ANTICANCER IMMUNITY IN ANIMAL MODELS
AND EXPERIMENTAL IMMUNOTHERAPY WITH
GLYCODENDRIMERS SPECIFIC FOR THE
LECTIN-LIKE RECEPTOR NKR-P1

PhD dissertation thesis

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Miloslav Pospíšil, DrSc, until January 2007, and then of Anna Fišerová, MD, PhD.

This work is dedicated to the memory of Prof. Miloslav Pospíšil, a cultured and creative
mentor, an unforgettable friend, and a clear gentleman.

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Luca Vannucci
SUMMARY

Anticancer immunity is a complex network in which innate immunity plays an critical role. The immune system can control the onset of tumors by recognizing the changed phenotype of the transformed cells as a non-self phenotype. The active control against cells not presenting self characters is defined by the paradigm of immune surveillance. In recent years, various studies have shown that the immune response against transformed cells start as a localized acute inflammatory response. The cooperation of natural-killer cells (NK) and phagocytes attracted by so-called “danger signals” (pro-inflammatory molecules and chemo-attractants delivered by stressed cells) can lead to total ablation of the harmful cell clone. If the intervention is not completely efficient, immunoediting of the tumor can follow with stimulation of chronic inflammatory responses, mainly mediated by macrophages. The systemic immunity attempt to terminate the uncontrolled inflammation stimulates the intervention of regulatory T lymphocytes and the shift from an antitumor cytotoxic Th1 response to a Th2 inhibitory response. This regulatory mechanism paradoxically assists the tumor development. All the described events needs the interplay and cooperation of both tumor cells and host cells and stromal elements, that altogether form the so-called “tumor microenvironment”. NK cells have various roles inside the tumor microenvironment, especially the possibility to act independently from MHC-restricted antigen presentation, necessary for the activation of the adaptive immunity cells (cytotoxic T lymphocytes CD8$^+$ – CTLs, and T helper CD4$^+$ lymphocytes).

The NK cell activation is based on the missing-self recognition: cells that not present regular MHC molecule expression (recognized by NK inhibitory receptors – Ly49 in rodents, KIR in humans) permit predominance of activation receptor signaling after binding to proper ligands on the target cell. In consequence to this activation, NK cells deliver cytotoxic granules within the cytotoxic synapse (place of direct contact with the target cell) inducing death of the target cell. The receptors involved in cell activation can be included in families of lectin-like receptors (NKR-P1 in rodents, NKG2 and CD69 in rodents and humans) with capability to recognize carbohydrate molecules. This property raises in importance the products of tumor aberrant glycosylation as a target for immune recognition. In our studies we evaluated multivalent glycoconjugates as possible glycomimetics of putative ligands for NKR-P1A receptor expressed by NK and NK-like cells.

Glycodendrimers based on a polyamidoamine (PAMAM) core, tetra- and octa-branched, decorated with GlcNAc molecules were developed and tested as possible immunotherapeutics in experimental cancers \textit{in vivo} (chemically induced colorectal adenocarcinoma in rats and B16F10 melanoma inoculated in C57BL/6 syngeneic mice). Reduction of tumor onset was observed in the colorectal cancer model. Melanoma-bearing treated mice showed reduction of cancer growth rate, increase in survival, increase of Th1 cytokines, and appearance of activated (CD69$^+$) cells inside the tumors within 24-48 hrs from the treatment. To trace the glycodendrimer diffusion and targeting of immune cells, the tetravalent glycodendrons (GN4) were linked to a molecule of either fluorescein or rhodamine. We found that the labeled GN4 molecule was targeting NKR-P1 positive cells, with following internalization of dendrimers and receptor. In the
spleen, fluorescence was localized in areas corresponding to CD69+ expressing cells.

The importance of environments on cancer immunity and progression was studied in my original model of colorectal carcinogenesis in the rat, both under conventional (with microbiota present in the bowel) and germ-free (animals completely sterile since the birth) condition. We considered the changes in immune cell subpopulations induced by cancer development, and the influence of the microbiota on the anticancer immune responses at the systemic level. The drop down of both cytotoxic cell number and function resulted as the key event for the cancer progression. The germ-free conditions resulted to permit a more active anticancer response leading to lower incidence of tumors, with increase in NK, NKT, CTL, B cells and cytotoxicity in the peripheral blood of cancer-resistant animals. This data suggest that the “physiological inflammation” sustained by the presence of intestinal microflora may negatively influence the anticancer immune response (increased tolerance?).

The results obtained from the glycodendrimer stimulation experiments, together with the evidence of an environmental impact on anticancer systemic immunity in CV vs. GF conditions, leaded to a new hypothesis of immunotherapy. To rescue the immune response from the inhibition produced by chronic inflammatory stimuli and Treg cell activity, a paradoxical treatment was performed in melanoma bearing mice (initial and developed tumors) either with antiinflammatory (nimesulide, a COX-2 inhibitor) or immune suppressive (azathioprine) drugs. They were used to temporarily block the deregulated immunity, followed by re-stimulation with administration of glycodendrimers. Results showed constant efficacy in reducing cancer growth by the immunosuppressive treatment, especially in association with glycodendrimers. Instead, the anti-inflammatory treatment showed efficacy only on early tumors and was worsened by the association with glycodendrimers. The functional rescue was evident evaluating the IFN-γ production ex-vivo in splenocytes derived from treated animals: after treatment with glycodendrimers alone and, especially, azathioprine + glycodendrimers important increase was seen when cells were stimulated with tumor homogenates obtained from each own animal. Untreated animals showed instead suppression as expected by the tumor microenviromental components.

The complex of all experiments demonstrated the importance of the local tumor environment as a target of anticancer interventions, its ability to condition the systemic immunity, and the possibility of its modulation by immunotherapies targeting lectin-like receptors of NK cells usány glycomimetics of putative physiological ligands.
AIMS OF THE STUDY

The aims of this study were:
1) to evaluate the possible biological and immunological effects of multivalent glycoderivatives (glycodendrimers) for the NKR-P1A lectin-like activation receptor as anticancer experimental treatment;
2) to assess models of carcinogenesis in vivo useful for following studies on cancer immunology;
3) to evaluate local environments as factors conditioning the cancer development and anticancer immune response;
4) to develop new approaches for immunotherapies rescuing anticancer immunity based on the results of glyco-immunobiology and cancer (micro)environment studies.
ABBREVIATIONS

ACIII: adenylyl cyclase III
ADCC: antibody-dependent cellular cytotoxicity
AIM: activation inducer molecule (CD69)
ANG2: angiopoietin 2
AOM: azoxymethane
AP-1: activating protein 1
APC: antigen presenting cell
Apc: adenomatous polyposis coli
BCR: B cell receptor
CC: chemokines with adjacent cysteine residue
CCL: CC chemokine ligand
CCR: CC chemokine receptor
CD: cluster of differentiation
CEA: carcino-embryonic antigen
CEACAM: carcino-embryonic antigen cell adhesion molecule
CLEC: C-type lectin-like receptor
Clr: C-type lectin-related proteins
COX-2: cytochrome c oxidase subunit 2
CSF1: colony-stimulating factor 1
CTL: cytotoxic T lymphocyte
CXCL: CXC chemokine ligand
CXCR: CXC chemokine receptor
DAB: diaminobenzidine
DC: dendritic cell
DC-SIGN: dendritic cell-specific ICAM-grabbing non-integrin
DMBA: di-amino-benzyl-antracene
DMH: dimethylhydrazine
DSS: dextran sulfate sodium salt
EGF: epidermal growth factor
FAP: familial adenomatous polyposis
Fc: fragment crystallizable
FITC: fluorescein isothiocyanate
Foxp3: forkhead winged helix protein 3
FUT7: fucosyltransferase VII
GlcNAc-TV: N-acetyl-D-galactosamine
Galt: gut associated lymphoid tissue
GFP: green-fluorescence protein
GN4: PAMAM-GlcNAc₄ dendron
GN8: PAMAM-GlcNAc₈ dendrimer
GN-T-III: β,1,4-N-acetylglucosaminyltransferase III
Gpx: glutathione peroxidase
GrB: granzyme B
HSA: human serum albulmin
HIF: Hypoxia Inducible Factor
HLA: human leukocyte antigens
HNPPC: hereditary non-polyposis coli
HSP: heat shock protein
IC: intracardiac
ICAM: intercellular adhesion molecule
IFN: interferon
IκB: inhibitor of NFκB
IKKβ: IκB kinase-β
IL: interleukin
ILT: immunoglobulin-like transcript
IM: intramuscular
iNOS: inducible nitric oxide synthase
IP: intraperitoneal
IR: intrarectal
ITGA5: alpha5-integrin
KIR: killer-cell immunoglobulin-like receptor
KO: knock-out
LAIR: leukocyte-associated Ig-like receptors / leukocyte-associated inhibitory receptor
LAK: lymphokine-activated killer cells
Lck: leukocyte-specific protein tyrosine kinase
Lea: Lewis a
LeX: Lewis X
LeY: Lewis Y
LFA: leukocyte functional antigen
LIR: leukocyte immunoglobulin-like receptor
L-PHA: Phaseolus vulgaris hemoagglutinin
LPS: lipopolysaccharide
LRC: leukocyte receptor complex
LTA: lipoteichoic acid
LTβ: lymphotoxin β
LVA: leucojum vernum agglutinin
MAM: methylazoxymethanol
MAPK: mitogen-activated protein kinase
MGL: macrophage galactose-type lectin
MHC: major histocompatibility complex
MIC: MHC class I chain-related gene
Min: Multiple intestinal neoplasias
MIP1α: macrophage inflammatory protein 1α
MMP: matrix metalloproteinase
NCRs: natural cytotoxicity receptors
NFAT: nuclear factor of activated T-cells
NFκB: nuclear factor kappa B
NK: natural killer cell
NKC: natural killer gene complex
NKDC: natural-killer dendritic cells
NKIS: NK immunological synapse
NKR-P1: natural killer receptor-protein 1
NKT: natural killer T cell
NO: nitric oxide
NSAID: non-steroidal anti-inflammatory
nu/nu: nude atymic mice
Ocil: osteoclast inhibitory lectin
PAMAM: polyamidoamine
PAMPs: pathogen-associated molecular pattern
PBMC: peripheral blood mononuclear cells
PET: proton-emission tomography
PGE: prostaglandin E
PIGF: placental growth factor
PIP3: phosphatidylinositol 3,4,5-trisphosphate
PIR: paired Ig-like receptors
PRRs: pattern recognition receptors
PTP: protein tyrosine phosphatase
Rae-1: retinoic acid early inducible 1
RNI: reactive nitrogen intermediates
ROI: reactive oxygen intermediates
SC: subcutaneously
SCID: severe combined immunodeficiency
SDC4: syndecan-4
SG: serglycin
SHP: Src homology phosphatase
sialyl-Tn: tumor-associated O-linked sialyl 2-6-alpha-N-acetylgalactosaminyl
Siglec: sialic-acid-binding immunoglobulin-like lectins
Src: sarcoma tyrosine kinase
Syk: spleen tyrosine kinase
ST3O: sialyltransferase ST3Gal-I
STAT: signal transducers and activators of transcription
TAA: tumor associated antigens
TBK1/NAK: TANK binding kinase-1/ NF-κB activating kinase
TCR: T cell receptor
TGF: tumor growth factor
Th: T helper lymphocyte
TLR: Toll-like receptor
TNF: tumor necrosis factor
TRAIL: tumor necrosis factor-related apoptosis-inducing ligand
Treg: T regulatory lymphocyte
TSA: tumor-specific antigens
UGT1: UDP-galactose transporter-1
ULBP: UL-16 binding protein
VEA: very early activation molecule (CD69)
VEGF: vascular endothelial growth factor
WGA: wheat germ agglutinin
PART 1

GENERALITIES AND BACKGROUND
1.1. THE IMMUNE SYSTEM AND THE IMMUNE SURVEILLANCE PARADIGM

1.1.1. The immunity: generalities

The immune system is constituted by cells and soluble molecules that permit to preserve the host’s integrity versus extraneous aggressors (bacteria, parasites) or altered (virus-infected, transformed) cells, and to maintain the homeostasis in the tissues (apoptotic/necrotic cell clearance, wound repair, tumor-onset control, balance of the immune cell populations).

The main activity of the immune cells is the recognition of structures that can either activate or inhibit their functions, according to receptor-ligand interactions. The immune cells express various receptors that determine their phenotype and their functional characteristics as cells either belonging to the innate (or natural) immunity or to the adaptive (or specific) immunity.

The immune cells belong from two lineages: lymphoid and myeloid lineages. Both lymphoid (natural killer - NK, T, natural killer T – NKT, and B lymphocytes) and myeloid (granulocytes, macrophages/monocytes, dendritic cells) cells are generated from a common progenitor in the bone marrow. B cells, myeloid lineage and NK cells maturate in the bone marrow, while T and NKT cells need to reach the thymus for completing their maturation.

The immune cells circulate in the organism through the blood and the lymphatic vessels and can migrate inside the tissues. This migration is regulated by modifications developing inside the tissues leading to inflammatory reactions. The lymphoid cells are also present as structured organs or infiltrates (thymus, spleen – central lymphoid organs; lymph nodes, tonsils, adenoids, appendix, Peyer’s patches – peripheral lymphoid organs; mucosal lymphoid infiltrates) with different specificity both in architecture and functions (1-3).

The innate immunity has effector cells that express non-antigen-specific receptors but a constitutive panel of germ-line encoded receptors that recognize pathogen-associated molecular patterns (PAMPs), but also other molecules not only on bacteria (1,4). For this reason (not necessity to be primed by a specific antigen to be activated) they are constitutively ready to promptly react to adequate targets. Consequently, the innate immunity represents the first line defense, and its cells are fundamental for mounting immediate reaction against infectious agents and infected or transformed cells. In cancer immunology, at date, the innate immunity activities are considered central, both for producing cancer cell elimination and tumor escape.
The innate immunity is the most ancient part of the immune system and has evolutionary maintained its basic functions becoming cooperative with and modulatory on the adaptive immunity when this last appeared (combinatorial immune system) (5, 6, 7).

Two properties are especially important: phagocytosis and carbohydrate structure recognition. A third property, the direct cytotoxic activity producing lysis and apoptosis of the target cells (e.g. infected cells, cancer cells) - partially exerted also by soluble molecular effectors (the complement) - is shared with the adaptive immunity (cytotoxic T lymphocytes – CTLs).

The innate immunity cells, in the mammals, can be functionally divided in three different groups:
- inflammatory → granulocytes (neutrophils, eosinophils, basophils), mast cells, monocyte/macrophages
- antigen presenting cells (APC) → monocyte/macrophages, dendritic cells (DCs)
- cytotoxic → natural killer cells (NKs)

Neutrophils, macrophages and DCs are phagocytes. The phagocytosis in the macrophages and DCs leads to the elaboration of the ingested materials in short peptides that can be bound to a major histocompatibility complex (MHC) molecule: class I, if the elaboration process is performed in the cytoplasm by proteasome complex; class II, if the material is digested in lysosomes (Fig. 1).

**FIGURE 1 - Schematic representation of the MHC class I and MHC class II pathways.**
*From: Anthony W. Purcell, James McCluskey & Jamie Rossjohn: More than one reason to*

Then, the MHC-peptide antigenic complexes are expressed on the cell surface to be presented to the cells of adaptive immunity: T lymphocytes are primed by the effective interaction between MHC-antigen complex and their receptors. The NK cells, instead, can exert or not their cytotoxic activity according to the level of expression of host MHC class I molecules on the target cell. They don’t need antigen presentation, and can recognize carbohydrate structures and non-classical MHC molecules expressed by cells under stress conditions (e.g. MICA). In this, the NK cells play a particularly important role in the cancer immunity according the tumor expression of MHC class I, stress molecules and products of aberrant glycosylation (see later) (1, 2).

Differently, the adaptive immunity cells express antigen-specific receptors able to recognize only one short, non-repetitive peptidic sequence (one cell – one receptor). The T (thymus-dependent) and the B (bursa-dependent) lymphocytes are the effectors of the adaptive immunity (1).

The T lymphocytes are characterized by the expression of a receptor complex consisting in a disulfide-linked heterodimer of the highly variable α and β chains (αβ TCR), CD3 invariant chains - γε, δε -, a disulfide-linked homodimer ζ chain, and some co-receptors (e.g. CD4, CD8, CD28) that altogether allow the effective triggering of the cell after the receptor-ligand interaction (1, 2, 3). A particular subset of T cell, interfacing with innate immunity and present in special districts (e.g. colonic mucosa, skin) with possible regulatory activities, express with the CD3 an alternative variable TCR with γδ chains (8, 9, 10). As above cited, the ligand (specific antigen) can be recognized if presented bound to an MHC molecule (MHC class I for cytotoxic CD8+ T cells, MHC class II for helper CD4+ T cells).

The T lymphocytes can be subdivided in various subpopulations according their phenotype and functions:

CD4+ (indicated also as T helper cells)
CD8+ (indicated also as cytotoxic T lymphocytes or CTL)

Both express CD3 and the αβ TCR heterodimer (also if not exclusively depending the differentiation).
The CD4⁺ cells are generally indicated as T helper (Th) cells, because able to release cytokines that can address the function of other immune cells (some authors like Janaway prefer to restrict this denomination to the CD4⁺ T cells that help the B cells to produce antibodies by producing IL-4).

According their phenotype and cytokine production, the CD4⁺ cells can be subdivided in the following subsets:

**Th1**: CD4⁺ CD3⁺ αβ TCR⁺ producing IFN-γ and IL-2. They lead the Th1 response sustaining the cytotoxic cell activity and activation of macrophages, important for the elimination of the non-self elements;

**Th2**: CD4⁺ CD3⁺ αβ TCR⁺ producing IL-4, IL-6 and IL-10. They lead to the Th2 response being involved in the stimulation of B cells and in the control of inflammatory processes to return to the local homeostasis

**Th3**: CD4⁺ CD3⁺ αβ TCR⁺ producing transforming growth factor-β (TGF-β) and at a lesser extent, IL-4 and IL-10 after antigen specific stimulation. They are a special subset present mainly in mucosal tissues and involved in oral tolerance as well as autoimmunity. They also reduce antibody levels inducing apoptosis in B cells (11, 12, 13; Fig.2).

FIGURE 2 – Differentiation of a naive Th cell after priming by dendritic cells (DC), according the cytokine stimulation. In this classic picture are indicated the Th3 cells that, according their cytokine production, can be included in the Treg cells. A further and
recently defined regulatory subset, the Th17 cells, is not represented here (from: Bendtzen 2004).

**T regulatory cells (Treg):** CD4⁺ CD25⁺ Foxp3⁺ cells, indicated also as suppressor cells, produce TGF-β in an IL-10-dependent manner inhibiting the T responses especially of Th1 type, and the NK cell functions. They can also induce apoptosis of B cells affecting the Th2 response. The expression of Foxp3⁺ (forkhead winged helix protein 3) marker is necessary for the Treg function. These cells resulted involved also in oral tolerance (14, 15, 16).

**T helper 17 (Th17) cells:** the Th17 cells belong to a recently identified T helper subset characterized as preferential producers of interleukin-17A (IL-17A), IL-17F, IL-21, and IL-22. They are involved in host defensive mechanisms to various infections, especially extracellular bacteria infections, and are involved in the pathogenesis of many autoimmune diseases. Heat shock protein (HSP) 70 used as adjuvant of prostate related antigens elicited production of IL-6 and autoimmune response Th17 mediated against prostate tissue, including tumors. The effector cytokines of Th17 cells can have a crucial role in the crosstalk between immune system and tissues (tissue immunity), because the receptors for IL-17 and IL-22 are diffusely expressed on various epithelial tissues. Th17 differentiation in mice is induced by co-presence of TGF-β and IL-6. The Treg cells are indicated the main source of the TGF-β together with dendritic cells, suggesting a quite complex regulatory network. In humans, instead, IL-1 and/or IL-23 are considered, at present, the most important factors for Th17 development (17, 18).

**CD8⁺ cells** (cytotoxic T cells - CTL): CD8⁺ CD3⁺ αβ TCR⁺ can deliver cytotoxic granules, express membrane associated Fas ligand (Fas-L) – inducing apoptosis in cells bearing the Fas receptor (CD95), and produce IFN-γ sustaining their own and NK cell activity. They produce also TNF-α that together with the IFN-γ increase the sensitivity of target cells to CTL lysis by both granule- and FasL-mediated death pathways (Fig. 3).
CTLs present cytoplasmic granules containing perforins (proteins that polymerize to form cylindrical structures in the membrane of the target cell, creating holes to induce cell lysis) and granzymes (serine proteases that, penetrating through the holes inside the cytosol, induce cell death by caspase-3-mediated apoptosis). These granules are polarized and released in the place where effector and target cell take contact and receptor-ligand interactions occur (“kiss of death”, cytotoxic synapse). The NK cells present the same mechanism of cytotoxicity. (19, 20, 21).

Another subset of cells must be considered for its importance in participating to the development of Th1 or Th2 responses: the natural killer T cells (NKT).

Under the term NKT cells are generally included NK1.1⁺ CD3⁺ αβ TCR⁺ lymphocytes (NK1.1 is an NKR-P1 receptor in mice; see p. 40). Present in spleen, thymus and bone marrow, they are especially numerous in the liver (22).

