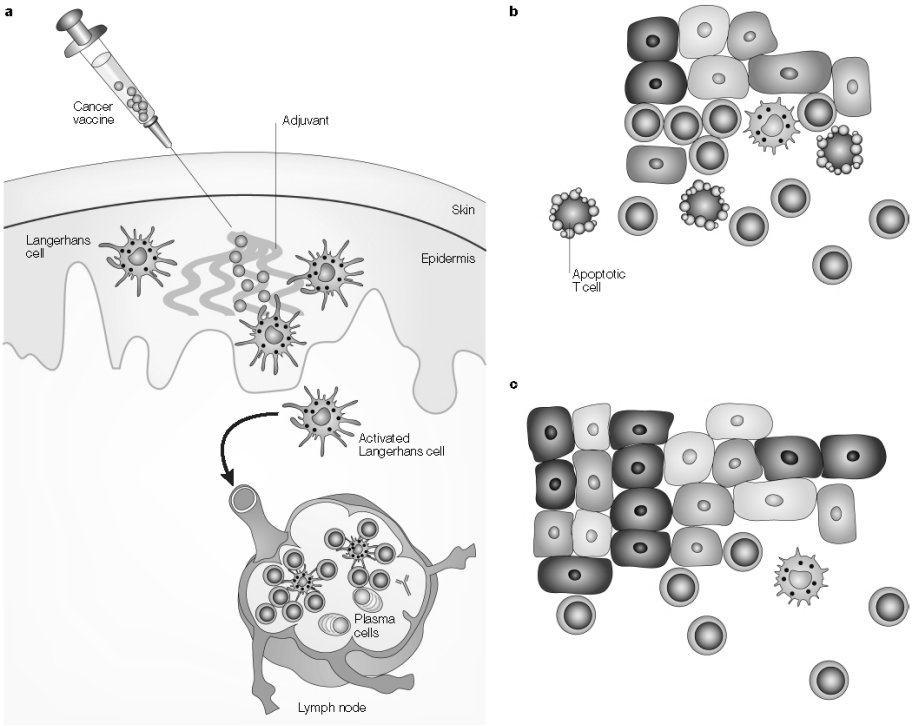

Figure 2 | **Manipulation of antitumour immune responses by therapeutic vaccination**. **a** | Therapeutic vaccines are administered after the tumour is diagnosed, at the time of interactions between the tumour and the immune system that correspond to parts **c** and **d** in FIG. 1. In the most optimal clinical setting, therapeutic vaccines intend to boost immunity against minimal residual disease and prevent the outgrowth of metastases shown in parts **b** and **c**. A vaccine based on autologous tumour or defined tumour antigens is administered in an immunostimulatory preparation (with adjuvant) that can activate Langerhans cells — dendritic cells (DCs) that reside in the epidermis. Activated Langerhans cells take up the tumour antigens and traffic to the draining lymph node in which they present antigens to T cells. B cells are also activated and the expected outcome is clonal expansion of tumour-specific T cells and the production of tumour-specific antibodies. **b** | Tumour-specific T cells migrate to the sites of tumour metastases where they attempt to kill tumour cells that express antigens contained in the vaccine. Their function is compromised by the immunosuppressive tumour microenvironement, which affects their function and leads to their death. Furthermore, tumour heterogeneity has been established over time. Some tumour cells have lost expression of antigens that are targeted by the immune response and others have become resistant to immune effector mechanisms. This allows many of the cells to evade the immune attack. **c** | Metastases that continue to grow are composed of tumour cells that lack antigens recognized by T cells and antibodies or are otherwise resistant to immune destruction.

tumour rejection in animal models, and are undergoing evaluation in the clinic at present. Results from a phase I study of a vaccine composed of DCs that were loaded with messenger RNA encoding prostate-specific antigen (PSA) have been reported recently. Vaccination of prostate cancer patients that had raised levels of expression of PSA induced T-cell responses against PSA in most patients and the log slope of PSA was temporarily decreased[110], indicating perhaps that growth of the tumour was slowing down.

Shared tumour antigens can be produced as synthetic or recombinant proteins and are, therefore, ideally suited for prophylactic vaccination of individuals who do not have a tumour, but are at high risk of developing a tumour. Yet, these antigens have, so far, been tested exclusively in therapy of advanced disease[17,111–121]. As with whole tumour-based vaccines, tumour antigen-based vaccines have shown impressive results in preventing tumours in animal models and only marginal results in therapy of advanced disease in both animals and patients[122].

Prophylactic cancer vaccines

Many of the potentially insurmountable problems that diminish the therapeutic effects of cancer vaccines, would not need to be considered in the setting of cancer prevention. An immune system that is primed to anticipate tumour antigens, would be expected to destroy the tumour before it becomes clinically obvious, heterogeneous, and can suppress and evade the immune response (FIG. 3). In 2002, Merck&Company Inc. announced preliminary results of a study testing the company's vaccine against human papillomavirus type 16 (HPV16)[123]. Infection with HPV is a known cause of most cervical cancers and HPV16 is found in over 50% of these tumours. HPV is a common infection in the general population and the immune response to the virus protects against chronic infection that can lead to cancer[124]. In a minority of individuals, the immune response seems not to be strong enough or of the right type, allowing the establishment of chronic infection. The results showed, after the first 2 years of a 4-year study on 2,392 women aged between 16 and 23 years who were randomly assigned to the vaccine or a placebo, that in the placebo group, 3.8% of women were infected with HPV16 annually compared with no infections in the vaccinated group. These are spectacular results considering that 150,000 women in developing countries die annually of cervical cancer that might now be reduced by preventing the initial infection with virus. If a world-wide HPV vaccination programme were to start in 2010, it is estimated that there would be no cases of cervical cancer by 2050 (REF 125). Results from vaccines against hepatitis B virus (HBV), which is also known to cause cancer in chronically infected individuals, already supports the expectation of lowered cancer rates. In Taiwan, where a national vaccination programme against HBV was started in 1986, there has been a marked reduction in the incidence of childhood liver cancer[126]. In the HBV vaccination programme in The Gambia, vaccination of

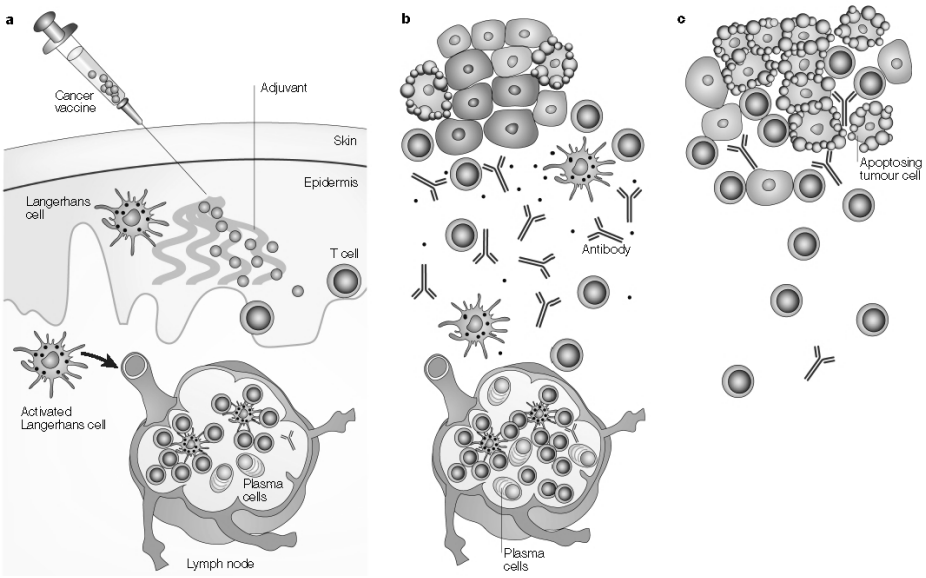

Figure 3 | **Manipulation of antitumour immune responses by prophylactic vaccination**. **a** | Prophylactic vaccines would be administered before the occurrence of tumours to individuals who are at high risk for developing tumours or have been diagnosed with premalignant changes in target tissues. A vaccine based on antigen/s that are expected to be expressed by the anticipated tumour is administered in an immunostimulatory preparation (with adjuvant) that can activate Langerhans cells — dendritic cells (DCs) that reside in the epidermis. Activated Langerhans cells take up the tumour antigens and traffic to the draining lymph node in which they present antigens to T cells. B cells are also activated and the expected outcome is clonal expansion of tumour-specific T cells and the production of tumour-specific antibodies. This clonal expansion of effector cells is followed in time by the generation of a pool of memory cells that are specific for the tumour antigen/s. **b** | If a tumour begins to grow sometime in the future, tumour antigens that reach the draining lymph node will reactivate tumour-specific memory cells and elicit a swift secondary immune response. This response will be characterized by large numbers of effector T cells, high titre of antibodies and continuous activation of DCs at the tumour site, for continuous processing and presentation of tumour antigens and further amplification of the immune response. **c** | The incipient tumour has not been allowed to grow large and heterogeneous and is easily eliminated by the prepared immune response. Moreover, the memory compartment is further expanded by this tumour-mediated boost.

Box 1 | Prophylactic vaccines for breast cancer

Many young women with hereditary risk of breast and ovarian cancer, especially those with mutations in the gene encoding breast cancer 1, early onset (BRCA1) or BRCA2 (REF 152), are, at present, offered prophylactic mastectomy and/or oophorectomy. Several large studies show that these procedures decrease the risk of both cancers[152–156]. Other presently available options are chemoprevention or frequent surveillance. All of these options are associated with considerable risks[157]. Breast cancer vaccines have not been one of the prophylactic options, in spite of the fact that promising breast-cancer antigens have been defined and, to the extent possible, shown to be safe in phase I and II clinical trials in patients with breast cancer[17,111,115,119,158]. The two main arguments put forward against the vaccines are: first, safety (potential cross-reactivity of the elicited immune response with normal tissues); and second, the need for a large number of patients and long-term observation to establish efficacy. In my view, neither of these arguments is valid. In the most extreme case of cross-reactivity, autoimmune destruction of normal breast or ovarian tissue should have no more serious consequences than their surgical removal. Similarly, if the statistical approaches that are used now to select an appropriate number of individuals at high risk allow evaluation of efficacy of prophylactic surgery or chemotherapy, the same statistics, the same number of patients and the same follow-up time can be applied to the evaluation of vaccine efficacy.

newborns has had 83% efficacy against acute infection and 95% efficacy against chronic infection[127]. The effect on liver cancer is still unknown, because the vaccinated individuals have not reached the advanced age at which the cancers arise. Knowing the strength of the association between chronic HBV infection and liver cancer, it is highly probable that the results will match the expectation of a markedly reduced incidence of cancer.

There are numerous cancers without a known virus cause that have a bigger impact in terms of human suffering, which could also be prevented with vaccines[122,128,129]. Viral antigens are no different from tumour antigens in that they both fail to elicit good immune responses in a therapeutic setting. An HPV16 peptide-based vaccine in women with advanced cervical carcinoma elicited only minor responses in the face of progressive disease[114]. A similar vaccine in women with an earlier stage of disease — a high-grade HPV16-positive cervical intraepithelial neoplasia — elicited slightly more convincing immune responses that did not translate into eradication of the virus[130]. Results from these trials are exactly the same as results that are obtained in many similar trials with other tumour antigens. The antigen, the formulation and the delivery of vaccine are all important for its efficacy, but the appropriate timing of administration might be the most important predictor of success for cancer vaccines.

The antigen has an important role, however, in assuring the safety of the vaccine-elicited immune response. Viral antigens that function as tumour antigens are expected to elicit a response that is specific only for the tumour cells that harbour them. However, many of the well-defined tumour antigens are also expressed by normal tissues, albeit in a reduced or modified form, and these tissues could potentially be damaged. This potential has to be considered most seriously in the setting of cancer prevention. Many pre-clinical studies of vaccines based on tumour antigens have put a special emphasis on defining tumour-specific epitopes and vaccine formulations that will prevent tumour growth, but not damage normal tissues. Results with several antigens indicate that they could be safely administered to individuals at risk for developing cancer. For example, mucin 1 glycoprotein is expressed by normal epithelial cells and by adenocarcinomas of the breast, pancreas, colon, lung, ovary, prostate and several others. It is also expressed by many myelomas and some B-cell lymphomas. Learning how to target the immune response against mucin 1 to tumours expressing mucin 1 could potentially be used for the prevention of all these tumours. Many groups are exploring this potential by defining various epitopes on mucin 1 that can be used to elicit tumour-specific immune responses[131–137]. There are quantitative and qualitative differences in the expression of mucin 1 between normal and malignant cells. Tumours overexpress mucin 1 and they also markedly underglycosylate this otherwise heavily O-glycosylated molecule. The immune system recognizes both differences and, as seen in animal models from transgenic mice to chimpanzees, it can destroy mucin-1-expressing tumours at the same time as ignoring normal tissues that express mucin 1 (REFS 12,138–142). Similar examples can be provided by reviewing the work on other well-known antigens, such as CEA[26,143–145] and HER2 (REFS 11,17,119,146). In addition to these antigens, which can be used safely without risk of autoimmunity, antigens, such as the melanoma antigens and PSA, are known to induce autoimmunity that can be tolerated, such as vitiligo or autoimmune prostatitis.

Box 2 | Prophylactic vaccines for pancreatic cancer

Patients with hereditary pancreatitis caused by the common mutations in the gene encoding trypsin have a median age of onset of the disease around 10 years of age. Half of these patients develop chronic pancreatitis and are at increased risk of pancreatic cancer[159]. At present, screening is recommended to patients with hereditary pancreatitis of aged 40 years and over, and if cancer is suspected, removal of the entire pancreas is the prophylactic option. This is a drastic measure with significant and lasting co-morbidities, such as brittle diabetes mellitus.

