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MOLECULAR SIGNATURE OF TUMOR INFILTRATING NATURAL KILLER CELLS

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1. Zusammenfassung

Natürliche Killerzellen (NK) sind Effektorzellen des angeborenen Immunsystems, die in der Tumorabwehr eine wichtige Rolle spielen. Die Aktivierung von NK-Zellen wird durch ein Zusammenspiel von Signalen hemmender Rezeptoren, die zumeist MHC Klasse I erkennen, und aktivierender Rezeptoren bestimmt. Neben zielgerichteter Eliminierung von Zellen, können NK-Zellen zusätzlich Botenstoffe freisetzen, die Entzündungen bewirken und somit Immunantworten des angeborenen und des adaptiven Immunsystems auslösen.

In unserer Studie charakterisierten wir NK-Zellantworten gegen MHC Klasse I defiziente Lymphome in Mäusen. Unsere Untersuchungen zeigten, dass nach subkutaner Injektion von RMA-S Zellen NK-Zellen, die in den Tumor eingewandert sind, einen geringen Reifungsgrad ($CD27^{high}$) aufweisen. Zusätzlich zeigte diese Population einen aktivierten Phänotyp, der sich durch hohe Expression von B220, MHC Klasse II und zytotoxischen Effektormolekülen widerspiegelt. Genexpressionsanalysen mittels Microarrays machten deutlich, dass Tumor-infiltrierende NK-Zellen ein stark unterschiedliches Transkriptionsprofil aufweisen als NK-Zellen im Blut von Tumor-tragenden Mäusen. NK-Zellen im Tumor zeigten neben einem verringerten Ausmaß von aktivierenden NK-Zellrezeptoren zusätzlich einen Anstieg von hemmenden Molekülen. Die meisten dieser Änderungen auf Transkriptionsebene konnten ebenso auf Proteinebene bestätigt werden.

Tumor-infiltrierende NK-Zellen exprimierten ein höheres Ausmaß von Mitgliedern der B7-Proteinfamilie verglichen mit NK-Zellen aus dem Blut. Hierzu zählen der aktivierende Rezeptor CD28 und die hemmenden Rezeptoren CTLA-4 und B7-H1, die alle an B7-1 binden. Die Funktion dieser Moleküle ist in T-Zellen gut verstanden, in NK-Zellen allerdings größtenteils unbekannt. sere Daten zeigten, dass die Expression von CD28 und CTLA-4 mittels IL-2 auf NK-Zellen induzierbar ist bzw. sich die Expression von B7-H1 erhöhen lässt. Eine Stimulierung von in vitro expandierten NK-Zellen mit einem B7-1 IgG Fusionsprotein führte zu Proliferation von NK-Zellen und deren IFNγ-Produktion. Mit Hilfe von knockout Mäusen zeigten wir, dass nach Stimulation von NK-Zellen mittels B7-1 IgG IFNγ-Produktion durch CD28 vermittelt und diese durch die CLTA-4 und B7-H1 Rezeptoren negativ reguliert wird. Im Gegensatz dazu wurden B7-1 exprimierende Zielzellen durch IL-2 expandierte NK-Zellen unabhängig von CD28, CTLA-4 und B7-H1 lysiert. Zudem beobachteten wir, dass in einer Ko-Kultur von NK-Zellen und B7-1 exprimierenden Zellen, darunter transduzierte Tumorzelllinien sowie reife dendritische Zellen, einen interzellulären Transfer von B7-1 der Zielzellen zu NK-Zellen bewirkt. Dieser Prozess ist teilweise abhängig von CD28. Schliesslich wiesen unsere Experimente darauf hin, dass wiederholte Applikation von IL-2 das Wachstum von $B7\text{-}1^{+}$ Melanome in T- /B-Zell defizienten Mäusen hemmt. Dieser Effekt konnte durch gleichzeitige CTLA-4 Blockierung noch geringfügig verbessert werden.

Zusammenfassend zeigen unsere Daten, dass Tumor-infiltrierende NK-Zellen einen unterschiedlichen Phänotyp als NK-Zellen aus dem Blut aufweisen. Dies spiegelt sich sowohl in ihrem Reifungsgrad, der Expression von aktivierenden und inhibierenden Molekülen als auch in ihrem umfassenden

Transkriptionsprofil wider. Wir beobachteten, dass bestimmte Rezeptoren der B7-Familie unterschiedlich exprimiert sind, die die Effektorfunktionen von NK-Zellen in vitro regulieren. Unsere Experimente deuten darauf hin, dass Mitglieder der B7-Proteinfamilie während der Tumorentwicklung eine wichtige Rolle bei der Kontrolle von NK-Zellantworten spielen. Unsere Ergebnisse sollten bei Immuntherapien gegen Tumore, die auf der Wirkung von NK Zellen beruhen, mit einbezogen werden.

2. Summary

Natural killer (NK) cells are innate immune effector cells that play an important role in anti-tumor defense. A delicate balance of signals delivered by activating ligands and inhibitory molecules, mainly MHC class I, expressed by target cells, determinates the NK cell activation. Besides direct killing of target cells, NK cells release mediators that induce inflammation and exert immunoregulatory effects influencing both innate and adaptive immune responses. Although efficient at eliminating metastazing cells and small tumor grafts, the eradication of larger solid tumors by NK cells is usually not efficient, despite the low expression of MHC class I by tumor cells in many cases.

In our study, we characterized the *in vivo* NK cell response against the MHC class I deficient mouse lymphoma. We demonstrate that after subcutaneous injection of RMA-S tumor cells, NK cells infiltrating the tumor tissue (TINs) correspond to the less mature $CD27^{high}$ NK cell population. At the same time, the subset of TINs displays a phenotype of activating state, which is reflected in the elevated expression of B220, MHC II and cytotoxic effector molecules. Gene expression profiling using whole genome microarrays, revealed a strikingly different transcription profile of tumor infiltrating compared to blood NK cells of tumor bearing mice. TINs downregulated activating NK cell receptors, which was accompanied by upregulation of inhibitory molecules. We confirmed that most of the molecules that were differentially regulated at the mRNA level were also differentially expressed at the protein level.

As compared to NK cells from the blood, expression of several members of the B7 protein family was elevated in TINs, including the activating receptor CD28 and the inhibitory receptors CTLA-4 and B7-H1, which all bind the B7 family ligand B7-1. Our data demonstrate that the expression of CD28 and CTLA-4 can be induced and expression of B7-H1 elevated on NK cells by IL-2. Stimulation of *in vitro* expanded NK cells with a B7-1 IgG fusion protein induced NK cell proliferation and IFNγ production. Using gene deficient NK cells, we demonstrated that B7-1 dependent IFNγ production was mediated by CD28, and negatively regulated by CTLA-4 and B7-H1 receptors. The lysis of B7-1 expressing target cells by IL-2 expanded NK cells was independent of CD28, CTLA-4 and B7-H1 expression. We observed that co-incubation with B7-1 expressing cells, including transduced tumor cell lines and mature dendritic cells, led to the intercellular transfer of B7-1 from target cells to the NK cells, in a process that partially depended on CD28. Our experiments indicated that therapeutic responses to $B7\text{-}1^{+}$ melanoma, mediated by IL-2 in T and B cell deficient mice, could be moderately improved by CTLA-4 blockade.

In summary, our data reveal that NK cells infiltrating solid tumors display a phenotype different from NK cells found in blood, including their maturation state, expression of activating and inhibitory molecules and global transcription profile. We demonstrate that certain receptors of the B7 family that were differentially expressed by TINs, regulated NK cell effector responses *in vitro*. Our results suggest that members of the B7 protein family can be involved in the complex control of NK cell responses during tumor progression and should be considered as potential targets in NK cell based anti-cancer immunotherapy.

3. Introduction

3.1. The immune system

The immune system represents the complex network of molecules, cells, tissues and organs evolved to protect the organism from pathogens. The main property enabling the immune system to accomplish its function is the ability to recognize non-self molecules as well as "altered-self" state. "Altered-self" recognition and possibility to sense any kind of cellular stress enables the immune system to detect both infected and transformed cells and potentially prevents the development of malignancy. Any molecule that can be recognized by the immune system leading to the activation of protective responses is considered an antigen. Two main components of the immune system, the innate and the adaptive, are distinguished in vertebrates based on the type of antigens they recognize, recognition strategy and kinetics of response.

3.1.1. The innate immune system

The innate immune system consists of distinct subsystems that prevent the entrance and establishment of infectious agents. Its main property is fast activation of pre-existing defense mechanisms (Table 3.1), thereby is considered as the first line of defense against potential danger. The principal components of innate immunity are physical and chemical barriers (epithelia, anti-microbial substances), cells (phagocytes, natural killer cells), blood proteins (complement) and cytokines. The receptors of the innate system are germ-line coded molecules that recognize so called pathogen-associated molecular patterns (PAMPs), as well as molecules expressed by host cells upon infection or any kind of cellular stress, including malignant transformation. PAMPs are evolutionary conserved structures unique to pathogens and often shared by particular classes of microbes. In addition, they usually represent essential evolutionary preserved components necessary for pathogen survival. Typical examples are complex lipids and carbohydrates found in bacterial (LPS, peptidoglycan, lipoteichoic acid) and fungal (β-glycan) cell walls, some viral proteins (m157 from mouse CMV) or specific modifications of viral and bacterial nucleic acids (double-stranded RNA, unmethylated CpG DNA sequences). The best characterized pattern-recognition receptors (PRRs) are the Toll-like receptors (TLRs), which recognize a multitude of pathogen-derived molecules [1]. TLR triggering stimulates pathogen uptake by phagocytic cells (macrophages, dendritic cells, neutrophils), which results in their destruction and secretion of factors (proinflammatory cytokines and chemokines) that recruit and activate other immune effectors. Pathogen detection and uptake by dendritic cells (DC), which are termed professional antigen-presenting cells (APC), lead to their maturation and migration to the local lymph node, where they initiate the adaptive immune response. In general, many mechanisms employed by the innate immune system function to not only eliminate infectious agent but also to prime, enhance and polarize the proper adaptive immunity

tailored according to the pathogen type. The effective response to the invading pathogen depends on the proper activation of both arms of the immune system.

Innate subsystem	Principal function
Barriers	
Epithelial layers	Prevent microbial entry
Defensins	Microbial killing
Intraepithelial lymphocytes	Microbial killing
Circulating effector cells	
Neutrophils	Early phagocytosis and microbial killing
Macrophages	Efficient phagocytosis and microbial killing Secretion of cytokines that stimulate inflammation
NK cells	Lysis of infected cells, activation of macrophages
Dendritic cells	Antigen uptake and processing Priming of adaptive immune system
Circulating effector proteins	
Complement	Microbial killing and opsonization Activation of leukocytes
Mannose-binding lectin (collectin)	Microbial opsonization, activation of complement
C-reactive protein (pentraxin)	Microbial opsonization, activation of complement
Coagulation factors	Walling off infected tissues
Cytokines	
TNF, IL-1, chemokines	Inflammation
Type I interferons	Resistance to viral infections
IFNγ	Macrophage activation
IL-12	Induction of IFNγ production
IL-15	NK cell proliferation and survival
IL-10, TGFβ	Control of inflammation

Table 3.1. Subsystems of the innate immune system. The principal components of the innate immunity are physical and chemical barriers that prevent pathogen entry and establishment of infection, and cells, blood proteins and cytokines that participate in the clearance of microbes from the organism. Representative components of innate subsystems are listed in the table. [2]

3.1.2. The adaptive immune system

The hallmarks of adaptive immunity are delayed responses compared to the innate immune system upon the first encounter with pathogen, high diversity of receptors formed by the process of somatic recombination and the ability to develop immunological memory. Memory response is raised upon any subsequent recognition of the previously encountered antigen and it is faster and stronger in intensity, thereby more efficient. The diversity of the antigen receptors of adaptive immune cells is formed through the process of somatic recombination in which sets of germ-line coded DNA sequences are randomly brought together to form functional B cell (BCR) and T cell receptor (TCR) genes [3]. RAG-1 and RAG-2 are the genes encoding the enzymes, which are the main components of the recombination machinery [4]. Since the expression of functional receptors is the prerequisite for B and T cell maturation, mice deficient in RAG coded enzymes lack B and T cells [5-6]. Every B and T cell expresses only one type of receptor with unique specificity, forming a clonally distributed repertoire. The receptors of adaptive immune system are not able to distinguish non-self from self. Rather, non-reactivity to self is achieved through the complex sequences of mechanisms called tolerance induction, which includes deletion or specific inactivation of self reactive clones, differentiation of regulatory cells and others [7-8].

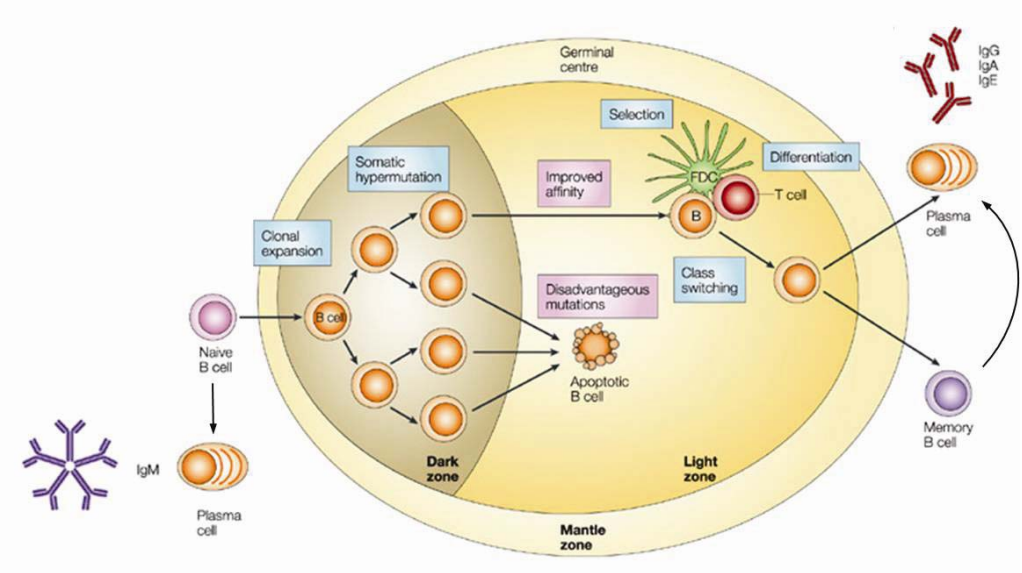


Figure 3.1. Differentiation of B cells into specialized effector subsets after antigen recognition. Activated B cells differentiate into antibody-secreting plasma cells or memory cells. In the germinal centers formed in the peripheral lymphoid organs activated B cells undergo a process of proliferation, isotype switch and affinity maturation that enables the differentiated plasma cells to secrete antibodies with high affinity for encountered antigen. Various isotypes of produced antibodies are specialized for different functions. Memory cells reside in bone marrow and are capable of fast differentiation into plasma cells upon subsequent recognition of the same antigen. [9]

The humoral and cellular effector arms are the two main systems operated by adaptive immunity. Humoral immunity is mediated by B cells and it is targeted against extracellular antigens. B cell receptors (BCR) are membrane bound immunoglobulins that can recognize diverse microbes and microbal toxins and activate B cells to differentiate into either antibody-secreting plasma cells or memory cells (Figure 3.1). In addition, activated B cells can accomplish the function of APCs and contribute to the development of cellular immunity. Antibodies represent isotype-switched soluble immunoglobulin molecules secreted by B cells. Different classes of antibodies mediate specific responses that include antigen neutralization, which prevents the host cell invasion, and opsonization of pathogens enabling their phagocytosis and/or destruction by the innate cellular effectors. In addition, antibodies activate the complement system, mediate mucosal immunity and stimulate mast cell degranulation.

Intracellular pathogens that are not accessible to antibodies are attacked by cell mediated immunity operated through T cells. T cells, like B cells, originate in the bone marrow (BM), but unlike B cells, migrate to the thymus to complete their maturation. T lymphocytes recognize protein antigens via the T cell receptor complex only when displayed as peptides bound to self major histocompatibility complex (MHC) molecules and presented by APCs. Based on the expression of membrane glycoproteins, which are the essential parts of TCR complex, T cells are divided into $CD4^+$ and $CD8^+$ subsets. $CD4^+$ T cells recognize MHC class II associated peptides, which are derived mainly from endocytosed proteins, while $CD8^+$ T cells recognize MHC class I associated peptides, which are mainly derived from cytosolic, endogenously produced proteins. To become fully activated, in addition to TCR engagement, costimulatory signals provided by APCs are crucial to trigger the functional T cell responses. In addition to the quality of costimulation, cytokines produced by innate immune cells at the site of antigen recognition polarize T cell differentiation towards effector cells with specific functional properties (Figure 3.2).

$CD4^+$ T cells differentiate into one of three T helper cell (Th) lineages - Th1, Th2 and Th17, or to the induced regulatory T cells (iTreg) (Figure 3.2) [11]. IFNγ and IL-12 potentiate differentiation of Th1 cells, which produce IFNγ and activate macrophages, DCs, B cells and $CD8^+$ T cells to perform their functions. IL-4 triggers the differentiation of the Th2 subset. They produce IL-4, IL-5 and IL-13 and control multicellular parasite infections through B cell, mast cell and basophil activation. Finally, transforming growth factor β (TGFβ) in the presence of proinflammatory cytokines, such as IL-6, IL-21 and IL-23, triggers the differentiation of Th17 cells. In the absence of inflammation, a high concentration of TGFβ favors the differentiation of regulatory T cells. Th17 cells produce IL-17 and IL-22 and play an important role in the clearance of extracellular bacteria and fungi, especially at mucosal surfaces. Regulatory T cells inhibit responses of other effector cells. They are involved in the control of ongoing immune responses, but also in the prevention of autoimmunity.

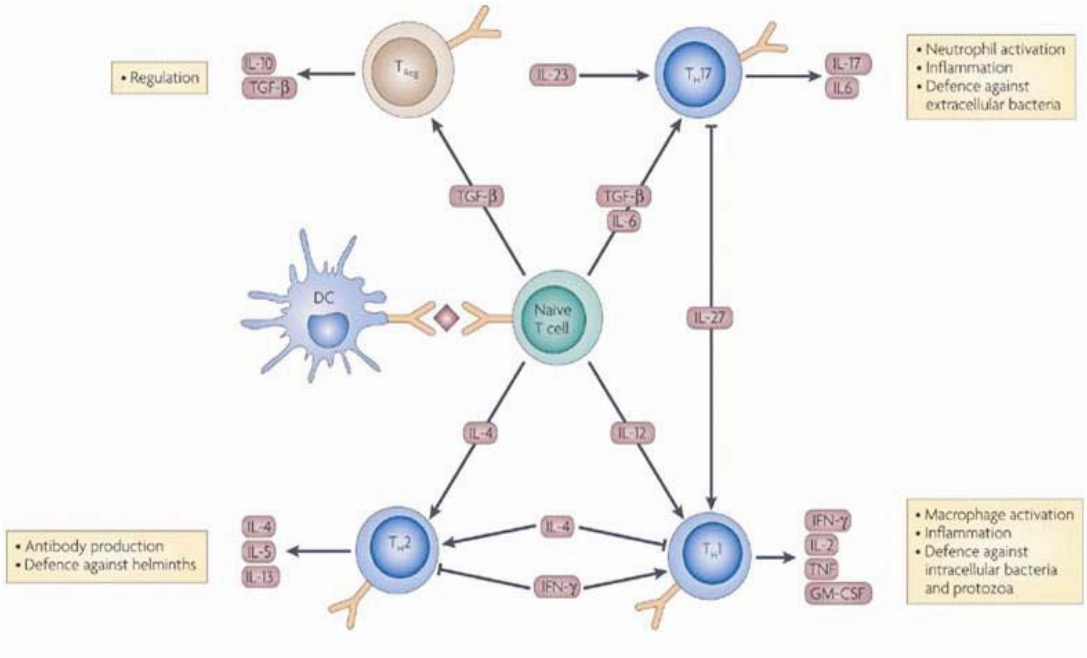


Figure 3.2. Differentiation of CD4$^+$ T cells into specialized effector subsets after antigen recognition. Activated CD4$^+$ T cells can differentiate into four functional subsets driven by cytokines present in the microenvironment. Th1 cells support cell-mediated immunity and develop under the influence of IL-12 and IFNγ. Th2 cells differentiate in the presence of IL-4 and support humoral immunity. TGFβ in the presence of IL-6 induces differentiation of Th17 cells, which stimulate the clearance of extracellular bacteria and fungi. In the absence of IL-6, regulatory T cells, which control the responses of multiple immune effector cells, are differentiated. [10]

Naïve CD8$^+$ T cells differentiate into cytolytic T lymphocytes (CTLs) upon antigen recognition and additional stimuli provided by Th1 cells. Their main effector functions are the elimination of target cells expressing the peptide-MHC I complexes against which response has been raised and production of IFNγ.

Thus, specific effector functions of different components of the adaptive system are developed based on initial pathogen encounter by innate immune recognition mechanisms and mediators produced dependent on the pathogen type. One of the functions of specialized adaptive responses raised in such way is further enhancement of innate mechanisms necessary for pathogen clearance.

3.2. NK cells

Natural killer (NK) cells represent a lymphocytic population that provides the first line of defense against diverse pathogens through the direct elimination of infected cell and production of cytokines and chemokines. In addition, NK cells are recognized to be important for the control of graft rejection and pregnancy. They also play an important role in tumor growth control and prevention of metastatic dissemination [12-15].

NK cells are classified as innate immune effector cells based on the observations that they lack typical T and B cell surface markers, and, more importantly, do not depend on the process of somatic recombination. Therefore, they are present in mice and humans unable to rearrange their antigen receptor genes. Instead, NK cells express several classes of germ-line coded receptors, which are used for recognition of potential danger signals. Initially, NK cells were discovered as a spleen cell population of naïve mice and rats and peripheral blood cell subset of healthy human donors that mediate spontaneous cytotoxic activity against different tumor cell lines [16-19]. Although very often defined as killing effectors with the capacity to eliminate dangerous cells without prior sensitization, there is emerging evidence that NK cell activation is regulated by integrated signals provided by surface receptors recognizing both potential targets and sensing local microenvironment [20]. In response to activating signals, besides cytotoxicity, the production of multiple cytokines and chemokines is triggered. Through these effector responses NK cells can eliminate potentially dangerous target cells, but also impact on ongoing innate and emerging adaptive immune responses [21].

3.2.1. NK cell activation - recognition of target

3.2.1.1. Inhibitory NK cell receptors

Primary targets of NK cells are infected, transformed as well as cells experiencing any kind of stress, including heat shock or irradiation. To be able to perform their effector functions upon target encounter, it is essential for NK cells to distinguish potentially dangerous from normal cells. Kärre and colleagues formulated the “missing-self” hypothesis describing a strategy for recognition based on the observation that MHC class I deficient congenic tumor cells are very efficiently lysed by NK cells [22-24]. Cells that express MHC class I on the cell surface at sufficient density would be then protected due to the engagement of inhibitory receptors expressed by NK cells that specifically recognize self MHC class I (Figure 3.3A). C-type lectin Ly49 receptor family in mice, killer cell immunoglobulin-like (KIR) and leukocyte immunoglobulin-like (LIR) receptor family in humans and CD94/NKG2A complex in both species interact with self MHC class I ligands (Table 3.2). The principal mechanism of action of MHC I specific inhibitory NK receptors is based on the presence of immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic tail, which recruits inhibitory signaling molecules and terminates putative activating signal transduction (Figure 3.3B). Most of NK cells express at least one MHC class I specific receptor. Inhibitory receptor repertoire formed over the individual cells allows the global NK cell pool to successfully monitor self and reacts when pathological conditions interfere with MHC class I expression [25-26].

NK cells expressing no inhibitory receptors for autologous MHC I alleles have been identified in healthy individuals and mice. However, they are rendered hyporesponsive in order to prevent possible autoreactive responses [27-28]. Similarly, in humans with transporter-associated antigen processing (TAP) deficiency or in mice with no MHC I expression (β_2m, TAP1, H2-K^b, H2-D^b gene deficient mice), NK cell

effector responses are reduced [29-30]. The need of NK cell to recognize self MHC class I during maturation in order to develop full effector competence is referred to as "licensing" [31].

In addition to MHC I specific receptors, NK cells express several other inhibitory receptors that recognize ligands widely expressed on normal cells. Their level of expression is often modified upon infection or malignant transformation. For example, the KLRG1 inhibitory receptor that recognizes cadherins can be considered as receptor providing an alternative strategy of sensing "altered-self" [32-33].

Receptor	Species	Ligand
NKG2A/CD94	Mouse, Human	HLA-E in human Qa-1 in mouse
KLRG1	Mouse, Human	Cadherins
2B4	Mouse, Human	CD48
NTB-A	Mouse, Human	NTB-A
Ly49A	Mouse	H-$2^{d, k, p}$
Ly49C	Mouse	H-$2^{b,d,k,s}$
Ly49I	Mouse	H-$2^{r, b, d, k, q, s, v}$
Other Ly49	Mouse	Various mouse MHC I alleles
NKR-P1B	Mouse	Clr-b
CD161	Human	LLT1 (Clec2D)
Various KIR	Human	HLA-A/-B/-C
KIR2DL4	Human	HLA-G
LIR1	Human	HLA I, UL18 HCMV protein
CAECAM1	Human	CAECAM1
Siglec-7/9	Human	Sialic acid

Table 3.2. Inhibitory NK cell receptors and their ligands. [34-35]

3.2.1.2. Activating NK cell receptors

MHC class I deficiency is not sufficient to trigger full NK cell activation that requires in addition the engagement of activating receptors, including members of the Ly49 family in mice, KIR-S family in humans and natural cytotoxicity receptors (NCR), NKG2D, DNAM-1 and CD94/NKG2C complex in both species (Table 3.3). Those receptors recognize diverse ligands that can be derived from invading pathogens or

induced on infected, transformed and stressed cells. The necessity for the existence of a positive signal for NK cell activation in addition to the absence of inhibition represents the basis for the "induced-self" recognition strategy. A delicate balance of inhibiting and activating pathways that govern NK cell activation is further hard-wired with cytokine, chemokine and costimulatory signals, which together influence the final effector response defined both by the nature of the target and the local microenvironment.

Receptors for MHC class I often exist in pairs of activating and inhibitory receptor with a highly homologous extracellular domain. The inhibitory receptors have a significantly higher ligand affinity compared to the activating counter receptors. In steady state conditions, inhibitory signals dominate over the interaction of NK cell and autologous target cell. This inhibition is based on the recruitment of SRC homology 2 (SH2)-domain-containing protein tyrosine phosphatases to the ITIM motifs of inhibitory receptors, such as SHP-1 and SHP-2. They dephosphorylate and inactivate signaling molecules, which mediate activation downstream of activating receptors (Figure 3.3B) [36-38].