NKT are T cells that typically express the natural killer cell receptor NK1.1, however they can be positive also for other NK cell receptors: KIRs (humans), Ly49A (mouse), DX5, CD69. NKT cells are modulated in their responses by the balance of signals produced by

FIGURE 3 – CTL-mediated cytotoxicity: a) by releasing TNF-α and IFN-γ; b) Fas-ligand mediated; c) granzyme-mediated.
the activation and inhibition receptors of NK type and they can immediately respond to antigen challenge: by this way, they are included in the innate immunity. The most important subset of NKT cells presents a particular semi-invariant antigen receptor, a Vα14 Vβ 8.2 TCR with invariant α chain due to rearrangement of Vα14 Jα281 gene in mice, and Vα24JαQ in humans. The Vα14 chain is expressed only by these cells, it is fundamental for their development and it is not a variation of the typical αβ TCR (typical T cells do not present this chain) (23). The Vα14 TCR recognize glycolipid antigens (as glycosylphosphatidylinositol – GPI, and α-galactosylceramide) presented by CD1d, a monomorphic MHC-like molecule expressed on APCs (including DCs) that shows a deep hydrophobic pocket for binding glycolipid antigens. So, NKT differ from typical αβ TCR+ T cells because CD1d-restricted. The Vα14 NKT cells express also CD4 co-receptor, and start the secretion of IL-4, a Th2 cytokine, immediately after binding with the antigen. Minor NKT subsets are also described: a) CD8+ or DN DX5 dull Ly 49- CD62L- CD69+ that are CD1d-dependent non-Vα14 NKT cells; b) CD1d-independent T cells expressing NK markers can be considered as activated conventional T cells (non-Vα14, αβ TCR+) that under environmental conditioning can produce Th1 cytokines (DX5+ CD1d-independent CD8+ NK1.1+ CD3+ → IFN-γ). By this way, they are “borderline” cells able both to participate to the early immune responses like an effector of innate immunity, because they do not need priming for being activated, and to address the response of adaptive immunity. In fact, depending on the subset triggered by the environmental conditions, their cytokine production can sustain either a Th1 (IFN-γ) or a Th2 (IL-4) profile. When activated by IL-12, Vα14 NKT can exert an important role in stimulating a potent antitumor response through activation of NK and CD8+ T cytotoxic cells. However, inside the tumors, the functional inhibition of CD8+ and CD4+ cells can be modulate by the same NKT cells ongoing a Th2 differentiation (24, Tab. 1).
The above described T cell subpopulations are crucial both for the development of anticancer responses and the escape of cancer from the immune attack.

The B cells express the BCR receptor represented by an immunoglobulin (IgM) complex with two chains (Iga and Igβ) for signal transduction, and a co-receptor complex formed by CD21 (CR2), CD19, and CD81. They can recognize antigens expressed on cell surface or soluble, not previously processed. In the case of thymus-dependent antigen recognition, the antigen is internalized after linking the B cell receptor, processed and expressed on the B cell surface bound to MHC class II molecule. This will be presented to the CD4+ T helper cell for binding to its specific TCR. This interaction is completed by the binding of the CD40L (ligand) - expressed on CD4+ cells, to the CD40 receptor - expressed on B cells, leading to the Th cell activation. The Th cell assists the B cell maturation and antibody production by releasing IL-4 (Th2 response). Consequently, the activation of the humoral immunity and creation of memory B cells and plasmacells completes the adaptive immune response. Recently, some studies were developing about the possible importance of the B cells as modulators of T and inflammatory responses by cytokine release. (25). Moreover, B cell-NK cell direct interactions are documented (26).


<table>
<thead>
<tr>
<th>Type I cells (Classical NKT cells)</th>
<th>Type II cells (Non-classical NKT cells)</th>
<th>NKT-like cells (CD1d-independent NK1.1+ T cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1d dependent</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>α-GalCer reactive</td>
<td>No*</td>
<td>No</td>
</tr>
<tr>
<td>TCR α-chain</td>
<td>Vα14-Jα18 (mice) Vα24-Jα18 (humans)</td>
<td>Diverse, but some Vα3.2-Jα2, Vα6 (mice)</td>
</tr>
<tr>
<td>TCR β-chain</td>
<td>Vβ8.2, Vβ7 and Vβ2 (mice) Vβ11 (humans)</td>
<td>Diverse, but some Vβ3.2 (mice)</td>
</tr>
<tr>
<td>NK1.1 (CD161)</td>
<td>+ (fully mature)</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>− (immature or post-activation)</td>
<td></td>
</tr>
<tr>
<td>Subsets</td>
<td>CD4+ and DN (mice)</td>
<td>CD4+ and DN (mice)</td>
</tr>
<tr>
<td></td>
<td>CD4+, CD8+ and DN (humans)</td>
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</tr>
</tbody>
</table>

*In humans, some CD1d-restricted Vα24/Vβ11, α-galactosylceramide (α-GalCer)-reactive T cells have been identified. Although most natural killer T (NKT)-like cells express diverse T cell receptors (TCRs), some subsets (for example, mucosa-associated invariant T cells) express semi-invariant TCRs. In mice, NKT cells have been traditionally defined as NK1.1+ T cells. However, it is clear that expression of NK cell markers is not unique to classical, CD1d-dependent NKT cells, which has resulted in confusion in the literature. We compare and contrast the different populations of cells that are often referred to as NKT cells, by dividing these into three different cell types: type I NKT cells, type II NKT cells and NKT-like cells. DN, CD4+CD8+ double negatives; IFN-γ, interferon-γ; IL-4, interleukin-4.
The adaptive immunity cells are induced to clonal expansion after they find and efficaciously recognize a specific antigen. A part of the expanded T cells will actively participate to the immune defense migrating toward and inside the insulted site of the organism (cell-mediated immune response), and a part of the B cells, going to differentiate as plasmacells, will produce and release antigen-specific antibodies (humoral immunity). Another part of both T and B cells, instead, will undergo maturation as memory cells. In the case of a future challenge by the same antigen, these memory cells will produce a quicker and more intensive specific response than after the first challenge, efficaciously eliminating the recurred danger (27): this is the kind of response necessary for avoiding cancer recurrence after its ablation.

In both T and B lymphocyte populations, the activation (priming) is consequent to the effective link to the specific antigen. More, the B cells can work also as antigen-presenting cells. The different phases of the immune response are sustained by a panel of cytokines (Th1: IFN-γ, IL-2, TNF-β; Th2: IL-4, IL-5, IL-9, IL-10, IL-13) that, produced by the progressively involved immune and environmental cells allow a cooperative network to develop a valid defense following the scheme: tissue homeostasis → tissue stress (infection, tumor) → innate immunity activation, inflammation, granulocytes → Th1 cytokines: cytotoxic innate and adaptive immunity response → apoptosis/necrosis of the aggressed target cells, inflammation, macrophages → Th2 cytokines: B cell and inflammation down-regulating response → total ablation of the tissue stress → new tissue homeostasis. So, we can say that the functions of both T and B populations develop under the Th1/Th2 balance that mainly depends on 1) the activity of innate immunity (NK, NKT, DC, macrophages) and regulatory (Treg) cells, and 2) environmental conditions (as in tumor microenvironment). (11, 28, 29)

Because the adaptive immunity needs a specific interaction between the effector cell receptor and its specific ligand, the time for mounting the response is necessarily longer than for eliciting the innate immunity response. Consequently, the two types of immunity need to perfectly integrate their activities to effectively produce a protective response (4, 7).

1.1.2. The concept of “self” and “non-self” and the immune surveillance paradigm

As above indicated, the immune system needs ability for discriminating the targets of its activities. Paul Erlich, as the first, introduced the concept of “self” and “non-self” to distinguish what is constitutive of an organism (to be preserved by immune system) and what is extraneous (to be eliminated by the immune system) (30). The cells of an organism
express specific molecules that define their immunological identity and reciprocal histocompatibility. By this way, they permit to the immune system to distinguish the unique “self” constitution of an organism (host) versus the “non-self” elements that do not match with the characterizing immune phenotype of the host.

The most important molecules expressed by the cells to permit the self versus non-self recognition are represented by the MHC molecules. The MHC is a cluster of genes on human chromosome 6 (known also as human leukocyte antigens or HLA genes) or mouse chromosome 17 (known as H-2 genes). They encode highly polymorphic glycoproteins which permit antigen presentation to T lymphocytes (MHC class I to CD8\(^+\) T cells and MHC class II to CD4\(^+\) cells). The two complexes (classical MHC molecules) have a different structure, as shown in the Fig. 4:

![MHC molecules structure](gsbs.utmb.edu/microbook/images/fig1_9.JPG)

**FIGURE 4 – MHC molecules structure.** A) MHC class I molecule; B) MHC class II Molecule. From: gsbs.utmb.edu/microbook/images/fig1_9.JPG

The genes encoding the \(\alpha\) chain of MHC class I molecule and the \(\alpha\) and \(\beta\) chains of MHC class II are within the MHC cluster, while the \(\beta_2\)-microglobulin and the invariant chain that complete these molecules are instead encoded by other genes, respectively on chromosomes 15 and 5 in humans and chromosomes 2 and 18 in mice.

The MHC complex include also less polymorphic genes that encode other molecules: MHC class IB for \(\beta_2\)-microglobulin-associated cell-surface molecules, MIC family (5 genes but only 2 are transcripted under stress conditions: MICA and MICB, ligands for NKG2D activation receptor on NK and CD8 cells), HLA-G (important in pregnancy for inhibiting -
through binding with the inhibitory receptor ILT-2 - the cytotoxic activity of NK cells against the placental cells, but also expressed by cancer cells), HLA-E (ligand for CD94/NKG2A/C inhibitory receptors on NK cells) (1-3).

The MHC class I is fundamental in the mechanisms for maintaining the homeostasis of an organism, and represents the ligand by which the cytotoxic effector cells of the innate (NK cells) and adaptive immunity (CD8+ CTL) can recognize self from non-self. As we will see later, NKs participate to the immune surveillance by the “missing-self” sensing, mainly based on quantitative changes in expression of classic MHC class I molecules on the target cell, and by recognition either of activating stress-induced non-classic MHC class IB molecules or inhibitory ligands (also included in the non-classic MHC class IB molecules). The lectin-like and the immunoglobulin-like (KIR) receptors expressed by NKs (but not only) cooperate in the recognition process and in tuning the following response leading to the activation or inhibition of cytotoxic activity (31).

Such a fundamental activity of the immune system, i.e. discrimination between self and non-self antigens and consequent activation or inhibition of the cytotoxic immune response against the sensed target, plays a central role in the anticancer immunity. In fact, it is assumed that the immunity is able to recognize the cells of the host that, transformed by progressive genetic mutations, reshape their original phenotype becoming cancer cells.

According the characteristics of the various carcinogenetic factors and the personal genetic constitution, it is supposed that during the life span of every individual many cells of the organisms can develop mutations possibly leading to the cancer phenotype. However, only some individuals develop tumors. This means that the organism has systems for controlling and inhibiting the development of cancers by the elimination of the transformed cells at their onset, and cell transformation can continue after the cellular mechanisms to correct genetic errors are surpassed (32, 33). On these bases, it was hypothesized that how bacteria, viruses and all extraneous structures challenging the integrity of the organism are continuously checked and aggressed by the immune system to be cleared, similarly the immune system can control the onset in the host of cells that loose their original (self) phenotype. This hypothesis generated the theory of the “immune surveillance against cancer”, as enunciated by Burnet F.M. (1957) (34, 35). Epidemiological studies about the occurrence of cancer in immune suppressed patients versus the regular population, according the age; experiments with experimental animals with severely impaired immunity by knocking-out genes that regulate immune cell development and functions (RAG2-/-; RAG2-/-STAT1-/-; p53+/+pfp-/- mice); the studies on T lymphocyte development (positive and negative T cell selection); and the expression of stress molecules by cells with DNA damage as ligand for activating receptor NKG2D gave support to this
theory, now considered a paradigm. This protective function is exerted by both innate and adaptive immunity cells, actively collaborating together (36-41).

1.1.3. The immune recognition

The cells of both innate and adaptive immunity express on their surface receptors that, by binding molecules exhibited on the surface of other cells, permit the recognition of self from non-self elements.

The recognition function is fundamental for the elicitation/regulation of the immune response and for the maintenance of the homeostasis in the organism. The function of recognition to be exploited needs the physical interaction of receptor and ligand. An efficacious receptor-ligand interaction able to deliver signals through the proper intracellular pathways depends from various factors. Conditions that can permit the receptor to function are: the proper constitution of the receptor; its conformation; its association with an immunoreceptor tyrosine-based activation or inhibition motif (ITAM or ITIM, respectively) in the intracellular domain of the molecule or, alternatively in its absence, coupling with co-receptors and adaptor molecules bearing the motif. The ITAM motif is formed by the sequence: tyrosine-x-x-leucine, where x is an unspecified amino acid; and the ITIM motif is formed by the sequence: isoleucine-x-tyrosine-x-x-leucine, where x is an unspecified amino acid.

The modification of the receptor conformation after binding the ligand permits the activation through tyrosine phosphorylation and association with phosphotyrosine-kinases (ITAM) or inhibition by removal by phosphatases of the phosphate groups added by tyrosine kinases to a receptor site (ITIM) (1-3).

The balance between the signals delivered by activating and inhibitory receptors is particularly important for the NK cell function. The delivery of the activating or inhibitory signals follows appropriate pathways: activation involves a kinase cascade including Lck and Syc, or Zap-70, similarly to the TCR signaling pathway; inhibitory receptors recruit SHP-1 after phosphorylation of ITIM by Src kinases initially activated by the activating receptors, suggesting interaction and intermixing of the receptors in the NK immunological synapse (42).

The efficient interaction of a ligand with the proper receptor depends on the possibility for the ligand to be in the presence of the appropriate receptor; its affinity for the receptor; its adequate size, structure and conformation to permit efficient binding at the receptor binding groove. Once all these conditions are respected, the efficacy of the receptor-ligand
interaction can be yet challenged by environmental factors (e.g. local changes of extracellular pH, as in cancer conditions, can modify the binding affinity of the molecules), like it can happen in cancer tissues (1, 2).

As said above, the immune recognition needs the direct contact between effector and target cell (either APC or non-self element) during which more molecules, on both sides, can interact. To trigger the immune cell it needs the cooperation of receptors, adhesion molecules and co-receptors organized in complexes that stabilize and make efficient the receptor-ligand binding. Their interaction, with possible coupling with adaptor molecules at intracellular level, permits the generations of signals able to trigger the functions of the immune cell. The recognition complex on T and NK lymphocytes and on the target (APC) is described as an immunological synapse. This is a specialized supra-molecular structure that requests the relocation of the involved proteins on the cell surface. For example, the T cell/ APC synapse shows the remodeling on the superficial distribution of co-receptors and their ligands (like CD28/B7, CD2/CD58) and adhesion molecules (as ICAM-1/ LFA-1) around the TCR - as well as the peptide-MHC molecule complex. The process is only partially active, takes about 30 minutes, and follows sequential steps until concentric rings of co-receptors and adhesion molecules surrounding the T cell antigen receptor are organized (Fig. 5). The cytoskeleton participates to this remodeling and also to the capture and internalization of the ligand presented in the contest of the synapse (43-45).

The NK cells use a complex system composed by involvement of various invariant receptors for the recognition of tumor and virus-infected cells. As above cited, it imply exhibition of classic and/or non-classic MHC class I molecules by the target, and the synaptic organization of a panel of activating and inhibitory receptors on the NK cell membrane. Similarly to the T cells, an immunological synapse can be organized at the contact site between the NK cell and its target. While the inhibitory synapse has been described, the constitution of the activation synapse is still unclear. Some authors (Davis DM: Assembly of the immunological synapse for T cells and NK cells. Trends Immunol 23 (7): 356-63, 2002) refer to the possibility that activating and inhibitory receptors are intermixed at the immunological synapse to determine by their balance the delivery of activation/non-activation signaling. Consequently, supposing a sequence of supramolecular organization like at the T cell synapse (Fig. 6A), they suggest that the known structure of the inhibitory synapse might be a non-progressing intermediate of the activating (cytotoxic) synapse, blocked by the prevalence of inhibitory signals (44, 46-48).

FIGURE 6 - Immunological synapse organization in T and NK cells. From Davis DM: Assembly of the immunological synapse for T cells and NK cells. TRENDS in Immunology 23 (7): 356-363, 2002
In fact, the receptors that recognize the MHC class I molecules (KIR) are found in the external ring, while the inner receptors are represented by adhesion molecules (LFA-1/ICAM-1) as in the first step of the T-cell synapse assembling (Fig. 6B). It was observed on T cells that NKG2D (co-receptor in T cells, but activating receptor for NK cells) after binding its ligand induce assembling of a ring of LFA-1 independently from the presence of peptide-MHC molecule complex for TCR. This phenomenon might be the possible next step for the organization of an activating NK synapse. Recently, the activating receptor NKG2D and its ligands on target cells have been described to accumulate at NK cell synapses. If MHC class I expressing tumors have enhanced expression of NKG2D ligands, their susceptibility to NK cell cytotoxicity appears increased. This observation suggests that NKG2D activation can override inhibitory signaling in the presence of MHC I protein expression, though contrasting reports show the engagement of inhibitory receptors able to block NKG2D-mediated cytotoxicity (49-51). Endt J. et al have shown that inhibitory receptor signaling can override NKG2D-mediated activation by a mechanism blocking the accumulation of NKG2D in GM1-rich membrane domains at the NK cell immune synapse, also impairing Vav-1 phosphorylation (Vav-1 phosphorylation induce actin polymerization and the clustering of NKG2D and lipid rafts at the immunological synapse (52).

The CD94/NKG2A, an ITIM-containing inhibitory receptor on NK cells and some CD8+ T cells, was found to direct interfere with the definitive assembling of an activating synapse by affecting the actin cytoskeleton remodeling and excluding lipid rafts (51).

The immunological synapse is also suggested to have functional properties. Maldonado et al. (2004) demonstrated co-polarization of TCR and IFN-γ receptors (IFNGR) at the synapses of activated CD4+ Th cells, suggesting a possible role for immunological synapses in addressing the T cell development (in this case, toward Th1 maturation). At the point of initial conjugate formation between CD4+ Th cell and mature splenic DC, the TCR and IFNγR had a random distribution on the cell surface. After 30 minutes of cell–cell contact, TCR and IFNγR congregated at the cellular interface. Time-lapse microscopy of cells showed low Ca2+ levels and uniform receptor distribution prior to CD4+ Th cell –DC contact. Following Ca2+ influx, TCR and IFNγR progressively migrate towards the point of cell–cell contact. In CD4+ Th cell activated by antibody-mediated TCR cross-linking, co-localization specific to IFNγR and co-polarization of TCR and IFNγR were again observed. As additional observation, the authors reported that CD4+ Th cell activation induced significant receptor co-recruitment in cells from a Th1-prone mouse strain, B6. In contrast, cells from a Th2-prone strain, BALB/c, showed 200-fold less TCR and IFNγR co-polarization. These results are intriguing because suggest that genetic differences in CD4+
Th cell developmental tendencies may be mediated by changes in receptor trafficking. The presence of Th2-promoting IL-4 completely prevented TCR cross-linking-induced receptor migration. This suggested that IL-4 inhibit the assembling of a Th1-promoting complex (53).

Understanding the constitution of the immunological synapse is important for better enlightening the mechanisms of interaction between the NK cell and target, and the delivery of signals addressing the cell function after recognition. As we will see later, other receptors might be involved in this very complex function.

Finally, the immunological synapse is also the site that permits the direct cytotoxic attack by an effector cell against its target. NKs and CTLs are lymphocytes that can directly kill the cell that they recognize as non-self. In fact, as previously indicated, these lymphocytes are characterized by the presence of cytoplasmic granules containing cytotoxic proteins. These proteins (perforins) have capability to penetrate the cytoplasmic membrane of the target cell creating discontinuity, and to induce apoptosis (granzymes and -in humans- also granulysin) by direct activation of the caspase-3 (but also by a caspase-3-independent mechanism) (54, 55)

Granzyme B (GrB) and perforin were described to coexist as multimeric complexes with the proteoglycan serglycin (SG) in cytotoxic granules. Because cytotoxic cells were observed also to secrete exclusively macromolecular GrB-SG, alternative mechanisms explaining the penetration of the cytotoxic molecules in the target cell were hypothesized. Metkar S.S. et al. (2002) proposed that granule-mediated apoptosis can develop after a target cell perceives granule contents as a multimeric complex consisting of serglycin, perforin, and granzymes, respectively indicated as the scaffold, translocator, and targeting/informational components of this modular delivery system (56).

The granules to be released needs the contact between effector and target cell in a defined area (the so-called “death’s kiss”). This interaction is created within the immunological synapse. In this site, an accrual of receptors interacts with the target cell ligands and between themselves. If the contact produces an activating signal, the remodeling of the actin filaments induces polarization of the granules and the exocytosis in the closed area of interaction between effector and target cell (57, Fig. 7):

1.2. THE NK CELLS AND THEIR RECEPTOR REPERTOIR

1.2.1. NK cells

As previously discussed, the NK lymphocytes are cells of the innate immunity that find a special role in immune surveillance against virus-infected and transformed cells. These large granular cells (Fig. 8) appear to be a composite subpopulation with not only important cytotoxic function but also able to produce cytokines (IFN-γ and TNFα - Th1 cytokines, granulocyte-macrophage colony stimulating factor – GM-CSF, colony-stimulating factor 1 – CSF1, lymphotxin β – LTβ) and chemokines (IL-8 – CXCL8, macrophage inflammatory protein 1α – MIP1α, CCL3), displaying a regulatory profile. A particular subset present in the uterus during pregnancy in humans (uNKs) is able to deliver cytokines involved in
angiogenesis and vascular stability (vascular endothelial growth factor C –VEGFC-, placental growth factor –PIGF, angiopoietin 2 - ANG2). The NK cell function is enhanced by IFN-α, IFN-β (indicated in the panel of the so-called danger signals delivered as a very early response to transformed-cell clone growth), IL-2, IL-12, IL-15, and IL-18. Once activated, they can induce inflammatory responses, modulate hematopoiesis, control monocyte, granulocyte and dendritic cell growth and function, and affect the type of subsequent adaptive responses orientating the helper cell (CD4+) activity (58-61). In this view, it is interesting the description in human NK cells of two subsets, NK1 and NK2, developing from NKs cultivated in the presence of either IL-12 or IL-4, respectively. Similarly to the Th cells, the NK1 produce IL-10 and IFN-γ, and express Fas (CD95) molecule at a higher degree than NK2 subset. NK2 are much less sensitive to IL-12 than NK1 and produce IL-4, IL-5 and IL-13, but not IFN-γ. Not particular differences were found in their cytotoxic activity against NK-sensitive targets (K562 cells). It is possible that environmental conditions may address their development toward one or the other subset, and this is especially important in cancer conditions, where they are suggested to play modulatory activity on both inflammatory processes and adaptive immunity responses. An example is given by NK1 cells significant inhibition of IL-4 and soluble CD40-ligand-stimulated IgE production in peripheral blood mononuclear cells isolated from atopic dermatitis patients (62, 63).

The cross-talk between NK cells and DCs is considered crucial for the reciprocal activation and function of these cells, with effects extended to the regulation of the adaptive immunity.