Screening detects early mutations in premalignant lesions that are known to be precursors of pancreatic cancer, defined as pancreatic intraepithelial neoplasia (PanIN)[148]. The number of mutations that accumulate over time characterizes the stage of progression of these lesions towards malignancy. Pancreatic cancer vaccines have so far been tested only in patients with late-stage pancreatic cancer[4,111]. These same vaccines could be a reasonable prophylactic option for patients with chronic pancreatitis, who after screening show cancer-promoting mutations and advanced PanINs. Although a reduction of cancer incidence would be the ultimate end point, that might take a long time to reach, vaccinated patients could be screened as early as a year after vaccination for the disappearance of mutations as a way of evaluating the vaccine efficacy.

The future of cancer vaccines

Having done as much as is possible to show the efficacy and safety of several well-known tumour antigens, it is important to decide what will be the next step in developing these as effective cancer vaccines. One option is to continue testing vaccines in cancer patients in small phase I and II trials, with individual antigens in different forms, in different vaccine formulations and with different adjuvants, taking advantage of new technological developments and hoping for improvements in efficacy. The best example of a cancer vaccine that has followed this option is the anti-idiotype vaccine for B-cell lymphomas[147] — a prototype of a therapeutic cancer

Box 3 | **Prophylactic vaccines for colorectal cancer**

Of the 130,000 cases of colorectal cancer that are diagnosed in the United States each year, 15% are hereditary with 5% due to either familial adenomatous polyposis syndrome (FAP) or hereditary non-polyposis colorectal cancer syndrome (HNPCC). Mutations that are associated with these two syndromes are known, and individuals who have one or more of these mutations are at increased risk of colorectal cancer[162]. Large-scale clinical trials, such as those that use non-steroidal anti-inflammatory drugs (NSAIDs) have been carried out testing chemoprevention of polyps as a means of preventing colon cancer. If this prevention approach is to be effective, individuals at risk will need to take the drug for life. This brings up the issues of drug toxicity, drug resistance, as well as non-compliance. Alternatively, the same individuals could be immunized against a tumour antigen that is known to be differentially expressed by polyps versus normal tissue, and expressed by all colorectal adenocarcinomas. Dysregulated expression of mucin genes in polyps has been documented[163]. Colon-tumour antigen mucin 1 is not expressed by normal colon, but it is expressed by adenomatous polyps in the tumour-associated underglycosylated form[164]. The hope for a prophylactic vaccine containing mucin 1 would be that it would prevent the occurrence or recurrence of polyps. This is an end point that can be used to evaluate the efficacy of this vaccine in a relatively short period of time.

vaccine based on a unique tumour antigen. If the same approach is applied to shared tumour antigens, it will yield vaccines for the treatment of a limited number of patients at major medical centres in developed countries. However, the impact on cancer as a global health problem will be negligible.

The other option is to make a decision that cancer vaccines that have shown efficacy and safety in preclinical studies are relevant for the prevention of cancer and to begin to test them as such. Trials to test the ability of mucin 1, CEA or HER2 vaccines to prevent breast cancer in women at high risk should pose no bigger logistical and financial challenges than similar trials of other preventive modalities, among which randomized trials on hundreds of women that are treated with double mastectomies or oophorectomies are taking place (BOX 1). When prevention becomes a stated goal of at least some cancer vaccines, a different approach will be encouraged for the identification of new tumour antigens. Instead of continuing to focus on the tumour as a source of antigens, the emphasis could shift to premalignant lesions. Many such lesions are known for pancreatic cancer[148] (BOX 2), prostate cancer[149], colon cancer[150] (BOX 3), esophageal cancer[151] and others. Having vaccines that could prevent the progression of these lesions to cancer would make the cancer screening efforts much more useful than they are now and set the stage for a more general use of prophylactic cancer vaccines in the near future.

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MINIREVIEW

Monitoring of Antigen-Specific Cytolytic T Lymphocytes in Cancer Patients Receiving Immunotherapy

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Recent progress in molecular and immunologic approaches to discovery of tumor-associated antigens (TAA) in humans has resulted in the characterization of a number of new epitopes (3, 23). In most cases, the success of these efforts depended on the availability of tumor-specific T-cell lines or clones, which were used as probes for isolation and biochemical characterization of TAA (10, 55). Two types of methodologies have largely been used for antigen discovery: (i) biochemical fractionation of naturally processed and presented peptides derived from major histocompatibility complex (MHC) class I molecules expressed by tumor cells (16) and (ii) expression cloning of cells transfected with cDNA libraries derived from tumor cells (54). More recent introduction of the SEREX (serological analysis of tumor antigens by recombinant cDNA expression cloning) technology (47) and of computer-based modeling of peptides that best fit the relevant MHC class I molecules expressed on tumor cells (15) further expands the list of technologies available for antigen discovery and for identification of TAA which might be therapeutically useful. SEREX is based on identification of recombinant tumor antigens by immunoglobulin G (IgG) antibodies present in the patient's serum. To qualify for immunotherapy, e.g., as components of antitumor vaccines, TAA or their newly identified epitopes have to be immunogenic, that is, able to induce and sustain an immune response specifically targeted not only to the immunizing epitope but to the tumor itself. With the exception of the products of mutated genes, few if any TAA epitopes meet the criteria for therapeutic utility, largely because they are self-antigens rather than neo-antigens. As such, they are weakly immunogenic, and tolerance for self-epitopes in tumor-bearing hosts prevents generation of strong antitumor immune responses targeting these TAA. Most of the melanoma-derived peptides are normal differentiation antigens, which are overexpressed in tumor cells (3, 9, 23). The TAA encoded by mutated genes are the exception, of course, because they are truly new antigens, but their therapeutic usefulness is limited to individually tailored treatments that are not applicable to broad-scale immunizations.

Nearly all of the known TAA epitopes are ligands for T-cell receptors (TcRs) which are clonally expressed on T lymphocytes: on $CD8^+$ T cells expressing TcRs for nanopeptides associated with MHC class I molecules or on $CD4^+$ T cells responding to larger peptides presented by MHC class II molecules (32). The presentation of TAA-derived peptides to T cells could be accomplished by tumor cells themselves, provided they express MHC molecules (29). However, since most human tumors express abnormally low levels of class I molecules (17) and may have no or low expression of class II antigens (32), in vivo presentation of TAA-derived peptides to immune cells is likely to occur by the process mediated by dendritic cells (DC) and referred to as "cross-presentation." The importance of DC in immune responses to TAA has been emphasized in view of emerging evidence for frequent, if not universal, defective antigen processing in tumor cells (26, 50). This then means that DC can internalize and process TAA for presentation to T cells bearing the appropriate TcRs, bypassing the need for tumor cells to act as antigen-presenting cells (APC). Still, even if DC assume the role of TAA presentation in vivo and cytolytic T lymphocytes (CTL) are generated as a result of effective cross-presentation, these CTL have to be able to access the tumor site and recognize the relevant peptides expressed on the surface of tumor cells in the context of MHC molecules in order to initiate tumor cell lysis. Therefore, expression on the tumor cell surface of the MHC-peptide complexes is a prerequisite for immunologic recognition and immune cell-mediated tumor cell destruction.

TAA-specific T-cell responses following immunotherapy, and particularly after the administration of natural or synthetic anticancer vaccines, have been studied in patients with cancer (28, 34, 46). Early clinical trials evaluating such vaccines showed tumor regression even in patients with advanced disease (28, 34, 46). Quantitation of antigen-reactive T cells prior to, during, and after therapy is crucial for future development of antitumor vaccines. To detect the frequency of peptide-, protein-, or tumor-specific T cells in the peripheral circulation of patients treated with anticancer vaccines, several methods have been developed. The objective aimed for is a measure of effectiveness of therapy, as judged by the increased number of circulating specific T cells responsive to vaccinating antigens and, optimally, to autologous tumor cells as well. The assays available for measuring of TAA-reactive T cells include (i) cytotoxicity assays, which provide the assessment of the ability of T-cell populations to lyse tumor cells, (ii) cytokine expression or production assays, in which TAA-specific responses of T cells are evaluated based on antibody-mediated detection of intracellular cytokines or cytokines released by T cells following stimulation with the relevant antigen, (iii) direct quantitation in peripheral blood mononuclear cells (PBMC) of T cells able to recognize and bind to a labeled peptide-MHC complex, and (iv) enumeration of T cells expressing a specific type of TcR, using PCR-based amplification. The purpose of this review is to briefly consider advantages as well as disadvantages of these methodologies for monitoring of TAA-specific responses in patients with cancer treated with antitumor vaccines and other immunotherapies.

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CYTOTOXICITY ASSAYS

Cytotoxicity assays have been in use for many years (5) for measuring antitumor responses. Typically, they depend on the use of a labeled tumor cell target, which is susceptible to lysis by T cells recognizing an antigen-MHC complex present on the tumor cell surface. There are multiple formats for performance of cytotoxicity assays, but a chromium release microtiter plate method has emerged over the years as the most widely applicable and reliable for detection of tumor-specific CTL (59). In fact, the chromium release assay has been the "gold standard" for assessment of antigen-reactive T cells based on their cytolytic effector function. The assay is performed in wells of 96-well plates, with each well containing 1,000 tumor cells (or a surrogate target presenting the immunizing peptide) and a defined number of effector T cells. Usually, no more than 10^5 effector T cells are placed in a well, resulting in the effector-to-target ratio of 100:1, to avoid high levels of nonspecific lysis. It is necessary to perform the assay at several (at least four different effector/target ratios to ascertain linear kinetics (58). In order to observe lysis, 100 to 200 specific effector T cells have to be present in the well, assuming that each effector T cell can eliminate five consecutive targets during the 4-h incubation period. Thus, for a cytotoxicity assay to be positive, the frequency of CTL in the population has to be at least 1 in 1,000 cells, providing the detection limit of 10^3. The available data indicate that the frequencies in PBMC of CTL able to respond to some of the well-defined MHC class I-restricted epitopes are considerably below this limit of detection (11, 45) and imply that cytotoxicity assays are not sufficiently sensitive to be useful for monitoring of tumor-specific CTL in the peripheral blood. However, it is possible to stimulate PBMC in bulk cultures with antigen, using the procedure called "in vitro sensitization" (IVS). To expand specific CTL to the numbers detectable in chromium release assays, three to four rounds of consecutive weekly stimulations with the antigen are required. While IVS facilitates expansion of CTL from their precursors (CTLp), it yields only a qualitative estimate of the presence of specific CTL in PBMC or other lymphocyte populations. In general, methods for evaluation of CTL responses based on ex vivo expansion may greatly underestimate the number of specific T cells, because some T cells have a reduced proliferative potential, particularly in patients with cancer or certain infections (30, 36). The kinetics of CTL generation in IVS may allow for a distinction to be made between primary and secondary T-cell responses. However, for quantitative assessments of the frequency of CTL in cellular populations, cytotoxicity assays have to be performed following limiting dilution and clonal expansion of CTLp.

Limiting-dilution analysis (LDA) is a microculture technique in which lymphocytes, plated at various cell doses (e.g., 50,000 to 1.0/well) in wells of 96-well plates in the presence of antigen, APC, and interleukin-2, undergo rounds of antigen-driven replication, resulting in the formation of microcultures in a proportion of the plated wells (31). A statistical formula is then used to determine the frequency of proliferating CTLp in the population of plated cells (53). The obtained microcultures or clones (if they are derived from wells containing a single CTLp) of T cells can then be tested in cytotoxicity assays against the relevant target to determine the proportion of wells containing effector CTL. LDA has been extensively used in the past for the quantitation of both virus- and tumor-specific CTL (11, 12, 45, 53), and until recently it has provided the best available estimates of these effector cell numbers in various cellular populations. LDA is, however, very tedious and technically demanding. It is not easily applicable to monitoring of patients undergoing immunotherapy. Furthermore, the assay is notoriously variable and has been shown to grossly underestimate the size of the viral effector CTL population in murine studies (6, 13). For these reasons, the LDA has been largely replaced today by newer and more accurate technologies discussed below.

A multiple-microculture assay, involving stimulation of PBMC in a limited number of microcultures (e.g., 24 wells, each containing 10^5 responding PBMC or 10^4 enriched $CD8^+$ T cells), was introduced to avoid the labor-intensive LDA and to provide a semiquantitative estimate of peptide-specific T-cell frequencies (44). The cells are restimulated twice at weekly intervals with irradiated autologous PBMC pulsed with the peptide in the presence of cytokines, and on day 7 following the third stimulation the cells are tested in chromium release assays against suitable peptide-expressing targets. Cytotoxicity assays are performed following cold-target inhibition with K562 targets to block NK-like activity. Simultaneously, proliferation or cytokine production can be assayed in split wells, provided T-cell expansion yields adequate numbers of responding lymphocytes. Comparing the number of wells with CTL activity in pre- versus postvaccination specimens, it is possible to obtain a semiquantitative assessment of CTLp specific for single CTL epitopes and to use the assay for monitoring of effector cells in clinical trials (unpublished data). More recent reports suggest, however, that the multiple-microculture assay is not sufficiently reproducible and that it may grossly overestimate or underestimate the frequency of tumor-reactive T cells relative to LDA or to enzyme-linked immunospot (ELISPOT) (see below).

Overall, cytotoxicity assays remain firmly established in the repertoire of available CTL measurements. The ability to kill a tumor cell target is, after all, the key functional attribute of antitumor CTL. The specificity of killing, easily confirmed in this assay by the inclusion of anti-MHC and anti-TcR antibodies, may be in many instances more important than the assay sensitivity. Clearly, the assay is not acceptable for screening of CTLp frequencies in PBMC. As a confirmatory method, however, for measuring specific cytotoxicity, this assay is likely to continue serving as a gold standard for antitumor effector cell function until comparisons validate the equivalent performance for cytokine-based or tetramer-based technologies.