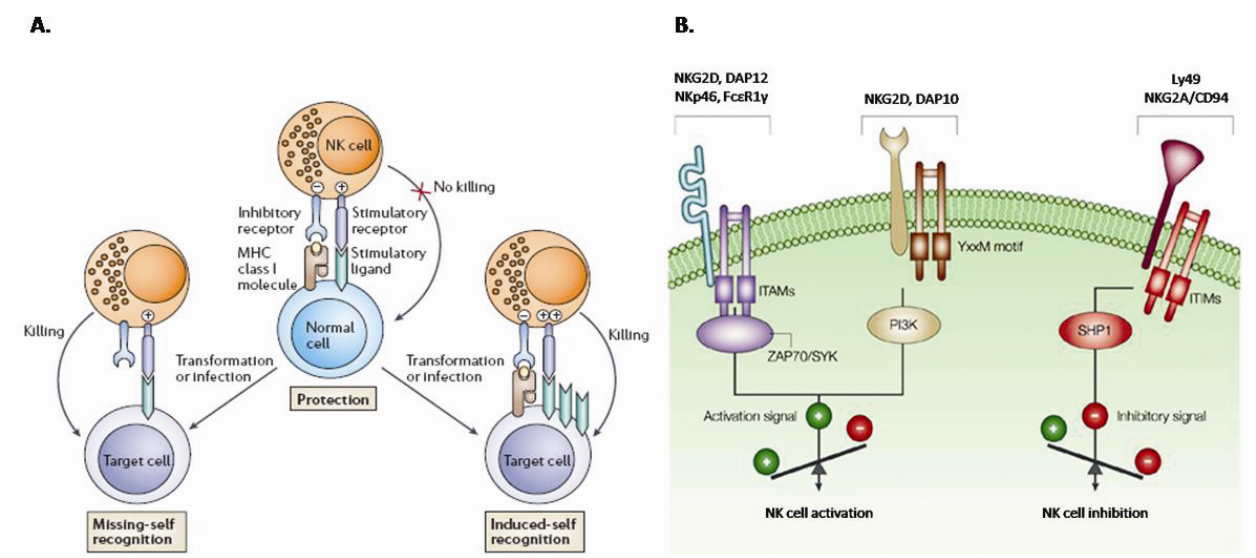


Figure 3.3. NK cell activation upon recognition of susceptible target cells. A) Normal cells typically engage both the activating and the inhibitory NK receptors. Signals delivered through the inhibitory receptors dominate and prevent NK cell activation and the lysis of normal cell. Infected or transformed cells dowregulate expression of inhibitory ligands (missing self recognition) or/and upregulate ligands for activating NK cell receptors (induced-self recognition) allowing NK cell activation. B) Upon ligand engagement, activating NK cell receptors recruit signaling molecules, such as protein kinases Syk, ZAP70 and PI3K that mediate NK cell activation. The inhibition of NK cell triggering is based on the recruitment of tyrosine phosphatases, such as SHP-1, to the ITIM motifs of inhibitory receptors or associated adaptor molecules, which dephosphorylate and inactivate signaling molecules downstream of activating receptors. [34, 39]

Several pathogen-encoded molecules are known to be directly recognized by NK cells and induce their activation. The MCMV derived m157 protein is detected via Ly49H, influenca A and Sendai virus derived haemagglutinins via NKp44 and NKp46, *Staphylococcus aureus* derived endotoxin B and ligands derived from *Mycobacterium tuberculosis*, *Plasmodium falciparum* and *Leismania spp.* via yet unidentified

receptors. However, the majority of pathogens are sensed by accessory cells, such as monocytes, macrophages and dendritic cells that upon encounter of pathogen transmit activation signals to NK cells via soluble mediators or in a contact-dependent manner [40].

Stress induced ligands are molecules that are usually not expressed or are present at very low levels in healthy cells, but strongly induced upon infection or malignant transformation. As such, they can be recognized by NK cell activating receptors and trigger lytic activity and/or cytokine production. Some of them can be induced on activated immune effectors, e.g macrophages incubated with LPS, where they could have a potential role in the control of an immune response [41]. The most extensively defined family of stress induced ligands engage the activating receptor NKG2D (Rae1α-ε, H60, Mult1 in mice; MICA/B, ULBP1-4, RAET1G/L in human). Their expression is induced through various forms of cellular stress, such as heat shock, viral infection, wound healing, UV radiation or DNA damage [42]. Many tumor cell lines and analyzed tumor tissues have been reported to express NKG2D ligands indicating their potential role in tumor growth control by NK cells and other NKG2D expressing immune effectors [43]. Similarly, ectopic expression of mouse Rae1β and H60 causes rejection of subcutaneously implanted tumor cells in a NKG2D dependent manner [44-45].

Receptor	Species	Ligand
CD16	Mouse, Human	IgG
NKp46	Mouse, Human	Viral haemagglutinin, ?
CD94/NKG2C/E	Mouse, Human	HLA-E in human Qa-1 in mouse
NKG2D	Mouse, Human	MICA/B, ULBPs in human Rae1, H60, MULT1 in mice
DMAM-1	Mouse, Human	CD112, CD155
2B4	Mouse, Human	CD48
CRACC	Mouse, Human	CRACC
NTB-A	Mouse, Human	NTB-A
NKR-P1C	Mouse	?
NKR-P1F	Mouse	Clr-g
Ly49H	Mouse	m157 MCMV protein
Ly49D	Mouse	H-2^{d,a,b,k,p,q,s}
NKp30	Human	B7-H6, ?
NKp44	Human	Viral haemagglutinin
NKp80	Human	AICL
KIR2DS	Human	HLA-C

Table 3.3. Activating NK cell receptors and their ligands. [46-47]

Activating NK cell receptors mainly couple to three signaling pathways: DAP12/Syk/ZAP70, DAP10/PI3K and SAP/Fyn that further activate a signaling cascade via PLCγ and Vav family members. When such a cascade becomes fully activated in contact with sensitive targets, it typically leads to the delivery of cytotoxic granules to the synapse with target cell and/or cytokine and chemokine release. [48] [49].

3.2.1.3. NK cell costimulation

Several receptors and ligands expressed on target cells have been described to costimulate other NK receptors rather than mediating direct activation. The term costimulation has been adopted from the T cell system. Naïve T cells have an absolute requirement for TCR engagement to be activated. However, in addition they need triggering of costimulatory receptors, mainly CD28. Nevertheless, CD28 engagement alone cannot induce T cell activation [50]. In the mouse system, NKG2D coupled to DAP12 can directly trigger NK cells, while coupled to DAP10 adaptor molecules rather enhance the response initiated via other activation pathways. Indeed, DAP12 mediated signaling resembles TCR (Syk/ZAP70), while DAP10 recruits similar signaling molecules as CD28 (Grb2, PI3K) [51]. Similar functional properties are attributed to the receptors that recognize CD40, CD70, B7-1 (CD80), B7-2 (CD86), ICOS-L, OX-40L and 4-1BBL, ligands previously described to costimulate T cell effector functions at the different stages of activation [52-53].

CD40 on APCs engages T cell expressed CD40L, which is the interaction crucial for T cell priming and development of humoral responses [54-55]. Its ectopic expression on tumor cell lines triggers NK cell cytotoxic responses [56]. However, it is not clear which receptor on NK cells recognizes CD40, since $CD40^+$ targets are lysed by CD40L deficient NK cells equally as by WT effectors. A relatively high proportion of human melanoma has been reported to express CD40 [57], thus its contribution to the NK cell activation might be relevant for anti-tumor responses.

B7-1 and B7-2, similar to CD40, induce NK cell cytotoxicity and IFNγ production. B7-1 and B7-2 positive lymphoma enhances NK cell mediated cytotoxicity *in vitro* being potent enough to overcome inhibition by MHC I [56, 58]. T cells recognize the B7-1/2 ligands via receptors CD28 and CTLA-4 [59]. B7-1 recognition by both human and mouse NK cells has been controversial. NK cells have not been reported to express CTLA-4. In mice, CD28 expression has been only shown by some studies on IL-2 expanded NK cells [60]. Proliferation and IFNγ production induced by $B7\text{-}1^+$ cell lines has been contributed to CD28 in studies of Kelly *et al* [61], while the lysis of B7-1 expressing tumor targets was shown to be CD28 and CTLA-4 independent [58]. Similarly, recognition of B7-2 could not be contributed to CD28 and CTLA-4 [56]. Human NK cells seem to express different variants of CD28 that mediates activation by NK cell sensitive ($MHC\ I^-$) but not NK cell resistant ($MHC\ I^+$) tumor cell lines [62]. B7 costimulatory ligands are expressed at different levels by cell lines derived from patients with gastric, esophageal and colorectal cancer [63], as well as on AML and B cell precursor ALL blasts [64-65]. Therefore, NK cell costimulation by B7-1/2 can be relevant

for direct recognition of tumor cells. In addition, APCs express high levels of B7-1/2 costimulatory molecules upon activation, which might be involved in their cross-talk with NK cells [66].

CD70 is recognized by the tumor necrosis factor (TNF) family receptor, CD27, which is constitutively expressed on resting, naïve T, B and NK cells, and further up-regulated following activation [67]. The function of CD27 seems to be controlled via availability of its ligand, CD70, that shows restricted, transient and activation dependent expression on T, B and dendritic cells. CD70 is positively regulated via TLR triggering and pro-inflammatory cytokines, such as IL-1β, TNFα, IL-12 and GM-CSF, while IL-4 and IL-10 reduce its expression. CD27 engagement is not absolutely required for naïve T cell activation, but it strongly enhances differentiation and function (IL-2, IFNγ production) of effector cells through the positive effects on survival and proliferation. To prevent possible immunopathology, prolonged CD27 costimulation utilizes negative feedback mechanisms, which includes reduction of receptor expression, receptor shedding from the cell surface and induction of negative mediators, such as IL-10, Fas, FasL and inhibitory receptor PD-1 [67]. Both human and mouse NK cells subsets differentially express the CD27 receptor. Its level of expression is widely used to define the functional and/or maturation status of NK cells (see 3.2.3). Functional analysis of CD27 revealed its role in the positive regulation of NK cell effector functions, mainly proliferation and IFNγ production, without direct impact on cytotoxicity [68].

Ectopic expression of CD70 and CD80 on implanted tumor cells facilitates NK cell anti-tumor responses, leading to tumor rejection and generation of functional immunological memory. Those effects are dependent on NK cells and both perforin and IFNγ effector responses [61, 69]. A recent study of Chan *et al.* showed that activating NK cell receptor DNAM-1 is costimulated via CD70 and CD80 mediating suppression of lung melanoma metastases after IL-2 application [70].

ICOS-L (ICOS ligand) engages costimulatory receptor ICOS that is expressed on T cells upon activation and enhances T cell cytokine production and effector functions [71]. Similarly, ICOS is not expressed on resting NK cells, but it is induced by cytokine stimulation [72]. Its cross-linking promotes NK cell IFNγ production, while ectopic expression of ICOS-L induced efficient killing of tumor cells *in vitro.* ICOS-L expressing tumor cells are better controlled *in vivo* in a NK cell dependent manner. Among human tumors, leukemia cells express ICOS-L [73]. In addition, it is expressed on the surface of B cells, APCs and certain nonhematopoietic tissues. Thus, ICOS dependent costimulation of NK cells may play an important role in NK cell activation through the interaction with both target cells and other immune cell.

OX40L and 4-1BBL are members of the TNF superfamily ligands primarily involved in the regulation of T cell responses and cross-talk between T cells and other cells [74]. Both the ligands and their receptors (OX40 and 4-1BB) show inducible expression following immune cell activation. The ligands are primarily expressed by professional APCs, but also by non-immune cells as smooth muscle and endothelial cells. NK cells were shown to express OX40 and 4-1BB receptors as well as their ligands, which enables their extensive cross-talk with other immune cells during ongoing response.

3.2.2. NK cell effector responses

3.2.2.1. NK cell cytotoxic responses

Upon proper NK cell triggering, susceptible targets can be eliminated through perforin/granzyme dependent or death receptor initiated apoptosis (Figure 3.4.) [75]. Perforin and granzymes are crucial cytotoxic effector proteins stored in specialized lytic granules with secretory lysosome characteristics. Both NK cells and cytotoxic T lymphocytes (CTL) exert their cytolytic functions through the delivery of granule content to the contact site with target cell. The fusion of secretory granules with the plasma membrane leading to the release of cytotoxic mediators or cytokines is termed "degranulation". The process is used to measure NK cell activity at the single cell level upon triggering of specific activating pathways or encounter of target cells. It is based on the appearance of lysosomal membrane glycoproteins 1 and 2 (CD107a and CD107b) on the cell surface due to degranulation [76-77]. CD107a/b are proteins found in the lysosomal membrane, but redistribute on the cell surface shortly after granules fuse with the cell membrane. This process is followed by fast internalization and CD107a/b removal from the surface.

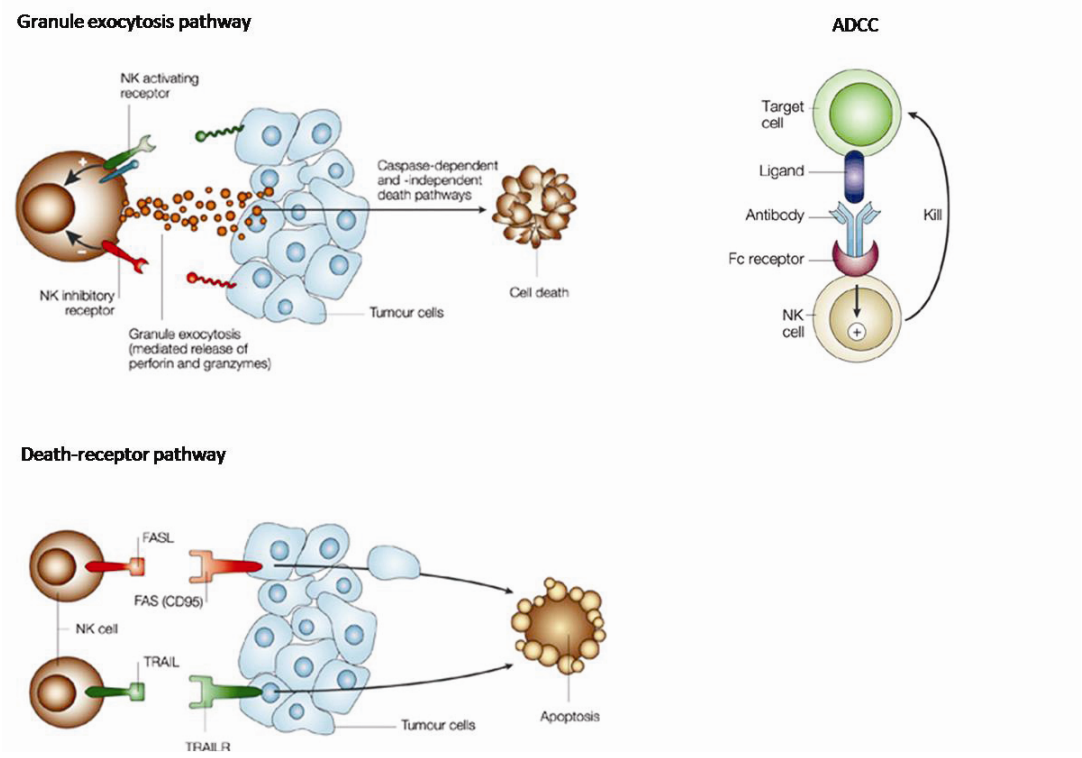


Figure 3.4. Cytotoxic mechanisms used by NK cells. NK cells use three distinct pathways to induce death of target cells. Granule exocytosis pathway mediates the release of the lytic granuli content including the membrane pore-forming protein perforin and serin-proteases granzymes that initiate target cell death. In antibody dependent cellular cytotoxicity (ADCC), NK cells recognize target cells opsonized with antibodies via Fc receptors that trigger NK cell activation and release of lytic granules. In death-receptor pathway, death inducing ligands (TNF, FasL, TRAIL) expressed by NK cells engage the cognate receptor on target cells inducing a series of events that lead to the activation of caspases and target cell apoptosis. [39, 78]

Perforin is a highly conserved membrane-disrupting protein vital for NK cell cytotoxicity [79]. Current models suggest that perforin polymerizes within the endosome- and/or plasma membrane and forms pores causing granzyme diffusion and necrosis of target cells. [80]. Perforin mediated cytotoxicity plays an important role in NK cell mediated tumor suppression [81-83]. Various anti-tumor therapies (e.g. IL-2 or IL-21 application) activate NK cells to destroy implanted tumors in a perforin dependent manner [84-85].

Granzymes represent structurally related serine-proteases with various substrate specificities. Apoptotic cell death triggered by granzymes is mediated via activation of caspases, induction of mitochondrial damage, which leads to the release of pro-apoptotic factors, and/or DNA damage pathway [80]. Besides induction of cell death, it was shown that certain granzymes could exert distinct biological roles. As an example, granzyme A can activate IL-6, IL-8 and IL-1β through the direct or indirect cleavage of their pro-peptides, thereby having a pro-inflammatory effect [86].

Antibody-coated target cells trigger a strong NK cell cytotoxic response named antibody dependent cellular cytotoxicity (ADCC), which can be seen as an example of coordinated actions of adaptive and innate effector mechanisms. When an antibody binds to a cell antigen, its Fc portion (carboxy-terminal constant region of an Ig molecule) can be recognized by low-affinity receptor for IgG (FcεRIγ), expressed by NK cells. Cross-linking of Fc receptors triggers NK cell activation and target cell destruction in a perforin dependent manner (Figure 3.4). ADCC is the dominant component of anti-cancer activity of currently applied antibodies in clinical settings against non-Hodgkin's lymphoma (αCD20, rituximab) and breast cancer (αHER2/neu receptor, trastuzumab) [87].

NK cells express at least three TNF family ligands – Fas ligand (FasL), TNF and TNF-related apoptosis inducing ligand (TRAIL), which mediate death receptor induced apoptosis of target cells (Figure 3.4). The engagement of the ligands induces a conformational change of death receptor and recruitment of adaptor proteins that initiate a complex series of events leading to apoptosis. The event crucial for apoptosis induction is the activation of signaling cascades whose main components are proteolytic enzymes caspases [88-89]. The usage of the specific lytic pathway depends on the tumor type, receptor/ligand expression by both effector and target cells and the way that NK cells are pre-activated. As an example, NK cell mediated metastases clearance and control of chemically induced tumors was shown to involve TRAIL [90-91]. Anti-metastatic effects achieved by IL-18 administration, rely on the FasL pathway [92]. Similarly, IFNγ produced by NK cells can induce Fas expression on cancer cells, followed by NK cell mediated target elimination [93].

3.2.2.2. NK cell cytokine production

Numbers of different cytokines are reported to be secreted by NK cells including IFNγ, TNFα, GM-CSF, IL-5, IL-10 and IL-13. IFNγ is seen as the most important effector cytokine produced by NK cells due to its crucial role in the early anti-viral and anti-bacterial response and orchestration of both innate and adaptive

cell activation against cancer [94]. IFNγ can target both tumor and host cells (Figure 3.5). Tumor cell responsiveness to IFNγ facilitates rejection via multiple mechanisms. IFNγ inhibits cell proliferation, induces sensitivity to apoptosis through its impact on caspase, FasL and TRAIL expression and makes tumor cell sensitive to CTL lysis via induction of MHC I and tumor antigen presentation. Host dependent anti-tumor effects are based on its role in polarization of Th1 responses, CTL maturation, macrophage activation and inhibition of angiogenesis and regulatory T cells generation [95].

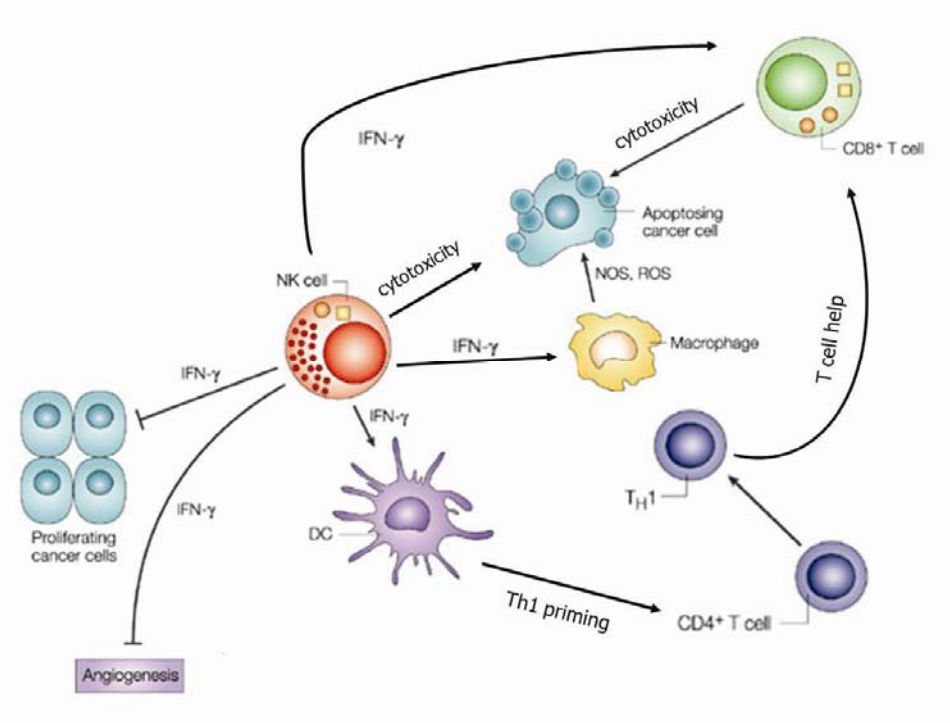


Figure 3.5. Central role of NK cell derived IFNγ in anti-tumor response. NK cell derived IFNγ inhibits angiogenesis and tumor cell proliferation and induces tumor cell sensitivity to apoptosis. IFNγ increases MHC I expression and activates CTLs and macrophages to destroy tumor cells. DC maturation and Th1 cell polarization is supported by IFNγ, while differentiation of regulatory T cells is inhibited. [96]

Complex microenvironmental signals control the cytokine production by NK cells. Cross-linking of activating receptors such as NKG2D, NK1.1, and NKp46 can induce IFNγ release *in vitro* [97-98]. Viral pathogens induce type I IFNs, which are also potent inducers of IFNγ [94]. IL-12, produced by macrophages, DCs and neutrophils at the site of infection, participates in the positive feedback loop by promoting IFNγ secretion, which in turn further potentiates IL-12 production. TNFα, IL-1 and IL-18 were described to further increase the IL-12 mediated release of IFNγ. In contrast, IL-10 and TGFβ are shown to suppress its production. When the amount of IL-12 and/or IL-18 is limited, co-triggering of activating NK receptors leads to maximal IFNγ induction. IL-18, initially described as IFNγ-inducing factor (IGIF) [99], exerts the most potent synergy with IL-12 regulating INFγ production by both NK and T cells [100]. The underlying mechanism of the synergistic action includes the reciprocal induction of the corresponding cytokine receptors and recruitment of different sets of transcription factors to the IFNγ promoter. The main

producers of IL-18 are macrophages, DCs, Kupffer cells and keratinocytes upon encounter of pathogens. The innate responses to intracellular microbes and viruses are amplified by IL-18 through its facilitation of cytokine (IFNγ, GM-CSF, IL-1β, IL-6, TNFα) and chemokine (IL-8) expression, induction of adhesion molecules and effector cell activation. IL-18 enhances neutrophil and NK cell cytotoxicity, Th1 responses in combination with IL-12, Th2 responses with IL-2 and Th17 responses with IL-23.

Due to the potent stimulating effects through both innate and adaptive immune effector arms, both IL-12 and IL-18 were widely exploited in therapeutic settings in mouse tumor models. Systemic IL-18 application has been shown to mediate the regression of poorly immunogenic B7-1 expressing mouse melanoma in a NK dependent manner [101]. IL-12 treatment induced suppression of metastatic growth by the enhancement of perforin dependent cytotoxicity, while in the same settings, IL-18 required tumor sensitivity to FasL [102]. However, IL-18 can have opposite pro-tumoral effects [100]. IL-18 dependent tumor growth promotion is correlated to its ability to support tumor angiogenesis through the induction of VEGF and hamper immune response by inducing FasL expression on tumor cells. Degradation of extracellular matrix by MMP-9 and expression of adhesion molecules on vascular endothelia, both induced by IL-18, support metastatic dissemination.

3.2.2.3. NK cell regulatory functions. Interaction with dendritic cells (DCs)

When a certain tissue is affected by pathogen invasion or malignant transformation, multiple immune effectors are recruited in response to released inflammatory mediators. Innate immune cells, which are first to invade the affected tissue establish multiple interactions both within each other and with resident cells, that can have multiple impacts on the quality and strength of ongoing innate and adaptive response that follows. It was shown that NK cell interaction with dendritic cells can shape both NK and DC effector functions (Figure 3.6) [66, 103]. The initial activation of tissue resident NK and dendritic cells is achieved via signals derived from tumor or virus infected cells in the environment and then further amplified through the NK/DC cross-talk. It was shown in the human system that the engagement of NKp30 in interaction with DCs induces TNFα release by NK cells, which in turn mediates DCs maturation. Activated DCs secrete IL-12 and IL-18, cytokines known to induce potent IFNγ secretion by NK cells. Such a cross-activation of NK and DCs is proposed to take place in invaded tissues at early time points when NK/DC ratio is relatively low. In addition, it can be highly relevant in lymph nodes, where the early release of IFNγ by NK cells plays a crucial role in T cell priming [104]. In a mouse model of MCMV infection, conventional DCs enhance NK cell lytic potential in IFNα and NKG2D dependent manner and IFNγ production through IL-12 and IL-18 production [105]. In tumor settings, adoptively transferred DCs promoted NK cell mediated control of MHC I deficient mesothelioma independently of IL-12 or type I IFNs [103].

Another possible result of the NK/DC interaction is the elimination of immature DCs by activated NK cells. In the human system, a major role in lysis of iDCs is played by TGFβ and the activating receptors NKp30 and DNAM-1 whose ligands are expressed by DCs. Since iDCs express MHC I molecules, killing is

mediated by CD94/NKG2A^{low} NK subset. Upon maturation, the increased density of MHC I protects mDC from NK cell mediated elimination. A proposed purpose of iDCs lysis is the elimination of DCs that might fail to mediate optimal T cell priming and might take place in the late phase of the response when NK/DC ratio is relatively high [106-108].