**FIGURE 8 – NK cell.** NK cell presents cytoplasmic granules (A) that are polarized after activation (B). (Vannucci, MBÚ, AVČR v.v.i.)
Freshly isolated NK cells, in resting conditions, rapidly acquire cytotoxic activity and produce IFN-γ when co-cultivated with DCs, but not with susceptible tumor targets. This phenomenon requests the direct contact between the NK cells and DCs, through the Nkp30 receptor expressed on NK cells. This contact triggers the maturation of the DCs (64). However, also the delivery of some DC-produced cytokines (IL-12, IL-18, type 1 IFNs) appears to assist the NK cell activation, and the IL-2 results necessary for IFN-γ production by NKS. Reciprocally, the NK-cell secreted TNF-α induces maturation of the DCs. The migration in the lymph nodes leads to definitive maturation of DCs with expression of co-receptors, like CD86, and release of IL-12 and TNF-α (together with the IFN-γ produced by the NKS) lead to the activation of T cells (assisting the CD8 CTL antigen-recognition and cytotoxic functions). The immature DCs (iDCs) unable to progress are eliminated by the NK cells after direct cell-cell interaction and absence of delivery of inhibitory signals (65-67).

As the CTLs, the NK cells have cytoplasmic granules containing perforins and granzymes. These granules are released within an immunological synapse with the target cell, which is lysed (necrosis) or killed by apoptosis. Perforin-independent cytotoxicity is exerted by the expression of Fas-L that binding the Fas-receptor (CD95) of the target cell triggers apoptosis. Finally, the NK cell cytotoxicity can be also triggered through CD16a receptor (FcyRIIIA receptor) by linking antibodies opsonising a target cell (antibody dependent cell-mediated cytotoxicity – ADCC) (68, 69).

NK cell subsets can produce IFN-γ but exert low cytotoxicity and vice versa. The CD56<sup>high</sup> NK cells have poor cytotoxic activity and when stimulated with IL-2 or IL-15 proliferate and intensively produce either IFN-γ (NK1) or IL-5 and IL-13 (NK2). Instead, the CD56<sup>low</sup> NK cells respond to the same stimulation with a not ready proliferation, low production of cytokines, but enhanced cytotoxic activity. Another difference is the response of CD56<sup>low</sup> NK cells to IL-8 (CXCL8) and soluble fractalkine with chemotaxis (as neutrophils do), while CD56<sup>high</sup> NK cells have high expression of CC-chemokine receptor 7 (CCR7) and L-selectin, associated with homing in parafollicular- Tcell zone of lymph nodes (10-fold higher number than in the blood), responding to CCL19 (ELC) and CCL21 (SLC) chemochines. The different response to homing factors makes the less aggressive but more modulatory CD56<sup>high</sup> NK cells to be the prevalent subset present in the placenta during pregnancy. These data are also interesting in relation with the antitumor responses mediated by NK cells and the inflammatory environment that accompany cancer development (70, 71).
1.2.2. NK cell receptors: generalities

The recently intensified research on NK cells has contributed to widely enlarge the panel of NK receptors and their identification is continuously growing, making quite difficult a precise update on the argument. However, the complete enumeration and description of all receptors is not in the finality of this dissertation, and it is not essential for describing the mechanisms to date known by which NK cells can be triggered or inhibited in their activity. So that, the following paragraphs will be centered on model receptors, with a particular focus on the lectin-like receptors and, inside this group, the NKR-P1 receptor family.

The large panel of receptors expressed on NK cell surface can be grossly divided in activation and inhibitory receptors. Then, the largest part of these receptors can be subdivided in lectin-like (e.g. NKR-P1, CD69, Ly49) and immunoglobulin-like (e.g. KIR, 2B4, CD16) receptors. Recently, also some Toll-like receptors were added (e.g. CCR7). Most inhibitory receptors genes are clustered together with genes for related receptors that display similar specificity but are unable to produce inhibition for lacking in ITIMs at cytoplasmic level. The ITIM-less receptors are often activation receptors. They present charged residues in the transmembrane domains facilitating association with signaling molecules (e.g. DAP12 or KARAP).

The inhibitory and activation receptor genes are located in two main clusters on different chromosomes, the leukocyte receptor complex (LRC) and the natural killer gene complex (NKC). The first encodes the immunoglobulin-like molecules (human) and the second the C-type lectin molecules (human, mouse, rat). Both loci are polymorphic and have plasticity in gene content, possibly due to their independent segregation from the genes for their highly polymorphic MHC ligands. However, MHC-class I restricted receptors do not appear to cover all NK cell specificities, as seen in TAP-deficient patients, indicating the existence of supplementary NK cell inhibitory receptors for other ligand specificities (Table 2). About these, an example could be the NKR-P1D and NKR-P1F with their particular ligands belonging from the Clr family (see below).
The inhibitory receptors that recognize MHC class I molecules are identified in two large families: the lectin-like receptor family Ly49, in rodents, and the immunoglobulin-like KIR receptors in human. Prevalently with inhibitory function, these receptor families include also activation receptors triggered by MHC class I molecules (classical and non-classical) expressed on the target and self cells (72, 73).

At genetic level, the C-type lectin genes are collected in the heterogeneous NK gene complex, identified in the laboratory of Seaman W.E. (1991), present with high level of homology on different chromosomes in the different species (mouse: chrom. 6; rat: chrom.4; human: chrom.12p12-13) (74, 75; Fig. 9).
FIGURE 9 - Schematic representation of the NK complex – Loci for activating and inhibitory receptors are encoded on different chromosomes in the three species, as a complex with high conservation of genes and their arrangement on the distal arm of mouse chromosome 6 and human chromosome 12p12-13. NKR-P1 result present in A but not in B. However, a very recent study identified NKR-P1 gene sequences present also in Balb/c genome encoding proteins with differences with the C57BL/6 NK1.1 (NKR-P1) receptor. NKR-P1 is present also in the rat cluster (Klrb1). Ly49 and NKG2 clusters are maintained in all three species, but in humans Ly49 is represented only by Ly49L not giving functional transcripts (The Journal of Immunology, 2001, 166: 5869-5873). In humans the Ly49 cluster is functionally substituted by the killer immunoglobulin-like receptors (KIRs). D - Orthologous complexes with similar structures have been identified on syntenic regions of rat chromosome 4 and human chromosome 12p13 (Immunological Reviews 2001 Vol. 181: 126–137).

Other immune receptors are encoded on other chromosomes. In human only one Ly49 receptor gene is identified in the NK complex, Ly49L, a pseudogene (none transcript identified to date). However, a family of receptors functionally homologues of Ly49 family, the killer cell immunoglobulin-like receptors (KIRs), is encoded on 19q13.4 within the leukocyte receptor complex (LRC), which contains many polymorphic genes of the immunoglobulin superfamily as well as multiple related sequences (immunoglobulin-like transcript – ILT, or leukocyte immunoglobulin-like receptor genes), leukocyte-associated inhibitory receptor genes (LAIR), NKP46, Fc alphaR and the platelet glycoprotein receptor VI locus, which encodes a collagen-binding molecule)
KIRs are expressed prevalently on NK cells and some T cells. The other LRC loci are more widely expressed. Additional loci with weak sequence similarity to the KIRs, including the extensive CD66 (CEA) and Siglec families are encoded further centromeric of the LRC. The LRC-syntenic region in mice contains no orthologues of KIRs.

1.2.3. Lectins and lectin-like receptors

The lectins were originally identified in plants. In a general definition, they are multivalent sugar-binding proteins of non-immune origin that agglutinates cells or precipitates glycoconjugates (Goldstein et al. Nature 285: 66, 1980). Though of no immunoglobulin nature, they are capable of specific-recognition reversible binding to carbohydrate moieties (or oligosaccharides, in animals) of complex glycoconjugates, without altering the covalent structure of any of the recognized glycosyl ligands. For example, Triticum vulgaris (Wheat Germ) Agglutinin (WGA) has affinity for \( N \)-acetyl-\( \beta \)-D-glucosaminyl residues and \( N \)-acetyl- \( \beta \)-D-glucosamine oligomers, while Leucojum vernum (Snowflake) Agglutinin (LVA) presents affinity for terminal \( \alpha \)-D-mannosyl residues. Multivalency appears to be an important condition for the receptor-ligand interactions, and the multimerization of the receptors a condition for releasing a proper signal, binding to multiple unites of a repetitive molecule (like glycans). Another possibility is that conformational changes or clustering of receptors at the cell surface (e.g. for integrins) may enforce the ligand binding.

The lectins contain at least two sugar-binding sites and may be soluble or membrane-bound. To date, also sugar-specific enzymes, transport proteins, and toxins may be qualified as lectins if they have multiple sugar-binding sites. Lectins are found in a variety of species from viruses to plants to man. For the animal lectins is more commonly used the term “lectin-like” to distinguish from the plant lectins (76, 77, 78).

Lectin and lectin-like receptors are diffusely represented on various types of immune cells with function as either adhesion molecules (e.g. selectins) or immune-recognition receptors (e.g. mannose-binding receptor, NKR-P1). As above described, the innate immunity cells use germ line encoded pattern recognition receptors (PRRs) to recognize conserved and common pathogen-associated molecular patterns (PAMPs) displayed on the pathogen cell surface (e.g.: peptidoglycan of bacteria, LPS of Gram-negative bacteria, lipoteichoic acid – LTA - of Gram-positive bacteria and \( \beta \)-glucan of fungi). The carbohydrate moieties of these molecules are the targets for lectin-like receptors expressed by the immune cells. The same receptors can be useful for recognition of aberrant glycosylation products of glyco-proteins and -lipids displayed on the surface of cancer cells. Moreover, lectin-like molecules have importance in the immunological synapse both as structural components for
cell-to-cell linkage (ICAM-1/LFA-1, ICAM-3/DC-SIGN interactions), and for the recognition of carbohydrate components of antigens (79-82).

The immune cells express C-type (calcium-dependent) transmembrane lectin-like receptors (Tab. 3).

### TABLE 3: General overview on C-type lectin-like receptors. The NK activating and inhibitory receptors are not included.

<table>
<thead>
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<th>Group and molecular structure</th>
<th>C-type lectin</th>
<th>Localisation</th>
<th>Ligand specificity</th>
<th>Function</th>
<th>Cytoplasmic tail motif (single letter amino acid code)</th>
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<td>Antigen uptake; cell adhesion</td>
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<td>Antigen uptake; cell adhesion</td>
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<td>DCEs, Mo, Mø, PMN, B</td>
<td>αm</td>
<td>Antigen uptake</td>
<td>LLY EED</td>
<td></td>
</tr>
<tr>
<td>Langenin</td>
<td>LC</td>
<td>Bound</td>
<td>Birbeck granules formation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCAL-1</td>
<td>DCs, germinal centre B</td>
<td>αm</td>
<td>T cell co-stimulation</td>
<td>EEE</td>
<td></td>
</tr>
<tr>
<td>BDCA-2</td>
<td>Plasmacytoid DCs</td>
<td>αm</td>
<td>Antigen uptake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV. Selectins</td>
<td>L-selectin</td>
<td>Leukocytes</td>
<td>sLex²</td>
<td>Leukocytes tethering; hormone and inflammation</td>
<td>YG/F</td>
</tr>
<tr>
<td>P-selectin</td>
<td>Platelets, endothelium</td>
<td>sLex²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td>Activated endothelium</td>
<td>sLex², sLex²</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Depending on the orientation of their amino (N) terminus, these molecules are indicated as type I and type II C-type lectins. The type I receptors has the N terminus pointing outwards the cytoplasm of the cell, while the type II into the cell. C-type lectin receptors contain the prototype lectin fold, consisting of two anti-parallel β strands and two α-helices. The cytoplasmic domains of the C-type lectins are diverse and contain several conserved motifs that are important for antigen internalization: a tyrosine-containing coated-pit intracellular targeting motif, a triad of acidic amino acids and a dileucine motif. Other type II C-type lectins contain other signaling motifs: ITIM associated to inhibitory signalling and ITAM associated to activation signalling. An example of this variety is illustrated by the C-type receptors express on DCs (83; Fig. 10)
FIGURE 10 - *C*-type lectin-like receptors expressed on dendritic cells. They are divided as type I C-type lectins and type II C-type lectins. The first group Type I C-type lectins (MMR and DEC-205) contain an aminoterminal cysteine-rich repeat (S–S), a fibronectin type II repeat (FN) and 8–10 carbohydrate recognition domains (CRDs), which bind ligand in a Ca2+-dependent manner. Type II C-type lectins contain only one CRD at their carboxy-terminal extracellular domain. P = proline-rich regions.

1.2.4. C-type lectin-like receptors on NK cells

NK cells present type II C-type lectins with either ITIM or ITAM motifs. However, some receptors present on other innate immunity cells (DC, macrophages) are also express, like CLEC. The following table shows some of the NK cell lectin-like receptors to date known (Tab. 4):

*From: Nature Reviews Immunology* 2, 77–84, 2002
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Gene</th>
<th>Ligand</th>
<th>Species</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLRG1(MAFA-L)</td>
<td>Klr1/KLRG1</td>
<td>?</td>
<td>M/H</td>
<td>?</td>
</tr>
<tr>
<td>NKRPA(KLRB1)</td>
<td>NKRPA</td>
<td>LLT-1</td>
<td>H</td>
<td>(Activation?) inhibitory</td>
</tr>
<tr>
<td>NKRPA</td>
<td>Nkrpa</td>
<td>?</td>
<td>M</td>
<td>Activation</td>
</tr>
<tr>
<td>NKRPA</td>
<td>NkrpAb</td>
<td>Ocil/Cib-b</td>
<td>M (SJL, SWR)</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>NKRPA(K1.1)</td>
<td>NkrpAc</td>
<td>?</td>
<td>M</td>
<td>Activation</td>
</tr>
<tr>
<td>NKRPA</td>
<td>NkrpAd</td>
<td>Ocil/Cib-b</td>
<td>M</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>NKRPA</td>
<td>NkrpAf</td>
<td>Ocilr/Cigr/Dcl-1/LCL-1</td>
<td>M</td>
<td>Activation</td>
</tr>
<tr>
<td>ClrB, F, G</td>
<td>Clrb, f, g</td>
<td>?</td>
<td>M</td>
<td>?</td>
</tr>
<tr>
<td>LLT1</td>
<td>LLT1</td>
<td>?</td>
<td>H</td>
<td>?</td>
</tr>
<tr>
<td>AICL(CLECSF2)</td>
<td>AICL</td>
<td>?</td>
<td>H</td>
<td>?</td>
</tr>
<tr>
<td>KLRF1</td>
<td>KLRF1</td>
<td>?</td>
<td>H</td>
<td>Activation</td>
</tr>
<tr>
<td>CLEC1</td>
<td>CLEC1</td>
<td>?</td>
<td>H</td>
<td>?</td>
</tr>
<tr>
<td>CLEC2</td>
<td>Clec2/CLEC2</td>
<td>?</td>
<td>M/H</td>
<td>?</td>
</tr>
<tr>
<td>LOX1 (ORL1)</td>
<td>LOX1</td>
<td>LDL,HSPAP</td>
<td>M/H</td>
<td>?</td>
</tr>
<tr>
<td>NKG2D (KLRK1)</td>
<td>Nkg2D</td>
<td>RAE,H60,Mult1</td>
<td>M</td>
<td>Activation, co-stimulation</td>
</tr>
<tr>
<td>NKG2D(KLRK1)</td>
<td>NKG2D</td>
<td>MIC,ULBP</td>
<td>H</td>
<td>Activation, co-stimulation</td>
</tr>
<tr>
<td>NKG2A(KLR1)</td>
<td>Nkg2A/NKG2A</td>
<td>Qa-1/HLA-E</td>
<td>M/H</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>NKG2C(KLRC2)</td>
<td>Nkg2C/NKG2C</td>
<td>Qa-1/HLA-E</td>
<td>M/H</td>
<td>Activation</td>
</tr>
<tr>
<td>NKG2E(KLR3a)</td>
<td>Nkg2E/NKG2E</td>
<td>Qa-1/HLA-E</td>
<td>M/H</td>
<td>Activation</td>
</tr>
<tr>
<td>NKG2F(KLR4)</td>
<td>NKg2F</td>
<td>?</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>Ly49A</td>
<td>Ly49a</td>
<td>H2Dd, Dk, Dp</td>
<td>M</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>Ly49C</td>
<td>Ly49c</td>
<td>H2Kb, Db, Kd, Dd, Dk</td>
<td>M</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>Ly49D</td>
<td>Ly49d</td>
<td>H2Dd, H m1-C4</td>
<td>M</td>
<td>Activation</td>
</tr>
<tr>
<td>Ly49E</td>
<td>Ly49e</td>
<td>?</td>
<td>M</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>Ly49G (LGL-1)</td>
<td>Ly49g</td>
<td>H2Dd</td>
<td>M</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>Ly49H</td>
<td>Ly49h</td>
<td>m157</td>
<td>M</td>
<td>Activation</td>
</tr>
<tr>
<td>CD94(KLRD1)</td>
<td>KLRD1</td>
<td></td>
<td>M/H</td>
<td>Activation/ inhibition</td>
</tr>
</tbody>
</table>

**TABLE 4 – NK cell receptors**

*(modified from: http://www.rheumatology.wustl.edu/NKC/NKCRepository.htm)*
As NK cells can express on their surface PRRs as other innate immunity cells, cells of the innate - but also adaptive – immunity can express NK cell receptors. The complete meaning of this shared, and sometime transient, expression of receptors needs to be better understood. Some receptors, like NKR-P1 or CD69, appear associated to developmental or functional moments of various cell types, and may have more complex meaning in the control of cell activity (83, 84).

To understand their possible role in the immunology of cancer, it is necessary a short description of the C-type lectin-like receptors that are considered more involved in the control of NK cell functions.

1.2.4.1. Ly49 family

Ly49 are non-lineage restricted C-type lectin molecules type II glycoproteins disulfide-bound homodimers expressed on natural killer (NK) cells and some T-cell subsets. They are represented in the mouse by Ly-49A (A1, YE1/48); Ly-49B; Ly-49C (5E6); Ly-49D; Ly-49E; Ly-49F; Ly-49G (LGL-1) and Ly-49H; in the rat by rLy-49.9, rLy-49.12, and rLy-49.29. In humans only a Ly49 pseudogene is present at the NK complex level (Ly49L). In rodents was observed strain variability of expression. Ly-49A, Ly-49C and Ly-49G have been shown to be expressed as disulfide-linked homodimeric Ca+-dependent type II glycoproteins. Homology between the proteins of the rat and mouse Ly-49 families was found between 46%-81%. All members have a similar general structure. They present an extracellular C-type lectin domain while the intracellular regions present either ITIM or ITAM motif. Ly-49A, C, E, F, G1 and G4 and rat Ly-49.9 are associated to inhibitory function, while Ly49D, and H in mouse, and rat rLy-49.9 and rLy-49.12 are associated to activating function. Putative Gi-binding motifs are found in mouse Ly-49A and D and rat Ly-49.9 (85, 86).

MHC class I molecules are identified as ligands for Ly-49A, Ly-49C, and Ly-49G. Because Ly-49A and Ly-49C interacts with carbohydrates, it is suggested that the involvement of carbohydrate may be essential for the recognition of MHC class I molecules on target cells. Their binding to MHC class I molecules inhibits NK cell cytotoxic function, suggesting Ly-49 molecules to be involved in the self recognition and protection from autoimmunity. Studies on the Ly49A indicate that phosphorylated peptides of the ITIM motif bind to cytoplasmic tyrosine phosphatases SHP-1 (PTP-1C) and SHP-2 (PTP-1D/Syp), leading to the inhibitory effect of this receptor (85, 87).
A Ly49s expressed by 129/J mouse strain (but not by C57BL/6) showed in individual cells both activating and inhibitory activity, functions transduced through either ITIM or kinase-associated adapter molecules (e.g. DAP10, DAP12).

Similarly, in the laboratory of J. C. Ryan (2005) a rat receptor with double function has been identified (Ly49.5). Ly49i5 is an inhibitory receptor that recognizes ligands encoded within the class Ib region of the u and l haplotypes, while the structurally related Ly49s5 is an activating receptor that recognizes class Ib ligands of the U haplotype (mitochondrial DNA). These results suggest the possibility of more complex recognition capability depending on the expression of the ligand on target cells (88).

Ly49 family in rodents results to exert analogous function than KIRs in humans. Moreover, Ly49 receptors present polymorphism on the strength or specificity of binding to MHC class I and different combinations on NK cells within an individual organism. Mouse MHC class I appears to influence the level of expression on cell membrane and the percentage of NK cells expressing Ly49 molecules. Consequently, an NK cell rarely expresses multiple inhibitory receptors specific for self MHC class I molecules (89, 90).

1.2.4.2. NKR-P1 family

NK cell receptor protein-1 (NKR-P1, CD161) family is formed by 60-kDa type II transmembrane C-type lectin-like glycoproteins, disulfide-linked homodimers. They are mostly expressed on NK cells, but also on some T-cell subsets (effector/memory CD4+ and CD8+, NKT, γδ-TCR cells), monocytes and DC. Transitory expression was described also in myeloid lineage cells. They are present in mouse (NKR-P1A-F) and in rat (NKR-P1A-F). In humans the non-polymorphyc gene KLRB1 encodes only NKR-P1A (CD161) (91-94bis).

In mouse the receptor NK1.1, identified by the ibrydoma product PK136, corresponds to functioning NKR-P1C in C57BL/6 (B6), CE, NZB, C58, Ma/My, ST strains, and to functioning NKR-P1B in NIH-Swiss (Sw), SJL FVP and CD-1 strains. BALB/c, AKR, CBA, C3H, DBA and 129 mouse strains result negative to anti-NK1.1 antibody (95, 96). The supposed negativity of other mouse strains to the expression of NKR-P1, based on the absence of anti-NK1.1 linking molecules (e.g. in the BALB/c strain), has been recently newly investigated by the laboratory of Makrigianni at the genetic level (97). The result of this study showed that both Nkrp1 and Ocil/Clr gene complexes are represented in the BALB/c genome, complete of all the known members as found in the B6 strain. They are functional genes with functional transcripts. Moreover, the NK cells of BALB/c have normal Nkrp1 expression like in B6 NK cells, and that the NKR-P1B receptor links to cognate Ocil/CLr-b ligand. The negativity of the NK1.1 antibody to Nkrp1 gene product in
BALB/c was explained by *Nkrp1* allelic divergence, specifically by a single amino acid substitution (S191T) present in the BALB/c NKR-P1B/C receptors. This divergence is localized to the *Nkrp1b/c* genes, the same that encode the NK1.1 transcript in the positive strains. The correction of this substitution re-established the positivity to the anti-NK1.1 antibody. The B6 *Nkrp1d* gene resulted as an allele of the *Nkrp1b* gene found in BALB/c and other mouse strains. The preservation of *Nkrp1* and *Ocil/Clr* gene complexes through different strains and species highlights their importance inside the immune system. The *Nkrp1e* is a pseudogene (not functional transcripts).