CYTOKINE-BASED CTL ASSAYS

Upon activation, T lymphocytes up-regulate expression of and secrete a number of cytokines (7). Polarization of the cytokine repertoire in Th1 and Th2 lymphocyte subpopulations has been well documented (33, 43). A number of methods have been introduced to measure cytokine expression in T cells responding to specific stimuli at the protein or mRNA level, as reviewed recently (42). Both the population-type and single-cell assays for cytokine expression are available (42). Here, the focus will be on the single-cell assays applicable to CTL frequency estimates, because these assays are increasingly frequently used for monitoring of responses to tumor vaccines in clinical trials.

Staining for intracellular cytokines involves in vitro stimulation of T cells with a relevant antigen in the presence of either monensin or brefeldin A to block secretion of the cytokine and enhance its accumulation in the cells. The cells are stained for surface markers (e.g., CD3, CD4, or CD8), fixed with paraformaldehyde, and then permeabilized in the presence of a detergent to allow for access of labeled anticytokine antibody inside the cell (21, 40). The positively stained cells are quantified by multicolor flow cytometry. This procedure has been

widely used for determining the numbers of antigen-specific T cells among human lymphocytes and especially for differentiating Th1 from Th2 responses (42, 56). In addition, by using appropriate monoclonal antibodies to surface antigens, it is possible to differentiate cytokine-expressing memory T cells from precursor T cells (see Table 1). The Fast Immune Cytokine System available from Becton Dickinson facilitates staining and permeabilization steps and provides all necessary control reagents for detection of intracellular cytokines. However, it is possible to purchase all the reagents separately and set up the assay independently of the kit. The only reservation about this method is that expression of a given cytokine cannot be always equated with its secretion and, therefore, the assay does not measure a cellular function. Preliminary comparisons performed in my laboratory showed that a considerable discrepancy existed between expression of gamma interferon (IFN-γ) as measured by flow cytometry and production of this cytokine by in vitro-stimulated PBMC obtained from normal volunteers and tested in ELISPOT assays (unpublished data). On the other hand, reports from other investigators indicate that there might be good agreement between flow cytometry and ELISPOT assays, although formal comparisons of these two methods are not yet available. The flow cytometry assay is also helpful in making a distinction between precursor and memory T cells: a positive assay after 4 to 6 h of stimulation with the relevant peptide suggests a memory response which needs little priming, while a longer period of stimulation (≥24 h) is usually necessary for primary responses. It is possible that the discrepancy in results between ELISPOT and flow cytometry assays for IFN-γ observed in my laboratory were related to the inability of the 24-h ELISPOT to discriminate between primary and memory responses (see Table 1).

More recently, a flow cytometry-based assay for measuring cytokine secretion by individual antigen-specific T lymphocytes was introduced (4). Called the "MACS IFN-γ secretion assay," this technology is designed for the detection, isolation, and analysis of T cells responding by IFN-γ secretion to brief (approximately 3- to 16-h) in vitro stimulation with a protein antigen or a peptide (4). The assay allows for capture and enrichment of antigen-specific T cells, thus facilitating the subsequent analysis as well as expansion of these cells. The IFN-γ-secreting cells are placed in the medium of low permeability for the secreted product (27). The secreted IFN-γ is retained on the cell surface of the secreting cell, using an affinity matrix for the secreted cytokine (the catch reagent) which consists of an antibody able to capture IFN-γ conjugated to a cell-surface specific antibody (4). The captured IFN-γ is then detected by the phycoerythrin-labeled second antibody specific for IFN-γ (a detection antibody). The subsequent analysis by flow cytometry allows for enumeration of lymphocytes secreting IFN-γ. Similarly to all other antibody-based assays, this one depends on the specificity and quality of anti-IFN-γ antibodies and on conditions set up for capture of the cytokine. It also offers a possibility for enrichment of IFN-γ-secreting cells by a special matrix consisting of paramagnetic MicroBeads conjugated to monoclonal mouse anti-phycoerythrin antibody by using the familiar MACS technology. The enrichment occurs by separation of magnetically labeled cells on a column, using a MiniMacs cell separator. The method has a wide range of applications, including monitoring and functional analysis of antigen-specific T cells as well as enrichment of IFN-γ-secreting cells for determinations of TcR epitope mapping. Depending on the conditions selected for this assay, it might be possible to discriminate between early (i.e., memory) and late (i.e., primary) IFN-γ expression in T-cell populations. Comparisons between this assay and ELISPOT have not yet been made.

The ELISPOT assay is another antibody-based technique for quantitation of single cells secreting cytokines in response to a challenge with antigen (2, 18, 20, 41, 48, 49). For detection of IFN-γ-secreting cells, nitrocellulose-lined or plastic microtiter plates coated with a capture antibody are used. Graded numbers of PBMC, enriched $CD8^+$ or $CD4^+$ T cells, or cultured T cells are plated in wells of the microplate together with the appropriate APC plus antigen to stimulate secretion of the cytokine. The number of cells plated is critical, because a uniform lawn of single cells, only some of which (not too few and not too many!) secrete the cytokine, is optimal for assay quantitation. After an incubation period of 24 h, a detection antibody labeled with an enzyme such as horseradish peroxidase is added, followed by a suitable substrate for color development. The cells secreting IFN-γ are detected as discrete colored spots, which are microscopically evaluated and counted, using a computer-assisted video image analysis system developed especially for this purpose (18). Under optimal assay conditions, each spot corresponds to a single cytokine-producing cell (18). In addition to objective enumeration of spots in this system, the spot area can be determined to obtain an indication of the level of produced cytokine and thus the strength of the response to an antigen. The assay has been found to be highly reproducible, convenient to use with cryopreserved PBMC, and sufficiently sensitive to detect 1 IFN-γ-secreting T cell among 100,000 (2). When used with autologous DC pulsed with lysates of tumor cells, for example, the assay can detect not only $CD8^+$ but also $CD4^+$ responses (19). This is important in view of accumulating evidence that $CD4^+$ T cells play a critical role in the induction and maintenance of antitumor responses (37). Responses to MHC-restricted peptides presented on correctly matched APC or to non-MHC-restricted antigens processed and presented by autologous DC can be measured in ELISPOT assays. Because of these attributes and its versatility, the ELISPOT assay has been widely used for monitoring of the frequency of antigen-reactive T cells in patients treated with cancer vaccines and especially of T cells responsive to MHC class I-restricted melanoma antigens, including MAGE, tyrosinase, Melan-A/MART-1, and gp100 (2, 20, 38). In addition, it has been successfully used for identification of a novel DR4-restricted Melan-A/MART-1-derived peptide (Melan-A/MART-1_{51-73} recognized by $CD4^+$ T cells obtained from HLA-DR4-positive patients with melanoma or normal donors (60). The ELISPOT assay, which measures cytokine secretion (a relatively late event following antigen stimulation), does not discriminate between primary or memory responses, unless it is performed with previously separated precursor or memory T cells (Table 1).

Assays based on detection of cytokine production, as opposed to cytokine expression, have been steadily gaining ground, largely due to the perception that they are functionally more relevant. Since these assays depend on the use of two antibodies recognizing distinct epitopes on the cytokine which is being measured, they are highly specific. They are also highly sensitive, because of the amplification step that is generally associated with the application of antibody-based techniques. Limited comparisons of ELISPOT with cytotoxicity assays performed in my laboratory indicated good agreement between the two (unpublished data). In comparison to cytotoxicity, ELISPOT assays are less labor-intensive, more reproducible, and more cost-effective. The choice of ELISPOT versus single-cell flow cytometry-based cytokine production assays, such as MACS IFN-γ secretion assay, depends on the availability of a flow cytometer for serial monitoring. The requirement for a dedicated flow cytometer may discourage some users from implementing the MACS IFN-γ secretion assays. On the other

TABLE 1. Assays for monitoring CTL

Assay	Sensitivity (no. of cells)	Specificity for the following CTL type:		
		Effector	Precursor	Memory
Function				
^{51}Cr release (cytotoxicity)	$1/10^3$	+	+ (after LDA frequency estimates)	+ (after IVS with MHC class I-restricted peptides)
ELISPOT (cytokine production)	$1/10^5$	+	−	+
Expression				
Single-cell flow cytometry (intracellular cytokine)	$1/10^3$	+	+ ($CD45^+$ RA^+)[a]	+ ($CD45^+$ RO^+)[b]
Tetramers (binding to unique TcRs)	$1/10^3$	+	+ ($CD45^+$ RA^+)	+ ($CD45^+$ RO^+)

[a] Time to response, 6 h.
[b] Time to response, ≥24 h.

hand, ELISPOT, whose general format resembles that of the enzyme-linked immunosorbent assay, lends itself remarkably well to monitoring of clinical protocols and offers an opportunity for quantitative assessments of $CD8^+$ as well as $CD4^+$ T-cell frequencies in freshly isolated or cultured cellular populations. It is, therefore, highly likely that ELISPOT will emerge as the assay of choice for the frequency analysis of tumor- or virus-specific effector T cells after comparisons with other assays are completed.

MHC-PEPTIDE COMPLEXES FOR DIRECT ASSESSMENT OF LIGAND-BINDING T CELLS

An attractive approach to isolation and quantitation of peptide-specific T cells in mixed lymphocyte populations was recently introduced based on the use of the fluoresceinated complexes containing the peptide itself linked to MHC class I molecules (1). Commonly referred to as "tetramer binding," this technology involves formation of oligomeric complexes of MHC molecules with the relevant peptide. Because a monomeric peptide-MHC has a very weak affinity for TcR, a strategy was devised by M. Davis and colleagues of labeling the MHC molecules in the complex with biotin and assembling such biotinylated complexes to form tetrameric arrays on a scaffold of avidin (1, 25). These oligomeric peptide-MHC reagents have increased avidity for T cells expressing specific TcR, and when they bind, a strong fluorescent signal detectable by flow cytometry is generated, thus marking the T cell which recognizes the peptide. Another approach uses genetic linking of MHC molecules to IgG1 to produce a dimer in which IgG1 serves as a scaffold (24). The specificity of peptide-MHC reagents is their greatest asset, and as long as binding properties of the peptide to TcR are preserved or improved by oligomerization, they represent valuable and unique probes for peptide-binding clones of T cells. Such probes have been successfully employed for both quantitation and then isolation by sorting of $CD8^+$ T cells binding melanoma peptides in PBMC of patients with metastatic melanoma (25). While peptide-MHC tetramers or dimers are promising and undoubtedly highly specific reagents, their application to monitoring or frequency analysis of clinical samples presents a number of problems. First, these are unique, custom-designed reagents, and their preparation requires that both the tumor peptide and its MHC restriction be known, limiting the use of this technology to a handful of peptides and a relatively small number of patients with cancer. The production of oligomers, their stability, levels of multimerization, and quality of the peptide to be incorporated into the complex are all important factors that determine success in implementing this method. Second, tetramer binding is temperature dependent in that staining at 4°C may result in a high background due to the binding of tetramers to TcRs that recognize the peptide-MHC very weakly (57). At 37°C, on the other hand, the specificity of tetramer staining for strongly recognized, non-cross-reactive ligands is increased (57). Third, because TcR can exhibit promiscuity for peptide-MHC class I ligands, the potential for cross-reactivity exists and has to be considered when the identification of antigen-specific CTL is desired. Finally, the sensitivity of T-cell detection by this technology may be well below that achieved with more conventional methods for single-epitope-specific T cells, as discussed above. The detection of ligand-binding T cells is based on flow cytometry, where a lower limit of detection is generally placed at 0.2%, which means that ~1 positive cell per 10^3 tested can be detected. Furthermore, down-modulation of TcR on some T cells in patients with cancer may also contribute to diminished sensitivity of detection. To increase sensitivity, it is possible to increase the number of total events collected or to combine the peptide-MHC oligomer staining with a selected set of surface markers on T cells, e.g., those expressed on the memory-effector population, which may be expected to contain the majority of antigen-reactive T cells (Table 1). However, when Romero and colleagues used fluorescent HLA-A*201 tetramers to characterize MelanA/MART-1-specific T cells in PBMC of normal donors and several patients with melanoma, they observed that these cells displayed a naïve CD45RA (hi)/RO(−) phenotype (39). In contrast, influenza matrix-specific CTL from these individuals had a memory CD45RA (low/RO (+) phenotype (39). Thus, tetramers are proving to be useful for phenotypic as well as functional characterization of antigen-specific T cells (14). Nevertheless, more extensive evaluation of these promising tetrameric peptide-MHC class I complexes is necessary before they are accepted for monitoring of CTL responses. A requirement for multicolor flow cytometry restricts tetramer use to laboratories with the capability to undertake this type of labor-intensive analysis.

While the peptide-MHC oligomer technology might not lend itself readily to monitoring at this time, it appears to be a valuable tool for confirmatory studies of antigen-specific T cell subsets and for "fishing out" small numbers of antigen-specific T cells from mixed populations of lymphocytes for their phenotypic and functional characterization. Furthermore, these cells can be cloned in vitro for further characterization (14). It is likely that future improvements of this promising technology will eliminate some of the limiting steps and facilitate its broader use in clinical laboratories.