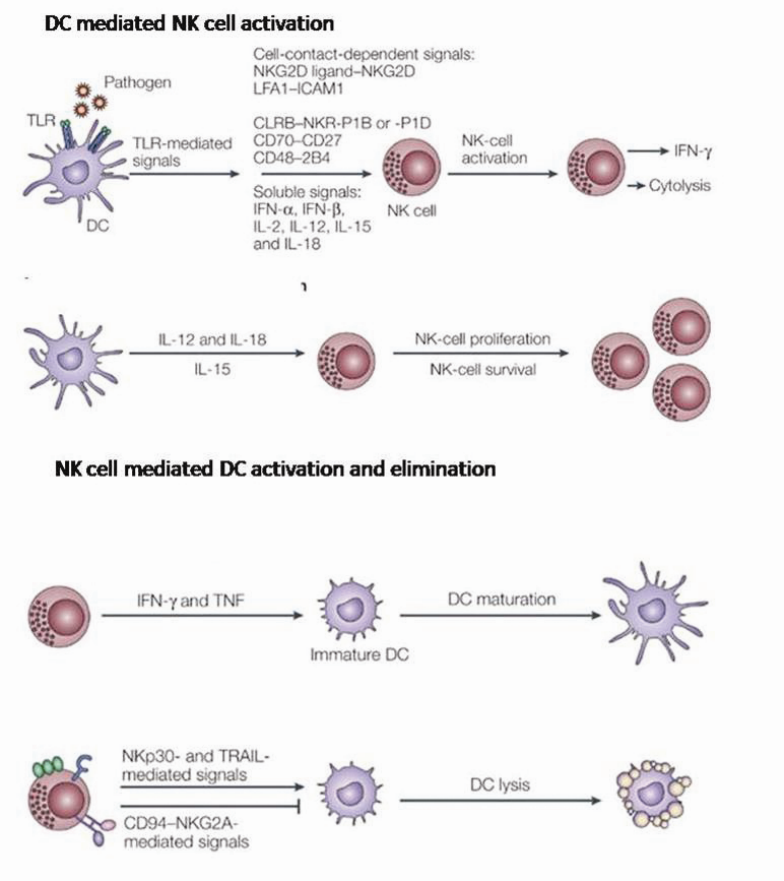


Figure 3.6. NK cell interaction with DC. DCs activated by the pathogen encounter increase NK cell cytotoxic response, IFNγ production, proliferation and survival through direct contact or via release of cytokines. In turn, activated NK cells support DC maturation or kill immature DCs providing optimal conditions for T cell priming. [109]

3.2.3. NK cell development, subsets and tissue distribution

The main pool of mouse peripheral NK cells develops from hematopoietic stem cells following a series of sequential stages of maturation, expansion and acquisition of specific NK receptors [110]. Every stage is defined by specific phenotypic and functional characteristics, although stimuli and limitations governing the progression through the stages are not completely understood. Committed NK cell precursors from bone marrow are lineage negative (Lin$^-$, CD3$^-$CD19$^-$Gr1$^-$) and express the IL-2/IL-15R common β subunit (CD122). Further NK cell development as well as survival in the periphery are completely dependent on IL-15. Committed Lin$^-$CD122$^+$ precursors at the next stage upregulate integrin α_v subunit and NK1.1 and

NKG2/CD94 receptors (Figure 3.7). Expression of tyrosine kinase c-Kit and acquisition of different Ly49 receptor family members are further events and precede the expansion stage in bone marrow. Fully mature NK cells are recognized by the expression of a variety of integrins like Mac-1 (α_M subunit associated with β_2, or CD11b) or DX5 (α_2 subunit associated with β_1 or CD49b), which are upregulated in the final maturation stage. In addition to integrins and in contrast to immature precursors, fully mature NK cells co-express CD43, known as leukosialin, low levels of c-Kit and no α_v, which are both downregulated as differentiation proceeds. Finally, fully mature NK cells show a functional competence concerning cytotoxic responses and IFNγ production.

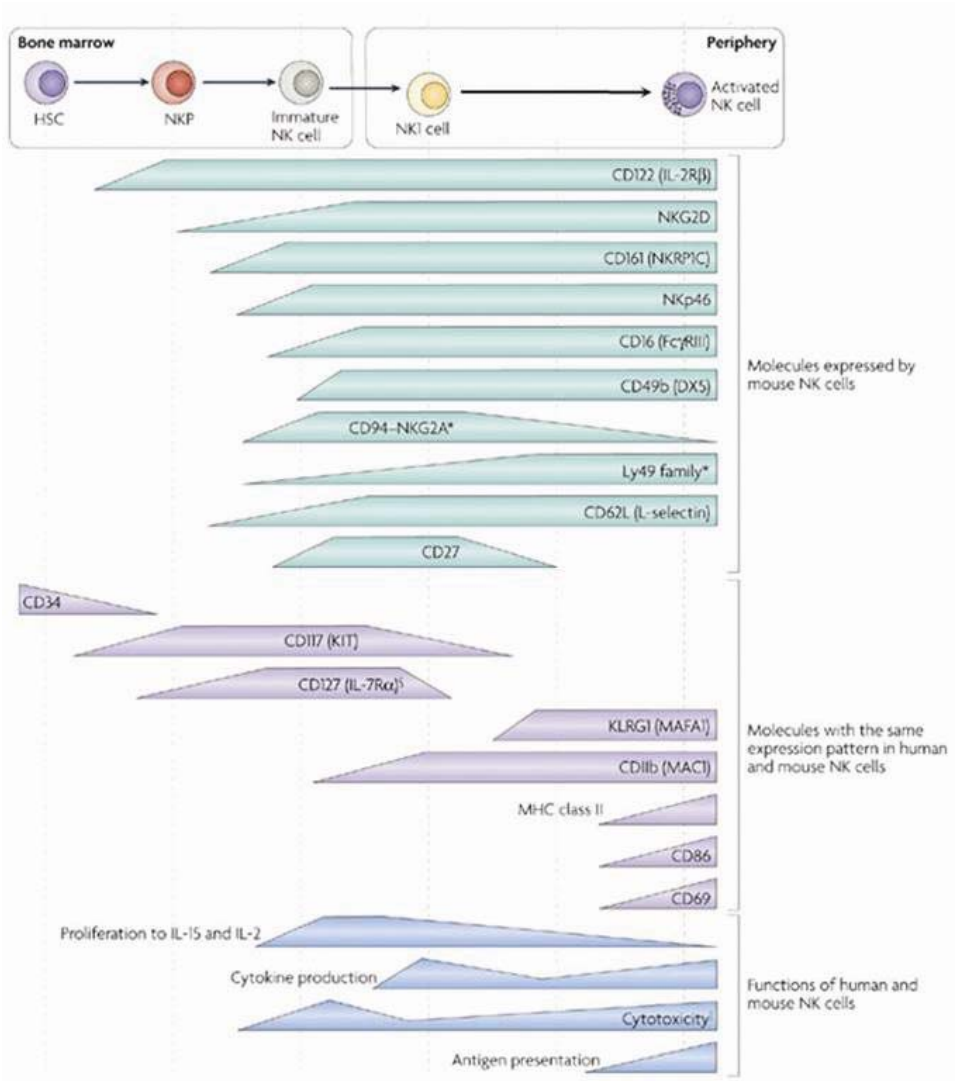


Figure 3.7. Model of murine and human NK cell development. NK cells originate from hematopoietic stem cell (HSC) and mature in bone marrow. Committed NK cell precursors (NKP) are lineage negative (Lin^-, $CD3^-CD19^-Gr1^-$) and express IL-2/IL-15Rβ (CD122). NK cell development and survival in the periphery are completely dependent on IL-15. The differentiation of the fully matured NK cells is characterized by the sequential acquisition of expression of NK cell receptor and adhesion molecules. Several molecules are expressed transiently during differentiation and therefore are used as the phenotypical markers for immature NK cells (c-Kit, CD117). The final step of maturation includes acquisition of functional competence for cytotoxic responses and IFNγ production. [111]

Although defined by different marker molecules, there is evidence that human NK cells follow a similar differentiation pathway *in vivo*. The maturation of human NK cells is characterized by the gradual upregulation of CD56 and CD11b and sequential upregulation of NK receptors: CD161 and NCRs, followed by CD94 and, at the final stage, CD16 and KIRs [112]. Human NK cells are defined as $CD3^{-}CD56^{+}$ and comprise two functionally distinct subsets. $CD56^{high}CD16^{-}$ NK cells express CCR7 and CD62L and thereby dominate in lymph nodes, where they can interact with DCs. They are very potent cytokine producers, while exerting poor cytotoxic responses against typical NK cell targets. On the other hand, they efficiently eliminate immature dendritic cells. The $CD56^{dim}CD16^{+}$ subset dominates in peripheral blood and inflamed tissue and shows high cytotoxic activity [113].

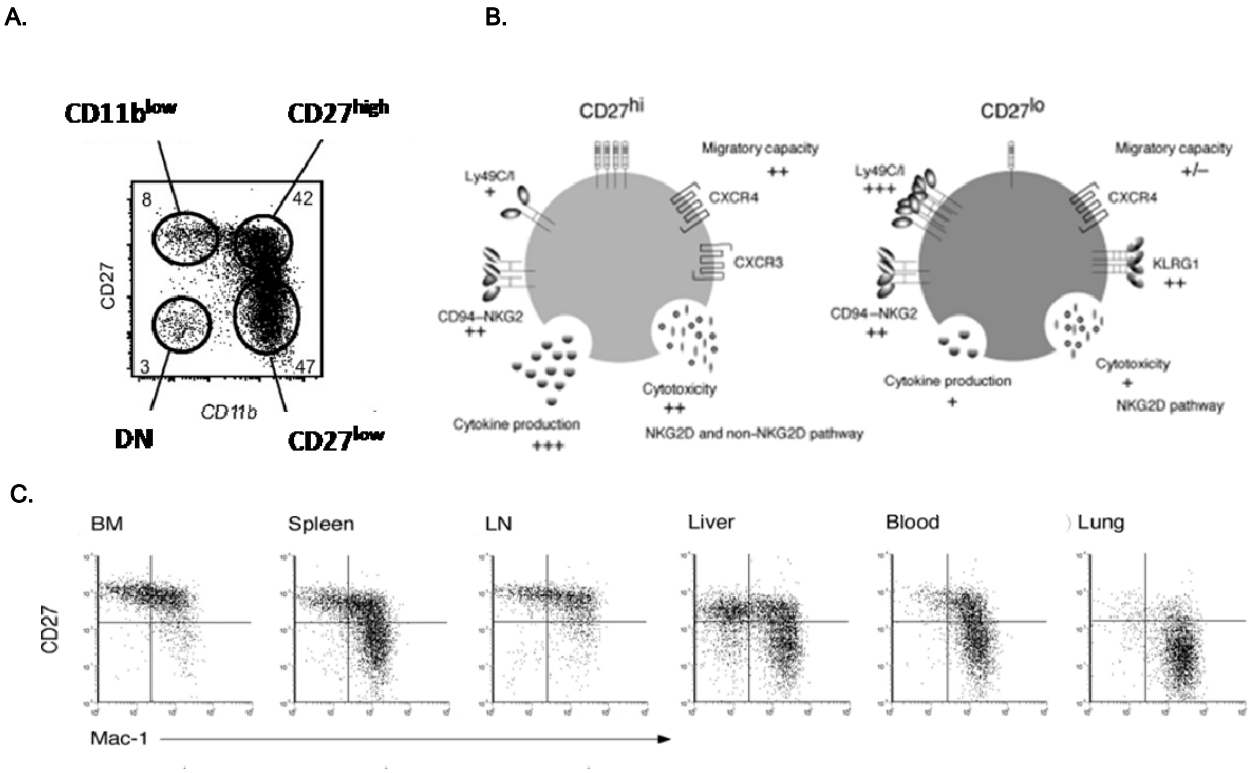


Figure 3.8. CD11b/CD27 based definition of NK cell maturation stages. (A) Four different subpopulations representing sequential maturation stages of NK cells: DN → $CD11b^{low}$ → $CD27^{high}$ → $CD27^{low}$. (B) Different phenotype and functional properties of mature $CD27^{high}$ and $CD27^{low}$ NK cell subsets. (C) Differential tissue distribution of various NK cell subsets; DN, double negative; BM. bone marrow; LN, lymph nodes

It has been recently described that the fully matured $CD11b^{high}$ NK cell pool can be further divided into two functional subpopulations according to the expression of the TNF family member receptor CD27 (Figure 3.8) [114]. $CD27^{low}$ NK cells represent the final maturation stage co-expressing inhibitory Ly49C/I receptors at high frequency, and KLRG1, inhibitory lectin-like receptor, but no early activation antigen CD69, c-Kit and CD127. This subpopulation dominates in non-lymphoid organs of adult mice, such as lung and peripheral blood, and has limited proliferation turnover. In contrast, $CD27^{high}$ subset still expresses low levels of CD69, c-Kit and CD127 markers indicating a less mature phenotype. Together with the immature

$CD11b^{low}$ subset, this subpopulation is dominant in bone marrow and lymph nodes, and represents the proliferating pool of NK cells. Surprisingly, the $CD27^{high}$ subset displays agreater capacity in migration towards certain chemokines, killing certain susceptible targets and producing IFNγ upon cytokine stimulation or co-culture with dendritic cells. It has been recently described that double negative $CD11b^{-}CD27^{-}$ NK cells comprise precursors of CD11b and/or CD27 expressing subsets, suggesting a linear model of NK cell development (Figure 3.8A) [115].

Among the total NK cell pool in the periphery, a population characterized by the expression of IL-7Rα (CD127) has been found to originate in thymus and strictly depend on the transcription factor GATA-3 and IL-7Rα [116]. Thymic NK cells are the most abundant in the thymus and lymph nodes. Compared to the mature bone marrow derived NK cells, they show a specific $CD11b^{low}CD16^{-}CD69^{high}Ly49^{low}$ phenotype, reduced cytotoxicity and enhanced cytokine production. Interestingly, a functionally similar human $CD56^{bright}$ NK subset, predominating in human lymph nodes, expresses CD127, while in blood and spleen a dominant $CD56^{dim}$ subset is highly cytotoxic and CD127 negative.

NK cell development can take place at some additional anatomical places from different precursor cell pools. NK cells can potentially arise from bipotent T/NK precursors found in fetal liver, spleen and blood [117]. In addition, a subset of immature thymocytes on both a double negative $CD4^{-}CD8^{-}CD44^{+}CD25^{-}$ and, to a lesser extent, double positive $CD4^{+}CD8^{+}CD44^{+}CD25^{+}$ maturation stage can differentiate toward NK cells [118]. A subpopulation of lymph node cells in mice resembling immature thymocytes is also found to differentiate into NK cells *in vitro* [119].

NK cells found in different organs can have different phenotype and exert different functional characteristics. However, it is still not clear whether mature NK cells modify their phenotypic and functional attributes in defined microenvironmental conditions or immature precursors mature in those conditions giving rise to divergent NK cell functional subsets. Based on the CD11b/CD27 subset definition, the spleen NK cell compartment is composed of all four subsets, immature NK cells dominate in bone marrow, while at peripheral sites such as blood and lungs, preferentially mature $CD27^{low}$ NK cells can be detected. Lymph nodes contain low percentages of NK cells that are mainly $CD27^{high}$ and express CD127. Since those cells have a high capacity of cytokine production, they could play a very important role during T cell activation by providing IFNγ for Th1 polarization [120].

3.2.4. NK cells with specialized functions

Liver NK cells

Mouse liver preferentially contains NK cells of the immature $CD11b^{low}DX5^{low}$ phenotype that express TRAIL, but no Ly49 receptors, perforin and granzymes. $TRAIL^{+}$ NK cells are predominant in fetal and neonatal mice being distributed in spleen and other peripheral lymphoid organs. Their number decreases with age and they are retained only in the liver. On the one hand, the liver can represent the pool of

immature NK cells that can differentiate into fully mature effectors in a short time period. On the other hand, TRAIL expressing NK cells can have very well defined but still not fully discovered biological purposes, including elimination of potentially dangerous targets arising in liver, including metastasing tumor cells [121]. In addition, evidence exists that the total pool of NK cells residing in the liver could contain cells with memory properties [122].

Uterine NK cells

Uterine NK cells in human were shown to invade the decidua early in gestation at the time of implantation of the fetal trophoblast. They constitute of up to 70% of the immune cells present in the deciduas during the first trimester of pregnancy. Human decidual NK cells cosist of the predominately $CD56^{bright}CD16^{-}$ phenotype with special functional properties. They promote the migration of trophoblast and vascularisation of placenta by the production of IL-8, CXCL10, VEGF and PDGF [123]. Mouse uterine NK cells have an unusual phenotype distinct from splenic NK cells [124]. Similar to the immature NK cell precursors, they do not express NK1.1 and DX5, while expressing low levels of CD11b and high levels of CD69 and c-Kit. However, unlike immature NK cells, uterine NK cells express Ly49 receptors with a repertoire qualitatively and quantitatively different compared to the NK cells found in the spleen.

Gut NK cells

Recent studies have shown that both human and mouse gut-associated lymphoid tissue harbor NK cells with unique phenotypical and functional properties, whose main characteristics are the production of IL-22. IL-22 is a member of the IL-10 cytokine family essential for host defense at the musical barriers. The IL-22 receptor is expressed on epithelial cells and its triggering induces the production of multiple anti-microbial mediators [125-126]. In mucosal areas of human tonsils and Payer's patches a specialized subset of $NKp44^{+}$ NK cells expressing the chemokine receptor CCR6 is identified to produce IL-22 [127]. In mouse, the analysis of the *lamina propria* and the intraepithelial surfaces of small intestine revealed a minor subpopulation of mature classical NK cells, expressing Ly49 receptors, perforin and IFNγ, and an additional IL-22 producing NK cell subset that express CD127 (IL-7Rα), CD117 (c-Kit), but not Ly49 receptors, perforin and IFNγ [128-130]. While conventional NK cells require IL-15 for their development and homeostasis, thereby are absent from $IL\text{-}15^{-/-}$ and $IL\text{-}2\gamma c^{-/-}$ mice, IL-22 producing NK cells depend on the transcription factor RORγt and commensal flora and develop normally in the absence of IL-15 signal.

Interferon-producing killer DC (IKDC)

A number of recent publications have described a cell population with dual nature, combining the properties of both DCs (antigen presentation, IL-12 secretion) and NK cells (lytic capacity and IFNγ production) [131-132]. The phenotypical characterization indicated that those cells could be found within the $CD11c^{+}$ (DC marker) $NK1.1^{+}$ (NK cell marker) spleen subpopulation. However, it is still not clear if the dual NK/DC function can be performed by a single cell type within the $CD11c^{+}NK1.1^{+}$ population and whether it is exerted at the same time or sequentially acquired during ongoing response. Indeed, the study

of Ullrich *et al.* indicated that upon IL-15 trans-presentation those cells lose their DC potential and become typical NK effectors [133]. Commonly named natural killer DC (NKDC) or interferon-producing killer DC (IKDC), this population is further defined by the co-expression of B220 (CD45R) and DX5, while T cell (CD3), B cell (CD19) and granulocyte-monocyte (Gr1) markers are absent. More recent studies revealed their close relationship with NK cells, supported by the finding that IKDC express the typical NK cell marker NKp46, as well as Ly49 receptors, NKG2D and CD122 [134-135]. In addition, their dependence on IL-15 and the fact that NK cells upregulate CD11c and B220 upon stimulation, support the view of IKDCs being a subpopulation of activated NK cells. Upon target encounter and elimination, IKDCs upregulate MHC II and costimulatory molecules (B7-1/2, CD40, OX40L), a process shown to depend on IFNγ, and are able to activate $CD4^+$ and $CD8^+$ cells [136-137]. Similar phenotypical and functional properties can be attributed to the NK cells, activated through target cell recognition and lysis [138].

In spleen of naive C57BL/6 mice, approximately 1% of all $CD11c^+$, and about 10% of $CD3^-NK1.1^+$ NK cells phenotypically correspond to IKDCs [139]. More importantly, IKDCs were found to accumulate in tumor tissue and mediate potent anti-tumor activities. B16 melanoma progression in mice can be markedly inhibited by the adoptive transfer of $CD11c^+B220^+$, but not $CD11c^-B220^-$ NK cells into $Rag^{-/-}IL\text{-}2R\gamma c^{-/-}$ tumor bearing mice. A current hypothesis suggests that the dual function of IKDCs (or activated NK cells) can be beneficial during early responses to tumor and pathogens, due to their fast activation and potential to eliminate targets, uptake antigens, accumulate in lymph nodes and prime T cell responses.

3.2.5. NK cells and cancer

3.2.5.1. NK cells and cancer immunosurveillance

Cancer immunosurveillance represents a concept, which predicts that the immune system recognizes and in most cases destroys precursors of cancer before it becomes clinically apparent [140]. There are numerous experimental data indicating that different components of immune response are implicated in cancer surveillance (Table 3.4), including both the adaptive and the innate immune system. The involvement of NKG2D, IFNγ, perforin and TRAIL mediated mechanisms in the process strongly suggests that NK cells could play a very important role in the control of tumor development. However, it is clear that the role of a single cell type and different mechanisms applied by a given cell, depends on the tumor type and the strategy that particular developing tumor uses to evade the response of the immune system. For example, MCA induced sarcomas show higher incidences in DNAM-1, but not in NKG2D deficient mice. On the other hand, NKG2D is implicated in the control of development of Eμ-Myc driven B cell lymphomas [42].

Mouse strain	Tumorigenesis promoting system	Tumor type	Defective immune component
Spontaneous tumors			
129/Sv	-	Colon, Lung	RAG2
129/Sv	-	Colon, mammary	RAG2 and STAT1
C57BL/6	-	B cell lymphoma	β_2m and perforin
C57BL/6	-	Lymphoma	TRAIL
C57BL/6	-	Lymphoma	perforin
Transgenic and knockout tumor models			
129/Sv	Tp53-/-	Lymphoid and other	STAT1
129/Sv	Tp53-/-	Lymphoid and other	IFNγR
C57BL/6	Tp53-/-	Lymphoid and other	TRAIL
C57BL/6	Tp53-/-	Lymphoid	perforin
C57BL/6	Tp53-/-	Lymphoid and other	TCRJα28 and CD1d
C57BL/6	TRAMP	Prostate	NKG2D
C57BL/6	TRAMP	Prostate	TCRδ
C57BL/6	Eμ-Myc	B cell lymphoma	NKG2D
C57BL/6	Eμ-Myc	B cell lymphoma	TRAILR
C57BL/6	Eμ-Myc	B cell lymphoma	RAG1
Carcinogen induced tumors			
129/Sv	MCA	Fibrosarcoma	RAG2
129/Sv	MCA	Fibrosarcoma	IFNγ
129/Sv	MCA	Fibrosarcoma	IFNγR
129/Sv	MCA	Fibrosarcoma	STAT1
129/Sv	MCA	Fibrosarcoma	RAG2 and STAT1
C57BL/6	MCA	Fibrosarcoma	IFNγ
C57BL/6	MCA	Fibrosarcoma	perforin
C57BL/6	MCA	Fibrosarcoma	TRAIL
C57BL/6	DEN	Hepatocarcinoma	TRAILR
C57BL/6	MCA	Fibrosarcoma	TCRJα28
FVB	MCA	Fibrosarcoma	TCRβ
FVB	MCA	Fibrosarcoma	TCRδ
FVB	DMBA and TPA	Cutaneous	TCRδ
C57BL/6	MCA	Fibrosarcoma	TCRδ

Table 3.4. Immune deficiencies associated with greater tumor incidence or tumor severity in mice. [42] MCA, methylcholanthrene; DMBA, 7,12-dimethylbenzanthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate; DEN, diethylnitrosamine

The broader concept of immunoediting states that the complex interplay between the cancer and immune system eventually leads to progressive tumor outgrowth. It is proposed that cancer usually develops through the series of steps characterized by events that all together can alert the immune system (Figure 3.9). Elimination, equilibrium and escape are the three phases of immunoediting [95].

Elimination. Activation of oncogenes or/and mutation affecting tumor-suppressor genes can induce DNA damage, heat-shock and other responses that result in cell cycle arrest, cell senescence and apoptosis. At the same time, those mechanisms have been shown to induce numerous ligands on the surface of affected cells (e.g. upregulation of NKG2D ligands upon DNA damage [141]) allowing the immune system to be alerted for potential danger and eliminate transformed cell.

Equilibrium. If some tumor cells are not destroyed in the elimination phase, they can persist, being prevented to expand by the immune system, in the so called equilibrium phase.

Escape. Under the selective pressure of immune response, further mutations in malignant cells, which affect the genes regulating metabolic processes, proliferation, survival and sensitivity to immune destruction, can help the evasion of protective mechanisms leading to the development of cancer. For example, tumor cells can downregulate MHC I, antigen-processing machinery, death receptors and other apoptosis mediators. The direct or indirect immune system inactivation involves the expansion and recruitment of suppressive cell populations and production of suppressive mediators, such as TGFβ, IL-10, IL-13, IDO, arginase-1 and others. In order to become invisible, tumor cells can differentially regulate the expression of ligands that can be recognized by the immune system. Expression of NKG2D ligands can be downregulated by shedding the proteins from the cell surface by metaloproteinases [142]. In other cases, high expression of NKG2D ligands can be exploited to downregulate the receptor on immune effector cells due to the chronic engagement, which can lead to the impairment of NKG2D as well as other activation pathways [142-147]. In patients with MICA positive tumors, soluble MICA ligands have been detected in serum, which correlated with the downregulation of NKG2D on effector cells and impairment in their responsiveness [143]. Similarly, AML patients show impaired NK cell activity correlated to low expression of natural killer cell triggering receptors (NCRs) [148].