NKR-P1A, C and F are activation receptors associated with p56<sup>lck</sup> (98, 99). These molecules present a charged transmembrane arginine (R) residue for the association with the FcγR adaptor protein (transductor of signals from FcγRIII – CD16, and FcεRI) necessary for activating the signaling pathway. The phosphorylation of the ITAM tyrosines on the FcγR adaptor protein by Lck induces recruitment of SH2 domain-containing Syk-family protein tyrosine kinase and activation of downstream second messenger. The cross-linking of NKR-P1A or C stimulates phosphatidylinositol turnover, arachidonic acid generation and calcium influx, activating cytotoxicity, antibody-induced redirected lysis, and cytokine production (IFN-γ) (69, 100, 101, Fig.11A).

NKR-P1B and D are inhibitory receptors possessing cytoplasmic ITIM motif (99, 102, 103). Murine NKR-P1 proteins include the Cys-X-Cys-Pro (CxCP) motif, also present in the cytoplasmic domains of CD4 and CD8, associated with the Src-related non-receptor protein kinase, p56<sup>lck</sup>. Human NKR-P1A doesn’t present this motif (99, 104, 105).

The mechanism of inhibition by NKR-P1B, like other inhibitory receptors presenting ITIM motif, results dependent from the recruitment of SHP-1 phosphatase by the receptor cytoplasmic domain in a phosphorylation dependent manner. In turn, this event induces dephosphorylation and inhibition of proximal kinases of activation pathways (99, 103, Fig.11B).
Carbohydrate moieties are indicated as possible ligands for NKR-P1 receptors (106). Rat NKR-P1A was shown to have affinity at different degree for various saccharides (GalNAc > GlcNAc >> Fuc >> Gal > Man) (107). The strength of the carbohydrate-receptor interaction resulted strictly dependent to Ca+ bound to the receptor.

Glycoderivatives were tested as mimics of the possible physiological ligands. The molecules containing multiple GlcNAc, either in linear sequence (chitooligomers) or coating multibranched scaffolds (dendrimers), demonstrated high affinity for the NKR-P1A in the rat and C in the mouse, triggering effector functions. Multivalency of the ligand is considered essential for effective interaction and function activation (108-111; see also chapter 4).

Recently (2006) it was also found that human NKR-P1A can bind to α-Gal epitope and NACLac of laminin with possible NK cell activation (112).

However, the identification of the physiological ligands for the various NKR-P1 receptors is still an open question, also if some ligands were described in the last few years.

To date the known physiological ligands are:
- lectin-like transcript-1 (LLT-1) for human NKR-P1A, with inhibitory activity
- Ocil/Clr-b (expressed by dendritic cells and macrophages) for mouse NKR-P1B/D inhibitory receptors
- Ocilrp2/Clr2/Dcl-1/LCL-1 for mouse activating NKR-P1F.

Both Nkrp1 and Clr genes are included in the same NKC, and this finding suggests conservation of gene order, with minimal allelic polymorphism and suppression of recombination. Ocil/Clr-b is displayed at high levels on nearly all haematopoietic
cells, with the exception of erythrocytes, in a pattern that is similar to that of class I MHC molecules. It is interesting that Ocil/Clr-b is expressed at high levels on nearly all haematopoietic cells (but not on erythrocytes), in a pattern that is similar to that of class I MHC molecules. Moreover, Ocil/Clr-b was found frequently down-regulated on mouse tumor cell lines, indicating a role for this receptor-ligand system in a different form of "missing self-recognition" of tumor cells outside the MHC-dependent regulation of NK cell function (113, 114).

Involvement of NKR-P1A receptor in transendothelial migration was described by Poggi A. et al., especially in NKT cells. They also described the effect of IL-12 on NKR-P1 expression. IL-12 resulted to up-regulate the expression of the receptor in a time and dose dependent manner. The maximum of the NKR-P1 expression was found after 7 days of culture in the presence of the cytokine, indicating a de novo transcription of NKR-P1A mRNA. Previously, the same authors found that the NKR-P1A molecule can be expressed by bone marrow and thymic precursors of monocyte/DC after cultivation in the presence of GM-CSF. During the differentiation, the expression of functional NKR-P1A molecule was not lost, and its stimulation produced intracellular calcium increase, as well as IL-1β and IL-12 production from resting monocytes and DCs. Taken altogether, these phenomena suggest the possibility of important involvement of the NKR-P1A receptor for the NK and NK-like cell function in activated microenvironments (inflammation, cancer) (115-118).

1.2.4.3. NKG2 family

The NKG2 gene family is part of the NK complex, and encodes several similar type II lectin-like receptors: NKG2A, B, C, E, D/F and H (A/B and E/H are identified as splice variations of the same gene). NKG2F is represented only in humans. The NKG2D is considered remotely related to the other members of this family, looking as a structurally (only 21% homology with the other members) and functionally more separated form of lectin-like receptor. In fact, while NKG2D is a homodimer, all the other receptors need to be coupled in a heterodimer with C-type lectin like receptor CD94, by disulfide bond, for being expressed on the cell membrane. Therefore, CD94 is suggested to be like a chaperone permitting transport of the NKG2 receptor to the cell surface. These receptors are expressed predominantly on NK cells and subsets of T cells. They were shown to play an important role in regulating responses against infected and tumorigenic cells (119, 120, 121)

Functionally, they are:
CD94/NKG2A, F(?) inhibitory receptors
CD94/NKG2C, E activation receptors
NKG2D activation receptors
NKG2D is an activation receptor expressed on NK, NKT, CD8+ αβ T cells, γδ T cells, and macrophages. It is an important molecule both in humans and in rodents as an activation receptor as well as a co-receptor. Stimulation of NK cells with an anti-NKG2D antibody was seen to redirect lysis. However, the same stimulation of lymphokine-activated killer (LAK) cells did not induce cytokine release. When the NKG2D stimulation was associated to the stimulation of CD16 or NK1.1 activation receptors with specific antibodies, the induced cytokine production resulted enhanced than upon the stimulation of the activation receptor alone. This result indicated that NKG2D can operate as co-receptor of activation receptors (121,122).

Functional NKG2D detects virally infected, stressed and transformed cells by recognizing stress inducible ligands including MHC class I chain-related gene A (MICA), MICB, and UL-16-binding proteins (ULBP) in humans; retinoid acid early inducible 1 (Rae-1) family of proteins, MULT and H60 protein encoded on chromosome 10 in mice. NKG2D ligands are found on many tumors and are up-regulated upon infection (50).

In humans, the receptor, after interaction with the ligand, activates the phosphatidylinositol 3-kinase pathway upon the association through a charged transmembrane residue with DAP10 adaptor, bearing an ITAM. In the mouse, NKG2D can be present also as NKG2D-S isoform with possibility to be alternatively associated to either DAP10 or KARAP/DAP12. The activation stimulates the formation of the NK immunological synapse (NKIS) with recruitment of NKG2D to the center synapse (see also above), the expression of CD25, NK proliferation and cytokine production (123).

Interestingly, structural studies have revealed the C-type lectin fold in the intact murine NKG2D to be highly similar to other C-type receptors: CD94, Ly49A, rat MBP-2, and CD69 (Fig. 12).

![FIGURE 12 - Activating NK lectins and their associated adapter molecules.](image-url)
Activating NK lectins have positively charged residues (R or K) in their transmembrane domains, which allow for their functional associations with negatively charged (D) adapter proteins. Activating NK1.1 (NKR-P1) molecules associate with FcεRIg, while activating Ly-49 and heterodimeric CD94/NKG2 receptors associate with DAP12. The monomorphic NKG2D receptor associates with DAP10. Both FcεRIg and DAP12 signal via immune tyrosine-based activation motifs (ITAMs) through which they recruit the Syk and ZAP70 tyrosine kinases. DAP10 contains a cytoplasmic motif (YxxM) for recruitment of the regulatory p85 subunit of PI-3-kinase. From: Immunological Reviews 2001 Vol. 181: 126–137

The NKG2D orthologues are present in human, chimpanzee, rhesus monkey, cattle, pig, rat, and mouse.

The heterodimer CD94/NKG2 recognizes non-classical MHC class I molecule HLA-E in human (for NKG2A and C) and its homolog, Qa1 in mouse (for NKG2A, C and E). CD94 is an invariant molecule with a short cytoplasmic domain apparently without functional motifs (124, 125).

CD94/NKG2A is an inhibitory receptor with two ITIMs in the intracellular tail of NKG2A. This molecule can be alternatively spliced to give NKG2B. CD94/NKG2C and E/H are ITIM-less activation receptors. For their function, they associate with DAP12 through their charged transmembrane residue. NKG2F, present in human, encode a molecule that has charged transmembrane residue, cytoplasmic ITIM-like sequence, and absence of C-type lectin-like domain.

The HLA-E in human and Qa1 in mouse mainly present peptides derived from the leader sequences of classical MHC class I heavy chains. In this way they offer to the receptors the possibility to monitor both the expression of MHC class I and of the HLA-E or Qa1 molecules itself, requiring an intact MHC class I assembly pathway. Both in mouse and humans the inhibitory receptor appears to exert dominant effect on the activation receptor, blocking the reorganization of the cytoskeleton and inhibiting the migration of lipid rafts at the immunological synapse by recruitment and activation of SHP-1 e dephosphorilation of VaV1, guanine nucleotide exchange factor and regulator of actin. By this way the cytotoxicity is impeded. (51, 52) The level of expression of CD94 appears also inversely correlated with the level of apoptosis in NK and CD8+T lymphocytes, and the levels of CD94/NKG2A are enhanced after specific antigen recognition (120).

1.2.4.4. CD69

Receptor not exclusive of the NK cells, CD69 is a constitutively phosphorylated glycoprotein (Ser/Thr), type II membrane protein with a C-type lectin domain. Group V C-
type lectin is expressed as a disulfide-linked homodimer of 60 kDa. It is closely related to the NKR-P1 and Ly-49 NK cell activation molecules. Known also as activation inducer molecule (AIM) or very early activation (VEA) molecule, CD69 is a cell activation marker, involved in early events of lymphocyte, monocyte and platelet activation (126, 127). The CD69 gene is encoded in the NKC (human chromosome 12 p13-p12, mouse chromosome 6) (74, 75). In the T cells, CD69 expression produces Ca\(^{2+}\) influx, synthesis of cytokines and their receptors, induction of the expression of c-myc and c-fos protooncogenes. In activated NK cells, CD69 has also a functional role in redirected Ca\(^{2+}\) dependent target lysis. The NF\(\kappa\)B regulates TNF alpha-mediated induction of CD69 gene expression, and promoter consensus sequences include AP-1, NFAT, and NF\(\kappa\)B. Cross-linking of CD69 induces cytotoxic activity and costimulates cytokine production of activated NK cells and selected T cell clones (128, 129). Highly expressed on T cells derived from inflammatory infiltrates, CD69 is transiently expressed on activated leukocytes including T cells, thymocytes, B cells, NK cells, neutrophils, and eosinophils, while it is constitutively expressed by a subset of medullary mature thymocytes, platelets, mantle B cells, and certain CD4\(^+\) T cells in germinal centers of lymph nodes, platelets, and epidermal Langerhans cells (126, 127). CD69 ligands are still not defined, though as a lectin-like molecule present particular affinity for certain carbohydrate moieties (Gal, GalNAc > GlcNAc). It was seen that chitosan and alginate biopolymers up-regulate CD69 expression on B-cells and CD4+ T-cells. CD69 triggering involves Lck to induce rapid and selective activation of the tyrosine kinase Syk. Syk and Src family tyrosine kinases control the tyrosine phosphorylation and activation of phospholipase C\(\gamma\)2 and the Rho family-specific exchange factor Vav1 (involved in the actin-dependent cytoskeleton modifications leading to the formation of the immunological synapse) and are responsible for CD69-triggered cytotoxicity of activated NK cells (130, 131).

Paradoxically, CD69 can be also a negative modulator of autoimmune reactivity and inflammation through the synthesis of TGF-\(\beta\)1, and its block resulted in enhanced antitumor immunity. These peculiar findings needs a wider investigation to better understand the receptor functions and its interplay with other receptors (both activation or inhibitory) leading to possible divergent immunomodulatory results (132)

1.2.4.5. Immunoglobulin-like superfamily of cell surface receptors

Within the many immunoglobulin-like superfamily receptors discovered on NK cells, need to shortly describe two important families: the natural cytotoxicity receptors (NCRs) with activation function, and the killer cell immunoglobulin-like receptors (KIRs) with prevalent inhibitory function.
1.2.4.5.1. Natural cytotoxicity receptors (NCRs)
The natural cytotoxicity receptors (NCRs) are molecules, firstly identified in human, that result to be expressed exclusively on NK cells. They include NKp30, NKp40 and NKp46. The NKp30 and NKp46 receptors are expressed on the surface of activated and non-activated NK cells, while the NKp44 receptor is expressed on the surface of activated NK cells only. The NCRs trigger NK cell lysis of tumor and virus-infected cells on interaction with cell-surface ligands of these target cells. While NKp44 and NKp46 appear to be involved in the recognition of viral hemagglutinins expressed on the surface of virus-infected cells, NKp30 and NKp46 can recognize heparan sulfate epitopes expressed by tumors. The fact that non-microbial endogenous carbohydrate structures contribute to this recognition is important for the role that these receptors (possibly together with the lectin-like receptors) may play in antitumor immunity as well as autoimmunity and inflammation outside of the MHC molecule recognition. The expression of the three NCRs on the surface of NKs is coordinated, with variable surface density in different individuals and also in the NK cells isolated from a given individual. The level of NCR expression can affect ability of the NK cells to lyse the tumor cells. NKp46 resulted the only NCR involved in human NK-mediated killing of murine target cells; a homologue of NKp46 has been found also in mouse. Coupling of NCRs to different signal transducing adaptor proteins, including CD3ζ, FceRI gamma, and KARAP/DAP12 is necessary for their function. Cooperation of NKG2D and NCRs for determining tumor lysis was also described.

In a very recent publication, Walzer T. et al (2007), showed that NKp46 is expressed by NK cells in humans, all mouse strains analyzed in the study, and in three common monkey species. This result suggested NKp46 cell surface expression to be used as a unifying phenotypic definition of NK cells across species (133-135; Tab. 5).
### TABLE 5 - Activation receptors on NK cells and their ligands. Several receptors are still orphan for ligand.

#### 1.2.4.5.2. KIR superfamily

Human killer immunoglobulin-like receptors (KIRs) are type I transmembrane glycoproteins and with 2-3 extracellular C2-type immunoglobulin-like domains. They are MHC class I – specific receptors prevalently with inhibitory function, like the mouse Ly49 receptors. They exert their activity through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) on the cytoplastic tail that are phosphorylated subsequently to the ligand binding. This event produce recruitment and activation of intracellular tyrosine phosphatases (e.g. SHP1) with possible dephosphorylation of molecules involved in the activation signal cascade. They are encoded as approximately 12 genes in the LRC region on chromosome 19q, with 90% sequence identity. Similar genes were identified also in other primate species (Tab.6)
Homologous receptors (immunoglobulin-like transcript – ILT; leukocyte immunoglobulin-like receptor – LIR; leukocyte-associated Ig-like receptors – LAIR; paired Ig-like receptors – PIR; gp49) with 35-50% sequence identity and sharing a common fold with KIR are also identified and included in the KIR superfamily (136).

The KIR receptors are not expressed in rats and mice, like Ly49 is represented only by a non-functional gene (pseudogene). This may suggest equivalence and a specificity of function that does not admit redundancy.

While the largest part of the KIR has inhibitory function, the KIR2D and KIR3D families are ITIM-less, suggesting them to be activation receptors. They have a positively charged

---

**TABLE 6 – KIR receptors and their nomenclature.**

<table>
<thead>
<tr>
<th>Common names</th>
<th>HGNC(^1) Nomenclature</th>
<th>CD designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIR3DL2(^2)</td>
<td>p140, NKAT4/4a/4b</td>
<td>CD158k</td>
</tr>
<tr>
<td>KIR2DS2</td>
<td>p50.2</td>
<td>CD158j</td>
</tr>
<tr>
<td>KIR2DS4</td>
<td>p50.3, NKAT8</td>
<td>CD158i</td>
</tr>
<tr>
<td>KIR2DS1</td>
<td>p50.1</td>
<td>CD158h</td>
</tr>
<tr>
<td>KIR2DS5</td>
<td>NKAT9</td>
<td>CD158g</td>
</tr>
<tr>
<td>KIR2DL5</td>
<td></td>
<td>CD158f</td>
</tr>
<tr>
<td>KIR3DL1/S1</td>
<td>p70, NKAT3/NKAT10</td>
<td>CD158e1/e2</td>
</tr>
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<tr>
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<td>KIRX</td>
<td>CD158c</td>
</tr>
<tr>
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<td>NKAT1, p58.1</td>
<td>CD158a</td>
</tr>
<tr>
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<td>p58.2/p58.3, NKAT6/NKAT2</td>
<td>CD158b1/b2</td>
</tr>
<tr>
<td>KIR3DL7</td>
<td>KIRC1</td>
<td>CD158z</td>
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<tr>
<td>ILT10</td>
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<td>CD85n</td>
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<td>ILT9</td>
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<td>LIR5</td>
<td>LILRB4</td>
</tr>
<tr>
<td>ILT2</td>
<td>LIR1, MIR7</td>
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<tr>
<td>ILT5</td>
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<td>CD85n</td>
</tr>
</tbody>
</table>

\(^1\)HGNC, HUGO (the human genome organization) gene nomenclature committee.

\(^2\)KIR and ILT genes are located on human chromosome 19qter and 19q13.42 respectively.
residue in the transmembrane region capable to be paired with adaptor molecule activating motif. A 12-kDa dimer (DAP12) is the putative molecule to be associated to these receptors, in association with ZAP70/Syk. However, it is still unknown the possible biological function of such activation receptors.

KIRs recognize multiple alleles of MHC molecules. As receptors of the innate immunity ready to fast responses, they are germ-line encoded, with limited adaptability to the diversities of the MHC-peptide complexes and to the number of all MHC alleles. The possibility to recognize conserved residues within polymorphic regions of MHC enable a KIR to recognize multiple related MHC molecules. The otherwise polymorphic region of HLA presents highly conserved residues in its contest, and a variable amino acid (in position 80 in HLA-C). By this way, individual KIR can recognize multiple HLA class I molecules and discriminate among various allotypes on the base of the identity of only one variable amino acid in the receptor interface. On the KIR side, a single residue in position 44 controls allotype specificity toward HLA (137). The inhibitory receptors collaborate to the self recognition and can have affinity for HLA inhibitory molecules like HLA-G. These receptors can influence both innate and adaptive immunity by their function and interplay depending on: KIR genotype; HLA genotype; effect of heterozygosis versus homozygosis on cognate recognition between the HLA and KIR products carried by an individual; and the specific modulation of HLA expression by infection, transformation or peptide binding. Clonal variations of KIR expression between individual NK cells can generate subsets with different ability to respond to targets. This situation can be particularly important in anticancer activities inside the tumor microenvironment (138, 139).

1.2.4.6. Fc receptors

The Fc receptors bind to various Ig isotypes and subtypes and are expressed on leukocytes. They can bind to the γ or ε heavy-chain classes of immunoglobulines. Two types of Fc receptors can be expressed also on epithelial cells to mediate transepithelial transport of Ig: one allows in maternal-fetal exchanges through the placenta and the neonatal intestinal epithelium (neonatal Fc receptor – FcRn); the second is the adult homologous expressed in various epithelia with function of recycling Ig to protect from fast degradation. FcI is a class of high-affinity receptors, while Fc II and III are low-affinity receptors. The cross-linking and clustering of these receptors permit efficient triggering (72).

The leukocyte Fc receptors are involved in phagocytosis and activation of phagocytes (expression on macrophages, DC, neutrophils, platelets, eosinophils; IgG receptors FcγRI – CD64, FcγRIIA – CD32, FcγRIIIB – CD16; FcαRI – CD89 an IgA receptor), feedback inhibition of B cells, but also down-regulation of immune complex activation of mast cells,
macrophages and neutrophils (FcyRIIB – CD32 with ITIM sequence in the intracellular domain), antibody-dependent cell-mediated cytotoxicity (FcyRIIA – CD16 on NK cells), cell degranulation (FcεRI IgE receptor on mast cells, basophils, eosinophils). Another receptor (FceRII – CD23) expressed on B cells, Langerhans cells, eosinophils is a C-type lectin. It binds with low affinity to IgE, inducing phagocytosis of IgE-coated particles (140).

CD16 or FcyRIII is a 50–80 kDa transmembrane glycoprotein that is expressed in two isoforms, A and B. The FcyRIIIA is encoded by NK cells. As Fcγ receptor, CD16 recognize the Fc portion of IgG, presented either as immune complexes or as free antibody. The Fc-binding polypeptide α chain is a member of the Ig-like superfamily and it is generally associated with a disulfide-linked homodimer FcR γ chain. However, it is possible also an association with either TCR ζ chain homodimer or a γ ζ heterodimer, all including ITAM sequence. This γ chain presents a short extracellular amino terminus and a large cytoplasmic carboxyl terminus with certain homology with the ζ chain in the TCR. The cytoplasmic domain includes an ITAM that couples receptor clustering to the activation of protein tyrosine kinases.

CD16 is the NK receptor that mediates the antibody-dependent cellular cytotoxicity (ADCC), integrating the cytotoxic function induced by the lectin-like or natural cytotoxicity activation receptors. The extracellular Ig-binding domain recognizes IgG1 or IgG3 molecules bound to a target cell surface. Clustering of the receptors activate the killing response by the NK cell through ITAM phosphorylation → PI-3 kinase activation → recruitment of adaptor molecules (SLP-76, BLNK), phospholipase cγ and Tec family kinase → formation of PIP3 and diacylglycerol → calcium flux.

For the NKR-P1 activation resulted essential the association with the Fcγ chain and, consequently, needs investigation the interplay with the CD16 receptor. (69, 72, 140)

1.2.5. General mechanism of NK cell activation

NK cells are able to recognize both proteic and carbohydrate antigens. Whether the contemporary presence of carbohydrates on a protein (or lipid) is necessary for the correct recognition of the antigen is still unclear.