IMMUNOSCOPE ANALYSIS OF CDR3 DOMAINS IN T CELLS

The complementarity-determining regions (CDR) of TcR are the most variable parts of the receptor protein, endowing it with diversity. The CDR are found on six loops at the distal end of variable domains, with three loops protruding from each of the two variable domains of TcR. The CDR3 are the most variable of the three. The CDR are in direct contact with the binding ligand and determine the receptor specificity. Molecular cloning of TcR genes and sequencing of hypervariable CDR3 indicated that a broad range of specificities exist in the TcR repertoire of an individual (35). However, for certain antigens, the TcR repertoire is quite restricted, in the sense that a few closely related TcR are recognized by responding antigen-specific clones of T cells (8, 52). In principle, clonotypic Vβ-specific primers can be used to detect the presence of antigen-specific T cells (i.e., T cells with a restricted Vβ repertoire) among mixed lymphocyte populations by reverse transcriptase-PCR-based methodology. One quantitative approach involves an initial PCR with unlabeled Vβ- and Cβ-specific primers to determine the length of the CDR3 region. The PCRs are set up to amplify the cDNA of interest, using primers to the regions on either side of CDR3 (a Cβ-specific primer and 1 of 24 Vβ-specific primers). The product of each amplification is then visualized by performing a runoff reaction, which includes an additional fluorescently labeled probe. The runoff products are sequenced on an automatic sequencer in the presence of fluorescence size markers. The size and fluorescence intensity of the fragments are then analyzed using Immunoscope software (35). The Immunoscope analysis provides results in the form of a bell-shaped curve with an average of eight peaks for PBMC of a normal donor. The emergence of one prominent peak signifies the presence of one or a few cDNAs with identical or similar CDR3 regions. This means that the T cells utilize a restricted repertoire of Vβ genes and may be clonal or oligoclonal. While this technique allows for the detection of restricted TcR repertoires of T cells, it does not identify the ligands recognized by these T-cell clones. It now appears that antigen-specific T cells can utilize quite diverse TcR repertoires (8, 52, 22). Thus, this technology cannot be applied to monitoring of antigen-specific CTL responses, simply because it is impossible to predict a priori whether a TcR repertoire for a given antigen will be diverse or restricted. However, the method is applicable to following changes in the TcR repertoire in individual patients during therapy (57).

CONCLUSIONS

Several new methods have been identified for monitoring of antigen-specific CTL. A better understanding of the processes of antigen processing, presentation, and recognition by T cells has significantly contributed to the development of these technologies. The availability of these technologies has focused attention on monitoring activities and frequencies not only of antigen-specific effector T cells but also of memory and precursor T cells. Table 1 lists the assays that are currently available for monitoring of these populations in humans and provides estimates of the limits of detection for each assay. The advantages and disadvantages of these assays most relevant to their application in patient monitoring are discussed above. The possibility for quantitation as well functional characterization of antigen-specific T cells in populations of lymphocytes has provided new opportunities for monitoring immune responses to individual antigens in vitro and in vivo. Application of these methods to monitoring of patients with cancer treated with biologic therapies is likely to result in a definition of new immunologic end points. However, to meet criteria for monitoring, the current available methods have yet to be validated. Work is currently in progress to compare the performances of various assays in the clinical setting, and before long it should be possible to recommend those that are biologically and clinically most relevant and economically acceptable.

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FcγRIIB is differentially expressed during B cell maturation and in B-cell lymphomas

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Summary

FcγRIIB, a low affinity receptor for the Fc portion of immunoglobulin G (IgG), is thought to drive negative selection of B cells in germinal centers (GC) by inducing apoptosis upon interaction with immune complexes. Its expression was investigated by immunohistochemistry in 22 reactive lymphoid tissues and 112 B-cell lymphomas. Pre-GC mantle cells, marginal zone cells and their neoplastic counterparts expressed FcγRIIB. The B chronic lymphocytic leukaemia (B-CLL)/small lymphocytic lymphomas were also positive. Not detected in GC, FcγRIIB was expressed in 52% of follicular lymphomas and in 20% of diffuse large B cell lymphomas (DLBCL). In DLBCL, FcγRIIB expression was linked to transformation ($P < 0{\cdot}001$). Re-analysis of a gene profile data set from the Lymphochip microarrays showed that FcγRIIB expression in the activated B-like DLBCL subgroup was higher than in the GC-like one ($P < 0{\cdot}04$), and was associated with an adverse prognostic both in univariate ($P < 0{\cdot}003$) and in multivariate analysis including the International Prognostic Indicator (IPI) ($P < 0{\cdot}01$). Thus these results challenge the potential role of FcγRIIB during B-cell selection in GC, and suggest a prognostic value of FcγRIIB expression in DLBCL.

Keywords: non-Hodgkin's lymphoma, FC receptor, B lymphocytes, immunophenotype.

After leaving the bone marrow, naïve B cells encountering antigen in secondary lymphoid organs undergo crucial differentiation steps that transform them into antibody-secreting plasma cells or memory cells (Tarlinton & Smith, 2000). In the extrafollicular T-cell area, B cells can enter two different pathways (MacLennan, 1994). The first pathway results in local proliferation of immunoblasts and transformation into low affinity immunoglobulin M (IgM) secreting plasma cells. The second pathway, which requires B cell migration into the follicular area, gives rise to germinal centers (GC). B cell-derived centroblasts located in the GC dark area proliferate extensively and undergo a process of somatic mutations, allowing diversification of their V-genes. Selection and differentiation of centrocytes into high-affinity antibody-secreting plasma cells and B memory cells then occurs in the GC light zone, driven by antigen retained on the follicular dendritic cells (FDC) in the form of immune complexes (Nossal, 1994; Rajewsky, 1996; Healy & Goodnow, 1998). In the GC, DNA recombination leads to the deletion of Ig constant regions, allowing Ig class switching.

Most of the major effector functions of antibodies involve their interaction with Fc receptors (FcR) present on hematopoietic cells. Three categories of FcR for IgG (FcγR) exist: FcγRI (CD64) has a high affinity for monomeric IgG, whereas FcγRII (CD32) and FcγRIII (CD16) bind avidly IgG-containing immune complexes (Fridman *et al*, 1992; Gessner *et al*, 1998). Depending on their cytoplasmic region and/or their associated chains, FcγR display activating or inhibitory roles during immune responses (Daeron, 1997). In humans, two major FcγRII isoforms exist, FcγRIIA and FcγRIIB, which differ principally in their cytoplasmic sequences. FcγRIIB contains an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) in its cytoplasmic tail. When co-engaged with ITAM-bearing receptors, such as FcγRIIA on monocytes (Pricop *et al*, 2001) or the B cell antigen receptor (BCR) Igα or β chains on B cells (Muta *et al*, 1994; Ono *et al*, 1996; Coggeshall, 1998), it down-regulates activation signals. As the unique engagement of FcγRIIB by immune complexes generates an apoptotic signal in chicken B cells, it has been suggested that FcγRIIB may also play a role in negative

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selection of B cells in the GC (Pearse *et al*, 1999). Thus, those B cells that express hypermutated BCR of low affinity for antigen may be eliminated by FcγRIIB-dependent apoptosis triggered by FDC-bound immune complexes whereas the high affinity B cells may be protected from apoptosis and positively selected. However, this hypothesis is challenged by the down-regulation of FcγRIIB expression in GC B cells described recently (Macardle *et al*, 2002; Rao *et al*, 2002) and in the early studies of Pulford *et al* (1986) using the pan-anti-FcγRII mAb KB61.

B cell lymphomas correspond to a heterogeneous set of malignancies reflecting proliferation of B cells at various stages of differentiation (Jaffe *et al*, 2001). In addition to chemotherapy regimens, anti-CD20 is currently successfully used to treat some of these malignancies but its mechanism of action remains unclear (Nguyen *et al*, 1999). Among the proposed mechanisms, a potential role for FcγR expressed by host effector cells has been suggested from the association of response with FcγRIII polymorphisms (Cartron *et al*, 2002) and from the FcγR-dependent effect of anti-CD20 therapy of human B cell lymphomas grafted in nude mice (Clynes *et al*, 2000). FcγR expressed by the tumour cells themselves may also interfere with anti-CD20 therapy. However, little is known about the expression of FcγR in the different subtypes of lymphoma.

In the present study, we have analysed a series of human reactive lymphoid tissues and mature B-cell neoplasms using polyclonal antibodies raised against the intracytoplasmic portion of the receptor and which works on paraffin-embedded tissues. Whereas a profound down-regulation of FcγRIIB was found in GC B cells, FcγRIIB was expressed by a subset of follicular lymphomas and DLBCL. Our study suggests that FcγRIIB expression may be linked to transformation in follicular lymphoma and associated to adverse prognostic in DLBCL.

Materials and methods

The anti-FcγRIIB Antibodies

The anti-FcγRIIB/IC polyclonal antibodies, previously described (Cassard *et al*, 2002), were prepared by hyperimmunization of rabbits with a recombinant Glutathione S-transferase (GST) fused to the IC1 to IC3 sequence of human FcγRIIB1 (GST-IC). A reverse transcription polymerase chain reaction fragment obtained from FcγRIIB1 cDNA by using the following amplimers 5′-gctctcccaggataccctgagtgc-3′ (sense) and 5′-AATACGGTTCTGGTCATCAGGCTC-3′ (antisense), was cloned into the pGEX-2T expression vector (Pharmacia Biotech, Uppsala, Sweden) after addition of restriction sites. Expression of the GST-IC protein in *E. coli* was induced by Isopropyl bD-thiogalactopyranoside (IPTG) and the protein was purified from periplasmic fraction by affinity chromatography on glutathione agarose column (Sigma). Anti-GST antibodies were adsorbed by three successive passages onto GST-coupled sepharose until there was a lack of enzyme-linked immunosorbent assay reactivity as tested on GST-coated plates.

In contrast to the anti-CD32 mAb KB61 (Pulford *et al*, 1986), anti-FcγRIIB/IC polyclonal antibodies recognize specifically FcγRIIB (CD32) and not FcγRIIA. Moreover, they recognize intracytoplasmic sequences, located outside the IgG-binding site, making a potential masking effect of FcγRIIB-bound IC very unlikely.

Detection of FcγRIIB by immunohistochemistry

The cases were analysed by immunohistochemistry using paraffin-embedded material sections prepared conventionally or as a multi-tissue block for 11 cases of DLBCLs. The latter cases were prepared on a single paraffin tissue block, using a microarrayer kindly provided by G. Szekeres (Histopathology Ltd., Pécs, Hungary), to analyse 2×2 mm diameter punch sections for each case. For each block analysed, 4 μm slices were deparaffinized in a routine manner. Antigen retrieval was performed with microwave pretreatment followed by 1 h of incubation with 5 μg/ml of anti-FcγRIIB/IC antibody. After two washes, the sections were covered with 10 μg/ml of horseradish peroxidase-conjugated $F(ab')_2$ fragment goat anti-rabbit IgG (Immunotech, IM0831, Marseille, France) for 30 min. The staining was visualized with diaminobenzidine and the nuclei counterstained with haematoxylin. In all cases, the quality of staining was controlled by internal positive controls, such as macrophages and/or plasma cells. For lymphoma cases, samples were considered to be positive when at least 10% of tumour cells were stained, but the results usually produced a clear-cut positive pattern with the majority of tumour cells strongly positive at their plasma membrane. All cases were independently reviewed by two pathologists.

The polyclonal anti-FcγRIIB/IC serum gave good IHC positivity of paraffin-embedded pellets of FcγRIIB1-transfected melanoma cell lines but not in the non-transfected or FcγRIIA-transfected controls, confirming the specificity of this reagent (Figs 1A and B). Good immunohistochemical technical quality was obtained using material that was fixed with the following solution: 10% formalin, 10% formalin-phosphate-buffered saline, AFA (Alcohol, Formalin, Acetic acid) and Bouin's fixative (data not shown).

Patients

Twenty-two reactive lymphoid tissues of various origin and 112 cases of B-cell lymphomas were analysed (Table I). Reactive lesions and systemic cases of B-cell lymphomas were retrieved from the files of the department of Pathology, Hôtel Dieu, Paris, France. Central Nervous System (CNS) lymphomas were obtained either from a series included in the SNC98 GOELAMS trial ($n = 15$) or a previous work using samples obtained from the Groupe hospitalier Pitié-Salpêtrière ($n = 7$) and Hôpital du Val de Grâce ($n = 8$), Paris, France (Camilleri-Broet *et al*, 2000). Among the latter, the eight human

COLOUR FIG.

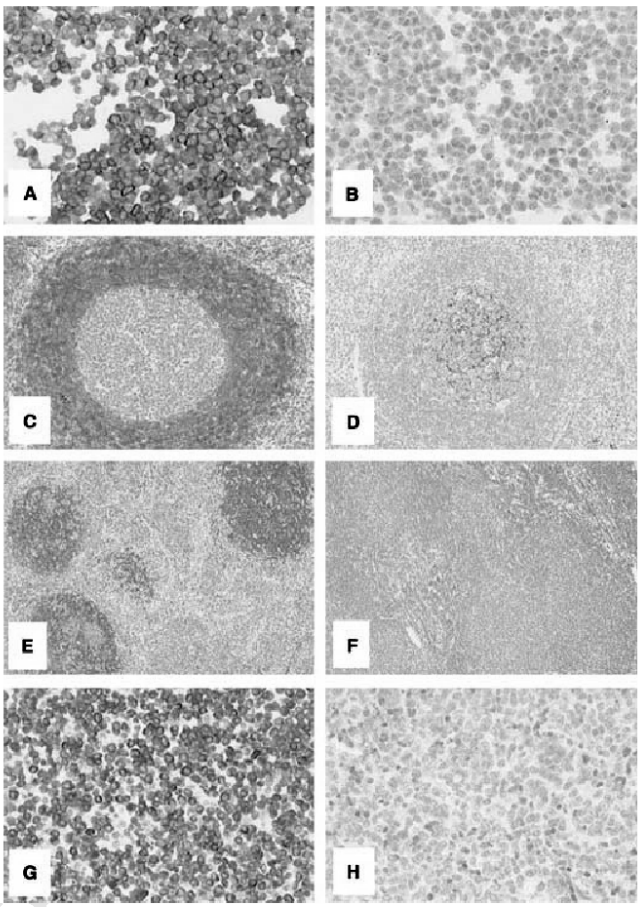

Fig 1. Immunohistochemical staining with the polyclonal anti-FcγRIIB/IC antibodies. (A) Strong plasma membrane staining of fixed- and paraffin-embedded pellets of FcγRIIB1-transfected melanoma cell line. (B) No staining of fixed- and paraffin-embedded pellets of melanoma cell line transfected with FcγRIIA. (C) Absence of detectable expression of RFcγIIB in reactive GC while mantle cells strongly express this protein. (D) Staining of follicular dendritic cells with the anti-CD23 antibody. (E) Strong expression of RFcγIIB in a case of follicular lymphoma. (F) Absence of detectable expression of RFcγIIB in a case of follicular lymphoma. (G) A case of diffuse large B cell lymphoma (DLBCL) showing strong RFcγIIB expression at the plasma membrane. (H) A case of diffuse large B cell lymphoma that does not express RFcγ IIB.