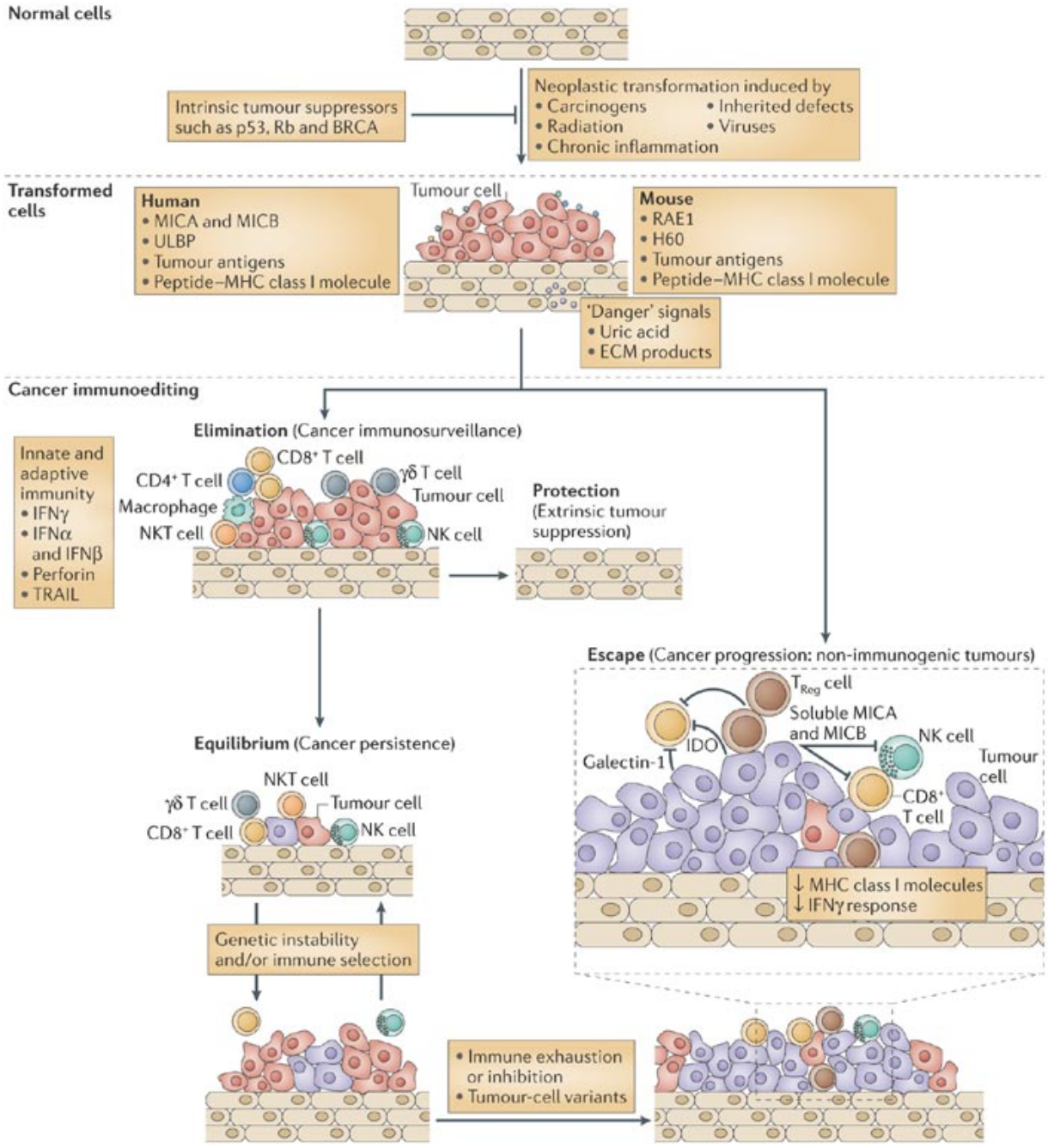


Figure 3.9. Cancer immunoediting. [95] The process of malignant transformation leads to the upregulation of danger signals and the expression of various molecules that alert the immune system. Transformed cells are destroyed by immune effector cells in the phase of elimination. In the equilibrium phase, small numbers of cancer cells that were not eliminated persist, but do not proliferate due to the control established by the immune system. Genetic instability and immune selection result in the evasion of protective mechanisms leading to the development of cancer, which further inhibits immune responses through various mechanisms. ECM, extracellular matrix; IDO. Indolamine 2,3-dioxygenase

3.2.5.2. NK cell based cancer immunotherapy

NK cells are recognized as potent anti-tumor effectors *in vitro* and in mouse models *in vivo*. Multiple NK cell effector mechanisms can be exploited for anti-cancer therapy, both direct (destruction of tumor targets) and indirect (activation of adaptive responses via DCs). Up to date, various strategies have been designed to implement NK cells in anti-tumor therapeutic settings (Table 3.5).

Treatment		Clinical application	Clinical effects	Effects on NK cells
Recombinant cytokines				
IL-2	i.v.	Melanoma	16% OR	High toxicity
IL-2	i.v. or s.c.	RCC	15% OR	
IL-2	+ LAK	RCC	21% OR	
IL-2	+ IFNα	RCC	20.6% OR	
IL-2	+ BMT	BC, NHL	No improvement	↑ IFNγ in serum, ↑ PBMC cytotoxicity
IL-2	s.c.	Advanced cancer	-	↑ NK number, cytotoxicity, IFNγ, ADCC
IFNα	i.v.	CML	60-80% OR	↑ NK cytotoxicity, IFNγ
IL-12	i.v.	Melanoma, RCC	1PR, 3SD (n=14)	↑ IFNγ, IL-15, IL-18 in serum
IL-12	i.v.	Advanced cancer	-	↑ NK cytotoxicity, LFA-1
IL-12	i.v. + IL-2	Melanoma, RCC	1 PR (n=28)	↑ NK number, ↑ IFNγ, CXCL10 in serum
IL-18	i.v.	RCC, melanoma, HD	2 PR (n=28)	↑ NK FasL ↑ IFNγ, GM-CSF, IL-18BP in serum
IL-21	i.v.	Melanoma	1 CR (n=29)	↑ NK cytotoxicity ↑ Prf, GzmB mRNA in PBMCs
Flt3L	s.c. + BMT	HD, BC	-	↑ circulating iDCs
Vaccines				
Hsp96	s.c.	Colorectal cancer	33% DFS 79% 24-months OS	↑ NK cytotoxicity, IFNγ, NKG2D, NKp46, CD69, CD25 ↑ CD40, CD83, IL-12 by PBMCs
Hsp96	+ IFNα + GM-CSF	Melanoma	11 SD (n=38)	↑ NK IFNγ, ↑ T cell activity
CpG	i.v.	NHL	2 PR (n=23)	↑ NK cytotoxicity
MD-DC+CEA		Metastatic CEA^+ cancer	5 SD (n=9)	↑ NK number, cytotoxicity
DEX	s.c.	Melanoma	1 PR, 2 MR, 2 SD (n=15)	↑ NK number, cytotoxicity, ↑ NKG2D

Antibody therapeutics				
Rituximab	+ IL-2 s.c.	B cell NHL	53% OR	↑ NK number, ADCC
Rituximab	+ IL-2 s.c.	Indolent NHL	8.8% OR	↑ NK number, ADCC
Rituximab	+ IL-2 i.v. + LAK	B cell NHL	1 PR, 4 SD (n=10)	↑ NK number, ADCC
Trastuzimab	+ IL-12 i.v.	BC	1 CR, 2 SD (n=15)	↑ NK IFNγ, ADCC
αCD16/CD30 bispec Ab	i.v.	HD	25% OR	↑ NK cytotoxicity
αCD16/CD30 bispec sAb	+ IL-12 + GM-CSF	HD	29% OR	↑ NK number, ADCC
Daclizumab		Uveitis		↑ NK number, IL-10
Daclizumab	+ IFNβ	MS	70% CNS lesion reduction	↑ NK number, cytotoxicity against activated T cells

Table 3.5. Clinical trials using strategies to modulate NK cell function. [87] i.v., intraveneous; s.c., subcutaneous; LAK, lymphokine activated killer cells; BMT, bone marrow transplantation; RCC, renal cell carcinoma, BC, breast cancer; NHL, non-Hodgkin's lymphoma; CML, chronic myeloid leukemia; HD, Hodgkin's lymphoma; MS, multiple sclerosis; OR, overall response; PR, partial response; SD, stable disease; CR, complete response; DFS, disease-free survival; OS, overall survival; CNS, central nervous system; Prf, perforin; GzmB, granzyme B

Use of autologous NK cells

The systemic or local application of multiple factors previously known to enhance NK cell functions has been shown to be beneficial in mouse tumor models. IL-2 suppresses many experimental tumors by NK cell dependent mechanisms that require perforin and/or IFNγ [90, 102]. Positive effects are reported for IL-12 [84], IL-15 [149-150], IL-18 [102] and IL-21 [151-152].

In humans, Rosenberg and colleagues performed pioneering studies in advanced renal cancer and melanoma patients applying the adoptive transfer of *ex vivo* expanded autologous NK cells together with IL-2, however with no benefits compared to IL-2 alone [153]. In addition, high dose of IL-2 used for the activation of both endogenous NK and T cells, although beneficial, has been shown to have toxic side effects and can exert additional negative action through T regulatory cells expansion or induction of activation-induced cell death (AICD) of NK cells [154-155]. Moreover, toxicities have precluded the use of IL-12, although striking effects have been achieved in mouse models [156]. In contrast, IL-21 was shown to be effective with no adverse effects [157-158]. Therefore, it has a good potential to be implemented in the future therapy designs.

NK cell proliferation, expression of activating receptors as well as lytic and secretory potential can be enhanced by other cytokines, such as IL-15, IL-18 and type I IFNs, which are shown to be efficient when

used in combination therapies with other agents, e.g. IL-15 together with haploidentical NK cell transfer in patients with poor-prognosis AML. Essentially, any treatment that harness the ability of DCs to mediate NK cell activation (e.g. TLR agonists such as CpG) is potentially mediated by IL-12, IL-15 and IL-18, produced by DCs and macrophages [159].

Many drugs used in current anti-cancer clinical protocols are proven to work at least in part via NK cells. Examples include *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) treatment of bladder cancer [160-161] or tyrosine kinase inhibitor imatinib mesylate (Gleevec), used for patient with gastrointestineal stromal tumors [162]. The efficiency of antibody based therapeutics is to a large extent contributed by NK cell mediated ADCC [163-164].

Use of allogenic NK cells

The best example of the use of allogenic NK cell is provided in the haploidentical stem cell transplantation in patients with AML that greatly improves disease free survival and reduces relapse rates [165]. Unlike T cells, donor-derived NK cells in these settings do not mediate GVHD, but exert a strong GVL effect. Together with NK cells developed from donor hematopoietic stem cells, they do not only eliminate residual tumor cells, but also recipient's APCs, preventing antigen presentation and activation of alloreactive T cells. A prerequisite for the successful treatment is the KIR-ligand mismatch or in other words, the recipient should lack one or more HLA I ligands that engage donor inhibitory KIRs. A similar approach, called NK cell based donor lymphocyte infusion, is used in high risk leukemia patients after relapse. It is based on the infusion of highly purified haploidentical NK cells that can mediate potent anti-tumor effects [166-167].

Haploidentical NK cell infusions together with IL-2 were given by Miller and colleagues in non-transplantation settings to renal cell carcinoma, Hodkin's disease and AML patients. When high doses of IL-2 were applied, donor NK cell proliferation has been observed. A long term survival in this study has been correlated with KIR mismatch [168].

Trials with adoptive transfer of the NK cell line NK-92 have also been performed and found to be safe. The advantages of the NK-92 line could be seen in easy production, no expression of inhibitory KIR molecules and the possibility of *in vitro* manipulation and adjustments. The efficacy of the treatment is to be analyzed in future studies [169].

Crucial issues for NK cell based anti-cancer therapy

Although the knowledge about NK cell anti-tumor effects is increasing, the clinical efficacy in human trials using NK cell effector responses in different ways has been modest. A possible reason can be that most of the studies have been performed in patients with heavy tumor burdens for whom conventional therapeutic approaches were ineffective. It is known from the studies in mice that NK cells have a very limited capacity to impact on established malignancies. The most effective use of NK cells is considered to be in situations when tumor burdens are relatively low, e.g. in patients with minimal residual disease, post surgery,

through other treatments or following relapse. In addition, approaches used so far are to be further improved via the manipulation of NK cell numbers, activation state and activating and inhibitory receptor repertoire, choosing subpopulations with desired functional capacities and enhancing trafficking to the tumor site. NK cell based therapies against large solid tumors still remain a challenge, until sufficient knowledge is acquired about their homing to the tumor site, migration through tumor tissue, interactions with both tumor cell and other immune effectors, including inhibitory populations, and all other forces that can impede or improve NK cell anti-tumor actions.

3.3 B7 family

Naive T cells require two signals for activation [170]. The first signal is delivered by the engagement of the T cell receptor (TCR) by proper MHC-peptide complex. Naïve T cells provided solely by a TCR signal (if this signal is quantitatively not very strong) are rendered anergic and become inactivated. The second signal required for activation is delivered through costimulatory receptors, primarily CD28, and cytokines provided by the microenvironment. CD28 belongs to the B7 receptor-ligand family, which is mainly implicated in the regulation of T cell activation and effector function [171]. While positive regulators of the family support T cell activation, negative regulators are important for the prevention of inappropriately directed responses, e.g. to self-antigens, and for limiting the size and duration of an immune response, thereby preventing immune mediated tissue and organ damage.

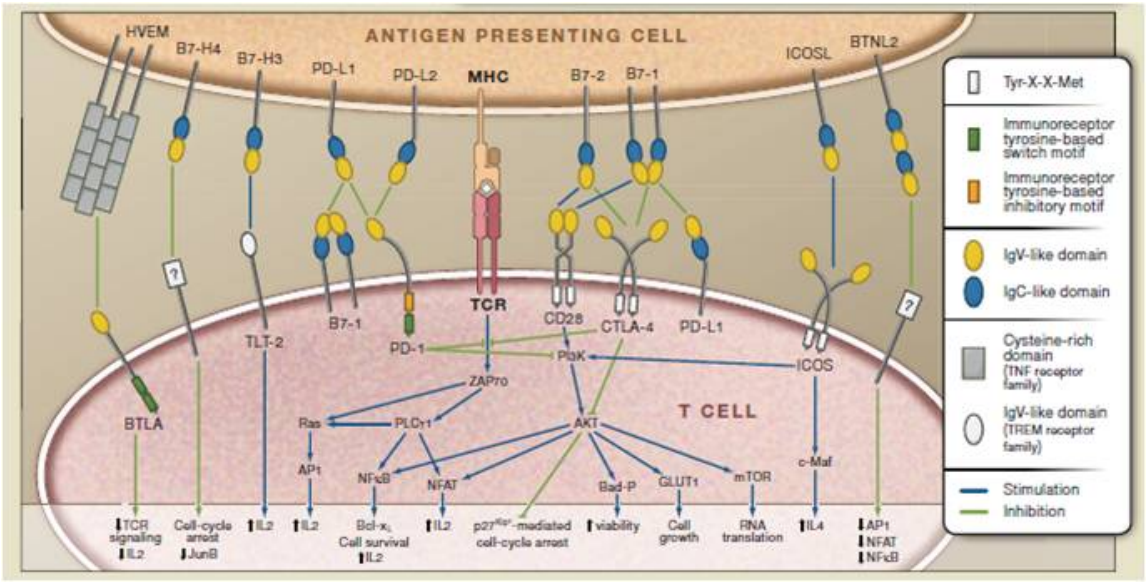


Figure 3.10. Structural characteristics, binding partners and the functional consequences of B7 family activation [172]. The prototypic B7 family receptors CD28, CTLA-4 and PD-1 engage B7-1/2 and B7-H1/PD-L2 ligands and deliver activating (CD28) or inhibitory (CTLA-4, PD-1) signals. However, reverse signaling of the ligands has been also reported. Some B7 members interact with non-B7 molecules, such as HVEM (TNF family), NKp30 (IgG family) and TREML2.

The members of the CD28/B7 family do not function independently but rather modify primary signals delivered by the TCR. The receptors of the B7 family are type I transmembrane proteins with a conserved IgV domain, while ligands are type I transmembrane molecules with both IgV and IgC domains (Figure 3.10) [173]. Within the family a single receptor can bind to multiple ligands and a single ligand can engage more than one receptor. In addition, bidirectional signaling has been reported for several interaction partners [52]. The final outcome of regulation through B7 family members will then depend on the established interaction type and the integration of the signals delivered from different family members with TCR derived signals. In addition, tight spatial and temporal regulation of the expression of different receptor-ligand pairs contributes by adding a new level to the diversity of B7 driven regulation.

3.3.1. Costimulation through B7 family members

3.3.1.1. CD28

CD28 is constitutively expressed by both resting and activated mouse T cells [171]. In the absence of TCR signaling its engagement does not play a significant role. When the TCR is triggered simultaneously, CD28 decreases the threshold for T cell activation and promotes T cell survival, expansion and proliferation. The main effects of CD28 co-engagement are increased transcription and stabilization of IL-2 mRNA that supports proliferation [174] and up-regulation of anti-apoptotic protein Bcl-X_L that supports survival [175]. CD40L, ICOS, OX40 and CD137 (4-1BB) are also induced by CD28 and further contribute to T cell activation providing the costimulatory signals of different quality. In addition, the increased expression of a glucose transporter and glycolytic enzyme activity as a consequence of CD28 triggering provides metabolic requirements for the cell division and effector function [171]. Recruitment and activation of phosphoinositide 3-kinase (PI3K) appears to be essential for CD28 mediated costimulation. The function of inhibitory B7 family receptors PD-1 and CTLA-4 is partially based on the counteraction of CD28 effects by targeting proximal signaling molecules in its pathway. PD-1 targets PI3K, while CTLA-4 inhibits more downstream Akt activation via the phosphatase PP2A [176]. CD28 deficient mice exhibit impaired T cell responses [177] and reduced disease severity in murine models of arthritis, EAE and musosal lung inflammation [178-180].

CD28 engages two ligands, B7-1 and B7-2, that have different spatial and temporal regulation of expression, and are also recognized by the B7 inhibitory receptor CTLA-4. B7-2 is expressed constitutively at low levels on B cells, DCs and macrophages, but it is rapidly up-regulated following activation and plays an important role in the initiation of immune responses. B7-1 expression is induced in the span of several days upon activation, which makes it more important for the regulation of sustained T cell activation in the later phases of effector responses [181]. Although binding to the same receptors, due to the different binding affinities and expression patterns, B7-1/2 ligands induce different functional outcomes.

For example, while B7-1 engagement preferentially favors Th1 responses, B7-2 engagement augments IL-4 production and Th2 differentiation [182].

There is evidence that both B7-1 and B7-2 can deliver reverse signals that differentially affect DCs. CD28 engagement by B7-1/2 expressed on DCs leads to IL-6 production and subsequent T cell immunostimulation [183]. On the other hand, binding of CTLA-4 induces IFNγ, which acts in an autocrine manner and induces indolamine 2,3-dioxigenase (IDO) synthesis by DCs [184]. Released in the microenvironment, IDO degrades tryptophan to kynurenines, which inhibit T cell proliferation and induce apoptosis [185]. Its induction is one of the mechanisms used by Treg to repress T cell function, owning to their constitutive expression of CTLA-4 [186].

3.3.1.2. ICOS

ICOS is a costimulatory receptor expressed as a homodimer on activated and memory T cells [187]. The expression of ICOS is positively regulated, although not absolutely dependent, upon TCR and CD28 engagement. It typically functions distally to the CD28 delivered costimulation. While the CD28 costimulation is critical in the priming phase and necessary for IL-2 production and T cell expansion, ICOS provides the signal for the sustained regulation of previously activated T cells. Its engagement leads to the enhancement of cytokine (IFNγ, TNFα, GM-CSF, IL-10, IL-4, IL-5 and IL-13, but not IL-2) production and effector T cell function [71]. Although upregulated on both Th1 and Th2 cells, ICOS is found to costimulate Th2 responses more effectively. In addition, ICOS plays a very important role in antibody responses and germinal center formation. The engagement of ICOS by ICOS-L expressed on B cells could be a critical event for the activation of the CD40:CD40L pathway, necessary for the development of humoral immune responses [188]. Its ligand is constitutively expressed by B cells, macrophages and dendritic cells and can be further modulated by inflammatory stimuli such as IFNγ, TNFα, GM-CSF or LPS. Interestingly, ICOS-L is highly expressed on immature DCs and B cells and its expression is reduced in the presence of bacterial stimuli and BCR triggering, respectively [59].

3.3.2. Inhibition through B7 family members

3.3.2.1. CTLA-4

In both mouse and human, the *ctla-4* gene is mapped to the same chromosomal region as *cd28* and considered to arise by duplication of an ancestral costimulatory gene. On protein levels these two molecules share 30% identity. Interestingly, the cytoplasmic tail of the CTLA-4 protein is 100% conserved among mammalian species [189]. CTLA-4 is encoded by 4 exon gene encoding leader peptide, ligand-binding site, transmembrane region and cytoplasmic tail, respectively. In human and mouse the CTLA-4 transcript undergoes alternative splicing giving rise to full-length CTLA-4 (flCTLA-4), soluble

CTLA-4 (sCTLA-4), a transcript coding only exon 1 and 4, and, in mouse only, ligand-independent CTLA-4 (liCTLA-4) [190]. Upon TCR engagement, CTLA-4 mRNA is detected within 1h peaking at 24-36h post-stimulation. The stability of the transcript increases in the presence of CD28 costimulation. CTLA-4 protein can be detected on T cell surface after 24-48 h [191]. However, most of the protein is retained intracellularly and only about 10% is exposed on the cell surface [192]. The trans-Golgi network (TGN) as well as endosomal and lysosomal compartments were identified to comprise CTLA-4 intracellular pools (Figure 3.11). Intracellular trafficking of CTLA-4 is tightly regulated by association with adapter proteins. At the Golgi site, CTLA-4 cytoplasmic tail interacts with TRIM, which mediates its transport to the surface, or AP-1, which targets it to lysosomes. Surface exposed CTLA-4 has a very short half-life, since it is internalized upon binding of AP-2, and targeted to endosomes and lysosomes for degradation. Upon T cell activation, the CTLA-4 cytoplasmic tail is phosphorylated, which prevents AP-2 binding and stabilizes CTLA-4 surface expression. In addition, the level of surface expression can be further enhanced by the activation induced secretion of CTLA-4 enriched lysosomes [192-194].

A. B.

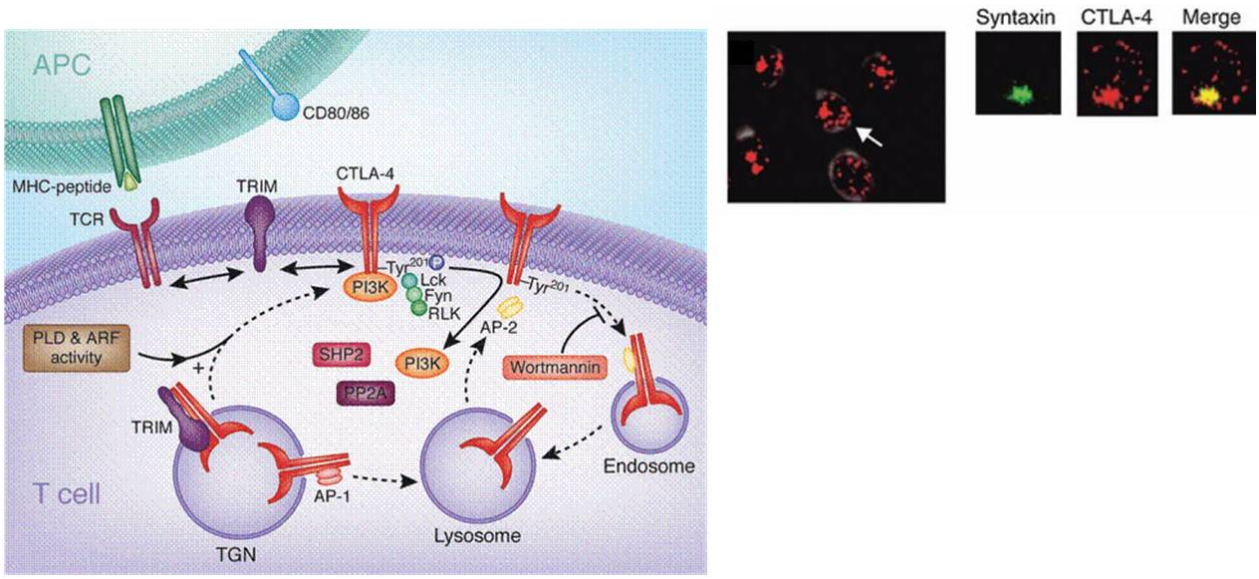


Figure 3.11. Intracellular trafficking of CTLA-4. [195] (A) Newly synthesized CTLA-4 binds to the transmembrane adapter TRIM in the TGN promoting the formation of CTLA-4-containing vesicles and their transport to the cell surface. On the cell surface, CTLA-4 and TRIM no longer associate allowing TRIM to interact with other receptors, possibly the TCR complex. Shuttling to the lysosomal compartment from the TGN occurs due to adapter AP-1 binding to CTLA-4. On the surface, CTLA-4 becomes phosphorylated by kinases Lck, Fyn, and Rlk leading to the association of PI3K and possibly other proteins. Phosphorylation retards internalization. Dephosphorylation allows binding to the clathrin adapter AP-2 and rapid internalization to endosomes and lysosomes. Upon T-cell activation, CTLA-4 enriched lysosomes and endosomes are recycled to the cell surface. (B) Pattern of intracellular CTLA-4 staining in WT T cells. Cells were labeled with anti-Syntaxin (TGN marker, green) and anti-CTLA-4 (red). TRIM, T-cell receptor-interacting molecule; TGN, trans-Golgi network; PI3K, phosphoinositide 3-kinase

CTLA-4 is a high affinity receptor for B7-1 and B7-2, ligands shared with the activating counter-receptor CD28. The avidity of the CTLA-4 binding to shared ligands is around 20 times higher when compared to CD28. As a consequence, in situations when ligand expression is limited, CTLA-4 might out-compete CD28 for binding, which results in overall inhibition. Structural analysis revealed that pairing of CTLA-4 with B7-1/2 results in the formation of extended organized periodic network at the interface of a T cell and APC (Figure 3.12). Such a structure might prevent diffusion of receptors and/or ligands from the contact site and favor recruitment of signaling molecules at a high concentration, but can also interfere with signals established by the initial interaction between two cells. All these mechanisms might underline possible CTLA-4 dependent inhibitory actions.

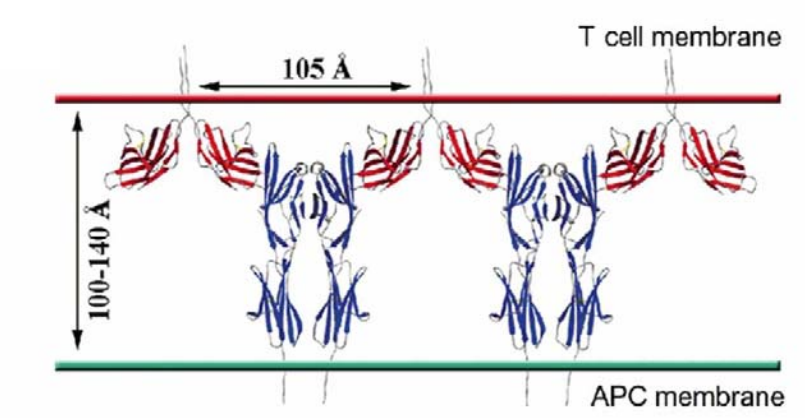


Figure 3.12. Extended periodic array formed by the CTLA-4:B7-1/2 complexes. [173] A lattice-like network is created by the interaction of a single CTLA-4 receptor with two different B7-1 or B7-2 dimmers.