The target cell to be addressed to the recognition process (self versus non-self) needs to take contact with the effector cell. In our case the effector cell is the NK cell with its wide panel of receptors, co-receptors and ligands. The temporary cell-to-cell connection is made possible by the multiple linking of adhesion molecules and receptors with their physiological counterpart on the target cell (see paragraph 1.3). The simultaneous
engagement of the various molecules can modify their distribution on the cell surface and their reciprocal interactions. The proportion of activation receptors is reported to be lower than the one of inhibitory receptors, and the activation signals less intense than the inhibitory signals. The receptor ligand interactions are generally non-covalent and the affinity can vary according the isotype of the molecule but also the environmental conditions (e.g. pH). For some receptors it is documented the necessity of clustering to obtain effective signaling. Especially for the lectin-like receptors, the multivalency of the carbohydrate ligands appears important for their triggering. The clustering of various receptors as well as adhesion molecules is documented in the organization of the immunological synapse. As seen in paragraph 1.3., the activation signals permit actin cytoskeleton reorganization through VAV1 activation, with accrual of lipid rafts, receptors and adhesion molecules, while the cytotoxic granules polarize. On contrary, the delivery of inhibitory signals blocks the evolution of the immunological synapse and induces disruption of the initially organized actin network.

The large part of inhibitory receptors (lectin-like in rodents, Ig-superfamily in humans) is specific for MHC (HLA) class I molecules of the host repertoire to preserve the self elements from destructive attacks. Their signaling, through the activation of phosphatases, blocks the eventually triggered activation pathways. This effect is reverted in front of altered MHC class I, either alone or in complex with peptides, where the inhibitory signal cannot be delivered upon the absence of correct receptor-ligand interactions. In this case, the activation signals will override the possible inhibitory signals, leading to the calcium influx, cytotoxic immunological synapse organization, cytokine production and delivery of cytotoxic granules in the intercellular linking area (kiss of death). According to the panel of involved receptors, the proportion of their expression in the clonal NK subsets, their stochastic distribution on the cell surface and engagement by the molecular repertoire exhibited by the target cell (mutated molecules, but also MICA or HLA-G, as an example), the final NK cell response (“to kill or not to kill”) will be the result of the summatory of the reciprocal and simultaneous interactions between activating and inhibitory signals evoked by the triggered receptors. Lectin-like activation receptors (NKR-P1A, C; NKG2D; Ly49D; CD69) are present on the NK cell membrane as well as the Ig-like Nkp40, 44, 46, and the inhibitory MHC class I restricted and non-restricted molecules. How all these receptors are represented in the initial contact area and, subsequently, in the immunological synapse, and how they interrelate it is not yet completely clarified and needs further studies (48, 50, 51, 68, 89, 104, 133, 141). The classic model of NK cell activation is shown in Fig. 12: A) the NK cell has contact with a target cell. The contact is stabilized by the adhesion molecules of the
immunological synapse. Activating and inhibitory receptors bind to molecules expressed on the target cell surface. If the inhibitory receptors (here, Ly49) find their ligand – e.g. a host self-MHC class I molecule – the delivered signal blocks further organization of the synapse initiated by the activation receptor binding to a target molecule: no cytotoxic granule release is delivered. B) Alteration of the MHC class I molecule pattern does not allow interactions with the inhibitory receptors. The signal delivered by activating receptor (here, NKR-P1 binding carbohydrates exhibited on target cell surface) after binding with its ligand is not impeded and triggers the cytotoxic response leading to polarization of the cytotoxic granules, which are delivered inside the cytotoxic synapse. The target cell goes to apoptosis/necrosis under the action of granzyme and perforins contained in the released granules.

FIGURE 12 – NK cell recognition of the target (see text). (Vannucci, MBU AVCR, v.v.i.)
1.3. THE CANCER

1.3.1. An overview

Cancer is a biologically multi-faceted pathology. For a long time researches have been principally focused on the biochemical and genetic aspects of the cell transformation and cancer cell metabolism. However, thanking recent advances both in molecular biology and in immunobiology, cancer results better described as an articulated process that derives its characteristics from the very active interplay between the transformed (cancer) cells and the host. According how this interplay evolves, a cancer can or cannot develop. In the interplay between the cancer cells and the host, the host immunity competence and its capability to mount an efficacious response against the tumor cells are fundamental. In this view, it is important to underline a critical factor: the cancer cell is fundamentally a self cell that acquires its special characteristics in a progressive mode (142). This is a central fact that makes the anticancer immune response very peculiar – directed against mutated but “still self” cells, almost in the early development of cancer - and different from the immune response against a true “non-self” like a microorganism.

Typically, a cancer grows as an expanding mass that infiltrates the surrounding tissues and spreads to replicate in distant organs (metastasis). It kills the host as the result of its volumetric expansion, invasiveness, metastatic spread, delivery of biologically active products (e.g. TNF-α, IL-1β) affecting the general metabolic activity and immunity of the host, when not treated possibly in early stages of development.

The biological evolution of a cancer can be summarized in the following stages:

1) it starts with sequential mutations of a cell under the pressure of carcinogenic agents (e.g. chemicals, radiations, viruses, chronic inflammatory environment, etc.) and/or a favorable genetic background (143, 144);
2) the mutated cell generates a clone that, while growing, produces stress in the surrounding tissue (cells, stroma) with alteration of the local homeostasis (145, 146);
3) the developing tumor mass generates, together with the host participation, a complex structure (tumor microenvironment) allowing its own survival, progression, invasion and spread in the host organism (147, 148, 149) (see also later).
The characteristics that make a cell a “cancer cell” were clearly defined by Hanahan and Weinberg (2000, ref. 150). They focused in six points the hallmarks of a cancer, stating that cancer cells are able to

1. provide their own growth signals (by autocrine/paracrine mode)
2. ignore growth-inhibitory signals (loosing cell-cell and cell-stromal regulations)
3. avoid cell death (by anti-apoptotic mutations e.g. of p53)
4. replicate without limits
5. sustain angiogenesis (e.g. by VEGF production under the pressure of Hypoxia Inducible Factor 1 – HIF-1, produced and delivered by tissue in hypoxic conditions)
6. invade tissues through basement membranes, capillary walls and nerves (e.g. by release of matrix metalloproteinases – MMPs)

This definition is complete on the side of the cancer cell but does not consider its interaction with the host. To include also this aspect, the group of R. D. Schreiber has recently proposed the addition of a seventh hallmark, concerning the interplay between cancer and the immunity of the host:

7. avoidance of immunosurveillance (tumor escape) (151)

When the immune system fails to eliminate all tumor cells, tumors with reduced immunogenicity may emerge able to escape immune recognition and destruction (142). This combination of host-protective and tumor-promoting functions of the immune system throughout tumor development has been termed “cancer immunoediting” (151, 152) and has been envisaged as a dynamic process composed of three phases: elimination, equilibrium, and escape. Elimination embodies the classical concept of cancer immunosurveillance; equilibrium is the period during which cancer growth balance the aggression by the antitumor immune response with incomplete tumor destruction; and escape reverts to the final outgrowth of tumors that have outstripped immunological restraints of the equilibrium phase.

All tissues of our body can originate a cancer when a repeated and/or intensive exposition to mutagenic agents produces effective damages at DNA level. The action of a carcinogen can be increased by both the individual genetic background (e.g. congenital impairment of DNA-reparation mechanisms) and the activity of promoting agents (e.g. chronic inflammation) (153, 154, 155).

The sequence of events that characterize the carcinogenetic process is typically defined in two phases:

1. initiation (the carcinogen produce a DNA instability and genetic damage)
2- promotion (factors that do not damage the DNA but stress the genetically damaged tissue, pushing the transformation)

The carcinogenesis of colorectal and skin tumors represents a typical example of this sequence (156, 157).

A first defense against cancer is represented by the different cellular pathways that can buffer the negative effects produced by the carcinogenic agents (153)

The DNA damage can be repaired by different mechanisms (base-excision repair, nucleotide-excision repair, homologous recombination, non-homologous end-joining) (158)

When these mechanisms are congenitally incomplete (154) or the action of the carcinogenic agents is so intensive to override the attempts to repair, mutations appear and a progressive genetic instability allows further mutational events (159). These mutations affect genes regulating the cell replication (e.g. ras, myc, fos) and the mechanisms of apoptosis (e.g. p53). The sequence of carcinogenic events is indicated with the term transformation, which is the consequence of and consists in progressive modification of gene expression, alteration of metabolic pathway activities (e.g. glycosylation, MHC molecule synthesis, expression of stress molecules, etc.), and changes in cell phenotypic characteristics (e.g. impaired expression of MHC class I molecules, and neo-expression of aberrant carbohydrates structures, tumor-specific antigens – TSA, and tumor associated antigens – TAA, on cell surface) (154, 160, 161).

The cumulative effect of these changes progressively impedes the transformed cell to correctly adapt to the environmental conditions of and controls by the surrounding tissue. The uncontrolled cell replication creates a clone which generate increasing stress in the tissue structure (cells and stroma) inducing various local phenomena: 1) the host tissue react expressing stress proteins and mediators collected under the name of danger signals (162), initiating the activation of the immune response; 2) the cancer clone sustains its own development by overexpressing receptors to growth factors and ligands to inhibitory receptors of immune cells (both for its own characteristics and in response to tissue and immunity reaction to its growth - see later), and by conditioning the surrounding environment (e.g. secretion of VEGF, growth factors, MMPs, inhibitory cytokines). The complex formed by cancer cells, host tissues, host immune cells and related molecular products constitutes the tumor microenvironment. (148, 163, 164, 165, 166)

1.3.2. Glycosylation and cancer

Carbohydrates are important both for protecting the molecules to which they are bound from rapid degradation by environmental enzymes, but also to assist their correct folding
and receptor-ligand interactions. Nearly all the key molecules involved in natural and adaptive immunity are glycoproteins and specific glycoforms are essential elements during immune recognition (167). The carbohydrate structures expressed on the surface of normal and cancer cells can be directly involved in the immune recognition through lecitin-like receptors with modulation of the immune response and regulation of intercellular interactions (168). Moreover, it was sustained that glycosyl epitops in microdomains associated with various functional membrane proteins can have importance as mediators of cell adhesion, signal transduction events, and capable to influence cellular phenotypes (169). The presence of the N-glycan biosynthesis pathway in metazoans, depending on GlcNAc transferases, and its absence in the protozoa is suggested to represent as an evolutionary response to the needs for intercellular interactions in multi-cellular organisms (168, 170)

Aberrant glycosylation of glycoproteins and glycolipids is one of the many molecular changes that accompany malignant transformation. The abnormal expression and activity of glycosylation enzymes during the carcinogenesis and cancer progression can lead to a significantly altered glycosylation pattern on the cell surface, thus disturbing the normal functions of the glycocalyx and producing effects on immunogenicity, intercellular regulations and mobility of the cancer cells (171, 172).

The alterations that can affect the expression of the carbohydrate structures include loss or excessive expression, appearance of truncated, incomplete or new structures:

- changes in N-glycans by altered branching can result from overexpression of N-acetylglucosaminyl-transferase V - GlcNAc-TV;
- O-glycans alterations produce truncated structures – simple mucin-type carbohydrate antigens Tn, sialyl-Tn and T, over-production and shedding of mucins;
- glycosphyngolipids can have over-expression and shedding, e.g. in melanoma and neuroblastoma;
- aberrant glycosamino-glycans can derive from altered expression of some heparin-sulfate-proteoglycans, and hyaluronan – CD44 ligand;
- finally, increase and/or alteration of sialic acids can be also observed (171, 173).

As an example, it was found that the changes occurring during malignant transformation produce in the breast and colon cells appearance of a different pattern of glycosylation, rich in ligands for DC-SIGN (LeX, LeY, Lea), MGL (Tn), and probably Siglec (sT, sTn). On contrary, the normal breast and colon epithelial cells are rich in N-linked glycans with disialyl-Lea, 6-sulfo-sLeX and 30sulfo-Lea epitopes, whereas the O-glycans are either of the core 3-, or elongated core 2-type. GlcNAc-branched N-glycans and terminal Lewis antigen sequences, observed to increase in some cancers, appear to correlate with poor prognosis (174, Fig. 8).
The role of sialic acid, neuraminidases, and changes in the mucins was argument of active investigations since a very long time (the earliest approach was by histochemical studies about the modification of the staining due to different acidity of the mucins in colon and breast tissues during carcinogenesis and cancer evolution). However, what was known over 30-40 years ago by the histologists and clinicians about this argument, only recently has found evidence at molecular level. Nevertheless, fundamental questions about correlation of cancer biological expressions to interventional approaches are still not solved. The expression of sialyl-X on tumor cells has been correlated to better sensitivity to NK cell aggression but the pattern of recognition is not clear (CD94?) (175).

As above cited, the over-expression of N-acetyl-glucosaminyl-transferase V (GlcNAc-TV) allows the production of overbranched N-glycans on the cell surface by β 1-6 branching. Staining with the lectin L-PHA is increased in cancer tissues presenting this aberration. Hyperbranched carbohydrate structures contribute directly to cancer progression and transformed cell motility, changing the intercellular interactions and adhesion. Morphological transformation, tumor growth and induction of a metastatic capacity were obtained after cell transfection with the Mgat5 gene encoding the GlcNAc-TV.
Interestingly, *Mgat5* expression is regulated by RAS-RAF-MAPK, a signaling pathway commonly activated in tumor cells (171, 176, 177). Another enzyme, the β-1,4-N-acetylgalcosaminyltransferase III (GnT-III), catalyzes the addition of a bisecting N-acetyl-d-glucosamine (GlcNAc) to N-glycans. GlcNAc-III is an enzyme that competes with GlcNAc-TV for acceptor. When ectopic expression was induced in B16 melanoma cells, it suppressed their metastatic potential. Li W. and al. (2007) reported that establishing stable expression of GnT-III in the B16 melanoma cell line produced a variation of the adenylyl cyclase III (ACIII) with upregulation of its function. This experiment demonstrated how the structure of N-glycans of ACIII regulates not only its enzymatic activity but also its downstream signaling, an example of the importance of carbohydrate structures not only for external cell phenotype but also for regulation of cell function (178).

Aberrant glycosylation was also found to be induced by hypoxia, a quite frequent condition in the growing cancer tissue. Colon cancer cells cultivated under hypoxic conditions presented increased expression of selectin ligands, the sialyl Lewis X and sialyl Lewis a, at the cell surface, leading to an increased adhesion of cancer cells to endothelial E-selectin. The hypoxia induced enhanced transcription of genes for fucosyltransferase VII (FUT7), sialyltransferase ST3Gal-I (ST3O), and UDP-galactose transporter-1 (UGT1), involved in the synthesis of the carbohydrate ligands for E-selectin. Also a remarkable induction of the genes for syndecan-4 (SDC4) and alpha5-integrin (ITGA5), molecules involved in the enhanced adhesion of cancer cells to fibronectin, was caused by the hypoxic culture through involvement of hypoxia inducible factors (HIFs). These findings once more point out the critical influence of tumor microenvironment in conditioning cancer evolution (179).

The identification of glyco-structures involved in the self/non-self recognition is of the greatest importance. In fact, carbohydrate molecules able to trigger activating receptors of cytotoxic cells could be perspective for possible therapeutic applications. The ligands for the innate immunity receptors involved in anti-infective responses are largely identified. More elusive, instead, is the identification of carbohydrate physiological ligands for lectin-like activating receptors (104, 180).

Nearly all the tumor associated antigens are heavily glycosylated molecules:

- CEA
- CA125
- CA19.9/Sialyl Lewis A
- Sialyl Lewis X
- MUC1,2,4,5Ac,6
- TF(Thomson – Friedenreich) blood group antigen
- Tn (addiction of N-acetylgalactosamine to serine or threonine residues form the Tn)
- Sialyl Tn
- Globo H
- MAGE

In many of these antigens, GlcNAc is well represented, necessary for the aberrant branching, and it is constituent of glycans, hyaluronans and heparan sulphate widely present in the organism and also in the inflammatory and cancer microenvironments. The GlcNAc can be organized in repetitive sequences, giving the possibility for multivalent link to putative receptors. The only C-type lectin-like receptor that is known, at date, to bind GlcNAc-rich molecules is the dectin-1(CLECSF12) (181). This receptor, expressed by dendritic cells, macrophages/monocytes, neutrophils, and some subpopulation of T cells, is a type II transmembrane glycoprotein receptor containing one lectin-like carbohydrate recognition domain able to recognizes β-(1-3)- and/or β-(1-6)- linked glucans as well as fungi particles (182). In addition to these ligands, dectin-1 can also recognize naïve T cells, stimulating their proliferation. The mechanisms of these stimulations are not yet identified (183, 184). The cytoplasmic tail of dectin-1 contains an ITAM-like motif. The intracellular signaling pathway triggered by this receptor is mostly unknown. Interestingly, the production of proinflammatory cytokines and chemokines by dectin-1 also requires signals generated from the TLRs (particularly, TLR-2, TLR-6) including MyD88 and NFκB. TLR-2 and MyD88 are expressed also by NK cells (185, 186).

1.3.3. The tumor microenvironment

The concept of tumor microenvironment is relatively recent and today is generally accepted. It represents an important working-model because able to interconnect the various elements that collectively form a cancer. In fact this model combines the host elements that, locally, are in direct relation with the growing clone of cancer cells, to constitute an interactive and interdependent network. In this view a cancer is constituted by:
- cancer cells
- fibrous-recticular connective (stroma) of the host
- vascular endothelium of the host, neo-generated vessels and blood
- lymphatic endothelium of the host, neo-generated vessels and lymph
- immune cells of the host
- nervous fibers of the host
- intercellular matrix
- molecules expressed and released by all the involved cells

In this articulated network, three major phenomena occur:

1- invasive expansion of the tumor mass and cancer cell heterogeneity
2- generation of hypoxic conditions and neo-angiogenesis induction
3- immune response and tumor escape

The mass of the cancer cells is continuously growing because the deregulated cell replication (mutated ras, myc, fos, cyclins, bcl2, p53, etc.). In solid tumors, the development to larger volumes can determine an inadequate blood perfusion caused by insufficient vascular net development in proportion to the tumor mass. As a result, oxygenation and supplement of nutrients within these malignancies can be inadequate and chaotic, generating regions that become chronically exposed to severe hypoxia, low pH (values around pH 5), and nutrient deprivation. Consequently, signaling via growth factors, oncogenes and hypoxia inducible factors (HIFs), promotes the up-regulation of glycolysis, intracellular pH (pHi) and vascular endothelial growth factor (VEGF) via cooperative mechanisms. Recently it was shown that HIF can target to genes involved in angiogenesis, glucose metabolism and vasomotor control, but also can affect functions in matrix metabolism, apoptosis, carbon dioxide metabolism and secondary cascades of transcriptional control. The activation of HIF consequent to the hypoxic conditions can assist the establishment of classical metabolic alterations in cancer cells. A wide range of cytogenetic changes are also described to be produced by the exposures to acidic and hypoxic environments (increases in mutation frequencies; deficits in DNA repair; DNA over-replication and gene amplification; induction of chromosomal fragile sites, triggering genomic rearrangements; changes in gene expression). In these conditions, the cancer cells can progress in genetic mutations to adapt to the progressively unfavorable environment, with increase of clonal heterogeneity. This contributes to progressive selection of more malignant clones with better environmental adaptability, escaping the immune response of the host and acquiring more resistance to chemotherapeutics. Acidity, in particular, can have effects on resistance to chemotherapy, proliferation and metastatic behavior of a tumor. For example, the extracellular pH of solid tumors, significantly more acidic than that of normal tissues, can induce impairment of weakly-basic chemotherapeutic drugs uptake, change the affinity of immune receptors for their ligands (e.g. lectin-like receptors and carbohydrate recognition) or modify the tertiary structures of ligands. Moreover, cancer cells can utilize some of the mechanisms that regulate the pH homeostasis in mammalian cells (e.g. protonic pumps in the plasmatic membrane) to protect themselves from the acidic
environment and to maintain acidity in an environment unsuitable for normal or more differentiated cells: an extracellular pH of 5.8 to 5.3 resulted in a nearly complete loss of the cytotoxic response by peripheral blood mononuclear cells, lymphokine-activated killer (LAK) cells, and natural killer cells (188, 189, 190).

The microenvironmental hypoxic conditions collaborate to stimulate the development of new vessels inside the tumor by inducing both tumor and host cells to express and release pro-angiogenic factors. The neo-angiogenesis is essential for neoplastic growth and metastases (191). Many molecules have been described as promoters of the angiogenesis: vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor (PDGF), basic fibroblast growth factor (βFGF), and interleukin-8, urokinase-type plasminogen activator and its soluble receptor, E-selectin and vascular cell adhesion molecule-1 (VCAM-1), and von Willebrand’s factor (vWF). At present, VEGF and βFGF are considered to play the most important roles like promoters of endothelial cell survival, proliferation, and differentiation. However, the new vessels present a conformation that is less structured than in the normally developed vessels, with higher permeability and reduced capability to dynamically respond to functional requests (modulation of their caliper to adapt the flow to physiological necessities) (192-197; Fig. 9).