Table I. Reactive lymphoid tissues analysed.

Reactive lesions	22
Spleen parenchyma	4
Follicular hyperplasia in digestive tract	3
Lymph nodes:	15
Reactive to a lung carcinoma	4
Castleman disease	1
Necrotizing lymphadenitis	1
Unspecified lymphadenitis	9

immunodeficiency (HIV)-primary CNS lymphomas were immunoblastic with plasmablastic differentiation and associated with the Epstein–Barr virus (EBV). Formalin-fixed material was available for all cases, except for the myelomas that were Bouin's liquid-fixed bone marrows.

For follicular lymphomas, the following information was collected when available: histological grade (grade 3: five of 24), foci with the appearance of marginal zone (four of 24), intracytoplasmic Ig expression (seven of 16), Bcl-2 and CD10 expression (18 of 24 and 13 of 17 respectively), bcl-2 gene rearrangement (MBR or mcr loci) (seven of 18), bone marrow involvement (12 of 17) and follow-up (death, transformation).

Among the 46 non-HIV DLBCLs, 12 corresponded to transformation from indolent lymphomas, which were either known previously by history ($n = 6$) or recognized histologically without history of indolent lymphoma ($n = 6$). Among them, seven had transformed from follicular lymphomas. Other cases corresponded to transformation of marginal zone lymphomas or lymphoplasmacytic lymphomas. FcγRIIB expression was compared with Bcl-2 and CD10 expression.

Statistical analysis of FcγRIIB gene expression in DLBCL, based on web site microarray data set

The lymphochip cDNA microarray data set gene expression patterns in DLBCL reported by Alizadeh *et al* (2000) was re-analysed (http://llmpp.nih.gov/lymphoma/). The available data set consists of 4026 values for each sample, each value reflecting the relative abundance of a given mRNA as compared with the mRNA pool used as a reference. The six cDNA clones of the lymphochip that were entitled 'IgG Fc receptor II-B and C isoforms' and their corresponding postprocessing values were selected. We restricted our analysis to the 38 of the 40 DLBCL patients for whom all values were given. Measurements were centered first and scaled. A FcγRII score was calculated for each sample, corresponding to a unique linear combination (with equal weights) of these values in order to produce simple, comparative and informative data from the spot intensities of the different clones. The FcγRII scores were then compared with the International Prognostic Indicator (IPI) and DLBCL subgroups (GC B-like or activated B-like) using classical *t*-test. Considering a median cut-off for the FcγRII score, survival curves corresponding to the low score FcγRII and high score FcγRII were derived from the Kaplan–Meier estimates (Kaplan & Meier, 1958). The relationship between overall survival and FcγRIIB score, IPI and subgroups (GC B-like or activated B-like) were tested separately or combined using Cox proportional hazards models (Cox, 1972). All analyses were performed with the S-Plus software package. The characteristics of the patients and the results of FcγRIIB expression in our series were compared using Fisher's exact test. Only *P*-values ≤0·05 were considered significant.

Results

FcγRIIB expression in reactive lymphoid tissues

The pattern of FcγRIIB expression in follicles was studied in 22 reactive lymphoid tissues (Table I), whatever their origin. Mantle cells of secondary follicles clearly showed a plasma membrane expression of FcγRIIB (Figs 1C and D). Notably, except on rare plasma cells, no labelling was detected in GC, neither on FDC, tingible bodies in macrophages or lymphoid cells. In the interfollicular area, the plasma cells often showed a strong membrane expression of FcγRIIB, but the immunoblasts were negative. Monocytoid B-cells, found in some cases of lymphadenitis, as well as splenic marginal zone cells, were positive. A cytoplasmic and/or membrane staining of FcγRIIB was detected on some histiocytic/macrophage cells located in the interfollicular area.

FcγRIIB expression in B-cell lymphomas

Results are summarized in Table II. The pattern of FcγRIIB expression in several types of small B-cell lymphomas closely resembled the pattern observed in their corresponding normal counterparts. Indeed, all mantle cell lymphomas, splenic marginal zone lymphomas and mucosa-associated lymphoid tissue (MALT) lymphomas strongly expressed FcγRIIB at their plasma membrane (five, eight and five cases respectively). It was also true for the seven B-CLL/small lymphocytic lymphomas. Notably, FcγRIIB was expressed by a subset of follicular lymphomas (13 of 24) and of DLBCLs (11 of 54). Among the DLBCLs, two of eight HIV-associated primary EBV+ CNS lymphomas and nine of 46 non-HIV DLBCLs were FcγRIIB-positive (detailed below). In contrast to mature plasma cells, which expressed strongly FcγRIIB, only two of nine myelomas were positive.

Table II. FcγRIIB expression in mature B-cell lymphomas.

B-cell neoplasms	No. of cases studied	No. of positive cases	% positive cases
B-CLL/Small lymphocytic lymphomas	7	7	100
Splenic marginal zone lymphomas	8	8	100
MALT lymphomas	5	5	100
Mantle cell lymphomas	5	5	100
Myelomas	9	2	22
Follicular lymphomas	24	13	54
DLBCLs	46	9	20
De novo	34	2	6
Transformed	12	7	58
Primary HIV-associated CNS lymphomas	8	2	25
Total	112		

MALT, mucosa-associated lymphoid tissue; DLBCLs, diffuse large B-cell lymphomas; CNS, central nervous system.

FcγRIIB in follicular lymphomas: marker of progression?

In contrast to reactive GC B cells, which were constantly negative, 13 of 24 (54%) cases of follicular lymphomas expressed FcγRIIB at their cytoplasmic membrane (Figs 1E and F). The expression levels were variable from one case to another and, in most cases, similar to those of mantle cells. Two cases showed a weak expression whereas one case showed a very strong staining (Fig 1E). No significant relationship was observed between FcγRIIB expression and the histological grade, Bcl-2 expression, CD10 expression, Bcl-2 gene rearrangement or bone marrow involvement. No significant association was found either between FcγRIIB expression and marginal differentiation. Intracytoplasmic Ig expression was more frequent in tumour cells from FcγRIIB-expressing cases than in those from FcγRIIB-negative cases, but this did not reach statistical significance in this small series ($P = 0{\cdot}1$).

Among the 17 patients followed-up (median follow-up: 4 years), 10 expressed FcγRIIB. Of the seven FcγRIIB-negative cases, one death was recorded without recognized transformation, two patients showed a transformation 5 and 6 years after

the biopsy and four patients were alive without transformation at a median follow-up of 5 years. Of the 10 FcγRIIB-expressing cases, three deaths were recorded, two of which followed transformation. Moreover, five of 10 cases showed transformation, at the time of biopsy for one patient, within the first year for three patients and after 4 years of evolution for the last one. Altogether, these results show that, within the first year, transformation occurred in four of the 10 FcγRIIB-positive cases but in none of the seven negative cases ($P = 0{\cdot}1$).

FcγRIIB expression in DLBCL: a marker of transformed lymphoma?

Nine of the 46 non-HIV DLBCLs (20%) expressed strongly FcγRIIB at their plasma membrane (Figs 1G and H). No significant association was found between FcγRIIB expression and the prognostic marker Bcl-2 ($P = 1$). Among the 42 cases tested for both Bcl-2 and FcγRIIB, six of the 32 (19%) Bcl-2-positive cases co-expressed FcγRIIB, whereas it was expressed in two of the 10 (20%) Bcl-2-negative cases. CD10 expression was more frequently found in the FcγRIIB-negative group without reaching statistical significance ($P = 0{\cdot}1$). Moreover, FcγRIIB was more frequently expressed in the systemic cases (seven of 24; 29%) than in the CNS cases (two of 22; 9%), but was not significant ($P = 0{\cdot}14$). One important level of heterogeneity of DLBCL is that some of the cases are in fact indolent B-cell lymphomas that have undergone transformation, often underestimated and usually with a poor prognosis. In our series, FcγRIIB expression was found in a significantly higher proportion of transformed DLBCLs (seven of 12, 58%) than in DLBCLs without recognized indolent B-cell lymphoma (two of 34, 6%) ($P < 0{\cdot}001$).

FcγRIIB gene expression in DLBCL microarray studies

To further investigate the potential influence of FcγRIIB gene expression levels on the overall survival in DLBCL, we re-analysed the data set used by Alizadeh *et al* (2000). Alizadeh *et al* (2000) described two molecularly distinct forms (subgroups) of DLBCLs on the basis of their gene expression profiles, the GC B-like DLBCLs and the activated B-like DLBCLs, with gene expression patterns characteristic of GC B cells and of B cells activated *in vitro* respectively. The GC B-like DLBCLs patients had better overall survival than the activated B-like DLBCLs ones. By selecting the values given by the six cDNA clones entitled 'IgG Fc receptor II-B and C isoforms' and transforming them into a 'FcγRIIB score', we found that the higher FcγRIIB score values were significantly linked to activated B-like DLBCL subgroup ($P = 0{\cdot}04$), whereas no significant relationship was found between the FcγRIIB score and the IPI, the clinical indicator of prognosis ($P = 0{\cdot}17$). Moreover, a high FcγRIIB score was significantly linked to worse overall survival in univariate analysis ($P = 0{\cdot}003$) (Fig 2) as well as after adjustment for IPI ($P = 0{\cdot}01$). To determine whether the FcγRIIB score could provide prognostic

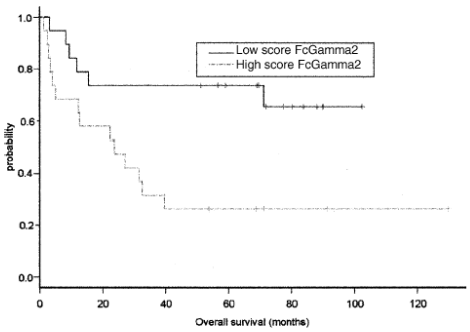

Fig 2. Kaplan Meier estimates for the calculated FcγRIIB score from microarray data set (http://llmpp.nih.gov/lymphoma/). Considering a median cut-off, a high FcγRII score is significantly linked to worse overall survival in univariate analysis ($P = 0{\cdot}003$).

information regarding the two subgroups (GC B-like DLBCL or activated B-like DLBCL), we introduced and tested this latter term in the previous multivariate Cox model. However, when taking into account subgroups, neither FcγRIIB score ($P = 0{\cdot}21$) nor subgroups ($P = 0{\cdot}31$) remained significant as compared with IPI ($P = 0{\cdot}013$). This probably relates to collinearity effect between subgroups (GC B-like DLBCL or activated B-like DLBCL) and the FcγRIIB score.

Discussion

This study has found a tight regulation of FcγRIIB expression *in situ* during B cell differentiation and in B cell malignancies. A profound down-regulation of FcγRIIB was observed in the GC of the 22 reactive lymphoid tissues studied, independently of their origin (lymph nodes, digestive tract and spleen) and the clinical situation. FcγRIIB down-regulation was transient as pre-GC and post-GC B cells were positive. Extrafollicular immunoblasts were negative as well, suggesting that down-regulation is not restricted to GC. The down-regulation of FcγRIIB observed in GC herein confirms and extends previous studies performed in mice (Rao *et al*, 2002) and humans (Pulford *et al*, 1986; Macardle *et al*, 2002). It is also compatible with the lower levels of FcγRIIB in GC B cells, as compared with resting/activated B cells, that were seen on the lympho-chip cDNA microarrays (http://llmpp.nih.gov/lymphoma/). Altogether, these results challenge the hypothesis that suggests that one of the major roles of FcγRIIB is to trigger negative selection of B cells expressing hypermutated BCR of low affinity for antigen (Pearse *et al*, 1999). If that is the case, FcγRIIB should be at least detectable on such cells. In contrast to mouse FDC (Qin *et al*, 2000; Rao *et al*, 2002), FcγRIIB was undetectable on human FDC. However, GC formation in mice is obtained after experimental immunization, a situation that is different from usual human clinical situations. Moreover in humans, immune complexes may be collected at least by

complement receptors (Barrington *et al*, 2002), human FDC being CD21 (CR2) and CD35 (CR1) positive.

In DLBCLs, although not significant in our series, there was a trend for a relationship between CD10 expression (GC marker) and FcγRIIB-negative cases. This was strengthened upon re-analysis of the lymphochip cDNA microarray data set from Alizadeh *et al* (2000), the results showing a significant relationship between high FcγRIIB scores and activated B-like profiles in DLBCLs. Furthermore, FcγRIIB gene expression levels are significantly associated with a poor prognosis in DLBCLs, independent of the IPI. This observation is compatible with a linkage between FcγRIIB-score values and the activated B-like profile, known to be of poor prognosis. We have found no significant relationship between Bcl-2 and FcγRIIB expression in our series whereas it has been shown that activated B-like DLBCLs had higher Bcl-2 mRNA levels than were observed GC B cells. This result most probably reflects that the full spectrum of activated and GC B-like DLBCLs as defined by microarray cannot be summarized by a single antigen expression.