The two main effects of CTLA-4 engagement are decreased IL-2 production and cell cycle arrest that finally results in the termination of T cell responses represented by cytokine production and/or cytotoxicity. There are several proposed mechanisms of CTLA-4 mediated inhibition of T cell effector functions (Figure 3.13) [196]. First one assumes that due to the much higher avidity for the ligands, CTLA-4 simply sequesters B7-1/2 molecules, reducing the CD28 engagement and thereby CD28 derived costimulation. This mechanism does not require an intact function of the cytoplasmic domain. However, it was shown that the cytoplasmic tail was necessary for the CTLA-4 recruitment to the site of immunological synapse [197]. In addition, CTLA-4 is able to inhibit the T cell function in the absence of CD28. Mice that express tail-less CTLA-4 develop lymphoproliferative disorder similar to CTLA-4 deficient animals [198]. A less aggressive appearance of the disease still indicates that ligand competition is a mechanism that plays a role in CTLA-4 dependent T cell inhibition and might be of crucial importance when amounts of ligands are limited. In addition to ligand sequestering, CTLA-4 excludes CD28 from the immunological synapse and blocks the formation of microclusters containing adapters and kinases needed for the effective signal transmission downstream of the TCR. The third mechanism implicates a direct inhibitory signal delivered by CTLA-4 expressed on the cell surface. Indeed, simultaneous cross-linking of CTLA-4 and TCR in the presence of CD28 costimulation is sufficient to inhibit IL-2 production and induce cell cycle arrest [199]. CTLA-4 lacks an intrinsic enzymatic activity and a classical immunoreceptor tyrosine-based inhibitory motif

(ITIM) frequently found in other inhibitory receptors. Despite this, SH2-containing thyrosine phosphatase-2 (SHP-2) and serine-threonine phosphatase protein phosphase 2A (PP2A) have been reported to associate with the cytoplamic tail of CTLA-4 (Figure 3.14A). However, their binding is not absolutely required for the inhibitory function. In addition, SHP-2 is rather implicated in positive signal transduction that leads to cell activation [195]. Both phosphatases associate with CD28 as well and differential recruitment to CTLA-4 during an ongoing immune response, which is regulated by phosphorylation, could be crucial for the final outcome of regulation (reviewed in [200]).

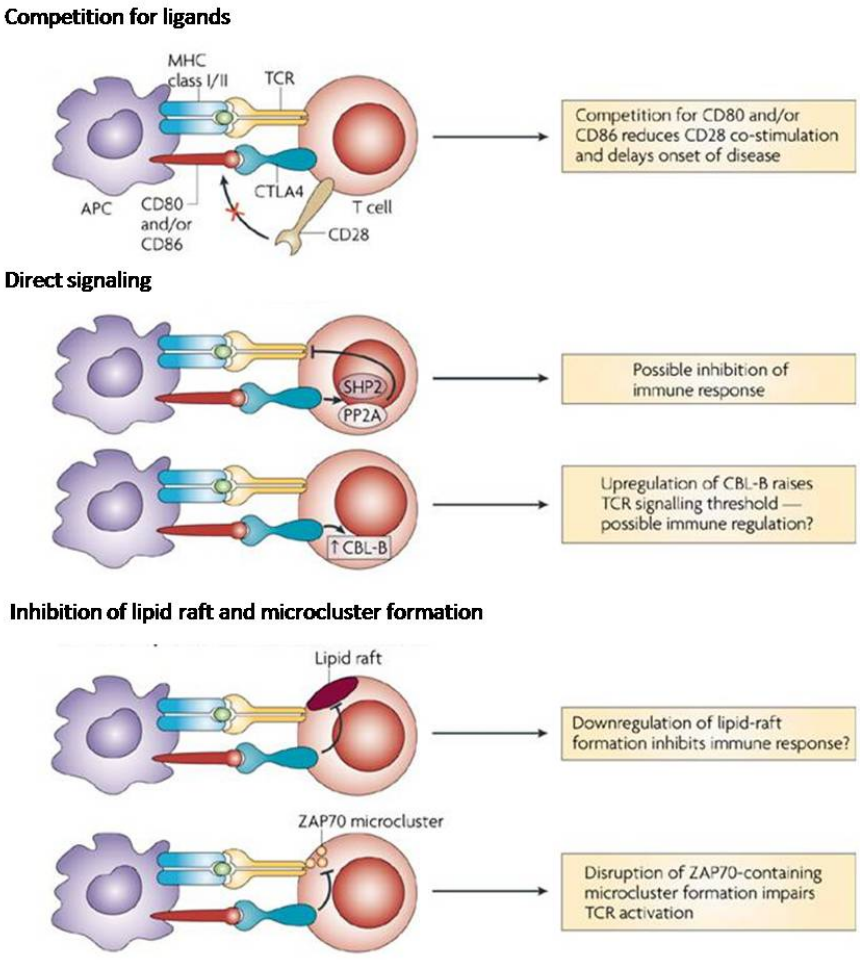


Figure 3.13. Mechanisms of CTLA-4 mediated inhibition of T cell function. [196] CTLA-4 inhibitory function is achieved by the combined action of several mechanisms: the direct competition for ligands with the activating receptor CD28, recruitment of inhibitory signaling components and prevention of lipid rafts and signaling microcluster formation. Under different conditions defined by the TCR signal strength, B7-1/2 ligand concentration, recruited signaling molecules etc, different mechanisms can play a dominant role in mediating inhibition.

Furthermore, CTLA-4 has been reported to induce positive signaling events in T cells (Figure 3.14B) [195]. CTLA-4 binds to PI3K with the same avidity as CD28 and activates JNK, while inhibiting extracellular signal-regulated kinases (ERKs). The inhibition of ERKs could account for negative signaling, while the activation of JNK could contribute to the positive effects on Th1 differentiation. Through the PI3K/Vav1

pathway CTLA-4 enhances LFA-1 integrin clustering and adhesion, which might stabilize the contact with interacting cells. In addition, CTLA-4 binding to PI3K leads to the activation of the PKB/Akt pathway, similar as CD28. CTLA-4 co-ligation in such circumstances acts to increase cell survival, while at the same time inhibits effector responses and/or induces state of non-responsiveness.

A. B.

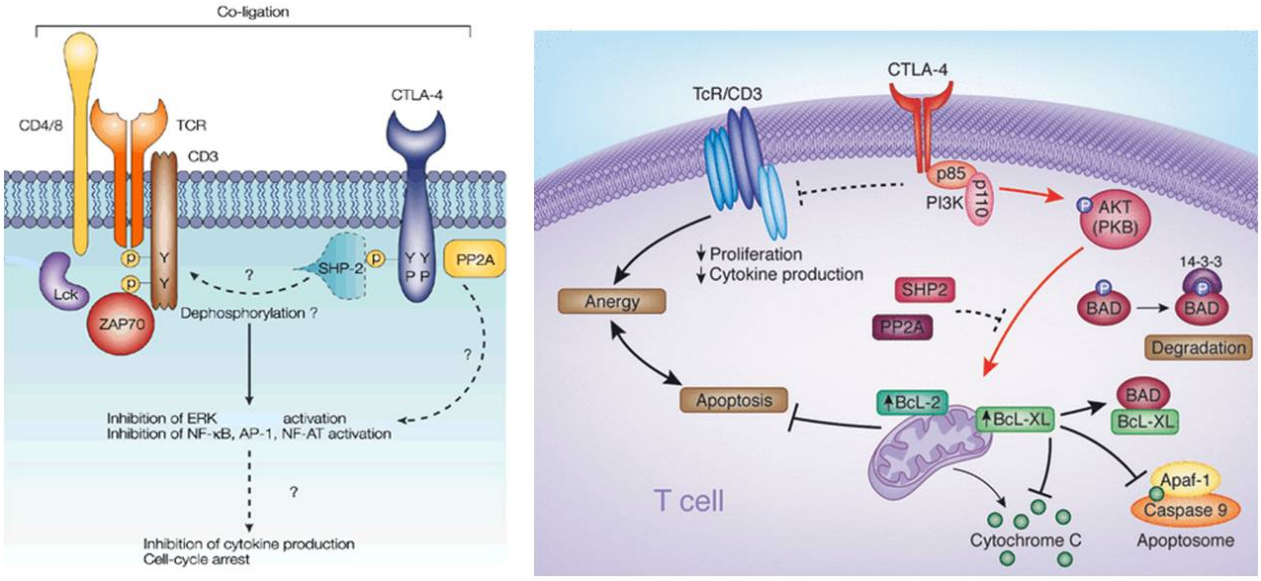


Figure 3.14. Functional consequences of CTLA-4 signaling. (A) CTLA-4 recruits two phosphatases that might be involved in direct dephosphorylation of TCR and/or CD28 proximal signaling molecules leading to the repression of T cell responses [201]. (B) Recruitment of PI3K leads to the activation of pro-survival pathways [195].

CTLA-4 deficient mice are born healthy. However, 5-6 days after birth, a large proportion of T cells becomes activated, causing splenomegaly, lymphoadenopathy and tissue destruction due to the infiltration of peripheral organs. The increased activation of B cells results in elevated levels of immunoglobulins in the serum. This pathology results in death at 3-4 weeks of age. T cells in CTLA-4 deficient mice show an activated $CD69^{+}CD25^{+}CD44^{high}$ phenotype, increased proliferation rate and cytokine production compared to WT counterparts. Both $CD4^{+}$ and $CD8^{+}$ T cells of broad specificity mediate the disorder [202-203]. Antigen-specific stimulation and costimulation via CD28:B7-1/2 pathway is required for the activation of CTLA-4 deficient T cells. The lymphoproliferative disorder due to the CTLA-4 deficiency is not cell autonomous. In the presence of WT counterparts, CTLA-4 deficient T cells do not cause the disease and undergo normal activation and expansion upon infection challenge [204-205]. In humans, several polymorphisms in the *ctla-4* gene have been connected to different autoimmune disorders, such are Graves' disease, multiple sclerosis, Addison's disease, type I diabetes, systemic lupus erythematosus (SLE) and rheumatoid arthritis, indicating its important role in the maintenance of peripheral tolerance and restriction of responses to self-antigens [206].

3.3.2.2. PD-1

PD-1 has been initially described as a receptor induced on a T cell line undergoing activation-induced cell death [207]. However, the expression of PD-1 is not a consequence of induction of cell death *per se*, but rather triggered by T cell activation [208]. In addition to activated T cells, PD-1 is expressed on activated B and NKT cells, monocytes and DCs, as well as on maturing thymocytes, playing a role in central tolerance induction [209-210]. Particularly high PD-1 expression is a characteristic of functionally exhausted effector $CD8^+$ T cells found in multiple persistent viral infections in both mouse and human [211-212]. The blockade of PD-1 or its ligand, B7-H1, leads to restored T cell proliferation and cytokine production. Regulatory T cells (Treg) show specific pattern of PD-1 protein expression: constitutively synthesized protein is stored intracellularly and transported to the cell surface upon stimulation [213].

B7-H1 (PD-L1, CD274) and PD-L2 (B7-DC, CD273) are two described ligands of PD-1 (PD-Ls). PD-1 engagement by its ligands leads to T and B cell inhibition [214-216]. However, it was reported that PD-Ls can also costimulate T cell responses, by increasing proliferation, IL-10, IFNγ and GM-CSF, but not IL-2 and IL-4 production [217-218]. Since costimulation by PD-Ls was observed in PD-1 deficient T cells, the existence of a second receptor has been postulated. The pattern of PD-Ls expression is significantly broader as compared to other B7 family ligands [219-220]. B7-H1 is broadly expressed on both hematopoietic (resting T and B cells, macrophages, DCs, NK cells) and non-hematopoietic (endothelium, epithelium, muscle cells, hepatocytes, pancreatic islets, placenta, eye) cells. It is further up-regulated by typical proinflammatory stimuli as type I IFNs, IFNγ or LPS. In contrast, PD-L2 is restricted to the hematopoietic lineage, more specifically to activated DCs, macrophages and resting peritoneal B1 B cells. It can be induced on monocytes and macrophages by GM-CSF, IL-4, IL-13 and IFNγ stimulation [221-222]. Due to the their distinct expression pattern and functions, PD-L2 might play a major role in the enhancement of T cell responses during the priming phase, while B7-H1 may act at the periphery by promoting Th2 cytokine production and down-regulating effector functions. The stimulatory role of PD-L2 is further supported by the increased immunogenicity of tumor cells that ectopically express this molecule [223]. In addition, its cross-linking on DCs leads to their activation and increased ability to stimulate T cells [224].

PD-1 attenuates T cell responses by mechanisms distinct from activation-induced cell death [176]. The delivered inhibition depends on the strength of the TCR signal with greater inhibition exerted at low levels of TCR stimulation. The cytoplasmic tail of PD-1 contains two tyrosines belonging to an ITIM (Immunoreceptor Tyrosine-based Inhibition Motif) and an ITSM (Immunoreceptor Tyrosine-based Switch Motif), respectively. Cross-linking of PD-1 alone does not transduce the signal. Simultaneous engagement of both TCR and PD-1 results in tyrosine phosphorylation of PD-1 ITIM and ITSM and in the recruitment of tyrosine phosphatase SHP-2, which mediates dephosphorylation of proximal TCR signaling components. Although both tyrosines are phosphorylated, only the ITSM motif was shown to be crucial for SHP-2 recruitment. ITSM is reported to transduce both positive and negative signals, which might indicate possible dual function of PD-1 receptor.

The functional consequences of PD-1 activation include decreased cytokine production and cell cycle arrest, mainly through the inhibition of IL-2 secretion, Bcl-X_L expression and repression of transcription factors associated with T cell effector function (GATA-3, Tbet and Eomes) [225]. Exogenous IL-2 or CD28 costimulation can rescue PD-1 mediated inhibition [214, 226]. Due to the broad expression of B7-H1 at the periphery, PD-1 is seen as a key regulator of peripheral tolerance and attenuator of self reactive T cells. In addition, the vigorous response to pathogens that can lead to immune mediated tissue damage is controlled by the PD-1 mediated inhibitory function [227].

PD-1 deficiency, consistent with its negative regulatory function, results in a late onset, chronic, progressive lupus-like glomerulonephritis and arthritis on C57BL/6 background [228]. On the other hand, Balb/c PD-1 deficient mice develop very rapidly autoimmune-dilated cardiomyophaty that leads to heart failure and premature death. B and T cells are required for the pathology to develop, since no disease is observed in Balb/c PD-$1^{-/-}$$RAG^{-/-}$ mice [229]. On an autoimmune-prone background, PD-1 deficiency negatively influences disease progression. Onset and severity of insulitis in NOD mice and EAE in a mouse model of MS are accelerated when PD-1/PD-Ls interaction is disrupted [230-231]. Similarly, in humans, several polymorphisms within the PD-1 encoding gene have been associated with autoimmune diseases, including SLE, type I diabetes, RA, MS and Grave's disease [232].

Various tumor cell lines [233-234] and established tumors [235-240] express PD-Ls, which are used by tumors to escape immunosurveillance by attenuation of effector cell responses via PD-1 engagement. This notion is supported by the observation that B7-H1 expression by tumor cells correlates with poor prognosis. In addition, tumor infiltrated lymphocytes upregulate PD-1, which correlates with decreased responses [241-242]. Apart from tumor cells, B7-H1 is expressed by vascular endothelial cells and myeloid cells, including DCs, in the tumor tissue, which can impair both T cell extravasation and activation at the tumor site. In mouse tumor models, treatment with αPD-1 or αB7-H1 blocking mAb as well as tumor implantation in PD-1 deficient hosts, augments anti-tumor responses [234, 243-245].

3.3.3. Other B7 family members

B7-H3 is a distant B7 family ligand initially found to costimulate IFNγ production and T cell cytotoxic response. It can be induced on dendritic cells by IFNγ and on monocytes by GM-CSF. Its receptor, expressed on T cell upon activation [246], has been recently identified as the triggering receptor expressed on myeloid cells (TREM)-like transcript 2 (TREML2) [247]. Alternatively, the inhibition of T cell function by B7-H3 has also been reported [248], but the receptor mediating inhibition is not known. It is also possible that in different conditions provided by the microenvironment the same receptor can have a dual functional role. Moreover, discrepancies were reported when mouse tumor models were compared with human malignances. While ectopic B7-H3 expression leads to the tumor regression in mice, its high expression in human tumors associates with higher dissemination of metastases and poor prognosis [249].

B7-H4, another distant B7 ligand member, has been detected on activated T and B cells, DCs, macrophages and monocytes and described as negative regulator of T cell responses [250-252]. Myeloid cells stimulated with IL-6 or IL-10 upregulate B7-H4, while DC differentiating cytokines GM-CSF and IL-4 decrease its expression. It was reported for different human cancers that B7-H4 was highly expressed by tumor cells and infiltrated macrophages, which correlated with poor prognosis [219].

B7-H6 has been recently identified as B7 family member that specifically binds and activates NK cell cytotoxicity via the receptor NKp30 [253]. Interestingly, B7-H6 was neither detected in normal tissues nor could be induced under various conditions of cellular stress. However, several tumor cell lines and primary tumor cells obtained from patients with hematological malignancies were found to express B7-H6, indicating that it can be upregulated by cell transformation.

BTLA (B and T lymphocyte attenuator) is a transmembrane B7 family receptor structurally similar to PD-1 and CTLA-4. It is expressed on most hematopoietic cells and increased upon stimulation [254]. Grb2 and PI3K binding sites were found in its cytoplasmic tail [255], as well as two ITIM motifs [256]. An inhibitory function of BTLA was supported by *in vitro* data [257] and the phenotype of the knock-out mice that develop a spontaneous autoimmune hepatitis-like disease at a late age. BTLA binds to the TNF-R superfamily member HVEM that is widely expressed through the lymphoid compartment. HVEM is also known for its ability to engage several TNF ligands, such as lymphotoxin α and LIGHT, acting as a positive regulator [258-259]. In addition, human T cells are shown to be inhibited through the HVEM interaction with CD160, another Ig superfamily inhibitory receptor mainly expressed on cytotoxic lymphocytes, including NK cells [260-261].

BTNL2 (butyrophilin-like 2) belongs to the family of butyrophilins, which are structurally closely related to B7 molecules [262]. Mouse BTNL2 is a type I transmembrane protein with two pairs of IgV-IgC domains, which are found in B7 receptors in one copy. Its mRNA is highly expressed in the lymphoid tissues as well as in the intestine. It recognizes a putative receptor whose expression on B and T cells is significantly enhanced after activation. BTNL2 engagement inhibits T cell proliferation and TCR activation of NFAT, NF-kappaB, and AP-1 signaling pathways.

3.3.4 B7 family members as therapeutic targets

Two main classes of disorders, malignancies and autoimmune diseases, are considered as the best therapeutic targets using approaches that modulate the activity of costimulatory and/or coinhibitory B7 family members. Autoimmunity, accompanied with deregulated inhibition towards self antigens, is usually treated in the way that blocks unleashed stimulation. On the other hand, multiple approaches are considered for the treatment of tumors. The self-nature of tumor antigens requires breaking of self tolerance by mechanisms which will dissociate therapy induced activation from possible adverse effects. In addition, proper costimulation should be achieved in order to prevent anergy induction. At the same

time, exhaustion and thereby inhibition of immune effectors due to the chronic stimulation should be avoided. Thus, the proper combination of therapeutic agents applied at the correct time points in the course of effector cell activation, within a window that will ensure a beneficial effect without detrimental side effects, should be designed to combat outgrowing tumors.

Providing costimulation

In several mouse models the introduction of B7-1 on tumor cells was sufficient to induce T cell mediated tumor rejection as well as subsequent memory responses. However, this strategy was not efficient for non-immunogenic tumors [263]. The mmunization with B7-1 irradiated transfectants can protect vaccinated mice from the subsequent challenge, but does not induce the rejection of established tumors. In general, B7-1 expression by tumor cells is thought to augment the effector phase of anti-tumor response, while efficient T cell priming rely on the cross-presentation and the proper expression of costimulatory molecules by APCs. B7-1 integrated in several vaccination strategies in phase I and II clinical trials confirmed the induction of specific responses with clinical benefits for some of the patients [263]. However, despite the induction of systemic immune response, the benefits were limited, since multiple layers of negative regulation are operating to hamper efficient responses within the tumor microenvironment.

Removing inhibition

So far, two B7 mediated inhibitory pathways were widely analyzed for their ability to suppress tumor immunity. Removing CTLA-4:B7-1/2 or/and PD-1:B7-H1 mediated inhibition was shown to be beneficial in enhancing T cell anti-tumor responses.

CTLA-4:B7-1/2

Mice challenged with colon carcinoma or fibrosarcoma cell lines treated with CTLA-4 blocking Ab demonstrated improved tumor rejection and the generation of protective T cell memory [264]. The effect was seen in both prophylactic and therapeutic settings with a relatively small tumor burden. Although similar results were obtained in lymphoma, prostate and renal carcinoma models [249], CTLA-4 neutralization was inefficient when applied at high tumor loads or with poorly immunogenic tumors. Improved rejection has been achieved in models of mammary cancer and melanoma by combining CTLA-4 blockade with immunization with an irradiated GM-CSF producing tumor vaccine (GVAX) [265-266]. This treatment was efficient only when applied at very early stages of tumor progression. Recently, a Flt3L producing tumor cell vaccine (FVAX) was also shown to be effective in controlling B16 melanoma and prostate cancer progression when combined with CTLA-4 neutralization [267]. Two fully human αCTLA-4 mAbs have been investigated in clinical trials with end-stage, treatment-resistant melanoma and renal cell carcinoma patients showing overall response rates around 10% with significant duration [268]. Anti-CTLA-4 monotherapy is thought to enhance pre-existing as well as to induce *de novo* anti-tumor responses. Evidence of immunological activity has also been

demonstrated in cases of prostatic, ovarian, breast and colon carcinoma, although objective response criteria have not been met. However, promising results were obtained from trials that combined CTLA-4 blocking with specific peptide vaccine, high dose IL-2 and GM-CSF producing tumor vaccine indicating that multimodal approach might be a better choice for the efficient treatment. However, patients that benefit from the treatment with αCTLA-4 mAb usually develop severe autoimmune side effects indicating the need for the improvement of therapeutic designs to reduce unfavorable responses and enrich responding patient population at the same time [268].

PD-1: B7-H1

Even when low or no expression is observed on tumor cell lines, most of human and mouse cancers express high levels of B7-H1. In addition, tumor associated immune cells are observed to upregulate surface B7-H1 expression [269]. B7-H1 exploits many different modes of action in order to suppress immune mediated tumor destruction including the induction of apoptosis, anergy and/or exhaustion of T cells, IL-10 production by DCs, promotion of Treg mediated immune suppression and formation of a "molecular shield" that protects tumor cells from direct lysis. Reverse signaling of B7-H1 into the tumor cells has been shown to induce resistance to apoptosis by multiple pathways [270]. Ectopic expression of B7-H1 into highly immunogenic P815 mastocytoma tumor cell line leads to progressive growth when injected in syngenic mice [233-234, 243]. However, B7-H1 expression by poorly immunogenic tumors, as B16 melanoma, does not influence tumor control [234]. Application of B7-H1 or PD-1 blocking antibodies can cause tumor regression in several mouse tumor models and treatment settings [244-245, 271-272]. Two humanized αPD-1 mAb have been developed and are to be further tested for their efficacy. So far, phase I trials documented clinical benefits for patients with hematological malignancies, but not for the cases with advanced solid tumors [263].

Crucial issues for B7 based anti-cancer therapy

The current knowledge reveals a very complex regulation of expression and function of B7 molecules. Dissection of the proper therapeutic targeting is proven to be difficult due to the multitude of possible interactions that can be established within the family and reverse signaling observed for most of them. In addition, in many cases both immune effector cells and tumor cells express the potential targets and can be affected in an opposed way by the given therapeutic agents. Although most of the studies dissect the clinical application with T cells as the main players in an anti-tumor immune response that is to be raised, it should be considered that other immune cells can potentially express targeted molecules and could respond to the therapy in different ways. In addition to adding and/or removing one or several B7 mediated interactions, from current studies it is clear that additional stimuli have to be provided to achieve efficient responses. Those stimuli are to enhance different modalities of global anti-tumor activities including cell priming, homing to the tumor site, extravasation, migration through tumor tissue, proliferation, cytokine production, cytotoxicity or other events that might synergize with B7 related therapeutics.

4. Aim of the Study

NK cells are recognized as potent anti-tumor effectors. They are particularly efficient in the recognition of tumor cells that lose the expression of MHC I molecules, which inhibit NK cell activation [23]. In cancer patients, many tumors downregulate MHC I in order to escape direct recognition by cytotoxic T cells, which are MHC I restricted [273]. At the same time, these tumors become susceptible to attack by NK cells. NK cells can efficiently eradicate disseminating cancer cells [39]. However, solid tumors usually grow progressively, despite the MHC I deficiency detected for majority of cases. Infiltration of the tumor tissue by NK cells represents a good prognostic factor [274-275]. Despite this, in cancer patients NK cells are found to be impaired in performing their effector functions [276].

In this regard, the aim of our study was to analyze NK cells responses against the MHC I deficient lymphoma, RMA-S. To better understand the nature and mechanisms that influence NK cell responses to progressively growing lymphoma *in vivo*, comprehensive analysis of NK cell phenotype in tumor bearing mice including the expression of surface markers used to define their maturation stage, subset composition, activation status and functional competence was performed. We took advantage of a global gene expression profiling of blood and tumor infiltrating NK cells. We aimed at the characterization of possible systemic changes in peripheral blood NK cells that could occur due to the tumor outgrowth and can be used as potential clinical biomarkers of tumor presence. In addition, we analyzed features of NK cells infiltrating the tumor to investigate possible mechanisms that operate within tumor tissue to inhibit NK cells anti-tumor responses. Our work describes some of the mechanisms involved in the complex control of NK cell responses, which should be considered for design of novel NK cell based anti-cancer therapies.