**FIGURE 9 – Tumor angiogenesis.** Comparison of the vascular net organization in the rat bowel, in normal and cancer conditions. Tumor stimulates a chaotic generation of blood vessels. Dextran 150000D-FITC, intracardial administration, confocal microscopy, x20 (Vannucci, MBÚ AVČR v.v.i., Kubinova, FGÚ AVČR v.v.i.)
Nevertheless, the development of a vascular (blood and lymphatic) net inside the tumor can give also an advantage to the host the local organization of the immune response by permitting the diffusion of tumor antigens, and assisting the circulation and intra-tumor translocation of immune effector cells. Another activity of VEGF is the participation to the immune suppression produced by cancer cells by suppressing the maturation of DCs through a STAT3 dependent mechanism and cooperating with IL-6 and IL-10 to inhibit both innate and adaptive immunity (198, 199)

1.3.4. Anticancer immune response and tumor escape

1.3.4.1 Anticancer immune response: generalities.

An efficient anticancer immune response needs the perfect collaboration between innate and adaptive immunity. Natural killer cells, DC and CTL are fundamental for the elimination of the cancer cells. In synthesis, the cancer cells are firstly aggressed by innate immunity effectors (neutrophils, macrophage/monocytes, eosinophils, NK cells). Dendritic cells present in the tissue (and macrophages) phagocyte killed cancer cells, elaborate in small peptides the antigens obtained from those, and express on the superficial membrane MHC class I-peptide complexes for the CD8\(^+\) T cells and MHC class II-peptide complexes for the CD4\(^+\) T cells. Then, DCs migrate to lymph nodes, where they maturate and express co-receptors to permit recognition of the tumor peptides to the adaptive immunity T cells. Primed CD8\(^+\) cells undergo clonal expansion, produce IFN-\(\gamma\), specifically attack and kill the tumor cells (Th1 response), but also establish memory cells to specifically repress the new appearance of cells expressing the targeted antigen. CD4\(^+\) helper T cells are also engaged both in assisting the CTLs activity by cytokines (IL-2, IFN-\(\gamma\)), and primed B cells (IL-10). B cells then proliferate and partly maturate to produce antitumor specific antibodies, partly constitute memory cells. In this way, a complete anticancer response is established, capable to prevent recurrence of the targeted tumor. Innate immunity and adaptive immunity effectors are linked through the DCs. This very schematic description needs to be integrated with the activity of other subsets of immune cells, very crucial to orientate the immune response: the NKT cells and the Treg. Other types of cells with regulatory or cytotoxic activities have been progressively described in these few years (\(\gamma\delta\) T cells, Th17, NKDCs, immature myeloid cells), while functionally different subpopulations (based on the Th1/Th2 model) are also described, for example, inside the NK cells (N1,N2), and macrophages (M1, M2) (10, 24, 62, 200-203). All these cell subpopulations can play a double-edged role in the cancer network, paradoxically assisting the tumor escape. The immune response against cancer is mounted both outside and inside the tumor,
intended as the whole of cancer cells and the other components derived from the host to create the tumor microenvironment. Therefore, the intricate interplay between the various components of the tumor microenvironment can deeply condition both the cancer development and the host immune response. Consequently, the knowledge about the anticancer immunity and the studies to better understand its mechanisms and its failure (tumor escape) have been reframed inside the microenvironmental prospective.

1.3.4.2. Inflammation and cancer

Genetic mutations and phenotypic changes primarily affect the behavior of the cancer cells and their relations with the surrounding cells. Reframing the problem in microenvironmental prospective, we can suggest that the very early modifications induced by an initial neoplasm on the stroma and the cells of the host tissue elicit host responses more addressed to the maintenance of local homeostasis than primarily directed against the cancer cells, as transformed cells. The release of molecules like IL-1β, IFN-α, HSPs, and breakdown products of hyaluronan can trigger and attract innate immunity cells (macrophages, neutrophils) that, surrounding and infiltrating the tumor, establish an inflammatory reaction. Dendritic cells as well as NK cells and CTLs will be activated. We can say that the initial process is an acute inflammation (162, 204). If the acute inflammation is not sufficiently effective against the tumor, the increasing mass of tumor cells and the products released by damaged host and cancer tissues will maintain the process, making the inflammation to become chronic.

The concept of inflammation as a carcinogenetic factor was proposed by Virchow in 1863, and re-proposed by Balkwill and Mantovani in 2001 (205). Virchow’s observed that a “lymphoreticular infiltrate”, indicating presence of inflammation, was detectable in tumors, and suggested that a chronic inflammation may assist tumor development. The Authors commented under this prospective recent data about the role of proinflammatory cytokines and cellular infiltrates as effectors of chronic inflammation in the tumor microenvironment promoting tumor development. They also retrieved a definition of H.F. Dvorak (1986, ref. 206) about the continuous tissue remodeling in a cancer: tumors, wounds that do not heal. This definition is helpful to underline that, while the healing of a wound results in restitutio ad integrum due to a self-limiting process of tissue remodeling guided by inflammatory response, the tumor evolution result in progression due to unresponsiveness to physiological regulation mechanisms and altered inflammatory network. Historical data from epidemiology have indicated that 1) dysplasia and tumors can be the consequence of chronic inflammation produced by infective agents (e.g. Helicobacter pylori > gastritis> gastric cancer; hepatitis viruses B and C > cirrhosis >
hepatocarcinoma) and estimate that about 15% of the worldwide cancer incidence is secondary to infectious agents (207; Tab. 7)

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### Table 7 – Associations between chronic inflammatory conditions and cancer.


2) the use of non-steroidal anti-inflammatory (NSAID) drugs is beneficial in reducing the incidence of many tumors (e.g. colorectal cancer, Familial Adenomatous Polyposis -FAP) in prevention protocols and in pre-cancerous conditions (208). An accrual of new evidence in cellular and molecular cancer immunobiology is progressively going to support the role of inflammation on tumor development.

First, the NFκB, important transcription factor in inflammatory responses, was found to regulate also the expression of genes that encode important proteins for the control of stress response, maintenance of intercellular communications, and regulation of cellular replication and apoptosis. A colorectal cancer model in mouse (induction by the carcinogen azoxymethane –AOM, and promotion by chronic inflammation agent dextran sulfate sodium salt -DSS) was used. The classical activation pathway of NFκB was blocked by specific deletion of IκB kinase-β (IKKβ) either in the colonic epithelial cells or in the myeloid cells. In both cases the incidence of cancer was reduced, but with different
mechanism. In the KO colon epithelial cells, apoptosis increased under the inflammatory stimulation as a consequence of reduced expression of anti-apoptotic protein Bcl-xL controlled by NFκB. On the other side, the IKKβ KO myeloid cells were unable to induce proliferation of the colon epithelial cells following inflammation as the consequence of inhibited expression of NFκB-controlled proinflammatory genes, the products of which act as paracrine tumor growth factors (209, 210).

The importance as tumor promoters of pro-inflammatory molecules was shown in models of cancer metastases in the mouse (by injection of CT26 colon cancer cells or by spontaneously metastatic 4T1 breast cancer). The injection of LPS was increasing the metastases, effect that was inhibited by the block of NFκB. By the expression in the tumor cells of a dominant negative IκBα “super-repressor”, the LPS effect was converted in tumor regression. The LPS mediator of inflammation was the TNFα. By this way LPS was inducing TRAIL expression on tumor surrounding immune cells and the the TRAIL receptor DR5 on tumor cells. When NFκB was blocked, TRAIL mediated tumor regression by reducing proliferation and inducing apoptosis. This was opposite to the antiapoptotic effect exerted in the presence of NFκB. The date confirmed that inflammation produced by LPS stimulates tumor growth by tumor microenvironment production of TNF-α (211).

Thus, NFκB looks to be key element linking inflammatory cell activity and tumor progression, by anti-apoptotic gene transcription in cancer cells. Moreover, the LPS-assisted tumor growth appears an interesting mechanism in all conditions that can associate a pro-carcinogenetic environment to the activity of microbial agents (212, 213).

Second, effector cells of the anticancer immune response (and their products) demonstrated a double-faceted activity depending from their interplay with the progressively organized tumor microenvironment. They can either elicit antitumor responses or help the tumor development and its immune escape. An example is given by the macrophages. The M1 type can delete the cancer cells, produce IL-12 with activation of cytotoxic lymphocytes (induction of IFN-γ production) and Th1 CD4+ cells, however, in response to the microenvironment, they can also inhibit the cytotoxic cells by the accumulation of nitric oxide (NO), and assume a suppressive phenotype (M2) (203, 204).

Myeloid cells and macrophages release free radicals. Reactive oxygen intermediates – ROI (hydroxyl radical - OH•, superoxide -O2•) and reactive nitrogen intermediates – RNI (nitric oxide - NO•, and peroxynitrite - ONOO-) are, in normal conditions, an important defense against microbes. They are synthesized by host enzymes such as myeloperoxidase, NADPH oxidase, and nitric oxide, under the regulation of inflammatory signaling pathways. In the case of chronic inflammation, the activity of ROI and RNI can produce negative effects on the host cells by oxidative damage and nitration of DNA bases which increases the risk of
DNA mutations. In a tumor microenvironment, they can sustain genetic instability and further mutational events in the transformed cells, and also produce damage on the immune cells of the anticancer response (214).

Third, the peculiar microenvironment created by the cancer cells, interacting with all the other components of the hosting tissue, is very dynamic and self-maintaining, driving changes from an originally acute inflammatory response (with release of danger signals) to a chronically stimulated inflammation (the “non-healing wound” model) (162, 165, 205). In their review, Balkwill and Mantovani indicated also another problem: the local inflammation produced by solid tumors leads to the contemporarily activation of a systemic anti-inflammatory response (205). To better understand how it is possible, we can summarize the evolution of the interplay between tumor and immunity in three stages:

1) During the early tumor development, the transformed cells starts deregulated replications. They induce expression of stress molecules and local delivery of “danger signals” (mainly pro-inflammatory cytokines) in the involved tissue (162, 165). Consequently, at the local level, the activity of the innate immunity cells can be elicited. If the immune surveillance process successfully works, the NK cells can kill the transformed cells and the neutrophils, M1 macrophages and dendritic cell can phagocyte the killed cells. The NK cell cytotoxicity is triggered by down-regulated or altered expression of MHC molecules on transformed cells (lacking of self molecule recognition by inhibitory Ly49 receptors –mouse, or KIRs - humans), and by changes in glycosylation of superficial structures (sensed by lectin-like activation receptors) as well as expression of stress molecules (MICA/B in the human, Rae-1 in the rat, and H60 in the mouse) (see §§ 1.5, 2.2). The DC-NK cell cross-talk will permit the priming and maturation of DC, maturation that will be completed with the migration in lymph nodes. Here they will present tumor peptides on MHC molecules for triggering adaptive immunity cells: MHC class I – peptide complexes prime and activate specific CD8+ CTL response with IFN-γ and perforin production; MHC class II-peptide complexes prime and activate CD4+ T helper cells in a Th1 polarized mode (IL-2 and IFN-γ production) sustaining the cytotoxic cell activity. However, transformed cells can escape from elimination and cancer can develop (67, 215).

2) During the cancer establishment and its progression inside the originating tissue/organ, the cancer pressure on host tissue, stroma, and surrounding cells induces a permanent stimulation sustaining the local inflammatory reaction and delivery of immunologically active molecules (cytokines, chemo-attractants, antigens, etc.) within the intercellular matrix. Contemporarily, the tumor cells deliver in the microenvironment cytokines and growth factors that modulate the immunity (146, 148, 164, 216).
This phenomenon can interfere with the organization of an appropriate anticancer immune response, by transition from a locally acute response to a chronic inflammation, mainly involving the activity of macrophages (Fig.10).

**FIGURE 10 – Experimental rat adenocarcinoma of the colon: infiltration by inflammatory cells (macrophages and neutrophils) (Vannucci, Rossmann, MBÚ AVČR, v.v.i.)**

In this contest, macrophages assume a different polarization (M2) becoming important tumor supporters in the cancer microenvironment, collaborating to impair the cytotoxic cell activities by delivering high concentrations of oxidation molecules (nitric oxide, free oxygen radicals, peroxide) and the release of other molecules (IFN-α, TNF-α, IL-1β, TGF-β, IL-10, PGE2) helpful to cancer establishment (217-220).

When these conditions occur, the initial antitumor cytotoxic response (sustained by CD4+ Th1 cells) shifts to a tolerant-suppressive response for the intervention of CD4+ Th2 cells, and CD4+CD25+Foxp3+ T regulatory (Treg) lymphocytes, producing IL-10, IL-6, IL-4, TGF-β. By this way the cancer progression is assisted (155, 221-225).

3) Finally, during the acquisition of full malignancy characteristics (tissue invasion and metastatic spread), tumor cells, immune cells and stromal/endothelial cells actively release of Fas-L and deliver pro-inflammatory factors and immunosuppressive cytokines (e.g. IL-
IL-1β, IL-4, IL-6, IL-10, TNF-α, HIF-1, VEGF) in the cancer microenvironment. This leads to an alteration of the immunity that can extend to the systemic level. All this factors can also interfere with the regular metabolism of the organism (221, 226-228).

Recent reports demonstrated the existence of intrinsic cancer pathways that can contribute to inflammation and carcinogenesis. RalB GTPase is a member of the Ras GTPase superfamily and inhibits apoptosis in tumor cell lines. The effector of this function is Sec5 (involved also in exocytosis as part of the exocyst) that is associated by RalB to TBK1/NAK (TANK binding kinase-1/ NF-κB activating kinase), an atypical IKK that activates NFκB exerting antiapoptotic function. The TBK1/NAK complex is a core component of the innate immune response mediated by TLRs, and TLRs are found also on cancer cells. This antiapoptotic pathway appears to be present in cancer but not in healthy epithelial cells. In the innate immunity cells the TBK1 pathway leads to cytokine expression including IFN-β. This function is triggered in non-tumorigenic human epithelial cells after TLRs stimulation and is also assisted by RalB. Chemokine production (CXCL8 or IL-8) by cancer cells is induced by activated ras in HeLa cells. In melanoma the activation of the oncogene BRAF induce cytokine production (IL-1β, TNF-α, IFN-α) that contribute to create a microenvironment favorable to tumor progression (229, 230).

The signal transducer and activator of transcription 3 (STAT3) represents another link between cancer proliferation and survival and cancer influence on immune cells (inhibition). STAT3 results constitutively activated in cancer cells and this activation can be propagated to immune cells by tumor STAT3-regulated factors, which include VEGF and IL-10. These factors mediate a cross-talk between cancer and immune cells with immunosuppressive effect, sustaining maturation of CD4+ CD25+ Foxp3+ Treg lymphocytes and Th17 (IL-17-producing T helper cells) (199).

1.3.4.3. Tumor escape

According to the data above reported, we can identify two critical periods in which cancer can escape the immune system response: 1) in the very early phases, as a clone of transformed cells in replication; 2) after an immune response is triggered against a developed tumor.

The reasons of this escape, in part previously discussed, can be summarized as following:

1. immune ignorance;
2. alteration of MHC class I and tumor antigen expression;
3. deregulated expression of adhesion / accessory molecules by tumor and/or antigen-presenting cells;
4. secretion of immunosuppressive soluble factors either by tumor cells or infiltrating T lymphocytes, or both type of cells;
5. induction of immune no responsiveness via anergy induction or clonal deletion of responding T cells;
6. induction of suppressor cells;
7. changes in T-cell signal transduction molecules;
8. tumor utilization of products of stimulated leukocytes, i.e. immunostimulation of cancer;
9. immune editing as the result of adaptive cross-talk between microenvironment and cancer cells, with reciprocal modifications including the downregulation, under the immune response pressure, of the expression on cancer cells of antigens targeted by host immunity.

The points 1, 2 and 3 are more important in the early phases of tumor development, while the others need a growing cancer and activated immune responses.

The immune ignorance stems from the fact that mutated cells or their antigens cannot properly have contact with immune effector cells (e.g. anatomical barriers) or cannot trigger the effector cell for defects in the recognition complex (e.g. absence of co-receptors and/or their ligands). Because, initially, the transformed-cell/immune-cell interactions are induced by the delivery of danger signals, it was suggested that tumor cells can lack danger signal production (231). Other impediments to cancer recognition during initial tumorigenesis may be due to: anatomical barriers; the limited dimensions of the initial clone; only late migration of tumor cells to the secondary lymphatic organs - exclusive place for the activation of naïve cytotoxic T cells; limited amount of expressed antigens in early phases; conditions that limit the migration to and maturation in the lymph nodes of naïve T-cells or naïve DCs (232, 233). Immature dendritic cells can help the tumor to be tolerated by deficits in the MHC-peptide complex – TCR complex interaction in the absence of necessary co-receptors. In fact, immune tolerance occurs when antigen presentation is weak leading to immune recognition without immune activation. Immune tolerance appears more frequent in advanced tumor stages (234, 142). Reduced antigenicity of TAA is another important factor leading to immune ignorance/tolerance. Additional alterations in the efficient production of tumor processed proteins (e.g. secondary to impaired function of the proteasome system) and altered structure in MHC molecule complexes can reduce the sensitivity to MHC-I dependent cytotoxic T-cell responses (235-238).

NK cells can play important roles in front of impaired CTLs. The expression by tumor cells of stress molecules (e.g. MICA, MHC class I chain related molecules) can trigger activation receptors on NKs (NKG2D). However, shedding of soluble MICA molecule can prevent this activity by inducing internalization of the receptor and lacking of its superficial
expression (239-241). The alteration and/or down regulated expression of MHC class I molecules and complexes would be a condition permissive for the NK cells to be triggered and activate the cytotoxic pathways (215, 242).

The NK cells can be also directly inhibited in their activation by the tumor. Cancer cells can express ligands for inhibitory receptors or release immune suppressive factors. The prevalence of inhibitory signals can block the NK cell-mediated killing. Examples of these mechanisms of escape are: the delivery of TGF-β, Fas-L, the expression of HLA-G and CEACAM (243-246).

As cited in previous paragraphs, tumor cells can release a wide panel of immunologically active molecules (cytokines, chemokines, Fas-L) conditioning the immune response in the cancer microenvironment, and, in more advanced stages, also in the organism. For example tumor-derived Fas-ligand, binding to the Fas receptor on immune cells, promotes their apoptosis (247).

The hypoxia produced by development of the tumor mass asymmetric with the development of stromal and vascular structures, leads to glycolysis and acidic pH in the tumor microenvironment with changes in affinity for receptor-ligand interactions, and production of HIF-1 which stimulates expression of VEGF. As above described, VEGF can induce angiogenesis but also inhibit immune cell functions (248). Metalloproteinases help the mobilization and migration of the cells, and IL-1β collaborates to the progression of the tumor also inducing adhesion molecule expression that helps the metastatic seeding. These molecules are originated both by inflammatory and cancer cells (165, 249)

Other cytokines (especially IL-4, IL-6, IL-10, TGF-β) both of cancer and immune cell origin, permit the creation of a local environment favorable to shift CD4 helpers from a Th1 to a Th2 response, and attraction of Treg lymphocytes (CD4+CD25+) that can complete their maturation inside the cancer by expressing Foxp3+ (15, 16, 221, 250). Other cells, more recently identified, can further complicate the regulatory network developing in the co-presence of TGF-β and IL-6: the Th17 cells (17, 18).

The importance of these cells in cancer is demonstrated in various studies in which the depletion or block of Treg cells rescued the efficacy of immunotherapies (e.g. increased competence of dendritic cell-based tumor vaccines) (251). Interestingly, studies about the maturation of Treg cells showed that activated NK cells can prevent the CD28-mediated conversion of naïve Treg (CD4+CD25-) to activated (CD4+CD25+Foxp3+) cells. Brillard et al. (2007) showed a molecular interaction between IFN-γ and Foxp3 downstream of CD28 signaling. This receptor signaling appears to be required in the homeostasis of Treg cells in their TGF-β-dependent conversion from CD4+CD25- to CD4+CD25+ (252, 253).
1.4. GLYCODENDRIMERS

Carbohydrate moieties and oligosaccharides, especially when organized as multivalent non-linear molecules, have been showed to have affinity for rat NK activation receptor NKR-P1A (GalNAc>GlcNAc) and CD69. From these observations, studies were developed to create and evaluate artificial multivalent glycoderivatives, as mimics of putative physiological ligands for these receptors for anticancer immunotherapy interventions (104, 106, 107, 109, 110, 111, 130, 187).

A variety of neoglycoconjugates using dendritic and polymeric scaffolds to mimic the complex multivalent scaffolds found in biological systems were developed in the past decade. Many factors can influence the carbohydrate receptor-ligand (natural as well as synthetic multivalent ligand) interactions: architectural features of the structures, including rigidity, spacing, topology, and density of saccharides; environmental conditions such as local pH (e.g. in tumor microenvironment) (254, 255).

Dendrimers functionalized with a “glyco-coat”, i.e. glycogedrimers, have been designed to mimic the hyperbranched example structures found in natural glycoconjugates (such as in mucines, tumor aberrant carbohydrate and bacterial carbohydrate molecules) to assist in chemical glycobiology (256-258). It has been shown that multivalent neoglycoconjugates such as glycoclusters, glycopolymers and glycodendrimers, exhibit higher affinities for their receptors with increasing number of sugar residues, such as in the case of GlcNAc- and mannose-containing glycoclusters (257, 259-63, 110). The weak interaction between a receptor protein subunit with its specific carbohydrate makes necessary the clustering of the carbohydrate moieties (multivalency) to effectively interact with the receptor. On the other side, it was shown how multivalent carbohydrates can also cluster receptors originating lattice structures both in isolated receptor suspensions and on the cell surface. (108, 264)

Glycodendrimers are multiantennary molecules constructed by assembling of basic units (generation zero –G0) in progressively more branched scaffolds (G1, G2, etc.). They can be classified as: (i) carbohydrate-coated; (ii) carbohydrate-centered; and (iii) fully carbohydrate-based. The most important coupling reactions used for synthesizing glycogedrimers include: amide and thiourea formation; glycosylation; photo-addition to allyl ethers; and reductive amination (265). Glycomimetics with non-natural glycosidic
linkages (e.g. C-glycosidic, thiourea bridges etc.) are resistant to the action of hydrolases, which is crucial for their stability when applied in vivo (266).

By repeated passages, it is possible to tailor the degree of carbohydrate clustering as required by the targeted carbohydrate-receptor. To produce defined molecules allowing the incorporation or decoration of a predetermined number of carbohydrate moieties is an important feature of glycodendrimers. In fact, it permits to use chemically defined and constantly reproducible molecules the synthesis, purification and analysis of which can allow the availability of pure compounds for biological use. Glycodendrimers appear to have similar binding properties than the glycopolimers (another type of synthetic multivalent glycoderivatives), but offer the advantage of a higher possibility to adapt their characteristics (flexibility, hydrophobicity, etc.) to the biological targets.

Although glycodendrimers may be branched into multiple-generation structures, the molecules bearing four to 16 terminal carbohydrate substitutions have been proved to be efficient ligands in most lectin systems (110, 267).

Different kinds of multivalent dendritic molecules were evaluated as synthetic ligands for the rat lectin-like receptor NKR-P1A. The GlcNAc-coated octavalent glycodendrimers (PAMAM-GlcNAc₈, GN8) displayed a very high affinity for this receptor, and they were chosen for our experiments of anticancer immunomodulation (106, 108, 110).