In our series, a significant relationship between FcγRIIB expression and transformed cases of DLBCL was found. This group of transformed DLBCL included patients with an indolent lymphoma component on histological biopsy, even in the absence of a previously known history of indolent disease. Thus, it is possible that the poor prognostic activated B-like group of patients may include a subset of unrecognized transformed lymphomas, which are known to be aggressive (Bastion *et al*, 1997). Moreover, the fact that a large proportion (40%) of the FcγRIIB-expressing follicular lymphomas were already transformed or in the process of transformation in the aggressive lymphomas, is also compatible with a possible association of FcγRIIB with transformation. Frequent abnormalities of chromosome 1q have been described in B cell lymphomas and are correlated with poor outcome in non-Hodgkin's lymphomas (Offit *et al*, 1991; Johansson *et al*, 1995; Whang-Peng *et al*, 1995; Zimonjic *et al*, 2001; Martinez-Climent *et al*, 2003). These abnormalities frequently involve the 1q21-23 region, containing the *FCGR2B* gene. Multiple and complex modifications such as duplication, triplication or translocation occur, probably reflecting the high level of instability of the region (Itoyama *et al*, 2002). In few cases, the translocation of this region was associated with hyperexpression of FcγRIIB2 protein (Callanan *et al*, 2000; Chen *et al*, 2001).

The question of the role of FcγRIIB in tumour development was addressed in mouse models. Its expression is associated with an inhibition of growth of human melanoma cells in nude mice, which occurs upon direct interaction of tumour cells with IgG3 anti-tumour antibodies (Cassard *et al*, 2002). However, FcγRIIB expressing polyoma virus-transformed 3T3 cells (Zusman *et al*, 1996) showed increased tumourigenicity in immunocompetent mice when compared with negative ones. These apparently contradictory results might be related to host immunoregulatory influences and/or tumour cells. In immunocompetent hosts, tumour expressing FcγRIIB may down-regulate effector functions of FcγR-positive cells present in the microenvironnement, by trapping immune complexes and thus leading to dominance of FcγRIIB expressing tumour cells. Tumour cells may also shed FcγRIIB under its soluble form by proteolytic cleavage or alternative splicing of the transmembrane-encoding exon, as demonstrated in the sera of mice bearing lymphoid tumours (Lynch *et al*, 1992). This soluble FcγRIIB may either inhibit B-cell proliferation or down-regulate the antibody-dependent effector functions by competition, leading to an escape from immune surveillance (Fridman *et al*, 1993). On the other hand, genes involved in the susceptibility of lymphoid tumours to promotor factors have been located in the *fcgr2* mouse locus. A direct role of FcγRIIB in transformation is difficult to predict on the basis of the known inhibitory functions of FcγRIIB in B cells. It cannot be excluded that FcγRIIB expression accompanies that of other gene(s) involved in the transformation process by co-amplification. Further studies have to be performed to draw a definite conclusion about the adverse prognostic values of FcγRIIB expression in follicular and DLBCL lymphomas. The anti- FcγRIIB antibody working in paraffin sections will enable this question to be addressed in large retrospective studies.

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Fc GAMMA RECEPTORS ***Review paper, in press***

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Most cells of the immune system express receptors for the Fc region of IgG (FcγR). This heterogeneous family of molecules (Figure 1) play a critical role in immunity, by linking the humoral to the cellular responses [1]. Depending on their cytoplasmic region and/or their associated chains, FcγR display coordinate and opposing roles in the immune system. The activating FcγR contain an Immunoreceptor Tyrosine-based Activation Motif (ITAM) in their cytoplasmic region or in their associated signal transducing units [2][3]. They initiate inflammatory, cytolytic and phagocytic activities of immune effector cells [4]. The inhibitory FcγR contain an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) in their cytoplasmic tail [5]. Upon cross-linking with ITAM-containing receptors, they down regulate responses. The inhibitory receptors negatively regulate Ag-specific proliferation and differenciation of B cells, IgE-triggered mediator release by mast cells or internalization of IgG-immune complexes by macrophages [6]. Three classes of FcγR exist (Figure 1). All of them belong to the Ig-Super Family. FcγRI are high affinity receptors (Kd = 10^{-8} M for monomeric IgG) whereas FcγRII and FcγRIII exhibit low affinity for monomeric IgG, with Kd for monomeric IgG ranging from 10^{-5} to 10^{-7} M. The high affinity FcγRI and the low affinity FcγRIIIa are composed of a ligand-binding chain associated to signal-transducing chains bearing an ITAM motif. The low affinity FcγRIIa is a single chain polypeptide which contains an ITAM motif in its cytoplasmic part. The low affinity FcγRIIb1 and FcγRIIb2 receptors are single chain receptors generated by alternative splicing. They contain an ITIM motif in their cytoplsamic tail. With the exception of NK cells and B cells which express exclusively FcγRIIIa and FcγRIIb respectively, most cell types express both activating and inhibitory receptors (Table I) [4]. Therefore the cellular response depends on the relative expression of activating and inhibitory receptors. This ratio is highly influenced by the cytokine environment. Thus Th1 cytokines (IFNγ, TNFα) up regulate the expression of activating receptors whereas Th2 cytokines up regulate the expression of inhibitory [7]. In contrast with the other FcγR, FcγRIIIb is anchored to the plasma membrane via a C-terminus-linked GPI moiety [8]. Present exclusively on neutrophils, it plays a predominant role in binding of immune complexes. Its co-aggregation with FcγRIIa activates phagocytosis, degranulation, and the respiratory burst leading to destruction of opsonized pathogens [9][10]. Activation of neutrophils leads to secretion of a proteolytically cleaved soluble form of the receptor corresponding to its two extracellular domains [11]. Soluble FcγRIIIB exerts regulatory functions by competitive inhibition of FcγR-dependent effector functions and via binding to the complement receptor CR3 [12], leading to the production of inflammatory mediators.

Recent progress in the FcγR field led to the concept that FcγR control the balance between autoimmunity and peripheral tolerance. In addition, because of their absolute requirement for antibody-dependent effector cell responses, they play a major role in the therapeutic effect of IgG-antibodies.

How Fcγ receptors bind IgG

The Fc region is separated from the antigen binding parts of the IgG molecule by a flexible hinge region and forms two structural domains, the CH2 and CH3 domains. Cellular and structural approaches have shown that the lower hinge region contains the major binding site for FcγR. It is established that cross-linking of FcγR membrane molecules is a prerequisite to IgG-mediated cell activation. Since the Fc portion is composed of two identical polypeptide chains which are related to each other by a two-fold axis, each IgG molecule may potentially bind two FcγR and initiate cellular responses even in the absence of multivalent antigen. However, equilibrium sedimentation experiments performed with soluble FcγRII and FcγRIII have shown that the stoichiometry of the interaction of low affinity FcgR with IgG is 1 : 1, in solution. Studies by NMR provided an explanation to this paradox, suggesting that a rearrangement occurs in the lower hinge of one heavy chain upon binding of one FcγR molecule. This small conformational change may preclude the binding of a second FcγR to the second heavy chain Fc [13]. The Fc-FcγRIII co-crystal structures confirmed the 1 : 1 stoichiometry and showed that the horse shoe-shaped Fc is slightly more opened at the N terminus of the CH2 domains in the FcγRIII-Fc complex compared with other unligated Fc structures [14].

All known FcR are members of the Ig Super Family, except for FcεRII. The crystal structures of the extracellular domains of FcγRII [15] [16] and FcγRIII [17] show remarkable similarity. The receptors consist of two extracellular Ig-like domains, D1 and D2, with acute interdomain hinge angles of 50-55°, unique to Fc receptors, and with Fc-binding region located in the D2 domain. The recent crystal structure of the FcγRIIIb-Fcγ1 complex [18][14] has revealed that the receptor-ligand interface consists of the BC, C'E, FG loops and the C β strand of the D2 domain, the hinge loop between the D1 and D2 domains of the receptor providing additional interactions with Fcγ (Figure 2). The receptor binds asymmetrically to the lower hinge region of both Fc heavy chains, creating a 1:1 receptor ligand stoechiometry [14]. Low affinity FcγR have a low affinity for monomeric IgG. Their biological role is indeed to bind immune complexes. Parallel FcγRIIIb dimers have been observed in the crystal lattice. Such dimerization may occur on the cell surface, increasing the avidity of the interaction and subsequently facilitating cell activation [17] [19].

Roles of Fcγ receptors in autoimmunity

Mice deleted for the ITAM-bearing signal-transducing γ chain associated to FcγRI and FcγRIII or for their respective ligand binding chains have impaired in vitro IgG-dependent phagocytic and ADCC responses (Table II) [20][21][22]. Since the γ chain is associated to the high affinity receptor for IgE, the γ-chain deficient mast cells are unable to respond not only to IgG but also to IgE. These mice are unable to mount type I and type III hypersensitivity reactions and are resistant to the induction of autoimmune diseases [23]. In contrast, mice deficient for the FcγRIIB gene exhibit enhanced inflammatory responses in vitro and are prone to spontaneous and induced autoimmune diseases (Table III) [24][25][26][27]. These data obtained in mice have led to the concept that many systemic autoimmune diseases are under FcγR control and that the complement plays an indirect role in such diseases, by regulating the ratio between activating and inhibitory receptors on inflammatory cells (Figure 3). Most probably, activating FcγR control autoimmune reactions by increasing the uptake of immune complexes and by triggering effector macrophages whereas the inhibitory receptors control the activation of autoreactive B cells and thus maintain peripheral tolerance. In addition FcγRIIb regulates the clearance of immune complexes by mononuclear phagocytes present in spleen and in liver. In the human, variants of low affinity FcγRIIa and FcγRIIIa exist, with reduced affinity for immune complexes due to mutations in or near the IgG-binding site [28][29]. Linkages between such FcγR polymorphisms and autoimmune

diseases such as SLE, RA, Guillain Barré syndrome and multiple sclerosis have been described. In addition polymorphism in the FcγRIIIb-NA antigens (related to FcγR glycosylation) seem also involved in systemic autoimmune diseases.

Roles of Fcγ receptors in antibody-based therapies :

There has been a renewed interest since few years in the use of mAbs in the diagnostic and treatment of various tumors [30](Table IV).The most impressive clinical results have been obtained with Rituximab, a chimeric anti-CD20 mAb in the treatment of B cell lymphoma [31]. A humanized mAb that recognizes the human oncoprotein HER-2/neu over-expressed in some breast cancers and other tumors, induces clinical responses [32]. Other mAbs such as Campath-1H or 17-1A produced encouraging results in the treatment of chronic lymphocytic leukemia [33] or colorectal carcinoma [34] respectively. There is increasing evidence that the Fc portion of the anti-tumor mAbs is a major component of their therapeutic activity, through binding to FcγRs expressed by effector cells present in the tumor microenvironment. The polymorphisms of FγRIIIb (Val/Phe^{158}) and FcγRIIa (His/Arg^{131}) that affect binding of IgG-immune complexes predict the response to Rituximab in patients with follicular lymphoma, supporting the hypothesis that ADCC by NK cells and macrophages plays an important role in the clinical effect [35][36]. As demonstrated in Fcγ receptor-deficient mice, the anti-tumor effects of Rituximab and Herceptin require the presence of the signal transducing γ chain to activate FcγRI and FcγRIII expressed on monocytes/macrophages and NK cells and are down regulated by inhibitory FcγRIIb at the monocyte/macrophage level [37]. The improved efficacy in tumor eradication of bispecific molecules (BSMs) that have one arm specific for tumor cells and the other specific for FcγRs on immune effector cells, further illustrates the major role of FcγRs in mAb immunotherapy [38]. This FcγR-dependent biological activities of therapeutic mAb's can indeed be extended to polyclonal IgG. As shown in experimental systems and in man, the efficacy of polyclonal IgG preparations from normal individuals (Intravenous Immunoglobulins) that are currently been used for therapy of many autoimmune diseases depends upon their interaction with host's FcγR [39][40][41], and is under control by inhibitory FcγR.

In view of their pivotal role in the activation and in the regulation of IgG-dependent effector responses, FcγR provide new tools not only to predict response to antibody-based therapies but also to manipulate patient's response to treatment.

Ectopic expression of FcγR on non-hematopoïetic tumor cells

The first studies indicating that non-hematopoïetic tumors may express FcRs were performed on a variety of experimental tumors [42] and of human cancers [43][44]. However, the ectopic expression of FcγRs by non-hematopoïetic tumor cells was a controversy because of the presence of FcγR positive inflammatory at the tumor site [45] and because FcγR expression was lost during short-term culture of tumor cells *in vitro* [46]. We reinvestigated the expression of FcγRs on human tumor cells of non-hematopoïetic origin. We found that tumor cells from about 40% of human metastatic melanoma tested express inhibitory FcγRIIb1 *in vivo* and *ex vivo* [47]. In earlier studies using Polyoma virus-induced mouse tumors expressing FcγRIIb1, it was shown that this receptor confers an *in vivo* growth advantage to tumor cells and increases their malignancy [48]. It was hypothesized that increased tumorigenicity mediated by FcγRIIb1 could involve immunological mechanisms. For example, FcγRIIb1 expressed by tumor cells could block complement-dependent lysis of tumor cells or could protect tumor cells from ADCC by binding Fc portion of Abs covering tumor cells [48]. However, in nude mice, we have shown that FcγRIIb1 expression by human metastatic melanomas has a profound down regulatory impact on tumor growth and uptake in nude mice. This effect is under the control of T-independent IgG anti-tumor antibodies [47]. The respective roles of the host immune system and of the tumor cell response in the regulation of growth and migration of metastatic tumors by ectopic FcγR are currently under study in our laboratory.

Conclusion

The knowledge on FcγR functions and structure has indeed progressed a lot since their original discovery by the 70's [49][50][51][52]. FcγR are important molecules not only to mediate and control the effectors functions of IgG antibodies, but they also control the autoimmunity-tolerance balance in the periphery. Furthermore, they are major actors of the efficacy of therapeutic antibodies. The recent description of the crystal structure of the FcγR/Fc complex opens new possibilities to manipulate FcγR/Fc interactions and hence the efficacy of such antibodies.