5. Materials and Methods

5.1. Materials

5.1.1. Laboratory equipment

Product	Company
Analytical scales, AE163	Mettler Toledo
Analytical scales, AG285	Mettler Toledo
Anesthesia machine, Vapor 19.1	Drägerwerk AG
Cell culture incubator, Heraeus BBD 6220 (CO_2)	Kendro
Centrifuge 5415 R (table)	Eppendorf
Centrifuge 5417 R (table)	Eppendorf
Centrifuge, Heraeus Biofufe Pico	Kendro
Centrifuge, Heraeus Multifuge 4 K-R/3 S-R	Kendro
Centrifuge, Sorvall Evolution RC	Kendro
DNA Engine PTC-200	MJ Research
ELISA microplate reader, GENios	TECAN
EPS 3501XL Electrophoresis Power Supply	GE Healthcare
FACS™ sorter, FACS™Diva	BD
FACS™ sorter, FACS™Vantage SE	BD
Flow cytometer, FACS™Calibur	BD
Flow hood, Heraeus Hera Safe BBD 6220	Kendro
Flow hood, LabGuard NU-437-600E	IBS Integra
Freezer -20°C, Premium/Comfort/Profi line	Liebherr
Freezer -86°C, VIP series	Sanyo
Fridge, Premium/Profiline	Liebherr
Gel Documentation System 2000	Bio-Rad
Heatblock	VWR/Scientific Industries
Ice machine	Hoshizaki
Magnetic stirrer, MR3001 K	Heidolph
Microscop, WIIovert 30	Hund Wetzler
N2 tank, CryoSystem 6000	MVE

Product	Company
PerfectBlueTM Horizontal Mini Electrophoresis Systems, Mini S and L	peQLab
pH meter	WTW
Photometer, Ultraspec 3100	Amersham Biosciences
Pump, Econo Pump	Bio-Rad
Scales, PB602-S	Mettler Toledo
Thermomixer, Compact/Comfort	Eppendorf
Vortex, VortexGenie2	VWR/Scientific Industries
Waterbath, Heraeus Julabo TW20	Kendro
Waterbath, SWB 20	Medingen
Gamma-counter, Cobra auto-gamma	Packard, PerkinElmer
Gamma cell 1000	Atomic Energy of Canada Ltd

5.1.2. Cell culture products

Product	Company	Catalog no.
Standard tissue culture flasks/filter screw caps – 25 cm2	TPP	90026
Standard tissue culture flasks/filter screw caps – 75 cm2	TPP	90076
Standard tissue culture flasks/filter screw caps – 150 cm2	TPP	90151
Tissue culture flasks/filter screw caps – 182 cm2	Greiner	660175
96-well U-bottom with lid – Standard TC	BD	353077
96-well flat-bottom with lid – Standard TC	BD	353072
48-well flat-bottom with lid – Standard TC	BD	353078
24-well flat-bottom with lid – Standard TC	BD	353047
12-well flat-bottom with lid – Standard TC	BD	353043
6-well flat-bottom with lid – Standard TC	BD	353046
6-well flat-bottom with lid – non-treated TC	BD	351146
24-well flat-bottom with lid – non-treated TC	BD	351147
50 ml conical tubes Falcon™	BD	352070
15 ml conical tubes	Greiner	188271
5 ml round-bottom polypropylene test tube	BD	352008
5 ml round-bottom polystyrene test tube w/ cell strainer	BD	352235
Serological pipettes, 5, 10, 25, and 50 ml, sterile	Costar	-
70 µm cell strainer Falcon™	BD	352350

Product	Company	Catalog no.
40 µm cell strainer Falcon™	BD	352340
Cryovial®, 2ml sterile	Roth	E309.1
NalgeneTM Freezing Container, "Mr. Frosty"	Nunc	5100-0001
0.5, 1.5 and 2 ml safe-lock tubes	Eppendorf	-
10, 20, 100, 200, 300 and 1000 µl filter tips	Biozym	-
50 ml Reagent reservoirs, sterile	Corning	4870
0.5, 1.5 and 2ml safe-lock tibes	Eppendorf	-
50 ml conical tubes with 0.22 µm filter top (Sterilflip)	Milipore	SCG00525
Syringe driven filter units, low protein binding, 0.22 and 0.45 µm	Milipore	-
1, 5, 10, 20 ml syringes	BD	-

5.1.3. Cell culture media and solutions

Product	Company	Catalog no.
RPMI 1640 (1x) w/o L-Glutamine	GIBCO-Invitrogen	31870
D-MEM (1x) (High Glucose) with L-Glutamine, 4500 mg/L D-Glucose, w/o sodium pyruvate	GIBCO-Invitrogen	41965
IMDM with L-Glutamine, 25mM HEPES and sodium pyruvate	GIBCO-Invitrogen	21980
D-PBS (1x) w/o Ca, Mg, sodium bicarbonate	GIBCO-Invitrogen	14190
RPMI 1640 (1x) w/o L-Glutamine with sodium bicarbonate	Sigma	R0883
D-MEM (1x) with L-Glutamine, 4500 mg/L D-Glucose, with sodium pyruvate and sodium bicarbonate	Sigma	D6429
IMDM with 25 mM HEPES, w/o L-glutamine	Sigma	51472C
D-PBS (1x) w/o Ca, Mg, sodium bicarbonate	Sigma	D837
Fetal Bovine Serum, Origin: EU Approved	GIBCO-Invitrogen	10270
Penicillin/Streptomycin-Solution 10000 U/ml penicillin, 10000 µg/ml streptomycin	GIBCO-Invitrogen	15140
L-Glutamine 200 mM (100x), 29.2 mg/ml	GIBCO-Invitrogen	25030
Non-essential amino acids (100x)	GIBCO-Invitrogen	11140035
Sodium pyruvate MEM 100mM	GIBCO-Invitrogen	11360088
β-mercaptoethanol 50mM	GIBCO-Invitrogen	31350010
Trypsin-EDTA (1x) HBSS w/o Ca^{2+}/Mg^{2+} w/ EDTA	GIBCO-Invitrogen	25300

Product	Company	Catalog no.
Dimethylsulphoxide Hybri Max® (DMSO)	Sigma-Aldrich	D2650
Cell Dissociation Solution Non-enzymatic (1x)	Sigma-Aldrich	C5914

5.1.4. Solutions

Solution	Indigrents
ACK lysis buffer	0.15 M NH4Cl 10 mM KHCO3 0.1 mM EDTA pH 7.2-7.4
FACS™ buffer	1X PBS 0.02 % NaN_3 (v/v) 1 % FCS 2 mM EDTA
Freezing medium	1X FCS 10 % DMSO (v/v)
MACS® buffer	1X PBS 0.5 % FCS 2 mM EDTA
Primary cell culture medium (PCM)	1X RPMI 10 % FCS 2 mM L-glutamine 100 U/ml penicillin 100 mg/ml streptomycin 1 mM sodium pyruvate 1X non-essential amino acids 0.25 mM β-mercaptoethanol
10X PBS	1.37 M NaCl 27 mM KCl 100 mM Na_2HPO_4 (anhydrous) 20 mM KH_2PO_4

5.1.5. Chemicals and biological reagents

Product	Company	Catalog no.
7-AAD	BD	559925
Carboxyfluorescein succinimidyl ester (CFSE)	Sigma-Fluka	21888
Chromium-51	PerkinElmer	NEZ030005MC
Collagenase, type IV from Clostridium histolyticum	Cell Systems	LS004188
CpG oligonucleotide ODN 1668	InvivoGen	tlrl-modnb
DNA mass ladder (high)	Invitrogen	10068-013
DNA mass ladder (low)	Invitrogen	10496-016
Dnase I	Sigma-Aldrich	DN25
dNTP Mix	Promega	U1511
FACS™ lysing solution (10x)	BD	349202
Golgi Stop	BD	554724
Heparin-Sodium B	Braun	1708.00.00
Hyaluronidase type V	Sigma-Aldrich	H6-254
Isofluran B	Braun	6724123.00.00
Lipopolysaccharide (LPS)	Sigma-Fluka	L4391
Lympholite® M	Cedarline	CL5035
Pellet Paint co-precipitant	Novagen	69-049-3
PMSF	Applichem	A0999
Polyinoside-polycytidylic acid (pI:C)	InvivoGen	tlrl-pic
Recombinant human IL-2	Chiron	50-4413 RN
Recombinant human IL-2	Hoffmann-La Roche	1104-0890
Recombinant mouse IFN-g	Peprotech	315-05
Recombinant mouse IL-12	Peprotech	210-12
Recombinant mouse IL-15	Peprotech	210-15
Recombinant mouse IL-18	MBL	B002-5
RNA Storage Solution	Ambion	AM7000
Rnasin® Plus Rnase Inhibitor	Promega	N2615
Triton X-100	Sigma-Fluka	T9284
Two-Cycle Target Labeling and Control Reagents	Affymetrix	900494
UltraPure™ DEPC-Treated Water	Invitrogen	750024
Nuclease-Free Water (not DEPC treated)	Ambion	AM9937

5.1.6. Antibodies

5.1.6.1. Fluorochrome-conjugated antibodies for flow cytometry

Specificity	Fluorochrome	Clone	Isotype	Company	Catalog no.
2B4	FITC	eBio 244F4	Rat IgG2a	eBioscience	11-2441
B7-1	PE	16-10A1	Hamster IgG	Biolegend	104708
B7-1	FITC	16-10A1	Hamster IgG	Biolegend	104706
B7-2	PE	GL-1	Rat IgG2a	Biolegend	105008
CD107a	FITC	1D4B	Rat IgG2a	BD	553793
CD107b	FITC	ABL-93	Rat IgG2a	BD	558758
CD11b	FITC	M1/70	Rat IgG2b	BD	553033
CD11b	APC	M1/70	Rat IgG2b	BD	553312
CD11b	FITC	M1/70	Rat IgG2b	Biolegend	101206
CD11c	FITC	HL3	Hamster IgG1	BD	557400
CD11c	FITC	N418	Hamster IgG	Biolegend	117306
CD127	FITC	A7R34	Rat IgG2a	eBioscience	11-1271
CD19	APC	1D3	Rat IgG2a	BD	550992
CD27	PE	LG.3A10	Hamster IgG1	BD	558754
CD27	PE	LG.3A10	Hamster IgG1	Biolegend	124210
CD27	FITC	LG.3A10	Hamster IgG1	Biolegend	124208
CD28	PE-Cy5	37.51	Hamster IgG	Biolegend	102108
CD3ε	FITC	145-2C11	Hamster IgG1	BD	553062
CD3ε	PerCP-Cy5.5	145-2C11	Hamster IgG1	BD	551163
CD3ε	APC	145-2C11	Hamster IgG1	BD	533066
CD3ε	FITC	145-2C11	Hamster IgG1	Biolegend	100306
CD3ε	PerCP-Cy5.5	145-2C11	Hamster IgG1	Biolegend	100328
CD3ε	APC	145-2C11	Hamster IgG1	Biolegend	100312
CD4	APC	RM4-5	Rat IgG2a	BD	553051
CD43	FITC	S7	Rat IgG2a	BD	553270
CD45.1	APC	A20	Mouse IgG2a	eBioscience	17-0453
CD45.1	FITC	A20	Mouse IgG2a	BD	553775
CD45.1	PE	A20	Mouse IgG2a	BD	553776
CD45.2	PerCP Cy5.5	104	Mouse IgG2a	Biolegend	109828
CD45.2	FITC	104	Mouse IgG2a	BD	553772

Specificity	Fluorochrome	Clone	Isotype	Company	Catalog no.
CD48	PE	HM48-1	Hamster IgG	eBioscience	12-0481
CD69	FITC	H1.2F3	Hamster IgG	BD	557392
CD8	PE	53-6.7	Rat IgG2a	BD	553033
c-Kit	FITC	2B8	Rat IgG2b	BD	553354
CTLA-4	PE	UC10-4B9	Hamster IgG	Biolegend	106306
CTLA-4	PE	UC10-4F10-11	Hamster IgG	BD	553720
F4/80	Alexa 488	BM8	Rat IgG2a	Caltag	MF48020
Granzyme B	PE	16G6	Rat IgG2b	eBioscience	12-8822
I-A/I-E	PE	M5/114.15.2	Rat IgG2b	BD	557000
I-A/I-E	Alexa 647	M5/114.15.2	Rat IgG2b	Biolegend	107618
ICOS	PE	7E.17G9	Rat IgG2b	BD	552146
ICOS-L	PE	HK5.3	Rat IgG2a	Biolegend	107406
IFN-γ	FITC	XMG1.2	Rat IgG1	BD	554411
IFN-γ	FITC	XMG1.2	Rat IgG1	eBioscience	11-7311
KLRG1	FITC	F1	Hamster IgG	Southern Biotec	1807-02
Ly49C/I/G/H	PE	14B11	Hamster IgG	Biolegend	108208
Ly49D	APC	eBio 4E5	Rat IgG2a	eBioscience	51-5782
Ly49A	PE	YE1/48.10.6	Rat IgG2a	Biolegend	116808
Ly49G2	FITC	eBio 4D11	Rat IgG2a	eBioscience	11-5781
NK1.1	APC	PK136	Mouse IgG2a	BD	557391
NK1.1	FITC	PK136	Mouse IgG2a	BD	553164
NK1.1	PE	PK136	Mouse IgG2a	BD	557391
NK1.1	PerCP-Cy5.5	PK136	Mouse IgG2a	Biolegend	108728
NK1.1	Alexa 647	PK136	Mouse IgG2a	Biolegend	108720
NK1.1	APC	PK136	Mouse IgG2a	Biolegend	108710
NKG2D	APC	CX5	Rat IgG1	eBioscience	17-5882
NKp46	PE	29A1.4	Rat IgG2a	eBioscience	12-3351
NKp46	Alexa 647	29A1.4	Rat IgG2a	eBioscience	51-3351
PD-1	PE	J43	Hamster IgG	BD	551892
PD-1	PE	RMP1-30	Rat IgG2b	Biolegend	109104
PD-L1	PE	MIH5	Rat IgG2a	BD	558091
PD-L2	PE	Ty25	Rat IgG2a	BD	557796
Perforin	PE	eBio OMAK-D	Rat IgG2a	eBioscience	12-9392

Specificity	Fluorochrome	Clone	Isotype	Company	Catalog no.
B220	FITC	RA3-6B2	Rat IgG2a	BD	553092
B220	APC	RA3-6B2	Rat IgG2a	BD	553087
CD3ζ	biotin	H146-968	Hamster IgG	Cedarlane	CL 7230 B
F4/80	Alexa 488	BM8	Rat IgG2a	Caltag	MF 48020
ICAM-1	PE	3E2	Hamster IgG	BD	553253

5.1.6.2. Affinity purified antibodies for functional assays

Specificity	Clone	Isotype	Company	Catalog no.
CD28	37.51	Hamster IgG	Biolegend	102112
2B4	eBio 244F4	Rat IgG2a	eBioscience	14-2441
Ly49D	4E5	Rat IgG2a	BD	555312
NKG2D	CX5	Rat IgG1	eBioscience	14-5882
NKG2D	A10	Rat IgG1	Biolegend	115602
NKp46	polyclonal	Goat IgG	R&D	AF2225
NKp46	29A1.4	Rat IgG2a	eBioscience	16-3351
NK1.1	PK136	Mause IgG2a	Biolegend	108712
CTLA-4	UC10-4B9	Hamster IgG	Biolegend	106308
B7-1	16-10A1	Hamster IgG	Biolegend	104710
TGFβ	1D11	Mouse IgG	R&D	MAB1835
CD16/CD32	93	Rat IgG2a	Biolegend	101310

5.1.6.3. Affinity purified fusion proteins for functional assays

Recombinant protein	Linker	C-terminus	Company	Catalog no.
Recombinant mouse B7-1/Fc Chimera	DIEGRMD	Human IgG1	R&D	740-B1-100
Recombinant mouse CTLA-4/Fc Chimera	IEGRMD	Human IgG1	R&D	434-CT-200
Recombinant mouse EphA4/Fc Chimera	IEGRMD	Human IgG1	R&D	641-A4-200

5.1.6.4. Antibodies for in vivo application

Specificity	Clone	Isotype	Company	Catalog no.
CTLA-4	UC10-4F10-11	Hamster IgG	BioXCell	BE0032
PD-1	J43	Hamster IgG	BioXCell	BE0033-2

5.1.6.5. Isotype controls and secondary reagents for flow cytometry

Isotype	Fluorochrome	Clone	Company	Catalog no.
Rat IgG2a, k	FITC	R35-95	BD	553929
Rat IgG2a, k	PE	R35-95	BD	554689
Rat IgG2a, k	APC	-	eBioscience	17-4321
Rat IgG2a, k	Alexa 647	R35-95	BD	557690
Rat IgG2b, k	FITC	A95-1	BD	553988
Rat IgG2b, k	PE	eB149/10H5	eBioscience	12-4031
Rat IgG2b, k	APC	eB149/10H5	eBioscience	17-4031
Rat IgG2b, k	Alexa 647	RTK4530	Biolegend	400626
Rat IgG1	FITC	eBRG1	eBioscience	11-4301
Hamster IgG	FITC	HTK888	Biolegend	400906
Hamster IgG	PE	HTK888	Biolegend	400908
Hamster IgG	PE-Cy5	SHG-1	Biolegend	402010
Hamster IgG	biotin	HTK888	Biolegend	400904
Goat anti-Human IgG $F(ab)_2$	PE	-	Jackson Immuno Res.	109-116-098
Streptavidin	FITC	-	BD	554060
Streptavidin	PE	-	BD	554061
Streptavidin	PerCP-Cy5.5	-	Biolegend	405214
Streptavidin	APC	-	BD	554067

5.1.6.6. Isotype controls and secondary reagents for functional assays

Isotype	Clone	Company	Catalog no.
Rat IgG2a, k	RTK 2758	Biolegend	400516
Mouse IgG2a, k	MOPC-173	Biolegend	400224
Hamster IgG	HTK888	Biolegend	400916
Goat IgG	polyclonal	R&D	AB-108-C
Goat anti-Hamster IgG	polyclonal	Jackson Immuno Res	127-005-160

5.1.7. Reagents for magnetic cell sorting

Product	Company	Catalog no.
anti-APC Beads	Miltenyi Biotec	130-090-855
anti-DX5 Beads		130-052-501
Streptavidin Beads		130-048-101
Pan-T-cell Isolation Kit		130-090-861
Anti-CD62L beads		130-049-701
LS columns		130-042-401
MS columns		130-042-201

5.1.8. Kits

Product	Company	Catalog no.
BCA Protein Assay kit	Pierce	23227
FITC BrdU Flow Kit	BD	557891
GeneChip Mouse Genome 430A 2.0 Array	Affymetrix	900499
Mouse IFN-g OptEIATM Set	BD	555138
OptEIATM Reagent Set B	BD	550534
Foxp3 Staining Buffer Set	eBioscience	00-5523
RNeasy® Mini Kit	Qiagen	74124
TURBO DNA-free™	Ambion	AM1907
Two-Cycle Target Labeling and Control Reagents	Affymetrix	900494

5.1.9. Cell lines

Name	Description	Medium*
RMA	Mouse T cell lymphoma	RPMI 1640
RMA-S	TAP2-deficient variant of RMA	RPMI 1640
RMA-S.B7-1	TAP2-deficient variant of RMA transduced to express B7-1	IMDM
B16	Mouse melanoma	DMEM
B16.B7-1	Mouse melanoma transduced to express B7-1	DMEM
B16.Rae1ε	Mouse melanoma transduced to express Rae1ε	DMEM

Name	Description	Medium*
B16.Rae1ε.B7-1	Mouse melanoma transduced to express Rae1ε and B7-1	DMEM
YAC-1	Mouse lymphoma	RPMI 1640
LL2	Mouse Lewis lung carcinoma	DMEM
2.4G2	anti-CD16/CD32 hybridoma	DMEM
X6310-GMCSF	GM-CSF producing cell line	DMEM

***** All cell culture media were supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin with the exception of medium for the 2.4G2 hybridoma, which was supplemented with 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate and 0.25 mM β-mercaptoethanol.

5.1.10. Mice

C57BL/6 (WT) mice were purchased from Charles River Laboratories (Sulzfeld, Germany and Erembodegem, Belgium). CD28-/- and B7-1-/- mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). Congenic C57BL/6-Ly5.1^{+} mice were purchased from Charles River Laboratories or bred in our animal facility. CTLA-4 -/- OT-I transgenic mice were kindly provided by Prof. M. Brunner-Weinzierl (Experimentelle Pädiatrie, Universitätskinderklinik, Otto-von-Guericke Universität, Magdeburg, Germany). B7-H1-/- mice were kindly provided by Prof. L. Chen (Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, USA). RAG2-/- mice were bred in our animal facility. Mice were housed under specific pathogen-free conditions and used in experiments at 8-16 weeks of age. All experiments were performed according to local animal experimental ethics committee guidelines and permission.

5.2. Methods

5.2.1. Cell culture methods

Thawing cells

Frozen cryovials were submerged in a 37°C water bath until ~ 10% of the cell suspension remained frozen. The cell suspension was immediately diluted into 10 ml of appropriate medium and centrifuged (1200 rpm, 5 min, RT). Cells were resuspended in the appropriate medium and cultured at 37°C, 5% CO_2.

Splitting of suspension cells

Cells in suspension reaching an optimal density were split in desired ratios by adding the appropriate volume of the required medium.

Splitting of adherent cells

Cell culture medium was removed from the flasks and cells were washed once with PBS. Pre-warmed 0.05% Trypsin-EDTA or non-enzymatic Cell Dissociation Solution was added in sufficient amounts to cover the cell layer. Cells were incubated at 37°C until a complete detachment from the flask was observed under the microscope. Cells were then resuspended in pre-warmed medium, collected in Falcon™ tubes and centrifuged (1200 rpm, 5 min, RT). Cells were subsequently diluted at appropriate ratios and distributed to new flasks.

Determination of cell numbers

Appropriate dilution of cell suspension was mixed in a 1:1 ratio with a 0.05 % trypan blue solution (w/v) to distinguish dead from live cells. Cells were counted using a Neubauer counting chamber (0.1 mm depth). The number of live cells per ml of cell suspension was calculated as: the average cell number per chamber square (0.1 mm^3) x dilution factor x 10^4.

Freezing cells

For cell freezing, cell suspensions were centrifuged (1200 rpm, 5 min, RT) and the pellet was resuspended in freezing medium (90% FCS / 10% DMSO) at a concentration of 2.8 x 10^6 cells/ml. 1.8 ml (5 x 10^6 cells total) was aliquoted in cryovials and placed in freezing containers. After initial 24 h storage at -80°C, frozen vials were transferred to liquid nitrogen for long term storage.

5.2.2. Organ dissection and preparation of single cell suspensions

Blood

Animals were sacrificed by asphyxiation with CO_2 and blood was obtained by heart puncture. Typically, 0.8-1 ml of the collected blood was mixed with 50 µl of heparin until treated with 25 ml of buffered ammonium chloride potassium phosphate solution (ACK-buffer) to obtain red blood cell lysis. Cells were further washed with PBS (1600 rpm, 10 min, 4°C) and resuspended in appropriate buffer.

Spleen

Animals were sacrificed by dislocation of the neck; the spleen was excised using sterile forceps and kept in ice-cold PBS. A single cell suspension was obtained by mincing the spleen through a 70 µm-pore cell strainer followed by washing with PBS (1400 rpm, 10 min, 4°C). To lyse erythrocytes, splenocytes were treated with buffered ammonium chloride potassium phosphate solution (ACK-buffer), washed with PBS (1400 rpm, 10 min, 4°C) and then resuspended in an appropriate buffer.

Lymph nodes

Animals were sacrificed by dislocation of the neck; the lymph nodes were excised using sterile forceps and kept in ice-cold PBS. A single cell suspension was obtained by mincing the tissue through a 70 µm-pore cell strainer followed by washing with PBS (1400 rpm, 10 min, 4°C).

Lung

Animals were sacrificed by dislocation of the neck and the the thoracic cavity opened using sterile forceps and scissors. The lungs were perfused with PBS by slow application of solution through the right chamber of the heart. Perfused lungs were dissected, cut into small pieces (~ 1-2 mm) and treated 30 min at 37°C with 10 ml digestion buffer per lung (1 mg/ml collagenase type I, 0.5 mg/ml DNase I). Single cell suspensions were obtained by mincing the digested lung through a 70 µm-pore cell strainer followed by washing with PBS (1400 rpm, 10 min, 4°C). Cells were subsequently resuspended in the appropriate buffer.

Tumor

Tumor bearing animals were sacrificed by dislocation of the neck. Tumor was excised using sterile forceps. Tumors were then cut into small pieces (~ 1 - 2 mm) and treated 30 min at 37 °C with 10 ml digestion buffer per tumor (0.5 mg/ml hyaluronidase type V, 0.5 mg/ml DNase I). Single cell suspensions were obtained by mincing the digested tumors through a 70 µm-pore cell strainer followed by washing with PBS (1500 rpm, 10 min, 4°C). Cells were subsequently resuspended in 7 ml of PBS and filtered through 40 µm-pore cell strainer. Live cells were obtained by centrifugation (1500 g, 20 min, RT) of cell suspension

loaded on Lympholite® M and subsequent collection of the middle cell layer. Cell were intensively washed with PBS (1500 rpm, 10 min, 4°C) before resuspension in the appropriate buffer.

Bone marrow (BM)

Animals were sacrificed by dislocation of the neck and the hind legs were dissected using sterile forceps and scissors. Under the sterile conditions bones were freed from all sinews and muscle tissue. The femur and tibiae were separated by breaking the knee and the heel, washed briefly in 80 % ethanol and placed in ice-cold DMEM. To rinse out the BM, the ends of the bones were cut and 5-10 ml of ice-cold PBS was forced through the bone cavity using 27G needle. The isolated BM was filtered through a 70 µm-pore cell strainer followed by washing with DMEM (1400 rpm, 10 min, 4°C). Cells are subsequently treated with buffered ammonium chloride potassium phosphate solution (ACK-buffer) to remove red blood cells, washed with DMEM (1400 rpm, 10 min, 4°C) and than resuspended in the appropriate buffer.

5.2.3 Cell separation

5.2.3.1 Magnetic cell sorting (MACS®)

NK cells

Single cell suspensions prepared from spleen were washed with MACS® Buffer (PBS 2mM EDTA 0.5% FCS) and resuspended at $1x10^8$ cells/ml. Splenocytes were first treated with 10% 2.4G2 supernatant for 15 min at 4°C to block Fc receptors and further incubated with APC conjugated αCD3 mAb. $CD3^+$ cells were then depleted using αAPC Beads according to the manufacturer's instructions. CD3 depleted splenocytes were then positively selected for $DX5^+$ cells using DX5 magnetic beads.

Naive T cells

Single cell suspensions prepared from spleen and lymph nodes were pooled, washed with MACS® Buffer (PBS 2mM EDTA) and resuspended at $1x10^8$ cells/ml. Cells were treated with 10% 2.4G2 supernatant for 15 min at 4°C to block Fc receptors. T cells were isolated via negative selection with pan T cell kit according to the manufacturer's instructions. Naïve T cells were then positively selected using CD62L magnetic beads.

5.2.3.2 Fluorescence activated cell sorting (FACS™)

Blood NK cells

Single cell suspensions prepared from blood were resuspended in PBS at concentration $5x10^6$ cells/ml and treated with 10 µg/ml αCD16/CD32 mAb to block Fc receptors. Cells were subsequently stained with

fluorochrome labeled αCD3 and αNK1.1 mAb for 30 min at 4°C. After washing (1500 rpm, 10 min, 4°C), cells are resuspended in PBS at a concentration $5x10^7$ cells/ml and filtered through a 30 µm cell mesh. 7-AAD solution was added to the cell suspension 10 min prior to sorting to label dead cells. 7-AAD$^-$CD3$^-$NK1.1$^+$ single cells were sorted using FACSDiva® or FACSVantage® cell sorter instruments.