The GlcNAc was chosen as reference molecule in consideration of: its good affinity for NKR-P1A receptor when tested as an oligosaccharide (107, 111); its presence in many structures of the organism (e.g. extracellular matrix and stromal structures, important in the cancer microenvironment); its crucial role for protein and lipid glycosylation; its presence and role in the aberrant glycosylation products in cancers; its commercially easy availability; and its low cost. Moreover, the binding groove of NKR-P1A receptor possesses four carbohydrate binding sites, as identified by direct binding experiments: 2-Acetamido-2-deoxy-d-glucose (β-N-acetyl-d-glucosamine, GlcNAc) had one strong, one intermediate, and two weak binding sites. Different single high-affinity binding site were found for 2-acetamido-2-deoxy-d-galactopyranose (GalNAc) and 2-acetamido-2-deoxy-d-mannopyranose (ManNAc) (268).

GN8 dendrimers are based on a first-generation (8 branches) polyamidoamine (Starburst™, Sigma-Aldrich, Seattle, USA) scaffold with GlcNAc terminal substitution. GN8 is a symmetric molecule that presents four branches per side, each one terminating with a GlcNAc molecule (Fig. 11A). The high affinity of GN8 for NKR-P1A was demonstrated by the formation of a lattice when added to suspensions of the recombinant receptor (108).
Therefore, this octadendrimer presented the characteristics for allowing binding inside the receptor groove through the four molecules of one extremity, and, possibly, linking to another receptor on the same or other cell with the other extremity.

Finally, to follow the fate of glycodendrimers both in vivo and at the cellular level, G0.5 PAMAM glycodendrons (four branches and a linking tail) coated GlcNAc moieties (GN4), were synthesized to be labeled with either fluorescein or rhodamine (Fig. 11B). Because the GN8 cannot allow the linkage to a fluorescent dye, the choice was to use the splitted form of the molecule (tetradendron) in consideration that each tetrameric side can be considered as a functional unit and the presence of the linking tail. After preliminary tests with GN4 that demonstrated biological effects similar to GN8 (in ex-vivo and in vitro experiments), though less intense, this molecule was chosen for binding to the fluorescent dye (see Part 2: 2.3). Tests showed that the labeled GN4 maintained the biological activity of the original molecule (Fig. 12).

FIGURE 11 – Polyamidoamine (PAMAM)-based glycodendrimers coated with N-acetyl-D-glucosamine (GlcNAc). A) Octadendrimer (Generation 1, GN8) (Th. Lindhorst); B)
tetradendrimer (Generation 0.5, GN4) linked with a molecule of fluorescein (GN4-FITC) or rhodamine (GN4-rho) – see Part 2: 2.2 and 2.3.

FIGURE 12 – CD69 (early activation marker) in splenocytes after in vivo administration of GN8 and labeled-GN4 dendrimers in melanoma-bearing mice. Sections of spleen from treated and untreated mice were stained with biotinylated anti-CD69 moAb, then developed in 3,3 diaminobenzidine-H2O2 (DAB), and counterstained with Harry’s haematoxylin. Negative control was obtained substituting the primary antibody with HSA alone. Cells were considered positive when DAB color designed their contour. The number of CD69 positive cell was obtained observing at x20 magnification 100 fields (10x10) from each slide, by a grid. Each field was 360 $\mu m^2$. GN4 dendrimers demonstrated activity like the GN8 dendrimers, but at a lower extent. The fluorescence of GN4 localized in the sites of higher CD69 activation (see Part 2: 2.2)
FIGURE 13 – Large granular cell uptakes GN4-rhodamine dendrimers. The other lymphocytes are negative (rat mononuclear cells, fluorescence microscopy, x40) (Vannucci, MBU AVCR, v.v.i.).

FIGURE 14 – GN4-FITC binding to NK cells. A) GN4-rhodamine uptake by granular CD3⁺ cell. B) GN4-rhodamine uptake by mouse freshly sorted NK cells (C57BL/6, CD3⁺ NK1.1⁺); C) GN4-rhodamine does not bind to NK1.1⁻ cells (CD3⁺ NK1.1⁻). D) GN4-FITC on the surface of a rat NKR-P1⁺ lymphocytes and is internalized in vesicles of regular dimensions. Fluorescence and contrast phase microscopy, various magnifications. (Vannucci, MBU AVCR, v.v.i.)

GN8 dendrimers, tested both in vitro and ex vivo on separated mononuclear cells, freshly sorted NK and NKT cells, were able to induce immune cell activation (expression of CD69 and production of IFN-γ) after they were internalized in the NKR-P1⁺ cells (see Part 2: 2.2, 2.4, 2.6). The labeled dendrimers resulted to transitory coat the cell surface of NKR-P1⁺ CD3⁻ (NK) cells and then be uptaken inside clatrin positive granules. Cell sorting of NK cells versus T lymphocytes demonstrated the targeting of NK cells (Fig. 13 and 14). Fluorescence and confocal microscopy confirmed the internalization of the labeled GN4 together with NKR-P1 receptor in NK cells (colocalization of the two fluorescences both on the surface and inside of sorted NK cells) (Fig. 15).
FIGURE 15 – Internalization of GN4-labeled dendrimers together with the NKR-P1 receptor (Vannucci L, unpublished data). A-C: confocal imaging of a section of freshly sorted mouse NK cell (from the spleen of an healthy animal). The sorted cells were mild fixed in 2% paraformaldehyde to demonstrate only superficial interactions. Then, they were co-incubated 30 min with GN4-rhodamine and anti-NK1.1 (PK136) monoclonal antibody, washed and observed. In A the FITC fluorescence of anti-NK1.1 Ab is shown (green); in B the fluorescence of GN4 rhodamine-labeled (red); in C is visible good colocalization of the two fluorescences (yellow). D: sequence of GN4-rhodamine dendrimer uptake in rat large granular lymphocytes at different time. The process is quite quick and leads to granule polarization in 15 minutes. Overlapping of contrast phase and fluorescence microscopy imaging, x40. E: mouse sorted NK cell in which is demonstrated the independence of DX5 (CD49b, pan-NK marker) from GN4-rhodamine internalization; anti-DX5-FITC MoAb binds to the cell surface, while GN4-rhodamine is internalized. Confocal microscopy, x40. F-G: a freshly sorted human NK cell after 20 min incubation with GN4-rhodamine, 15 min. fixation with paraformaldehyde 3.7%, permeabilization with Triton-X 0.1% for 5 min., and staining with anti-CD161-FITC MoAb. Colocalization (yellow) is present in the polarized granules, and it is possible to observe also few granules positive for a single fluorescence, confirming the effective colocalization in the others. F: constrast phase imaging; G: fluorescence microscopy, x40. H-I: confocal microscopy demonstration of extensive colocalization (yellow) of anti-NK1.1-FITC (green) and GN4-rhodamine (red) fluorescences in mouse sorted NK (incubation, fixation, permeabilization and staining as above described). H: NK cell section; I: 3-D deconvolution using all layers of the z-series; x40 magnification.

The administration of GN8 in cancer-bearing animals influenced the evolution of tumors slowing the growth of B16F10 melanoma in C57BL/6 mice and reducing the incidence of chemically induced colorectal carcinoma in rats (see Part 2: 2.1 and 2.2).
These results were presented as lectures or posters at international congresses (Part 4).

1.5. BASES OF EXPERIMENTAL CANCER MODELING

1.5.1. Generalities

To study cancer biology and immunity the in vivo studies are fundamental described processes in the fully biological contest in which they naturally develop: the complexity of the organism. Consequently, we need availability of adequate working models very closely reproducing the investigated human pathologies and immunologic conditions. The use of in vitro tests allows studying the molecular biology of the genetic and epigenetic changes of cancer cell, their responsiveness to treatments, and specific functions of the anticancer immune cell activity. Multiple systems that co-cultivate more than one type of cell together can be also prepared (e.g. cancer cells and cytotoxic cells). However, the results obtained in controlled laboratory conditions, necessarily artificial, need to be finally tested in vivo studies, as a definitive prove of the biological importance of the bench observations (in vivo veritas). By this way, the results can be more properly assessed, permitting development of translational applications for human patients (269-271).

Cancer models developed in experimental animals can be produced either by chemical (carcinogens), physical (radiations, microtraumas) or biological (toxins, parasites, bacteria, viruses) agents, with or without the utilization of promoters (substances or conditions amplifying the damage produced at the genetic level by the carcinogens). Another way to produce cancers is the creation of genetically mutated animals in which putative genes, involved in initiation and/or promotion of cancer, can be either introduced or knocked-out by genetic manipulations. Finally, animal strains that spontaneously result bearing particular genetic characteristics - heritable cancer traits - can be also used. Whatever of the models is chosen, it must try to reproduce as closer as possible the original human illness in its pathological-physiological events and pathologic features. Therefore, the choice of the adequate model is mandatory before to decide any kind of experimental investigation.

Cancer is a pathologic condition that, according to the recent scientific developments, needs in vivo studies. In fact, according to the tumor microenvironment model, it is necessary to perform investigation in conditions resembling the very tight functional interdependence
between all the elements that compose the cancer. Such a complexity is demonstrated by the recent studies on cancer immunity in which appear how the behavior of immune cells and cancer cells can be deeply modified by: the complexity of receptor-ligand interactions; interactions between cells and host stroma; and paracrine delivered products. The co-presence in certain proportions of various interleukins and growth factors inside a tumor microenvironment depends from the functional moment of cancer evolution in the specific host conditions, with important biologic effects (e.g. shifting cells from aggressive toward suppressive tolerant behavior as in the case of CD4+Th cell differentiation) that is difficult to fully reproduce in the *in vitro* systems. From this, the continuous increase of investigational technologies and methods looking to follow biological activities *in vivo* (e.g. confocal microscopy, proton-emission tomography - PET, spectrometry, green-fluorescence protein- GFP, etc.).

1.5.2. Development of carcinogenesis studies and chemical carcinogenesis modeling.

The induction of cancer by chemical carcinogenesis, eventually associated to promotion with agents considered pushing the transformation process by different mechanisms (e.g. fat-enriched diet or sodium dextran sulphate for colorectal cancers) is one of the oldest and most common methods for modeling cancers.

When we look to the clinical data about cancer evolution, they are referred to patients with a yet established illness and its progressive involvement of the organism leading to the death of the host. Progresses in epidemiological studies and in medical technologies have permitted: 1) the identification of risk factors in the affected population; 2) the definition of groups with enhanced risk (both on genetic and environmental bases); and 3) the description in various organs/tissues of lesions that represent favorable sites for cancer transformation (pre-neoplastic lesions, e.g. Barrett’s esophagus, leukoplakias in the mouth mucosa, aberrant glands in the colon mucosa, etc.). It is in the common knowledge of surgeons and practitioners that a tissue undergoing repetitive chemical and/or physical insults for long periods can develop alterations progressively changing both its structure (e.g. hyperplasia, vascular net, connective stroma) and functions (level of cell maturation, rhythm of cell turn-over, appearance of signs of inflammation). Under these conditions a cancer can arise. This knowledge belongs from practical observations since ancient times, but it started to find more precise and organic definition only between the end of the XVIII and the beginning of XIX centuries. Sir Percival Pott observations created the bases of the chemical carcinogenesis and importance of environmental/occupational conditions. He noticed in a memory published on 1808 in London, that the cymney-sweeps had higher
incidence of skin cancers than the general population, and the most frequent development of the cancers was localized on the scrotum (272). He wrote that:

"The disease, in these people, seems to derive its origin from a lodgment of soot in the rugae of the scrotum, and at first not to be a disease of the habit...here the subjects are young, in general in good health (at least at first), the disease brought of them by their occupation, and in all probability local; which last circumstance may, I think, be fairly presumed from its always seizing the same part..."

In these observations are contained all the hallmarks for chemical carcinogenesis:

1- an irritating agent (soot, the carcinogen)
2- the necessity of a continuously repeated application
3- the permanence in the place with capability to be adsorbed and cumulated
4- the localized arising of the cancer in the place of carcinogen application
5- the cancer as the result of a chronic irritation (inflammation)

Rudolf Virchow on 1863 (273), instead, observed that inflammatory infiltrates were present in many cancers, also when not primarily obtained from inflammatory sites, and he suggested for the first time a possible direct correlation between inflammation and cancer. This suggestion was in accordance with observation reported all medical manuals of pathology and surgery, linking chronic inflammation and alterations of the tissue repair (e.g. the higher risk for developing cancer in lesions due to basic caustics (caustic soda), characterized by retarded and complicated healing of the ulcerations; induction of tumors by long lasting parasitosis in the bladder - Chlonorchis sinensis, Schistosoma haematobium, or in the liver - Opistorchis viverrini).

Cancers were supposed to be caused either by “degenerative factors” (senescence) or chemo-physical causes. After P. Pott observations, aromatic compounds (hydrocarbons) were widely used for inducing cancer either by repeated applications on the skin of experimental animals (e.g. rabbit’s ear), or by singular/multiple dose treatments following various ways of administration: subcutaneous, intramuscular, intragastric, intraperitoneal, intracardial, intravenous, intrarectal. The cancers induced in such a way were found to develop in not less than 30 days, and in an average time of about 100 days (274).

In the 1911 Rous published the first demonstration, in chicken, of tumor development in a healthy animal by inoculation of filtered cancer material obtained from an affected animal. The tumor was developing in the place of inoculation. This experiment revealed the
existence of tumorigenic filterable infectious agents (viruses) and transmissibility of the illness following the Pasteur’s experimental rules (275).

In the 1940s-50s, C.B. Huggins gave the experimental demonstration of the hormonal dependence of the breast cancer and the possibility of its control by manipulation of the hormonal status in a pregnancy/menopausal-like mode. The initial idea derived during writing his M.D. thesis by reading a surgical report published on Lancet by Dr. G.T. Beatson on 1886 (276). The surgeon was describing two patients that after an operation for bilateral ovariectomy presented regression of synchronous inoperable mammary cancer. Huggins’s discovery opened the avenue to investigate the tumor sensitivity to hormones and other biologically active products of the host, the expression of functional receptors on cancer cells, and the possibility of biological control of tumor growth (277-279). He also created the first in vivo animal model of breast cancer by gavage with DMBA (di-amino-benzyl-antracene).

The idea that chromatin (DNA) anomalies could have a role in cancer cell development stemmed from 1929, when Boveri T. in his book on cancer stated:

“The essence of my theory is not the abnormal mitoses but a certain abnormal-chromatin complex, no matter how it arises. Every process which brings about this chromatin condition would lead to a malignant tumor” (280).

Anomalies at the chromosomal level studying tumor karyotypes were found associated with substances used for chemical induction of cancer, as well as associated to spontaneous tumors, especially in studies on leukemia cells (281-283).

From the discovery of the DNA structure (in the 1950s) to date, extraordinary progresses have been done in understanding the causes (radiations, chemicals, biological agents) and mechanisms that determine genetic damage and reparation. This had recently leaded to the discovery and description of molecular pathways regulating gene transcription common to both cancer cells and immune cells (STATs, NFκB, etc.), re-presenting under a new prospective centennial observations on cancer, surprisingly still valid in their fundamentals.

Finally, the discovery of the association of Helicobacter pylori to chronic gastritis, gastric and duodenal ulcer, gastric cancer (lymphomas and adenocarcinomas) reactivated the interest and the researches on the microbial agents and their possible role in the cancer process (284).
More recently, the evidence that chronic inflammation and activity of proinflammatory factors can have part in the carcinogenetic process have posed the problem of bacterial activity in procarcinogenic conditions (inflammatory bowel diseases - IBDs) and in the sporadic colorectal carcinoma development (212, 285).

All mucosas are site of persistent microbial colonization, with the partial exception of the gastric mucosa due to the very low pH of the stomach secretion. The bowel, especially the colon, is place were the microbiota are more represented, with predominance of obligate anaerobes (>90%; Bacterioides, Eubacterium, Bifidobacterium, etc). Escherichia coli and Lactobacillus are also present but in more limited proportion. Many studies have shown that the balance between the bacterial populations can influence the carcinogenic processes, inducing either its promotion (Clostridia) or inhibition (Lactobacillus) (286, 287). The eutrophism of certain bacterial populations follows the alimentary attitudes of the host, and the resulting metabolic products of the microbiota can act as tumor promoting (nitrosamine, polyamines, etc) or inhibiting (butyrate, etc) factors (288).

The possibility to produce germ-free reared animals and gnotobiologic animals (selectively contaminated with known agents) offers important models for studying the immunological and biological effects of bacteria in the gut, and bacteria-independent immune responses (289, 290). The continuous immunological solicitations exerted by the microbiota on the gut associated lymphoid tissue - GALT (the complex of lymphocytes present within and below the mucosal layer of enterocytes, either sparse or organized in submucosal follicles and patches) stimulate the persistent activation of the mucosal immunity. This phenomenon is also described as “physiological inflammation” and permits to control the risk of intramucosal translocation of the bacteria as well as to contribute to the tolerance toward the multitude of antigenic molecules that derive from the microbial membranes, the digested aliments, and the related bacterial metabolic products. This activated and tolerant immune environment may affect the level of responses in case of carcinogenic events (see Part 2, 2.7) (291-296).

The most used in vivo models of colorectal cancer are

1- chemical induction
2- hetero- or orthotopic (subcutaneous or in the colon wall) injection of cancer cells from cultured cell lines in syngenic or immunodeficient mice (nude atymic - nu/nu, SCID mice)
3- Implantation - either heterotopic or orthotopic – in immunodeficient mice of freshly harvested allo- or xenogeneic-tumor fragments, obtained from surgical specimens (including human tumors)

4- Genetically engineered (transgenic, KO) animals (e.g. Min Apc+/−)

The chemical induction is classically performed in rats and mice with the administration (IP, IM, SC, and IR) of carcinogens able to be metabolized with a good selectivity in the bowel mucosa. The most commonly utilized, both in rats and mice, are DNA alkylating derivatives of cycasin: methylazoxymethanol – MAM, dimethylhydrazine- DMH, and azoxymethane – AOM (Fig.16).

![Figure 16 - Azoxymethane](image)

Other used carcinogens include: aromatic amines (e.g. 3,2’-dimethyl-4-aminobiphenyl) applied in rat models; direct-acting carcinogens such as methylnitrosourea, N-methyl-N’-nitro-N-nitrosoguanidine; heterocyclic amines (e.g. 2-amino-3-methylimidazo[4,5-f]quinolone, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine). Notably, the types of lesions induced by these agents in the colon of the experimental animals resemble the types of lesions observed in human colon.

Azoxymethane-induced cancers in the rat present the advantage to develop a large number of genetic alterations observable also in human colorectal cancers (mutations in the codon 12 of K- and H-ras, increased expression of ras proto-oncogene family, enhanced COX-2 and iNOS, mutation in APC gene, β-catenin gene) (297, 298). Additionally, in tumors developed by mice treated with AOM, abnormalities in transforming growth factor beta (TGF-β) signaling were detected. Treatment with AOM activates intrinsic tyrosine kinase of EGF receptor while stimulating the synthesis of TGF-α (299-301).

The efficacy of these carcinogens is high, inducing tumorigenesis in the large bowel and, sometimes, in the small bowel and in the ear glands. The colon mucosa produces aberrant glands, adenomas, adenocarcinomas, not necessarily in a succession. The carcinogenetic effect can be increased by the use of a promoting agent. In some studies devoted to the evaluation of possible preventive strategies, DMH or AOM were associated to diet with
high fat content, biliary-pancreatic ileal diversion, cholic acids supplementation, and recently to dextran sulfate administration (inflammatory colon model) (302-311). In my model, based on AOM and biliary acids supplementation, the bile is directly injected inside the colon by puncture of a surgically created subcutaneous cecal hernia (Fig. 14). This permits to administrate the promoter in perfectly reproducible quantity, and only inside the target organ (large bowel). This model, firstly published on 1994 (312), have been re-evaluated, also in the immunological aspects, in these years and the results were published on 2004 (see Part 2- 2.5).

FIGURE 14 – The cecal hernia in the rat model of colorectal carcinogenesis. The bulky of the hernia is well visible and permits an easy access inside the cecum to directly introduce in the colon precise doses of a substance.

Transgenic and KO animals were generated trying to reproduce the genetic traits of intestinal tumor syndromes:

FAP (familial adenomatous polyps) model was developed in mice with APC gene mutation [Multiple intestinal neoplasias (Min) Apc<sup>−/−</sup> mouse models]. These animals spontaneously produce polyps, especially in the small bowel, but rarely in the colon. To increase the cancer onset in the colon is necessary the DMH or AOM administration (313).

HNPCC (Hereditary non-polyposis coli) was obtained in mice by inactivation of DNA mismatch repair genes. The Mlh1<sup>+/−</sup> mice presented 33% incidence of gastrointestinal tumors. An additional APC gene mutation into the Mlh<sup>−/−</sup> mice produced a 40-fold increase in the number of GI tumors, leading to 100% GI tumor incidence. However the efficacy on colon carcinogenesis remains low. The use of mutagens can enhance the carcinogenesis in these animals but reduce the target selectivity (induction also of extra-colic tumors) (314, 315).
Other genetic mutants were also produced in mice to evaluate the role of other possible genes involved in colorectal cancer onset (e.g. Smad3, p53, Gpx1/Gpx2). In some, it was evaluated the influence of the intestinal microbiota in conventional mice versus germ-free reared mice. For example, in a model of mice with disrupted Gpx1 and Gpx2 genes (glutathione peroxidases) conventional mice developed cancer while the germ-free did not, indicating the importance of redox stress produced by the intestinal flora on colon carcinogenesis. (316)

The main criticisms to these models is that the induced mutations are not always resembling the human equivalents, as demonstrated by the different sites of tumor development, and in some cases the generalized mutation may affect other biological pathways in the organism creating conditions not closely corresponding to the human pathology (317, 318).

The use of inoculation of cancer cells derived from established cell line either syngeneic with the recipient mouse strain or allo-/ xenogeneic (mouse/ human origin) can help to rapidly obtain tumor masses. Their quick availability permits in short time to achieve data about effects of treatments or biological stimulations. However, many steps of the carcinogenetic process are not represented in this model, making it not suitable for studying the early carcinogenetic events. The rapid development of these cancers allows only a partial constitution of the stromal component of the tumor, differently from the spontaneously arising tumors.

Other characteristics are present in tumor fragment transplantation, because the implanted fragments are composed by the all tumor components. However, the subcutaneous implantation offers a different environment than the original one, and can predispose to possible variations of the responsiveness of the cancer out of its original conditions.

Finally, the use of immunodeficient animals allows the in vivo growth of allo- or xenotransplants of cancer tissues, but contemporarily limits the immunological interventions or observations.