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legends of tables

Table I Expression of human FcγR on cells of the Immune System.

Table II Mice deficient in activating FcγR are resistant to systemic autoimmune diseases.
(ADCC: Antibody-Dependent Cellular-Cytotoxicity, ITP: Idiopathic Thrombocytopenic Purpura, AIHA Auto-Immune Hemolytic Anemia)

Table III Inhibitory receptor control inflammation and maintain peripheral tolerance
(IC: Immun Complexes, EAE Experimental Autoimmune Encephalomyelitis)

Table IV Clinical trials based on MAb therapy (source Sept 2003 http://clinicaltrials.gov)
(Percentage of the total MAb based trials, ITP: Idiopathic Thrombocytopenic Purpura)*

legends of figures

Fig. 1 FcγR are heterogeneous family of receptors
(ITAM: Immunoreceptor Tyrosine-based Activation Motif. ITIM: Immunoreceptor Tyrosine-based Inhibition Motif)

Fig.2 schematic view of interaction of low affinity FcγR with IgG

Fig.3 Tolerance-Autoimmunity: The FcγR equilibrium

	B Lymphocytes	Dendritic cells	Macrophages Monocytes	NK cells	Neutrophils	Mast cells
FcγRI		Antigen presentation by IC	Phagocytosis ADCC		Superoxyde production	
FcγRIIA					ADCC	
FcγRIIIA		Cytokine production		Cytokine production ADCC		Serotonin release Cytokines production
FcγRIIIB					Superoxyde production ADCC	
FcγRIIB	Down regulation of BCR activation	Down regulation of FcγR activation	Down regulation of FcγR activation		Down regulation of FcγR activation	Down regulation of FcγR activation
Remarks					FcγRI induced by IFNγ	

(Some FcgR are also express on Langherans cells, Eosinophils, Platelets, Endothelial cells, Mesangial cells, Melanoma)

Table I

PHENOTYPE OF MICE DEFICIENT IN ACTIVATING RECEPTORS		
Deleted gene product	***in vitro studies***	***in vivo studies***
	Fonctions impaired	***Fonctions impaired***
FcRγ chain	Phagocytosis ADCC Ag presentation Cytokine release Mast cell activation (IgE, IgG)	Hypersensitivity reactions Anaphylaxis (IgG, IgE) Anti bacterial survey ***Resistance to autoimmune diseases*** Spontaneous (NZB/W mice) induced by - Collagen (DBA1 mice) - Antibodies: •Glomerulonephritis • Vasculatis • Alveolitis • ITP •AIHA

Table II

PHENOTYPE OF MICE DEFICIENT IN INHIBITORY RECEPTORS		
Deleted gene product	***in vitro studies***	***in vivo studies***
	Fonctions Enhanced	***Fonctions Enhanced***
FcγRIIB	Macrophages response to IC Ca^{2+} flux Phagocytosis	Type I, II, III Hypersensitivity reactions Antibody production Anaphylaxis (IgE, IgG) ***Susceptibility to autoimmune diseases*** Spontaneous (C57Bl6 mice- Glomerulonephritis) induced by - Collagen (DBA1 mice) - MOG (EAE) - Antibodies: •Glomerulonephritis • Alveolitis

Table III

Molecular Target	Antibody	applications	Nbr of clinical trials
CD20	Rituximab	oncology	71 (24%)*
Her2/neu	Trastuzumab Pertuzumab	oncology	42 (15%) 4
VEGFr	Bevacizumab	oncology	32 (11%)
CD52	Alemtuzumab (CAMPATH-1H)	oncology	20 (7%)
IL2Rα (CD25)	Daclizumab (17.1A)	oncology / psoriasis / ITP	12 (4%)

Table IV

Fig1

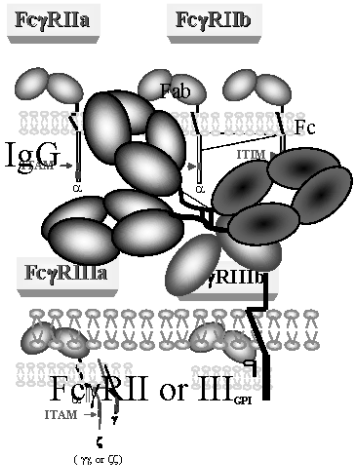

Fig 2

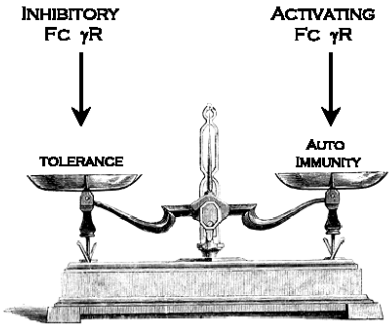

Fig3

Annexe A: Curriculum vitae – Wolf Hervé Fridman

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Present position

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President, Scientific Committee of ARC
VP, Scientific Committee of Paris 6 University
Member of AP Scientific Committee
Director of the INSERM Unit 255: Cellular and Clinical Immunology, Paris, France
Head Laboratory of Immunology - Hopital Europeen Georges Pompidou, Paris France

University titles

Medicine Doctor (M.D.) University of Paris, 1970
Doctor es Sciences (Dr Sci.) University Pierre & Marie Curie, 1981

Academic titles

Professor of Immunology - University Paris 6

Research experience

1966-1969	Fellow of Laboratory of Immuno Hematology (Pr J. DAUSSET) Institut de Recherches sur les Maladies du Sang. Paris, France.
1968-1970	Fellow of the Ligue Nationale Française contre le Cancer (Dr F.M. KOURILSKY) Institut de Recherches sur les Maladies du Sang, Hôpital Saint-Louis, Paris, France.
1970-1971	Scientific Expert. Institut A. FRAPIER - University of Montreal, Canada.
1972-1973	Post-doctoral Fellow of the Canadian Medical Research Council. Laboratory of Immmunochemistry (Pr RA. NELSON Jr). Lady Davis Institute for Medical Research of the Jewis General Hospital of Montreal, Canada.
1975-1976	Chargé de Recherche à l'Institut National de la Santé et de la Recherche Médicale (INSERM), Laboratory of Tumor Immunology, INSERM U.136 (Dr F.M.KOURILSKY) Institut de Recherches sur les Maladies du Sang, Hôpital Saint-Louis, Paris, France.
1976-1982	Chief, Group of Cellular Immunology. Institut de Recherches Scientifiques sur le Cancer (IRSC), Villejuif, France.
1982-present	Director. Laboratory of Cellular and Clinical Immunology, INSERM Unit 255, Institut Curie, Paris, France. Centre de Recherches Biomédicales des Cordeliers, Paris

Clinical experience

1967-1970	Fellow of Department of Hematology (Pr J. BERNARD) Hôpital Saint-Louis, Paris, France.
1973-1977	Consultant in Immuno Hematology, Hôpital Saint-Louis, Paris, France.
1977-1986	Consultant in Immunology, Institut Curie, Paris, France.
1986-present	Head Laboratory of Clinical Immunology, Institut Curie, Paris, France.
1992-2000	Chairman, Department of Clinical Biology, Institut Curie, Paris, France.
2000-present	Head Laboratory of Immunology – Hopital Europeen Georges Pompidou, Paris France

[1] INSERM is the equivalent of the American NIH

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Annexe B: Curriculum vitae – Catherine Fridman

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Present position

Senior scientist & Group leader, Inserm[2] U255, Centre de Recherches Biomédicales des Cordeliers

University title

Docteur *ès* Sciences (Ph.D) University of Paris, 1974

Academic titles

Professor of Immunology - University Paris 6

Research experience

1969-1971	Boursière de la Ligue Nationale Française contre le Cancer
1971-1972	Assistante Chargée de Recherche, Association Claude Bernard
1972-1975	Attachée de Recherche, INSERM
1975-1981	Chargée de Recherche, INSERM
1981-1983	Maître de Recherche, INSERM
1984-1990	Directeur de Recherche 2nd class, INSERM
1990-2000	Directeur de Recherche, 1st class INSERM
2000-present	Professor, University Paris 6

Research activities

1969-1976	Institut de Recherches sur les Maladies du Sang Laboratoire d'Immunologie des Tumeurs (Dir: Dr F.M. Kourilsky) *Hôpital Saint-Louis, Paris*
1976-1982	Institut de Recherches Scientifiques sur le Cancer *Laboratoire d'Immunologie Cellulaire, Villejuif* (Dir: Dr W.H. Fridman)
1983-2001	Institut Curie - *Laboratoire d'Immunologie Cellulaire et Clinique,INSERM U255, Paris* (Dir: Dr W.H. Fridman)
2001-present	Centre de Recherches Biomedicales des Cordeliers, *Laboratoire d'Immunologie Cellulaire et Clinique,INSERM U255, Paris* (Dir: Dr W.H. Fridman)

Honors

1984	Laureate of the Behring Metchnikoff prize
1992	Laureate of the Petit d'Ormoy prize

Research administration activities

1982-1986	Member of CSS nr 3 (Immunology) of INSERM
1982-1997	Member of various committees for international fellowships
1986-1990	Vice-president of the French immunological society (SFI)
1995-1999	Member of CSS nr2 (Cancerology) of INSERM
1997-2002	Member of CSS1 (Immunology) of ARC (Association pour la recherche sur le cancer)
2003-present	Member of CN1 (Ile de France) of ARC (Association pour la recherche sur le cancer)
2000-2003	President of the French Society for Immunological (SFI)

[2] INSERM is the equivalent of the American NIH

Editorial boards

Member of the editorial board of Immunogenetics, Journal of Immunogenetics.

Member of the executive board of Immunology Letters since 1996.

Major achievements

I am a biochemist directly involved in the characterization of membrane molecules and of their soluble products and in the analysis of their immunoregulatory roles.

I first worked on major histocompatibility antigens in the laboratory of Professor Jean Dausset at Hopital Saint-Louis in Paris. There I described the third major histocompatibilty locus (H2-L) and the association of HLA-antigens to β2 microglobulin.

In 1976 I founded an independant cellular immunology group with W.H. Fridman and switched to the study of receptors for IgG. My major achievements in that field have been the identification of the soluble forms of these receptors, the analysis of their functional roles and of their structure using recombinant molecules. I am now involved in tumor immunology, working on the expression and role of receptors for IgG on melanoma cells.

Publications – Journals (last five years)

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Annexe C: Curriculum vitae – Eric Tartour

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Present position

Associate Professor in Immunology at the Georges Pompidou European Hospital (Paris), University Pierre and Marie Curie.

Academic and Professionnal Education

1998	Senior Lecturer, University Pierre and Marie Curie, Paris.
1997	Ph.D. in Immunology, Université Denis Diderot, Paris.
1986-1991	M.D. in Internal Medicine, University René Descartes, Paris.
1989	Post graduate degree in Immunology (Institut Pasteur), Paris.
1990	Post graduate diploma in Immunology, Institut Curie, INSERM U255 (Dr. Fridman), Paris.

Academic and professionnal experience

1991-1992 **Resident** in the Department of Nuclear Medicine, Institut Gustave Roussy, Villejuif.

I worked under the supervision of Dr M. Schlumberger and was in charge of patients with endocrine and neuroendocrine tumors. I took part in protocols of radioimmunotherapy for colon carcinomas with radio-labeled monoclonal anti-ACE antibodies.

1992-1998 **M.D. Assistant in Immunology**. Clinical Immunology Laboratory (Director: Dr. Fridman), Institut Curie, Paris.

I was involved in the development of new clinical protocols of immunotherapy and in the monitoring of patients undergoing interleukin-2 therapy.

1999-present **M.D. Specialist in Immunology**. Clinical Immunology Laboratory, Georges Pompidou European Hospital (Director: Prof. W.H. Fridman).

I am in charge of the immunological monitoring of patients treated by gene therapy and cancer vaccines.

Publications – Journals (last five years)

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Benchetrit F, Ciree A, Vives V, Gey A, Fridman C, Fossiez F, Fridman W.H, **Tartour E.** *IL-17 inhibits tumor cell growth via a T cell dependent mechanism*. ***Blood*** 99. 2114 (2002)

Tartour E, Benchetrit F, Haicheur N, Adotevi O, Fridman WH. *Synthetic and natural non-live vectors: rationale for their clinical development in cancer vaccine protocols*. ***Vaccine***. 20 Suppl 4:A32-9 (2002).

Haicheur N, Benchetrit F, Amessou M, Leclerc C, Falguieres T, Fayolle C, Bismuth E, Fridman WH, Johannes L, **Tartour E**. *The B subunit of Shiga toxin coupled to full-size antigenic protein elicits humoral and cell-mediated immune responses associated with a Th1-dominant polarization*. ***Int Immunol***. 15:1161-71 (2003).

Gazagne A, Claret E, Wijdenes J, Yssel H, Bousquet F, Levy E, Vielh P, Scotte F, Le Goupil T, Fridman WH, **Tartour E**. *A fluorospot assay to detect single T lymphocytes simultaneously producing multiple cytokines* ***J Immunol Methods*** 283. 91-98 (2003)

Annexe D: Curriculum vitae – Sylvie Garcia

Sylvie GARCIA

Institut Pasteur
25, rue du Docteur Roux
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Fax: +33 1 45 68 86 39
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Present position

2002-present Chargée de recherche (Senior investigator) at the unit of Biology of lymphocyte populations, Institut Pasteur, Paris, France.