Tumor infiltrating NK cells

Single cell suspensions prepared from tumors of C57BL/6-Ly5.1 mice were resuspended in PBS at concentration $1x10^7$ cells/ml and treated with 10 µg/ml αCD16/CD32 mAb to block Fc receptors. Cells were subsequently stained with APC labeled αLy5.1 mAb. Ly5.1$^+$ cells, representing tumor infiltrating hematopoietic cells, were positively selected using αAPC Beads according to the manufacturer's instructions. Ly5.1$^+$ cells were further labeled with flurochrome conjugated αCD3 and αNK1.1 mAb for 30 min at 4°C. After washing (1500 rpm, 10 min, 4°C), cells are resuspended in PBS at concentration $5x10^7$ cells/ml and filtered through 30 µm cell mesh. 7-AAD solution is added to cells 10 min prior to sorting to label dead cells. 7-AAD$^-$Ly5.1$^+$CD3$^-$NK1.1$^+$ cells were sorted using FACSDiva® or FACSVantage® cell sorter instruments.

5.2.4 Primary cell culture

NK cells

Single cell suspension was prepared under sterile conditions from spleens of Rag2 -/- mice as described before (section 5.2.2). Cell were resuspended in the primary cell medium (25 ml per spleen) and incubated in 175 mm^2 cell culture flasks for 2h (37°C, 5% CO_2) to deplete adherent cells. A non-adherent fraction was harvested, washed with primary cell medium (1500 rpm, 10 min, 4°C) and resuspended at a concentration of $1x10^6$ cells/ml in primary cell medium containing 1700 U/ml rhIL-2. Suspension was distributed over round-bottomed 96-well plate (200 µl/well) and cultured for up to 12 days (37°C, 5% CO_2). Cell were routinely split 1:2 every second day with primary medium containing 1700 U/ml rh IL-2.

When NK cells were obtained from WT mice, single cell suspensions prepared from spleens were first depleted of CD3$^+$ cells by magnetic cell sorting through two-step procedure – cell labeling with CD3-APC mAb followed by αAPC magnetic Beads and subsequent separation. The CD3$^-$ fraction was then positively selected for DX5$^+$ cells using DX5 magnetic beads according to the manufacturer's instructions. CD3$^-$DX5$^+$ cells were cultured in primary cell medium with 1700 U/ml of rhIL-2 as described before. Day 7 cell culture routinely gave rise to ~99% pure CD3$^-$NK1.1$^+$ NK cells.

Bone marrow derived dendritic cells (BMDC)

Single cell suspension was prepared from BM under sterile conditions as described before (section 5.2.2). Cells were resuspended in primary DMEM (30 ml per mouse) containing 10% supernatant of GM-CSF

producing cell line X6310-GMCSF (DMEM-G). Cell suspension was incubated 24h (37°C, 5% CO_2) to deplete adherent cells. Non-adherent fraction was harvested, washed (1500 rpm, 10 min, 4°C) and resuspended at the concentration of $1x10^6$ cells/ml in DMEM-G. Suspension was distributed over cell culture non-treated polystyrene flat-bottomed 6-well plates (2.5 ml/well) and cultured at 37°C, 5% CO_2. Cells were split when full confluence was observed. Non-enzymatic cell dissociation buffer was used for cell detachment.

5.2.5 Cell staining

Surface staining

10^5-10^6 cells collected from the culture or single cell suspensions generated from organs or blood were first washed and than resuspended in 100 µl of FACS™ buffer. Fc receptors were blocked by incubation with 10% 2.4G2 supernatant for 15 min at 4°C. Appropriate fluorochrome labeled antibodies were added and cells were further incubated for 30 min at 4°C in the dark. When biotinylated antibodies were used cells were washed with FACS™ buffer and incubated with fluorochrome conjugated streptavidin for an additional 30 min at 4°C. At the end of the staining procedure, all samples were washed with FACS™ buffer to remove unbound antibodies. For discrimination of dead cells, 7-AAD was added to the samples 10 min prior to analysis. FACSCalibur® flow cytometer and CellQuest software were used for sample acquisition and data analysis, respectively.

Intracellular staining

10^5-10^6 cells collected from the culture or single cell suspensions generated from organs or blood were stained for surface markers as described above. At the end of the staining procedure, all samples were washed with FACS™ buffer and fixed using Fixation/Permeabilization Buffer (eBioscience, San Diego, CA, USA) according to manufacturer's instructions. Cells were further permeabilized with Permeabilization Buffer (eBioscience, San Diego, CA, USA) and incubated with 20% 2.4G2 supernatant for 30 min at 4°C in 100 µl total volume of Permeabilization Buffer. Intracellular targets were then stained with the appropriate mAb for 45 min at 4°C. Samples were washed with Permeabilization Buffer to remove unbound antibodies and resuspended in FACS™ buffer for analysis.

Prolonged surface staining of CTLA-4

A prolonged surface staining procedure was used to determine the surface expression of target proteins with a high rate of internalization. 6h before the cell collection from the culture 10% 2.4G2 supernatant was added to the culture and cells were incubated for 2h at 37°C. Appropriate mAb or isotype control Ab was then added and cells incubated for additional 4h at 37°C. Upon collection, cells were washed with FACS™ buffer and surface makers were stained as described before.

5.2.6 Functional assays

^{51}Cr release cytotoxicity assay

Target cells were labeled with 100µCi ^{51}Cr for 1.5h and washed three times with primary cell medium. A total of $1x10^3$ ^{51}Cr labeled target cells per well were distributed over the U-bottomed 96-well plate. NK cells were added at different effector to target (E:T) ratios to the final volume of 200 µl. The plates were centrifuged at 400 rpm for 3 min to obtain an optimal cell contact and then incubated for 4h at 37°C, 5% CO_2. At the end of the incubation, 100 µl of supernatant was collected from each well and the radioactivity was counted in a beta counter. The percentage of specific release was calculated as:

100 x [mean measured release – minimum (spontaneous) mean release] / [maximum (total) mean release – minimum (spontaneous) mean release],

Minimum (spontaneous) release corresponds to the amount of radioactivity released by tumor cells cultured in the absence of NK cells. Maximum release relates to the amount of radioactivity released by tumor cells cultured in the presence of 10% Triton X-100.

CD107 degranulation assay

100 µl NK cell suspension in primary cell medium at the concentration of $1x10^6$ cells/ml were distributed over the U-bottomed 96-well plate. Target cells were added at different effector to target (E:T) ratios to the final volume of 200 µl. 1 µl of GolgiStop with 1 µg of CD107a and CD107b mAb each or equal amount of Rat IgG2a, k isotype control was added to each well. The plates were centrifuged at 400 rpm for 3 min to obtain an optimal cell contact and incubated for 3 h at 37°C, 5% CO_2. Upon the end of incubation, cells were harvested, stained for surface markers and analyzed by flow cytometry.

NK in vivo kill assay

Single cell suspensions were obtained from spleens and lymph nodes of WT and tapasin-/- mice. After pooling spleen and lymph node fraction WT and tapasin-/- cells were labeled with 1.5 (high) and 0.1 µM (low) CFSE, respectively. Shortly, cells were resuspended in PBS at the concentration of $1x10^7$ cells/ml and incubated with CSFE for 15 min at RT in dark with constant slow shaking. Labeled cells were washed 3 times with PBS and resuspended at the concentration of $1x10^8$ cells/ml. WT and tapasin-/- cells were then mixed in 1:1 ratio and 200 µl of mix was injected in recipient mice ($2x10^7$ cells/mouse, $1x10^7$ cells/target) through the tail vain. As a control 200 µl of mix were incubated in 10 ml of primary medium at 37°C, 5% CO_2. Mice were sacrificed after 6h and single cell suspensions were made from desired organs. Differentially labeled $CFSE^+$ cells were quantified by flow cytometry and NK cell cytotoxic activity in given organ *in vivo* was calculated as:

$$\% \text{ specific kill} = 100-[(100 \times \text{ratio}_{\text{treated}}) / \text{ratio}_{\text{untreated}}]/ \text{ratio}_{\text{treated}}$$

ratio $_{treated}$ corresponds to the ratio of $CFSE^{high}$ and $CFSE^{low}$ labeled cells obtained from recipient mice. ratio $_{untreated}$ corresponds to the ratio of $CFSE^{high}$ and $CFSE^{low}$ labeled cells cultured *in vitro*.

Plate bound Ab stimulation assay

Flat-bottomed 96-well plates were coated for 18h at 4°C with 50 µl per well of appropriate mAb (10 µg/ml) or IgG fusion protein (2 µg/ml) dilution in PBS. Control wells were incubated with PBS only. At the end of incubation, wells were washed 3 times with 300 µl PBS. NK cells were first resuspended in PBS at the concentration of $5x10^6$ cells/ml and treated with 10 µg/ml αCD16/CD32 mAb for 30 min at 4°C. Upon washing, cells were further resuspended in primary cell medium at the concentration of $1x10^6$ cells/ml, seeded over coated wells and incubated for 8-12h at 37°C, 5% CO_2.

BrdU proliferation assay

Cells were stimulated as indicated. To analyze the frequency of the cells that actively enter cell cycle, BrdU was added to the cells 45 min before the end of stimulation at the final concentration of 10 µM. After harvesting, cells were stained for surface markers as described before followed by BrdU staining according to the manufacturer's instructions. Cell samples were analyzed by flow cytometry.

CFSE proliferation assay

Before stimulation cells were labeled with 1 µM CFSE. Briefly, cells were resuspended in PBS at the concentration of $1x10^7$ cells/ml. Appropriate amount of CFSE was added while vortexing the cell solution. After 10 min of incubation at 37°C, 5% CO_2, cells were washed 3 times with PBS and resuspended in primary medium at $1x10^6$ cells/ml. Upon stimulation, the frequency of proliferating cells and number of division were analyzed by flow cytometry.

5.2.7. Cytokine production determination

Quantification of cytokines

The amount of released cytokine over a given time period was determined by enzyme-linked immunosorbent assay (ELISA). At the end of incubation, cell free supernatants were collected and stored at -20°C until analysis. Cytokine concentration was measured using sandwich ELISA according to the manufacturer's instruction.

Quantification of cytokine producing cells

The frequency of cytokine producing cells was determined by intracellular staining. Prior to the collection from culture, cells were incubated 4-6h with Golgi Stop in order to prevent protein secretion.

5.2.8. Genetic expression profile analysis

Experimental setup

Groups of 20 mice were injected subcutaneously with PBS (naïve controls) or 10^6 RMA-S cells resuspended in 100 µl of PBS. Mice were used at 9 weeks of age. Naïve mice were sacrificed on day 3 post-injection. Groups of 20 tumor bearing mice were sacrificed on day 3, 10 and 17 post-injection. Single cell suspensions were prepared from blood and tumor as indicated in section 5.2.2. Blood and tumor infiltrating NK cells were isolated from the pooled blood and tumor samples as described in section 5.2.3.2. Typically, 5-10x10^5 NK cells were obtained from blood and 1-2x10^5 NK cells were obtained from tumor samples with a purity of ≥99% and ≥98% for blood and tumor, respectively. Biological triplicates with indicated purity of sorted NK cells were collected for every treatment (naïve/tumor bearing), every time point (day 3/10/17) and every organ (blood/tumor).

RNA isolation

RNA was isolated from NK cells sorted out of tumor or blood using RNeasy® Mini Kit with minor changes of the recommended protocol. Typically, cells were lysed in RLT buffer according to manufacturer's instructions and lysates stored at -80°C until continuing isolation. Obtained RNA was treated with DNase using TURBO DNA-free® kit in order to remove any possible genomic DNA contamination.

RNA precipitation

2 µl of Pellet Paint (Novagen), 0.1 v/v 3M NaAc pH 5.2 and 2 v/v 100% EtOH were added to the isolated and DNase treated RNA and incubated for 2 min at RT. RNA was pelleted (16000xg, 5 min, 4°C) and washed two additional times with 70% and 100% EtOH, respectively. The pellet was dried to remove any remaining EtOH and resuspended in RNA Storage Solution. Quantity and quality of obtained RNA was analysed using RNA Pico Assay performed on the Bioanalyzer 2100 Lab-on-a-Chip system. High quality RNA samples (RNA integrity number≥9) were chosen for microarray experiment.

RNA amplification

Identical starting amounts of RNA (35 pg) were amplified using Two-Cycle Target Labeling and Control Reagent kit according to the recommendations of the manufacturer. Two-cycle linear RNA amplification was performed.

Microarray experiment

Gene expression was detected using mouse genome 430 2.0 array from Affymetrix (Santa Clara, CA, USA). Five experimental groups were examined, with triplicates for each group. cDNA and cRNA synthesis, and hybridization to arrays were performed according to the recommendations of the manufacturer.

Microarray data analysis

Microarray data was analyzed based on ANOVA using a commercial software package JMP Genomics, version 4.0 from SAS (SAS Institute, Cary, NC, USA). Briefly, values of perfect-matches were log transformed, quantile normalized and fitted with log-linear mixed models, with probe_ID and sample group considered to be constant and sample_ID random. Hierarchical clustering of differentially expressed genes was performed using a build-in program.

Microarray pathway analysis

To identify pathways that are likely to be affected by differential expression, an ORA approach using Fisher's exact test as described by Manoli *et al.* was performed [277]. Analyzed pathways were collected from the KEGG database (Kyoto Encyclopedia of Genes and Genomes), or manually generated.

5.2.9 Mouse tumor models

Subcutaneous tumor cell inoculation

Tumor cells were harvested in the exponential growth phase after 5 days of culture and washed three times with PBS (1200 rpm, 10 min, 4°C). Cells were resuspended in PBS at the concentration of $1x10^7$ cells/ml (RMA-S, B16, B16.B7-1, LL2) or $1x10^6$ cells/ml (RMA). Mice were injected subcutaneously in the left flank with 100 µl of tumor cell suspension. Tumor growth was assessed every second day with a caliper measuring along the perpendicular axes of the tumors and expressed as the product of the three diameters. If not used for an experiment at a particular time point of tumor growth, mice were killed when the tumor surface exceeded 4 cm^2 or when became moribund.

Metastases model

For metastasis models 100 µl of tumor cell suspension in PBS were injected through tail vein. Mice were sacrificed two weeks post-injection. For the analysis of PD-1 and CTLA-4 expression in the lung of injected mice total of 10^5 RMA, RMA-S, RMA-S.B7-1, B16 or B17.B7-1 tumor cells were injected through tail vein. For the analysis of the IL-2 treatment effect on metastases formation (data not shown) total of $7.5x10^5$ B16.B7-1 tumor cells were injected through tail vein.

Intraperitoneal tumor cell inoculation

For short term experiments, mice were inoculated with 100 µl of $1x10^8$ cell/ml RMA-S or RMA-S.B7-1 tumor cell suspension in PBS and sacrificed 24h post-injection. For long term experiments total of $1x10^5$ tumor cells per mouse in 100 µl PBS was injected. The weight of individual mice was measured every two days. Mice were sacrificed 10-15 days upon tumor cell inoculation.

5.2.10. mAb application in vivo

αCTLA-4 and αPD-1

100 µl of 2.5 mg/ml αCTLA-4 or 3 mg/ml αPD-1 in PBS was injected i.p. in tumor bearing animals every three days starting one day before tumor cell inoculation. Control animals were injected with equal volume of PBS.

5.2.11. Cytokine application in vivo

IL-2

For subcutaneous tumor models 100 µl of 10^6 U/ml IL-2 was injected i.p. in tumor bearing animals for five consecutive days staring from the day of tumor cell inoculation. Control animals were injected with equal volume of PBS. In some experiments IL-2 application was combined with αCTLA-4 or/and αPD-1 treatments. In the case of metastases model 100 µl of 10^6 U/ml IL-2 was injected i.p. one day before tumor cell inoculation.

IL-18

100 µl of 20 µg/ml IL-18 was injected i.p., in tumor bearing animals for five consecutive days staring from day 8 after tumor cell inoculation. Control animals were injected with equal volume of PBS.

5.2.12. TLR ligand application in vivo

pI:C

Polyinoside-polycytidylic acid (pI:C) was applied i.p. resuspended in 100 µl PBS. For short-term experiments 200 µg of pI:C per mouse were injected and animals were sacrificed after 18-24h. For long-term experiments 50 µg of pI:C per mouse were injected every three days.

CpG

Mice were injected peritumoral with 100 µl of water solution of CpG at concentration 200 µg/ml. Typically, 2-3 spots around palpable tumor were chosen to apply the full volume of the resuspended reagent. Injections were repeated every three days.

6. Results

6.1. Phenotype of tumor infiltrating NK cells

6.1.1. Tumor infiltrating NK cells show an immature phenotype

Four subsets of NK cells can be distinguished in the periphery according to the expression of the TNF family receptor CD27 and the integrin α_m subunit CD11b [114]. Differential expression of the CD11b/CD27 marker combination characterizes maturation and the functional status, with $CD11b^{high}CD27^{low}$ being the most mature and $CD11b^{high}CD27^{high}$ being the most potent effector subset of NK cells. Peripheral organs are differentially populated with given NK cell populations. Solid tumors are complex tissues with their own blood and lymphatic system, composed of various cells including hematopoietic cells that can both support or hamper tumor progression [278]. We analyzed the expression of CD11b and CD27 in blood and tumor tissue of mice injected subcutaneously with tumor cells of different origin: B16 melanoma, RMA-S lymphoma and LL2 carcinoma cells (Figure 6.1A). The subset distribution in blood was not different between naïve and tumor bearing animals, with the most mature $CD11b^{high}CD27^{low}$ cells comprising the majority of gated $CD3^{-}NK1.1^{+}$ cells. In contrast, in all tumors $CD27^{high}$ NK cells were the dominant subset. To investigate if the distribution is changed during tumor progression, we analyzed the subset distribution in subcutaneous B16 melanomas of different tumor size. As shown in Figure 6.1B, even at early stages of tumor growth most of the cells in the tumor belonged to the $CD27^{high}$ subset. It is, however, still possible that, independently of the maturation state, CD27 is upregulated within the tumor tissue, owing to its potent costimulatory role for NK cell activity. Therefore, we analyzed the expression of several other markers that correlate with CD27 and characterize the less mature state of NK cells (Figure 6.1C). High expression of CD27 in the tumor was positively correlated with the expression of c-Kit and CD69 that are shown to be expressed by immature NK cells [109]. KLRG1 and CD43 that characterize mature NK cells showed reduced expression on tumor infiltrating $CD27^{high}$ NK cells. Thus, our data indicate that solid tumors of different origin are preferentially infiltrated by the less mature $CD27^{high}$ NK cell subset.

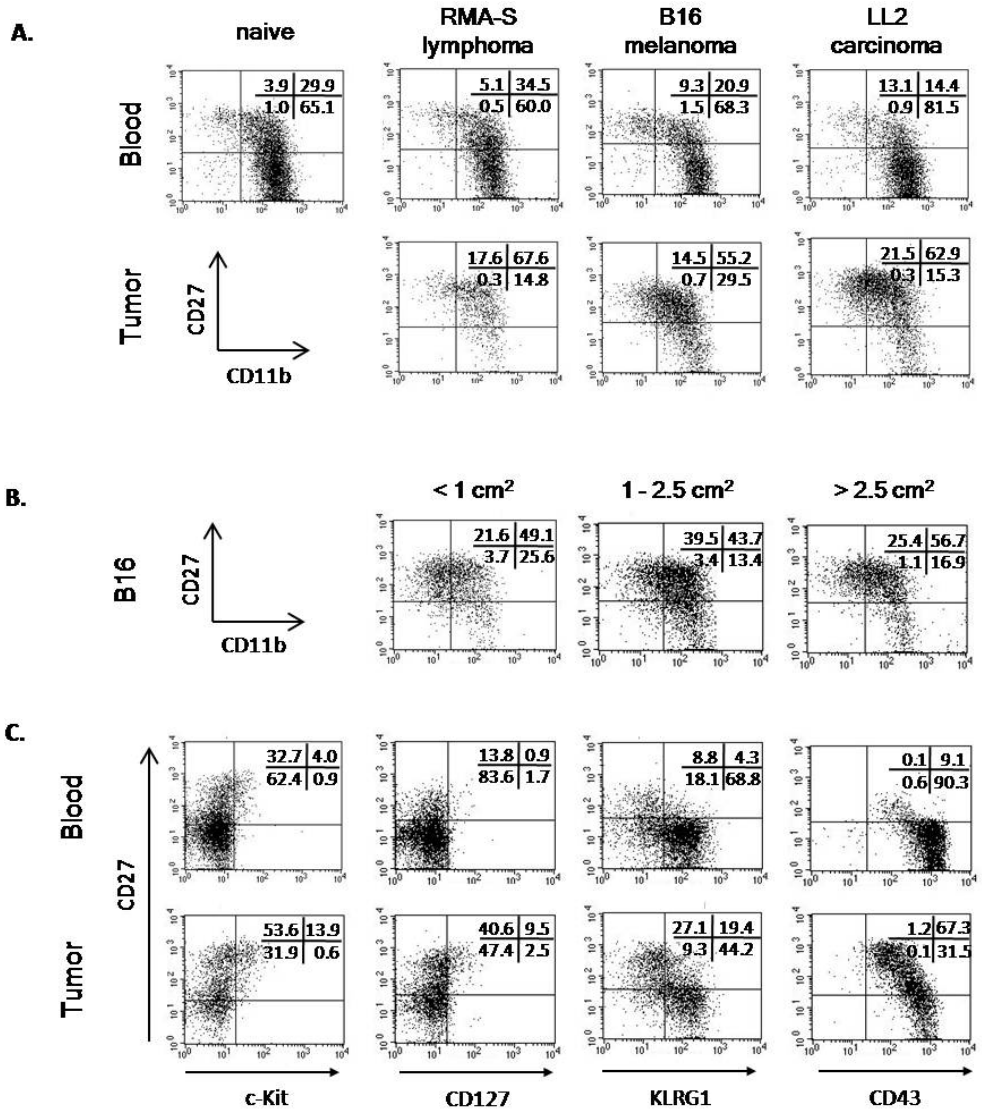


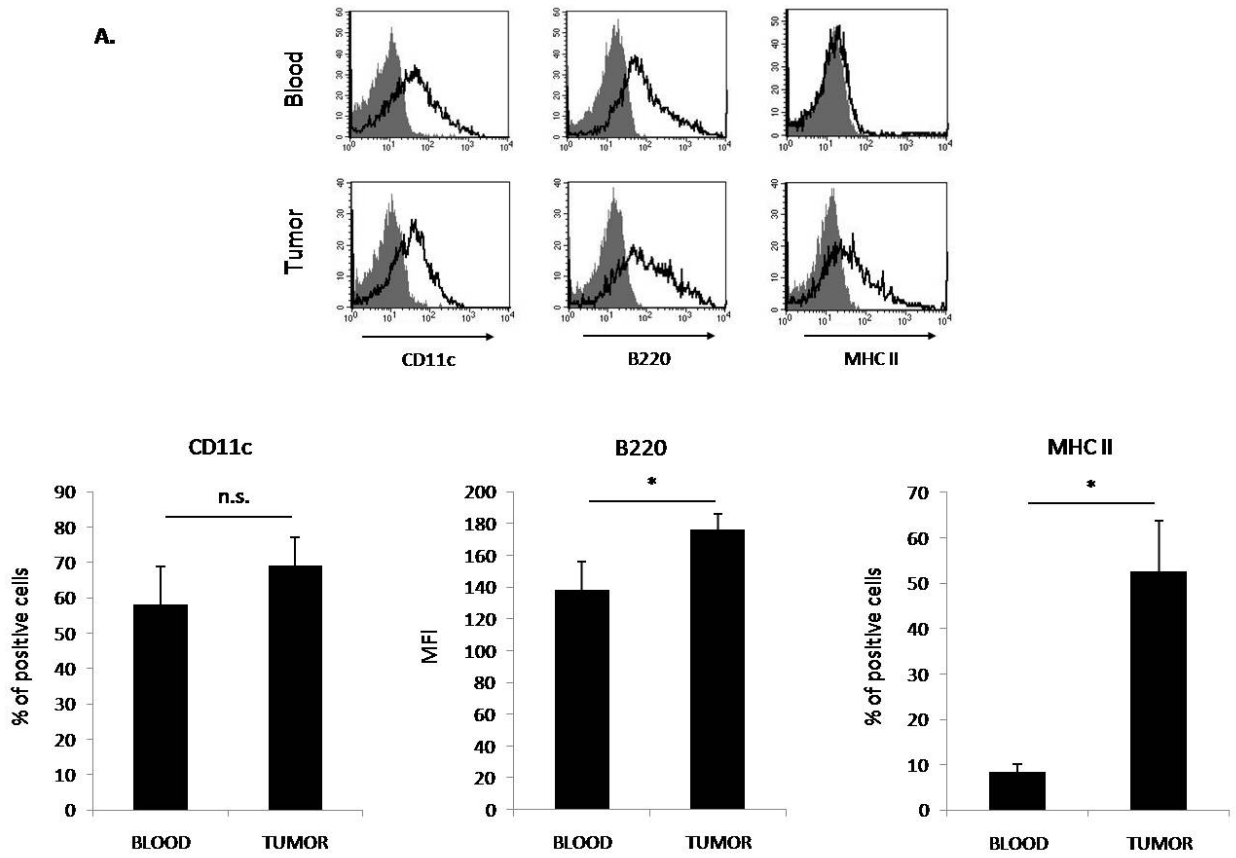
Figure 6.1. Tumor infiltrating NK cells show an immature phenotype. Mice were inoculated subcutaneously with PBS only (naive controls) or 10^6 tumor cells in 100 µl PBS. When tumor size exceeded 1 cm^2 (A and C), mice were sacrificed and single cell suspensions were prepared from blood and tumor. In B, mice were sacrificed when tumors reached the indicated size. Phenotype of blood and tumor infiltrating NK cells, defined as $CD3^-NK1.1^+$, was analyzed by flow cytometry. % of cells in the depicted quadrants are shown in the dot-plots.

6.1.2. Subset of tumor infiltrating NK cells show IKDC-like phenotype

Besides CD11b and CD27, which are mainly used to indicate the maturation stage, several other markers define NK cells with special functional features. Recently defined IKDCs (Interferon Producing Killer Dendritic Cells) were described as a cell population with the properties of both NK and DCs [139]. Due to their high potency of IFNγ production and cytotoxicity, as well as antigen presentation potential and release of IL-12, IKDCs have been shown to be very efficient anti-tumor effectors. Their main phenotypic

characteristics are expression of NK1.1, high levels of CD11c and B220, as well as MHC class II. To further characterize NK cells found within the tumor tissue, we analyzed the expression of IKDC markers on NK cells in blood and tumor of tumor bearing hosts.