For investigating the transformation and early event of cancer development, the chemically induced and the genetically manipulated models represent the choice (319, 320; Tab. 8).
In the presented studies, I used two principal models:

1- chemically induced colorectal carcinogenesis in the rat, following my model of initiation with AOM and promotion with bile (312). In this model, the tumors develop between 3 and 6 months from the beginning of the experiment. At the third month, the presence of early lesions permits evaluations and interventions on early stages of tumor development. Between the 6th and 8th month, 80-100% of animals have tumors and are suitable for established tumor evaluations and treatments. The inducing dosages of carcinogen are limited (9 mg/kg/dose) repeated 5 times/once a week, with difference from the largest part of the models using between 9 and 12 mg/kg for many weeks. Bile rich in secondary biliary salts was used as a physiological promoter. In my first experiment I used bile of human origin from drainages of non-septic non-neoplastic patients. Then, it was substituted with porcine bile (Sigma) for better standardization of the procedures. Directly introduced into the bowel by percutaneous puncture of a surgically produced cecal hernia, the bile stimulates the colon mucosa, and the colon peristalsis makes the promoter to physiologically progress and be elaborated along the complete colon-rectum.

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TABLE 8 – Tumor modeling in the mouse

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2- inoculation of B16F10 melanoma cells in syngeneic C57BL/6 (B6) mice. One million cells subcutaneously inoculated on the lower back of the mouse produce a palpable mass in about 10 days, with rapid growth.

The two models are different and each one corresponded to appropriate kinds of evaluations:

**In the rat:** carcinogenesis, importance of intestinal microflora, and capability of glycoderivatives to influence at different time the cancer evolution by NKR-P1 expressing cell modulation;

**In the mouse:** response of established tumors to immunotherapy by glycoderivatives; modulation and rescue of the immune response in established tumors

Moreover, the different immunogenicity and histological type of the two cancers, and the different species of the experimental animals permitted:

- to compare and better prove the efficacy of the glycoderivatives treatment;

- to test and compare in the two animal species the possibility of activating immune responses by NKR-P1 artificial ligands.
PART 3

DISCUSSION AND CONCLUSIONS
3.1. - DISCUSSION

The collection of studies presented in this dissertation is centered on the tumor immunology and immunotherapy.

A central problem in cancer immunology is to understand the causes of tumor immune escape and, consequently, to develop strategies to rescue an effective immune response with therapeutic effects. The many approaches and attempts to solve these questions have variously pointed out on the singular elements of the cancer-immunity interplay, but only recently a general view has been assembled. The introduction of the tumor microenvironment concept and the re-appraisal of the importance of inflammation both in anti-cancer immune response and in cancer development and immune escape have permitted the development of a more general and functional working model.

In the studies that we performed during these years, we started from cancer modeling (induced colorectal cancer in the rat) and evaluation of the carbohydrate modulation of NK cells through lectin-like receptors (particularly NKR-P1) in both in the colorectal cancer model and in experimental melanoma in mouse. The use of two different experimental cancers in different species permitted to evaluate the general effectiveness of multivalent carbohydrate-based molecules for inducing immune responses through the NKR-P1 receptor(s).

Then, we integrated our observations with the study of the tumor immune microenvironment modulation (in the melanoma model) and the effects of local (intestinal) environment on colorectal cancer. They served to confirm the role of innate immunity cells (particularly NK/NKT cells) in collaborating to anticancer responses and anticancer immunity rescue, and leaded to the hypothesis of a new therapeutic approach.

As described in the Part 1, and discussed in the collected papers (Part 2), we can summarize our comments and conclusions as following:

a) - We can assume the cancer development as a process during which the immune system is firstly activated only at the local level. Only in a second moment the systemic immunity is alerted and in such a moment is absolutely critical what kind of immune environment was locally established (immune microenvironment) (148, 149, 205, 226, 231). If a correct response was developed, the Th1 environment permit a regular activation and function of DCs, CD8+ and CD4+ cells with tumor elimination and antitumor immunization. Both NK and NKT cells collaborate in establishing the Th1 environment. Following the model of tumor immunoediting, if the elimination is impeded, the balancing of tumor growth with the tumor death induced by the immune effectors (equilibrium), the chronic immune
activation (immune pressure) can induce further genomic instability in the cancer cells with phenotypic in the tumor and elicitation of systemic immunity regulatory responses, with Th1 to Th2 shift. These phenomena permit the tumor escape and its further development (151). Such a model more clearly delineates microenvironmental events and systemic responses, assisting a more focused research to prove the singular phases, but also to address targeting for possible interventions. In our animal models of cancer we confirmed observation reported in human oncology about the importance of cytotoxic cells (NK cells, CTLs), regulatory cells (NKT and γδ TCR cells) and Th1 cytokines in controlling tumor development. The cancer progression was associated to the down-regulation of these components of the anticancer response (Part 2: 2.5, 2.7)

b) - Our investigations on experimental anticancer immunotherapy were addressed to evaluate the use of multivalent glycoconjugates (PAMAM-GlcNAc8, GN8) as mimics of putative carbohydrate ligands for rat NKR-P1A activation receptor (Part 2: 2.1-2.4). As discussed in the Part 1 (2.3, 2.4.2) NKR-P1 activation receptors are constitutively present on NK and NKT cells, γδ TCR cells, but also expressed by other cells when activated (effector/memory CD4+ and CD8+, monocytes, DC, myeloid lineage cells). The GN8 resulted able to stimulate NK and NKT cells in the rat and in the mouse (increase of CD69 expression and CD69+ cells, effector cell infiltration of tumor since 24 hours after GN8 treatment, Th1 cytokine production and increased cytotoxic response). The NK1.1 receptor in the C57BL/6 mouse strain corresponds to the NKR-P1C activation molecule and the in vivo administration of GN8 to the melanoma bearing C57BL/6 mice, but not (or at a very low extent) in Balb/c mice, which are negative for NK1.1 molecule as identified by the MoAb PK136. In fact, they express a different transcript (change of one amino acid) for NKR-P1C not recognized by the antibody (97). In the rats, NKRP-1C is an inhibitory receptor, like the human transcript NKR-P1A (CD 161). In both mice and rats NKRP-1B is an inhibitory receptor. In the rat, the analysis of NKRP-1C showed this molecule to be ~ 70 and 85% identical with the respective NKR-P1A and NKR-P1B receptors at the amino acid level. In the lectin-like domain, NKR-P1C and NKR-P1A or NKR-P1B are 82 and 78% identical, respectively. Identity resulted much lower for NKR-P1D (inhibitory?) receptor being only 48% (for the lectin-like domain 45%). The NKR-P1B and D (now indicated as B*) receptors resulted recognize Clr-b (C-type lectin related protein b), but differ in expressing an ITIM motif (present in B but not in D). NKR-P1D resulted similar to the mouse NKR-P1F, an activating receptor associates with Clr-g. In the mouse, B and D are quite identical, both presenting an ITIM motif. Although mice have multiple Clr family genes, only one ortholog,
**CLEC2D** (also named lectin-like transcript-1, LLT1), exists in humans, and LLT1 blocks osteoclast differentiation like mouse Clr-b, (103, 321). Both mouse and human Clec2 gene transcripts are type II proteins of the C-type lectin superfamily and are products encoded inside the NKC genes. The Clr-b molecules are ligands displayed at high levels on nearly all hematopoietic cells (but not on erythrocytes), in a pattern that is similar to that of class I MHC molecules. Interestingly, Clr-b has frequently down-regulated expression on mouse tumor cell lines, indicating a role for this receptor-ligand system in a new form of "missing self-recognition" of tumor cells by NK and NKR-P1\(^+\) cells. (113, 114, 103, 322, 323)

All ligands until now described are proteins. However, the existence of carbohydrate molecules as physiological ligands NKR-P1 receptors is consistent with their lectin-like nature, and needs to be more completely investigated.

As reported in the Part 1, the studies of Bezouska and Pospisil showed the capability of rat recombinant NKR-P1A to bind various oligosaccharides with different affinity, eliciting activation of the immune cells, leading to the elaboration of the multivalent carbohydrate-coated molecules (GN8, glycodendrimers) with in vitro high affinity for the receptor (104, 106-111).

The importance of carbohydrates as ligands triggering immune cells was confirmed by the results of GN8 administration in biological systems (tumors) as collected in the Part 2, with activation of anticancer immune responses and effects on tumor development (reduced incidence of cancers in the rat model, reduction of the tumor growth of mouse melanomas). A recent report by Christiansen D. et al. demonstrated that also the human NKR-P1A is capable to recognize carbohydrates (αGal epitope and NAcLac) (112).

These observations indicate that NKR-P1 receptors can have both proteins and carbohydrates as ligands, enlarging and refining their capability of immune recognition. Our results indicate that high affinity carbohydrate ligands can trigger immune responses with valuable biological effects also *in vivo* conditions.

Additionally, recent experiments were going to demonstrate an unexpected activity of GN8 on the cancer cells inducing changes in their glycolyslated structures on cell surface, as seen in fluorescence microscopy after staining the cells with fluorescent
dye-labeled lectins. Changes in the pattern of GlcNAc and Man expression on cell membrane were observed in vitro after prolonged incubation (≥1 week) of various types of cancer cells in cultures containing GN8. Conditioned melanoma B16F10 cells were expressing on the cell membrane more GlcNAc than Man, modifying their phenotype (unpublished data, discussed in congress presentations – Part 4, references 15, 16, 18, 20). Under this view, we can suppose the glycodendrimers to play a double role: by triggering NK/NKT cell activities through NKR-P1 receptor, and, indirectly, by modification of the glyco-phenotype (and, consequently, immunogenicity and behavior) of the tumor cells. Such an effect might be suitable for assisting the immune rescue in the impaired cancer microenvironment and is currently under study in our laboratory.

c) – Modeling tumors in vivo, as discussed in the Part 1, presents the fundamental problem of reproducing a multifactorial event re-creating the most similar biological conditions of the human disease. Every model that we produce in laboratory (from cellular to organism level) is an artifact affected by the experimental conditions and by the use of species other than the human one. Thanking the progresses in genetics, the knowledge about the genome constitution can assist a progressively better choice of the homologous model in the animals to reduce the possible gaps between the laboratory results and the human biology reality. However, working with mammalian, some fundamental molecules, structures, cell types and functions result evolutionary preserved and shared between the various species. This allows a sufficient level of comparability and generalization of the observed biological processes, that anyway need final prove in the human conditions. These philosophical considerations are necessary for ensuring a critical evaluation of our results (both positive and negative) to better address our researches and identify common patterns in the living organisms. Under this view, if the use of engineered animals can help for discovering the importance of genes functions at various levels in an organism, they risk to create an artificial reality producing a possibly wider and diffuse range of alteration in the organism biology than in nature. The generalized genetic misbalance is different condition from the mosaic nature of our tissues suitable to a more organ-limited elicitation of genetic instability by environmental carcinogens. An example is given by the tumor
production, different than expected, seen in Min Apc<sup>+/−</sup> and Mlh1<sup>−/−</sup> APC-mutated mouse models created for reproducing colon cancers, as cited in the Part 1 (314, 315).

Similarly, on another side, the tumor production based on the subcutaneous or orthotopic inoculation of cancer cells from established cell lines represent a quick and easily suitable model (similar to a metastasis development) but with some limitations (absence of the progressive transformation of resident cells and related interactions with the surrounding tissue and immunity, less stroma formation than in progressively developing tumors) that need to be known to plan and evaluate a cancer study. The current studies on tumor microenvironment have clearly indicated the importance of stromal components in orienting the tumor evolution. For all these reasons, I’m convinced that to properly study the cancer development <em>in vivo</em> (especially the colorectal adenocarcinoma) the chemical induction - adequately balanced to produce para-physiological effects, remains a fundament in tumor modeling, also according to other authors (317-319).

d – The re-issued importance of inflammation as a cancer inductor/promoter established new possible links between carcinogenesis and bacterial activities. As discussed in the Part 1, the composition of the intestinal microbiota can have a double-edged effect of the colorectal mucosa either protective (lactobacilli) or pro-carcinogenic (clostridia). This was experimentally demonstrated in various studies, especially in experimentally induced cancer in animals. Very intriguing is the condition of chronic activation of the mucosal immunity, involved in an intense and continuous interplay with the bacteria (Part 1, 5.2). In the study that we performed conventional and germ-free rats were induced to cancer by following an identical protocol. The differences in cancer development and immunity demonstrated a lower reactivity and a more impaired anticancer response in the conventional than in the germ-free animals. The presence of bacteria in the bowel is indicated by many authors as able to intensify the effects of the administrated carcinogen, the elaboration of the bile and aliments in co-carcinogenic components, and alteration of mucosal cell turn-over (324).

The germ-free animals represent a very special and important tool for studying not only the influence of microbiological factors on the bowel physiopathology, but also to test the role of constitutive environments on the immunity. Bacteria are part
of the body of every living form since the birth and evolutionary mechanisms have allowed the macro-organisms to survive and interplay with the bacterial environment. Immunity is developed creating the possibility of a symbiotic survival of both organism and bacteria with reciprocal advantage (selection of so-called non-pathogens to compete with the pathogen colonization, sustain of non-pathogens by products of the organism cells, production of vitamins by bacteria, etc). The total absence of bacterial presence, functions and products can deeply influence the development and functions of an organism, especially the immunity. Many studies along the last 40 years have investigated the immunological capacity of the germ-free animals, with special regard to the development of adaptive immunity cells (T and B repertoire) and capability to respond to infectious agents when reconstituted by selected bacterial strains (gnotobiologic animals) or by a pool of microbiota. These studies, mainly on the intestinal environment, have focused on the mucosal immunity and the mechanisms of immune tolerance toward the commensal bacterial flora. (325-328).

The presence of a continuous activation of the immune responses against the microbiota, together with the presence of regulatory cells and cytokines, permits a balanced control of the microbiota to attempt to invade the mucosa without elicitation of reactions dangerous for the host organ. This kind of attenuated immune response is particular in the mucosal system and was described also as “physiological inflammation”. Factors producing this condition (especially the Treg cells, the particular CD8+ lymphocytes and interleukins of the Th2 type) are also considered involved in the oral tolerance, important mechanisms allowing the nutrition without reaction of intolerance to foods (329-332).

These arguments are in large part discussed in the paper 2.7 (Part 2), with the corresponding references. It is necessary underline that all the effects produced by the constitutive interplay between resident bacteria and host organism must have influence on the systemic immunity, not only on the local (mucosal) network. Our study is the first that after many years is evaluating the development of chemically induced colorectal cancer in GF vs. CV rats. It is also the first time that the immunological modifications of cell subpopulations during the cancer development are described not at the mucosal but at the systemic level, to verify possible conditioning by the microbiota (especially intestinal) on more general immune
functions. The lower number of tumors in the GF (confirming previous reports by other authors) can be variously explained, including immunological and non-immunological factors (e.g. bile metabolism). The relevant result, however, is the increase of cytotoxic cell subpopulations and adaptive immunity cells in the GF induced to but not developing cancer versus the low reactivity of the same group of animals in the conventional conditions. Moreover, when cancer developed, the GF resulted still more immune competent than the CVs (lower decrease of the cytotoxic response). Our explanation, as in the paper, is that the germ-free conditions allow an earlier detection of initial immune reactions against the transformed cells (e.g. danger signals), indicating a higher threshold of reactivity in the CV animals mainly due to the presence of the “physiological inflammation, and of a wide antigenic challenge with consequent tolerance. This important result needs further evaluation, not only to be more completely proved, but also to demonstrate if a specific immunological memory is elicited in the GF animals with antibody production (a particular finding was the increased number also of B cells). On another side, the artificial condition of total sterility might increase possibility of self-reactivity or cross-reactivity against epitops expressed not only by transformed but also normal cells (as sometimes it happens in the melanoma). In this case, the GF models should be of the highest value to investigate the relationships between cancer and autoimmunity processes without environmental interferences (285, 330, 333).

The importance of environments in controlling the development and evolution of pathologies and immune responses seen in the GF vs. CV rat study leads considerations also on the cancer microenvironment. As many times reported, the tumor microenvironment demonstrates characteristics of an inflammatory network with evolution to a chronic-like inflammation. The locally stimulated but inefficient immune response elicits regulatory responses by the systemic immunity. Some parallelism could be found between the chronic microbiota-induced immune reaction in the bowel mucosa controlled by regulatory and tolerogenic mechanisms to do not develop dangerous effects (IBD) (293, 296). The local immune response continuously evoked and sustained by the tumor cell aggressive behavior paradoxically stimulate the immune system to down-regulate itself in an attempt to reconstitute the homeostasis in the pathologic tissue. The paradox is that, under this view, the immune system senses as the primary danger the locally deregulated
immune response but not the tumor (transformed cells). The dualism of these responses needs to be better investigated because it poses once again the question about “how much self if the tumor”. In this way, a vicious circle is created by: cancer growth $\rightarrow$ local immune reaction $\rightarrow$ insufficiency of the immune reaction $\rightarrow$ cancer growth $\rightarrow$ and so far, with increasing of the immune response inefficacy by the activity of the systemic immunity through T regulatory cells and Th2 cytokines. Such a particular condition stimulated suggested my hypothesis of a need to block the abnormal immunologic network to permit the reactivation of a proper anti-tumor-directed response when added an immune stimulation. In our case, GN8 was the chosen molecule because the advantages shown in the melanoma model (2.2). As shown by the results reported and discussed in the paper 2.6, a temporary immune suppression (but not an anti-inflammatory treatment) associated to GN8 is able to intensively trigger IFN-$\gamma$ production in splenocytes challenged with tumor homogenate from the same animal. Interestingly, the test showed also the suppressive capability of the tumor homogenate (microenvironmental components) confirming the inhibitory (tolerogenic?) network in the cancer. The splenocytes represent a lymphoid tissue that is outside the tumor microenvironment and not primarily inhibit by the locally suppressive cancer network. The presence of a developed melanoma resulted to have generated immune cell activation (higher level of IFN-$\gamma$ in the untreated control than in the immune suppressive drug treated mice). The COX-2 inhibitor used as an anti-inflammatory drug (334,335) showed to not affect an established cancer conditions, with levels of IFN-$\gamma$ comparable to the untreated melanoma. However, the splenocytes obtained from the immune suppressive drug (azathioprine- AZ) treated animals dramatically rescued their immune activity challenged by the specific target homogenate when in association with GN8. We suppose the AZ blocking the Treg cells through the CD21 receptor, with pro-apoptotic effects. The choice of the azathioprine was suggested by its recently described mechanisms of action and by its extensive use in IBD (ulcerative colitis), autoimmune diseases and transplantations. (336-338).

The GN8 can trigger the NKR-P1 receptor activating the cells expressing the receptor. In a developed tumor, CD$^8^+$ and CD$^4^+$ cells are primed by the tumor antigens, but, once in the tumor, they are inhibited in their functions. The intensive response could result from pre-activated cells and NK cells.
The validity of such a paradoxical approach has found a first confirmation in the experimental data (preliminary, and with necessity to be integrated by larger studies about Treg cells and cytokine network involvement), but also in numerous reports appeared in the literature of the last 15 years from which it clearly results the advantage of using low dosage immune suppressive drugs (in literature cyclophosphamide was described to inhibit Treg cells) associated to a following immune stimulation (e.g. dendritic cell vaccines, IL-12) to rescue primed CD8⁺ cell activity in developed cancers. Recently, a review by Mihich E (2007) has reported immunological activities of anticancer chemotherapeutics. These new aspects are promising for further advancements in immunotherapy of cancer (339).

3.2. CONCLUSIONS

We can conclude from the reported studies that:
1) lectin-like receptors (NKR-P1) are suitable for effective immunomodulation (activation) of NK/NKT cells with production of IFN-γ and expression of CD69 activation marker;
2) this evidence is confirmed by the results in the experimental melanoma treatment by multivalent glycoderivatives PAMAM based (glycodendrimers, PAMAM-GlcNAc8, PAMAM-GlcNAc4);
3) the glycodendrimers can be traced in vivo and at cellular level by the use of fluorescent dye labeling of the PAMAM-GlcNAc4 (GN4);
4) glycodendrimers are internalized by the targeted cell (NK) together with the NKR-P1 receptor (confocal microscopy co-localization);
5) the treatment with glycodendrimers (PAMAM-GlcNAc8, GN8) produced positive results also in colorectal cancer induced rats
6) the glycodendrimers can be considered mimics of putative ligands present in the cancer microenvironment
7) the evolution of cancer microenvironment and consequent immunological modifications can be replicated in the original model of colorectal carcinogenesis in the rat proposed by the author;
8) the model is suitable for studies about the intestinal environment influence on anticancer immunity in germ-free animals;
9) the comparison of colorectal cancer induction in germ-free (GF) versus conventional (CV) AVN rats confirmed a lower cancer production by the GF;
10) for the first time was shown an increased immune activation in the GF animals that were induced to but did not developed cancer (cancer resistant) in comparison with the same CV group;
11) the presence of intestinal microbiota affected both the cancer development (multiplicity in CV rats) and the systemic immunity (main changes in the blood);
12) the modulation of the cancer immune microenvironment is a suitable target for immunotherapies of developed cancers;
13) a temporary immune suppressive treatment associated to a subsequent immune stimulation can rescue the specific antitumor response, indicating participation of primed adaptive immunity cells.

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PART 4

REFERENCES OF PART 1 and PART 3

NOTE: these books, also when not directly cited, served as main references for the generalities on the various arguments treated in the dissertation thesis.


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PART 5

PRESENTATIONS OF THE DISCUSSED DATA IN INTERNATIONAL CONFERENCES


12. Vannucci L., Pospisil M., Fiserova A.: Glycomimetics as modulators of natural cell immunity. Invited lecture at the 9th World Congress on Advances in Oncology and 7th International Symposium on Molecular Medicine, October 14-16, 2004, Hersonissos, Crete, (Greece).


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on Advances in Oncology and 10th International Symposium on Molecular Medicine 11-13 October, 2007, Creta Maris, Hersonissos, Crete (Greece), Int J Mol Med 20 (Suppl.1); S19, 2007
PART 6

PUBLICATIONS OF THE AUTHOR
A) Papers on peer-reviewed journals with IF


B) Papers on peer-reviewed journals without IF


C) Popularization papers


D) Chapters of books


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**E) Conference papers published as chapters of Books of Proceedings**


F) Abstracts on indexed and IF journals