Academic Education

1986-1991 Master in Genetics and Immunology, Université Paul Sabatier, Toulouse, France.
1991-1992 D.E.A of Immunology, Université Paris VI, France
1992- 1996 PhD in Immunology, Université Paris VI, France

Professional position

1992-1996 PhD fellow at the Institut Pasteur, Paris, France
1997-1999 Post-doctoral position at the NIMR, London, England
2000-2002 Assistante de recherche at the Institut Pasteur, Paris, France
From June 2002 Chargée de recherche at the Institut Pasteur, Paris, France

Scholarship and fellowship

1992-1995 French Department of research fellowship, Unit of Retrovirus, Institut Pasteur, Paris, France
1995-1997 « SIDACTION » fellowship (France), Unit of Retrovirus, Institut Pasteur, Paris, France
1997 « Pasteur-Weizmann » fellowship (France), Unit of Retrovirus, Institut Pasteur, Paris, France
1997-1999 EMBO fellowship, (European Community), Division of Molecular Immunology, NIMR, London, England

Teaching experience

1992 Monitor for Practical course in Immunology, Université Paris VI, France
1993 Monitor for Practical course in Immunology, Université Paris VI, France
2002 Monitor for technical course in Immunology on cytokine detection, French Society of Immunology.

Publications – Journals (last five years)

E. Ledru, H. Lecoeur, S. Garcia, T. Debord, M-L. Gougeon. Differential susceptibility to activation-induced apoptosis among peripheral Th1 subsets: correlation with Bcl-2 expression and consequences for AIDS pathogenesis. *Journal of Immunology*, (1998), 160:3194.

L. Weiss, A. Roux, S. Garcia, C. Demouchy, N. Haeffner-Cavaillon, M-D. Kazatchkine, M-L. Gougeon. Persistent expansion, in a immunodefiency virus-infected person, of Vβ-restricted $CD4^+$ $CD8^+$ T lymphocytes that express cytotoxic-associated molecules and are commited to produce interferon-gamma and tumour necrosis factor-alpha. *Journal of infectious diseases*, (1998), 178:1158.

O. Akbari, N. Panjwani, S. Garcia, R. Tascon, D. Lowrie, B. Stockinger. DNA vaccination: Transfection and activation of dendritic cells as key events for immunity. J*ournal of Experimental Medicine*, (1999), 189:169.

S.Garcia, J. DiSanto, B. Stockinger. Following the development of a CD4 T cell response *in vivo*: from activation to memory formation. *Immunity*, (1999), 11:163.

N. Panjwani, O. Akbari, S. Garcia, M. Brazil, B. Stockinger. The HSC73 molecular chaperone: involvement in MHC Class II antigen presentation. *Journal of Immunology*, (1999), 163:1936.

C. Ferreira, T. Barthlott, S. Garcia, R. Zamoyska, B. Stockinger. Differential maintenance of naive CD4 and CD8 in normal and T cell receptor transgenic mice. *Journal of Immunology*, (2000), 165:3689.

H. Lecoeur, M. Février, S. Garcia, Y. Rivière, M.L. Gougeon. A novel flow cytometric assay for quantitation and multiparametric characterization of cell-mediated cytotoxicity. *Journal of Immunological Methods*, (2001), 253:177.

L.M. de Oliveira Pinto, S. Garcia, H. Lecoeur, C. Rapp, M.L. Gougeon. (2002) Increased sensitivity of T lymphocytes to TNFR1 and TNFR2-mediated apoptosis in HIV-infection. Relation to expression of Bcl-2 and active caspase-8 and caspase-3. *Blood*, 99 (5):1666-1675.

G. Kassioutis, S. Garcia, E. Simpson, B. Stockinger. (2002) Impairment of immunological memory in the absence of MHC despite survival of memory cells. *Nature Immunology.*, 3:344.

E. Ledru, M. Février, H. Lecoeur, S. Garcia, S. Boullier, ML. Gougeon ? (2003) A nonsecreted variant of interleukin-4 is associated with apoptosis: implication for the T helper-2 polarization in HIV infection, 101 (8):3102.

Publications – Book chapters, reviews… (last five years)

M-L. Gougeon, E. Ledru, H. Lecoeur, S. Garcia. (1998), T cell apoptosis in HIV infection: mechanisms and relevance for AIDS pathogenesis. *In "Results and problems in cell differentiation", 24:233.*

Annexe E: Curriculum vitae – Adrien Six

Adrien SIX
Immunophysiopathologie infectieuse
Institut Pasteur
25, rue du Docteur Roux
75015 Paris, France

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Present position

1996-present **Assistant-Professor**, University Pierre et Marie Curie – Paris 6. Research at Institut Pasteur, Immunophysiopathologie infectieuse, CNRS URA 1961, headed by Prof. Pierre-André Cazenave.

Education

1994-1996 **Post-doctoral position: Research Associate** at the Howard Hughes Medical Institute. Developmental and Clinical Immunology, University of Alabama at Birmingham, USA.

1990-1993 **Ph.D. in Immunology** from the University of Paris 6, Paris. Research at Immunochimie Analytique, Institut Pasteur, Paris.

1987-1992 **Ecole Normale Supérieure** de Paris

1989-1990 **D.E.A. d'Immunologie de Paris-Ile de France**, University of Paris 6.

1988-1989 **Master's Degree** of cellular biology.

Work Experience

Jan.-March 2003 University Diploma (DU) Biological and Medical Engineering on Valorization of Research and Biomedical Innovation, CHU Saint-Antoine, Université Pierre et Marie Curie, Paris, organized by Pr. Alain Sézeur.

September 2002 Co-organizer of the 1st PSU-IP International Teaching Platform on Cytokines, jointly organized by Prince of Songkla University and Institut Pasteur (September 9 – 14, 2002) at The Department of Biomedical Sciences Faculty of Medicine, Prince of Songkla University, Hat Yai, Thailand.

Invited speaker for the Molecular Immunology Course, Prince of Songkla University, Hat-Yai, Thailand. « B-cell development », « TCR and T-cell development », « Peripheral selection of T lymphocytes », « Immunology techniques », « Generation and Selection of T and B Cell Repertoire Diversity ».

January 2001 Invited speaker for the Molecular Immunology Course, Prince of Songkla University, Hat-Yai, Thailand. « B-cell development », « TCR and T-cell development », « Peripheral selection of T lymphocytes », « Immunology techniques », « Cytokines », « Generation and Selection of T and B Cell Repertoire Diversity », « Presentation of Institut Pasteur ».

February 1999 Invited speaker at the Biomedical Science Department, Faculty of Medicine, Prince of Songkla University, Hat-Yai, Thailand. Lectures on « Basics in molecular biology and application to neurosciences », « Development of T lymphocytes and expression of TCR »

Supervision & Administration Assignments

1997-present Group leader in the Unit Immunophysiopathologie infectieuse of a 5-6 people team working mainly on the analysis of lymphocyte repertoire diversity and perturbation during pathological situations.

2002-present Member of the executive board of the French Society for Immunology

2002-présent Deputy-director of the Immunology Department Valorization Committee, member of the Valorization Committee of Institut Pasteur

2001-présent Member of the Commission of Specialists n°65 for recruitment, University of Paris 6

2000-present Administrator of the laboratory teaching department "Ateliers de Biotechnologies", University of Paris 6

2000-present Member of the administrative committee of the Immunology Department, Institut Pasteur

2000-present Member of Valorization Committee of the Immunology Department, Institut Pasteur

Awards

March 1996 Joseph Reeves Award for Excellence in research by a research Fellow, Department of Medicine, University of Alabama at Birmingham, Birmingham, USA.

Membership to Associations and Scientific Societies

1997-present Euroscience

1991-present Société Française d'Immunologie.

1991-present Association des Anciens Elèves de l'Ecole Normale Supérieure.

Publications (last five years)

"T cell receptor gene deletion circles identify recent thymic emigrants in the peripheral T cell pool" (1999) Fan-kun Kong, Chen-lo H. Chen, Adrien Six, Richard D. Hockett, and Max D. Cooper. *Proceedings of the National Academy of Science, USA* 96, 1536-1540.

"Dental's patient database in the evaluation of a dental students' clinic management" (2000) Ngampis Aksornprai-Six, Adrien Six, Wichit Kaewsanit, Sayan Suriyothai, and Krassanai Wangrangsimakul. *Journal of the Dental Association of Thailand* 50, 221-225.

"ISEApeaks: an Excel platform for GeneScan and Immunoscope data retrieval, management and analysis" (2002) Alexis Collette and Adrien Six. *Bioinformatics* 18, 329-330.

"The Ig light chain restricted B6.$\kappa^{-}\lambda^{SEG}$ mouse strain suggests that the *IGL* locus genomic organization is subject to constant evolution" (2002) Yacine M. Amrani, Danielle Voegtlé, Xavier Montagutelli, Pierre-André Cazenave and Adrien Six. *Immunogenetics*, 54, 106-119.

"New methods and software tools for high throughput CDR3 spectratyping. Application to T lymphocyte repertoire modifications during experimental malaria" (2003) Alexis Collette, Pierre-André Cazenave, Sylviane Pied and Adrien Six. *Journal of Immunological Methods*, 178, 105-116.

"A profound alteration of blood TCRB repertoire allows prediction of cerebral malaria" Alexis Collette, Sébastien Bagot, Pierre-André Cazenave, Adrien Six and Sylviane Pied. In révision at the *Journal of Immunology*.

"Marginal zone B cell enrichment and strong follicular B cell reduction correlate with a delayed IgG response in a light chain diversity restricted mouse model" Yacine M. Amrani, Danièle Voegtlé, Eliane Barbier, Adrien Six and Pierre-André Cazenave. In révision at the *European Journal of Immunology*.

Patent, Statement of Invention

1999 Logiciel de traitement de données permettant la comparaison statistique d'ensembles de données et la recherche de critères de tri. Alexis Collette et Adrien Six. Déclaration d'invention n°99-92, Industrial Partnership Department, Institut Pasteur, Paris.

2002 ISEApeaks: Une stratégie d'analyse globale et statistique des répertoires immunitaires – Application du logiciel ISEApeaks (DI99-92) comme outil diagnostique ou pronostique de pathologies. Déclaration d'invention n°2002-48, Direction de la Valorisation et des Partenariats Industriels de l'Institut Pasteur. US provisional patent filed July 1st, 2002.

Annexe F: Curriculum vitae – Suvina Ratanachaiyavong

Suvina Ratanachaiyavong
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Faculty of Medicine
Prince of Songkla University
Hat-Yai, Songkhla 90110, Thailand.

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Present position

1993-present	Head of Department of Biomedical Science, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand.
2000-present	Program Director, M.Sc.-Ph.D. Program in Biomedical Sciences, Graduate School, Prince of Songkla University, Songkhla, Thailand

Education (degrees, dates, universities)

1971	B.Sc. (2nd Class honour)	Chulalongkorn University, Bangkok, Thailand.
1973	M.D.	Chulalongkorn University, Bangkok, Thailand.
1976	M.Med .	Singapore University, Singapore.
1977	Board of Internal Medicine	Thai Medical Council, Bangkok, Thailand.
1993	Ph.D	Univ. of Wales College of Medicine, Cardiff, UK.

Career/Employment (employers, positions and dates)

1977-81	Lecturer	Dept of Medicine, Fac of Med., PSU, Thailand.
1981-82	Fellow in Endocrinology	Department of Medicine, UWCM, Cardiff, UK.
1983-84	Lecturer	Dept of Medicine, Fac. Of Med., PSU, Thailand.
1984-89	Research Associate and Honorary Registrar	Department of Medicine, UWCM, Cardiff, UK.
1989-92	Senior Scientific Officer	Department of Medicine, King's College School of Medicine, Denmark Hill, London, UK.
1993-present	Head, Dept of Biomed. Sci.	Faculty of Medicine, PSU, Songkhla, Thailand.
2000-present	Program Director	MSc-PhD Program in Biomedical Sciences, Graduate School, PSU, Songkhla, Thailand

Specialization

(i)	main field	MHC polymorphisms
(ii)	other fields	Immunomodulators derived from traditional medicines
(iii)	current research interest	Medicinal plants and natural products
		Genetic Polymorphism and disease association studies

Honors, Awards, Fellowships, Membership of Professional Societies

Thai Medical Council
The Endocrine Society of Thailand
The Royal College of Physicians of Thailand
The Science Society of Thailand
The Thai Medical Technology Society
British Society of Immunology
Society for Free Radical Biology and Medicine

Publications

Patel A., Ratanachaiyavong S., Millward B.A. Demaine A.G.(1993) Polymorphisms of aldose reductase locus (ALR2) and susceptibility to diabetic microvascular complications. Adv Exp Med Biol.; 328: 325-332.

Ratanachaiyavong S., Fleming D., Janer M., Demaine A.G., Willcox N., Newsom-Davis J., McGregor A.M. (1994). HLA-DPB1 polymorphisms in patients with hyperthyroid Graves' disease and early onset myasthenia gravis. Autoimmunity;17: 94-104.

Ratanachaiyavong S., McGregor A.M. (1994) HLA-DPB1 polymorphisms on the MHC-extended haplotypes from families of patients with Graves' disease: two distinct HLA-DR17 haplotypes. Eur J Clin Invest; 24:309-315.

Shields D.C., Ratanachaiyavong S., McGregor A.M., Collins A., Morton N.E. (1994) Combined segregation and linkage analysis of Graves disease with thyroid autoantibody diathesis. Am J Hum Genet.; 55: 540-554.

De-Roux N., Shields D.C., Misrahi M., Ratanachaiyavong S., McGregor A.M., Milgrom E.(1996) Analysis of the thyrotropin receptor as a candidate gene in familial Graves' disease. J Clin Endocrinol Metab.; 81: 3483-3486.

Assanangkornchai S, Noi-pha K, Saunders JB, Ratanachaiyavong S. (2003) Aldehyde dehydrogenase 2 genotypes, alcohol flushing symptoms and drinking patterns in Thai men. Psychiatry Res 118, 9-17.