In RMA-S injected mice, all NK cells, defined as $CD3^{-}NK1.1^{+}$, from both blood and tumor expressed CD11c and B220. Of note, expression of B220 was increased on subset on NK cells in tumor (Figure 6.2A). We did not observe MHC II^{+} NK cells in blood. In contrast, tumor infiltrating NK cells increased MHC II expression with tumor progression. A similar phenotype has been observed in both B16 and LL2 tumor bearing mice (Figure 6.2B). These data indicate that subset of NK cells found in the tumor phenotypically resemble IKDCs. Functional analysis must be performed to confirm their potential ability to produce high amounts of IFNγ and/or IL-12. Importantly, NK cells within tumor tissue express MHC II, unlike their counterparts from blood, which indicates that they indeed might be able to present tumor antigens and activate adaptive immune responses.



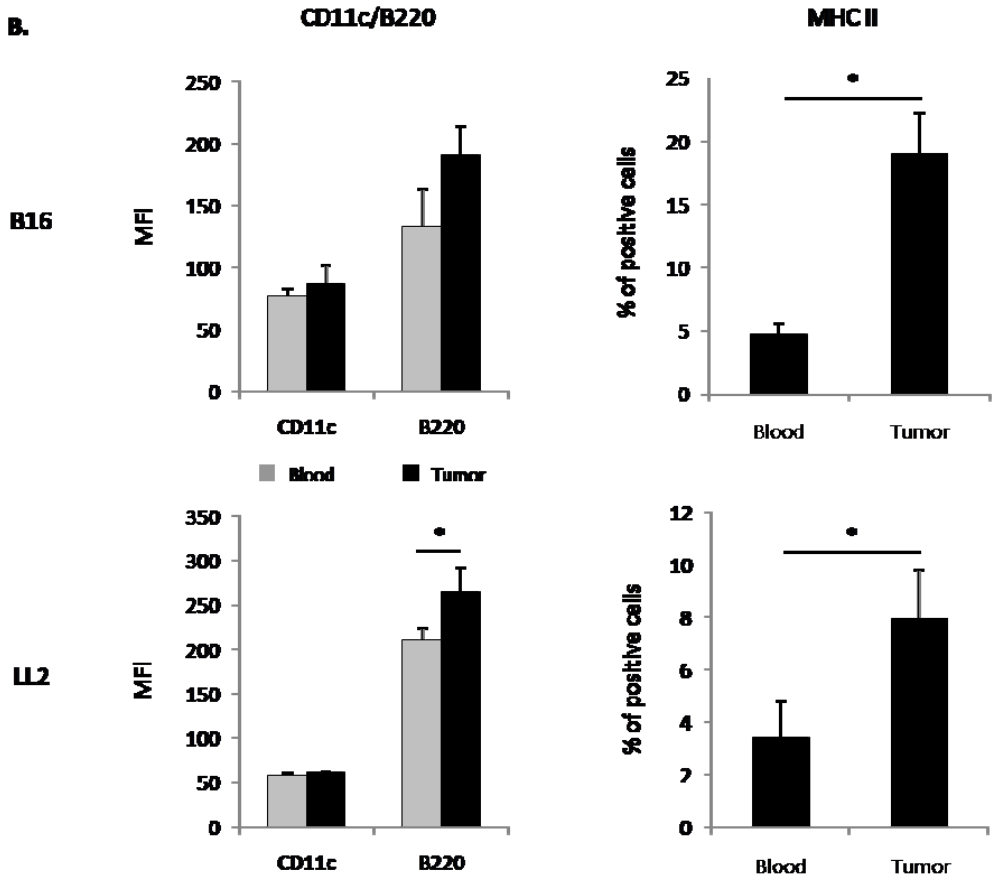


Figure 6.2. Tumor-infiltrating NK cells show IKDC-like phenotype. Mice were inoculated with 10^6 tumor cells in 100 µl PBS. When tumor size exceeded 1 cm^2, mice were sacrificed and single cell suspensions were prepared from blood and tumor. Phenotype of blood and tumor infiltrating NK cells, defined as $CD3^-NK1.1^+$, was analyzed by flow cytometry. Representative stainings and quantification of CD11c, B220 and MHC II expression on NK cells from blood and tumor of RMA-S lymphoma (A), B16 melanoma and LL2 carcinoma (B) tumor bearing mice are shown. Graphs indicate mean±SD of experimental group of 3 treated animals from one out of two experiments performed. *, $p<0.05$ by t-test

6.1.3. Tumor infiltrating NK cells express elevated amounts of granzyme B

Many transplanted tumors were shown to be controlled by NK cells in a perforin dependent manner indicating that direct cytotoxicity plays the central role in the NK cell anti-tumor response [81-82, 102]. However, when tumors efficiently evade immune surveillance mechanisms and progressive growth is established, NK cell functions might be altered. We compared NK cell cytotoxic response of blood and spleen NK cells derived from naïve or RMA-S tumor bearing animals. *Ex vivo* response was correlated to the level of NK cell degranulation, measured by CD107a/b expression, after the encounter with target cells *in vitro*. *In vivo* response was correlated to the clearance of tapasin deficient splenocytes that express reduced levels of MHC I, from the spleen of naïve and tumor bearing mice. Both *ex vivo* and *in vivo* NK

cells efficiently responded to target cells (Figure 6.3). Of importance, *ex vivo* response was measured against NKG2D sensitive target cells, YAC-1, while *in vivo* killing was directed against MHC I^{low} splenocytes. These data indicate that no systemic impairment of NK cell cytotoxic response involving different recognition pathways can be detected in tumor bearing animals.

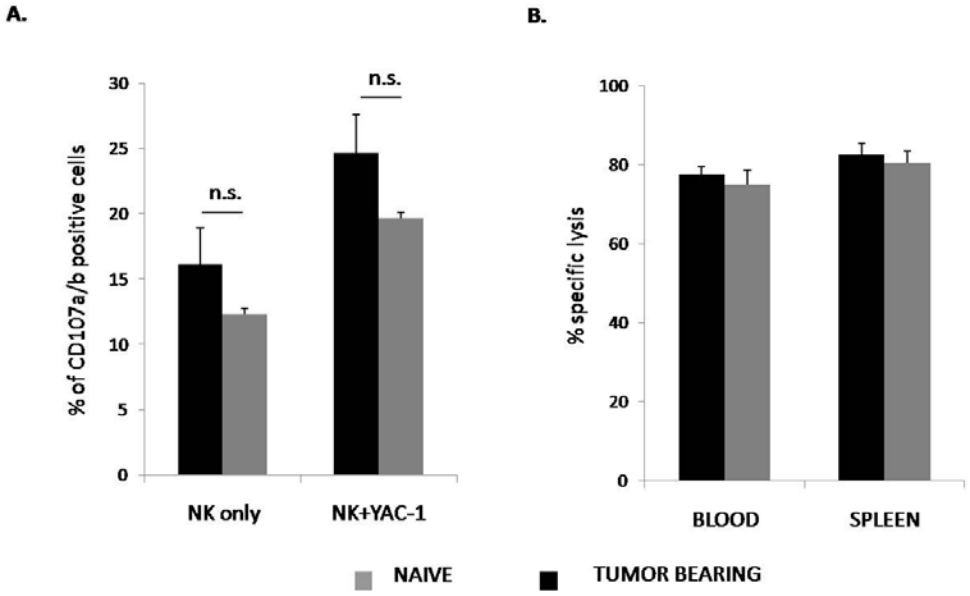


Figure 6.3. NK cells from tumor bearing animals exert normal cytotoxic responses. (A) Whole splenocytes from naïve or RMA-S inoculated animals (solid tumor, day 10 post-injection) were co-cultured with YAC-1 target cells in a 1:1 ratio in the presence of fluorochrome labeled CD107a/b mAbs or isotype control. Degranulation of NK cells in response to target was analysed by flow cytometry and expressed as % of $CD107a/b^+$ cells among gated $CD3^-NK1.1^+7\text{-}AAD^-$ cells. Graphs indicate mean±SD of triplicate culture from one out of two experiments performed. n.s., not significant by t-test. (B) WT or tapasin deficient splenocytes, labelled with different concentration of CFSE, were injected in naïve or RMA-S inoculated animals (solid tumor, day 17 post-injection) in a 1:1 ratio. After 6h, mice were sacrificed and % of differentially labelled $CFSE^+$ cells from spleen was measured by flow cytometry. NK cell cytotoxic response *in vivo* was expressed as % of specific lysis of tapasin deficient (MHC class I^{low}) compared to WT splenocytes. Graphs indicate mean±SD of experimental group of 3 treated animals from one out of two experiments performed.

We could not compare cytotoxicity of tumor infiltrating NK cells with those derived from blood or spleen due to the low cell numbers that were obtained from the tumor tissue. We, however, observed that NK cells from the tumor expressed perforin and granzyme B (Figure 6.4). Perforin levels were slightly elevated in TINs compared to spleen NK cells, although the differences were not always significant. In addition, tumor infiltrating NK cells were positive for granzyme B, which was not detected in spleen NK cells. Presence of perforin and granzyme B indicates that NK cells in the tumor are armed to perform cytotoxic responses.

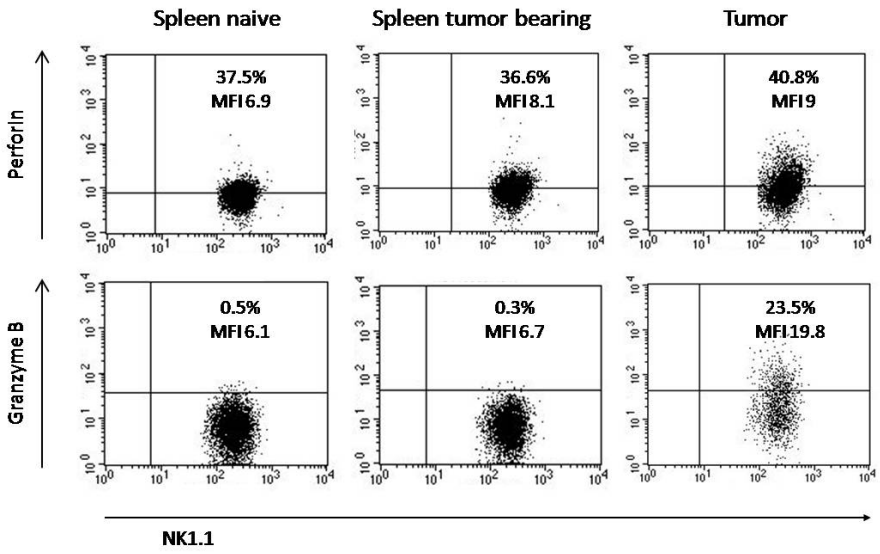


Figure 6.4. NK cells from tumor express perforin and granzyme B. Mice were inoculated with 10^6 RMA-S tumor cells in 100 µl PBS. 10 days post-injection, mice were sacrificed and single cell suspensions were prepared from spleen and tumor. Expression of perforin and granzyme B of spleen and tumor infiltrating NK cells, defined as $CD3^-NK1.1^+$, was analyzed by flow cytometry.

6.2. Gene expression profile of tumor infiltrating NK cells

Our previous data showed that tumor infiltrating NK cells might have special properties and exert functions different from NK cells found in the peripheral blood. Those properties could be the consequence of the natural response to the tumor targets and/or the microenvironment formed by the solid tumor. For the analysis of NK cells in tumor bearing animals we mainly used subcutaneous RMA-S lymphoma model. Owing to the low expression of MHC I by RMA-S tumor cells, when injected at low cell numbers, tumor formation is prevented in a NK cell dependent manner. When the initial tumor cell load is sufficient to drive tumor progression, we observed that tumor growth followed defined kinetics. Three phases of tumor growth can be recognized (Figure 6.5A). The first phase is represented by the slow kinetics of tumor progression and is followed by the intermediate phase in which tumor size does not change significantly for several days. While in the first phase innate immunity might play a dominant role in the anti-tumor response, in the second phase tumor growth might be efficiently controlled by combined actions of both the innate and the adaptive arm of the immune system. However, despite the fact that an initial control was established, all animals progress to the third phase characterized by tumor outgrowth that leads to death. When NK cells are removed from the system by application of αNK1.1 mAb, that depletes NK and NKT cells, the initial tumor control is lost and animals progress through the third phase-like tumor outgrowth (Figure 6.5B).

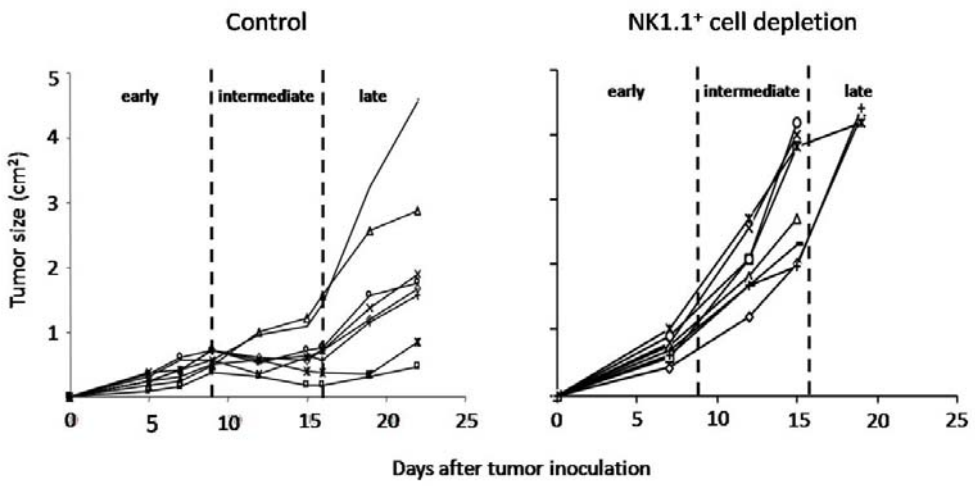


Figure 6.5. RMA-S tumor growth is controlled by NK cells. (A) Mice were injected with 10^6 RMA-S tumor cells subcutaneously and tumor growth was measured in regular time intervals. (B) Group of RMA-S inoculated mice was treated with αNK1.1 mAb on day -2, +2, +9 and +16 relative to the tumor cell inoculation, leading to efficient depletion of $NK1.1^+$ cells, which was sustained during experiment (data not shown). Every line represents tumor growth kinetics of an individual mouse.

We aimed to better understand the role of NK cells in tumor growth control during different phases of tumor progression. Therefore we performed global gene expression profile analysis of blood and tumor infiltrating NK cells from RMA-S tumor bearing animals at different time points of tumor progression. First, the experimental design (Figure 6.6) allowed us to compare blood NK cells from naïve and tumor bearing animals in order to characterize possible systemic changes that could occur due to the tumor outgrowth. If such changes were detectable from the early time points, they could be used as potential clinical biomarkers of tumor presence. Second, a comparison can be made between blood and tumor infiltrating NK cells in tumor bearing animals, allowing the analysis of potential special features of NK cells from the tumor tissue. Finally, the comparison between blood and tumor NK cell pools over time indicates how the NK cell effector functions and their potential to control the growth of MHC I deficient tumor changes while tumor progresses through the described phases (Figure 6.5). As shown in Figure 6.6A, we isolated NK cells from blood of naïve (day 0) and tumor bearing animals on day 3, 10 and 17 after tumor cell inoculation. NK cells from the tumor were obtained on day 10 and day 17 after tumor cell injection. Cells were sorted for high purity (≥99% from blood and ≥98% from tumor). RNA isolated from sorted NK cells was subjected to the quality test. High quality RNA was amplified, labelled and hybridized with mouse whole genome microarrays. Every experimental group and time point was represented as biological triplicate in microarray experiment.

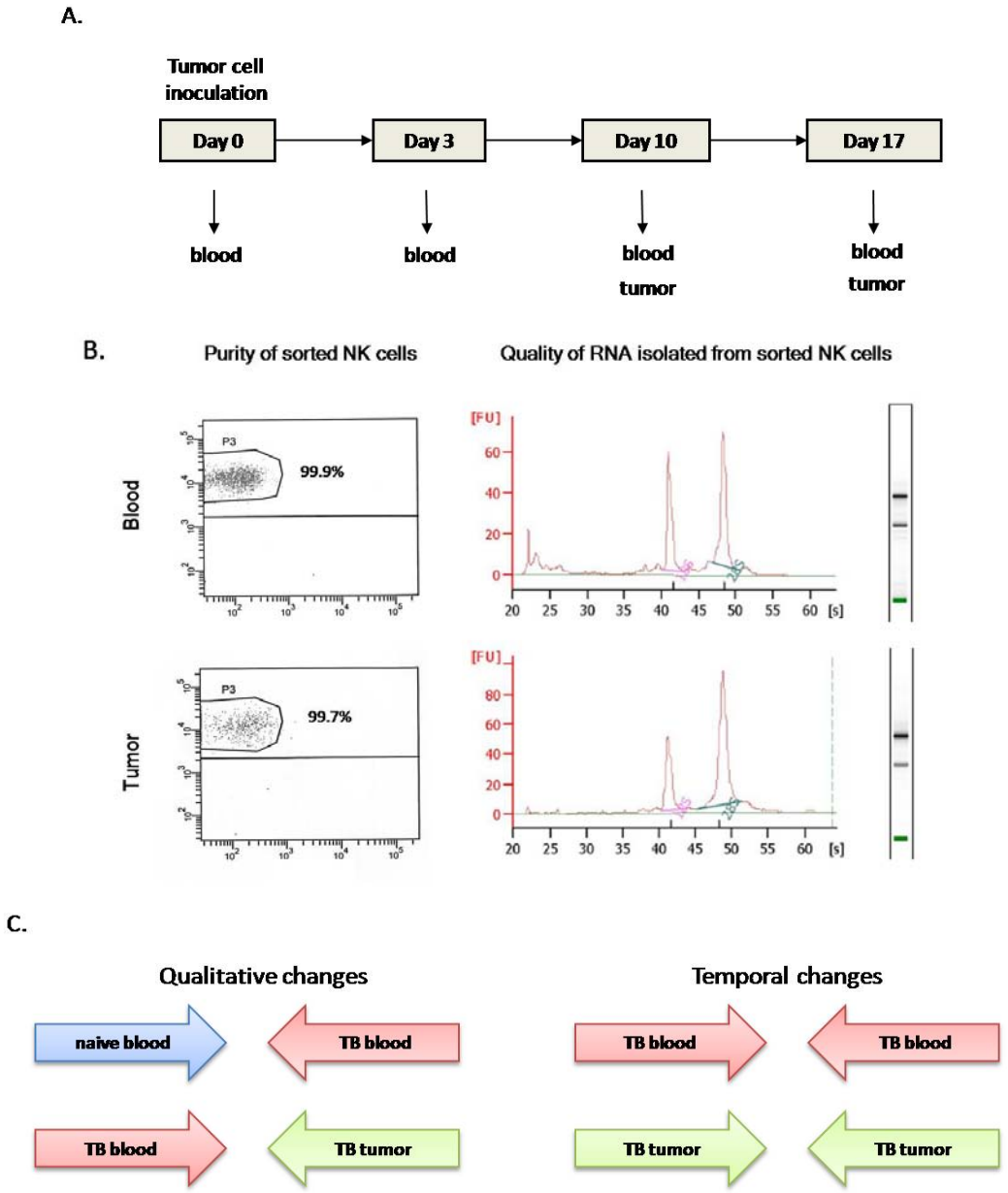


Figure 6.6. Experimental design of gene expression analysis of blood and tumor infiltrating NK cells. (A) Groups of 30 mice were injected subcutaneosly with PBS (naïve controls) or 10^6 RMA-S lymphoma cells (day 0). NK cells were isolated from blood and tumor tissue at indicated time points after tumor cell inoculation. Biological triplicates were provided for every treatment, every organ and every time point of tumor growth. (B) Highly purified NK cells were obtained by flow cytometry sorting from blood and tumor of naive and tumor bearing mice. One representative analysis of purity of sorted NK cells is shown (left). Blood NK cells samples with purity ≥99% and tumor NK cell samples with purity ≥98% were selected for RNA isolation. Total RNA isolated from sorted cells was analysed for quality using RNA 6000 Pico Assay performed on the Bioanalyzer 2100 Lab-on-a-Chip system (right). Similar starting amounts of high quality RNA were subsequently amplified, labelled and hybridized with Affymetrix® mouse whole genome microarrays. (C) Comparisons of gene expression profiles were performed between different treatments (naïve vs tumor bearing), different organs (blood vs tumor) or between same organ at different time point of tumor growth.

6.2.1. Tumor infiltrating NK cells show strikingly different transcription profile compared to blood NK cells

Nine different comparisons were performed over the transcription profiles of naïve blood, tumor bearing animal blood and tumor infiltrating NK cells isolated at different time points after tumor cell inoculation (Figure 6.7A). In total, 1387 transcripts were found to be differentially regulated between the various groups that have been compared. The heat map from Figure 6.7B shows clustering of top 50 genes differentially expressed in at least one of the comparisons, selected according to p-value. A similar pattern was observed when all changed genes were clustered together. The clustering pattern indicates that tumor infiltrating NK cells have strikingly different transcription profile when compared with NK cells isolated from blood of both naïve and tumor bearing animals. Less pronounced changes, reflected in the lower number of differentially regulated genes, were detected when the expression profile of NK cells from the blood of naïve mice was compared with the profile of blood NK cells of tumor bearing animals. Similar observation was made when expression profiles of different time points of tumor growth were compared for both blood and tumor infiltrating NK cells.

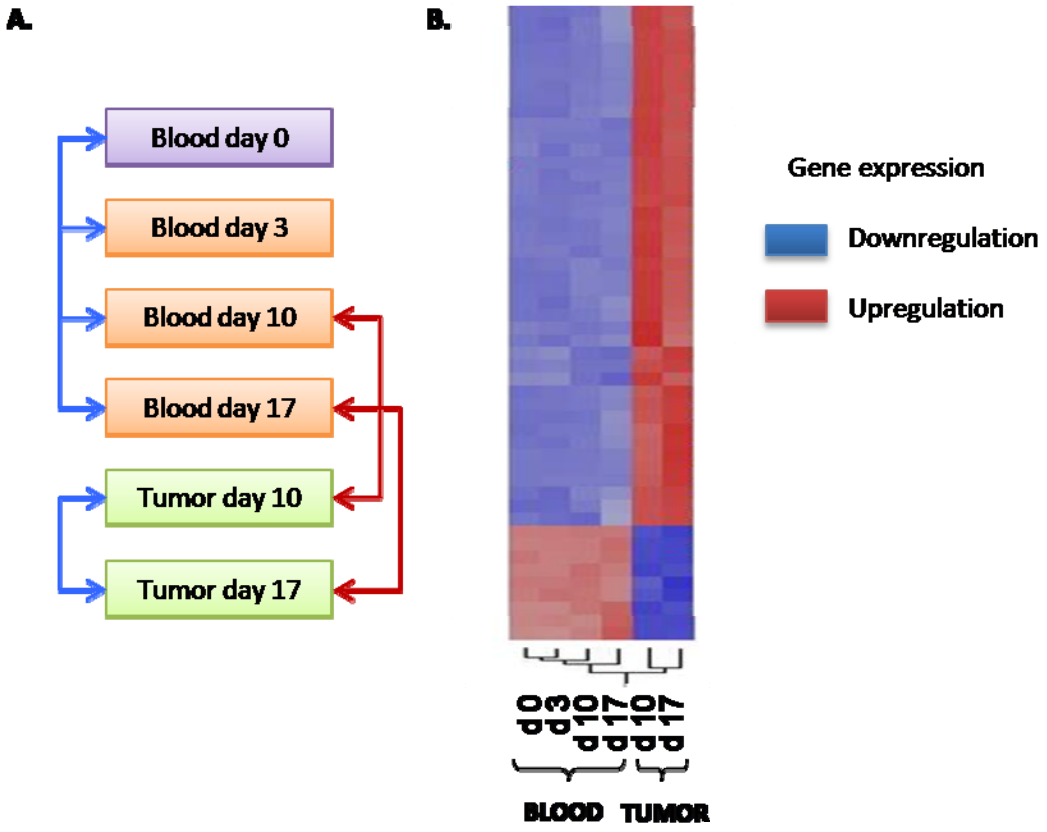


Figure 6.7. Tumor infiltrating NK cells show strikingly different transcription profile compared to blood NK cells. (A) Expression profiles were obtained in triplicates for every sample shown and compared according to the given scheme. Blue arrows indicate the comparisons of NK cells from the same organ, thereby measuring temporal changes, while red arrows show the comparisons of NK cell profiles derived from different organs at the same time

point of tumor growth. (B) Unsupervised hierarchical clustering algorithm for genes that showed significant change in the expression level in at least one comparison performed, was applied accross all samples. The length and branching pattern in the resulting dendogram reflects the similarity/difference in gene expression profiles between each of the NK cell samples. Top 50 differentially expressed genes, sorted according to p-value, are shown.

Functional classification revealed that differentially expressed genes between blood and TINs of tumor bearing mice were involved in the control of the function that might be highly relevant for anti-tumor responses (Table 6.1). Among the most significantly affected pathways are the BCR and TCR signaling pathway that utilize basically the same signaling components found downstream of NK receptors, such as Lck, Itk, Vav, PI3K, PLCγ, Cbl, MAP3Ks and NF-kB, all of which showed changed transcript levels in tumor infiltrating NK cells. This finding indicates that functionality of NK receptors might be differentially regulated within the tumor compared to blood. Effector functions triggered upon target recognition and NK receptor signaling include cytotoxicity and cytokine and chemokine production, all of which rely on vesicle trafficking and release of their components into extracellular space. Indeed, several genes regulating vesicle transport are positively regulated in TINs (pathway: SNARE interactions in vesicular transport), as well as some of the genes encoding cytotoxic vesicle components, such as cathepsins and several types of granzymes.

Pathway	Hits	Total	Hit percent	Significance	
TCR signaling pathway	25	75	33.3333	0.00000	***
Cell cycle	23	84	27.3810	0.00000	***
NK cell mediated cytotoxicity	18	65	27.6923	0.00002	***
Chronic myeloid leukemia	16	57	28.0702	0.00004	***
Cytokine-cytokine receptor interaction	29	155	18.7097	0.00020	***
MAPK signaling pathway	35	203	17.2414	0.00024	***
Streptomycin biosynthesis	5	8	62.5000	0.00030	***
Jak/STAT signaling pathway	20	95	21.0526	0.00039	***
BCR signaling pathway	12	45	26.6667	0.00062	***
Glycolysis and gluconeogenesis	10	35	28.5714	0.00122	**
Apoptosis	14	62	22.5806	0.00137	**
VEGF signaling pathway	13	57	22.8070	0.00182	**
Inositol phosphate metabolism	10	42	23.8095	0.00530	**
Hematopoietic cell lineage	13	64	20.3125	0.00533	**

Pentose phosphate pathway	6	19	31.5789	0.00690	**
Carbon fixation	5	15	33.3333	0.00933	**
Adipocytokine signaling pathway	12	61	19.6721	0.00944	**
FcεRI signaling pathway	12	61	19.6721	0.00944	**
Pancreatic cancer	12	61	19.6721	0.00944	**
Glyoxylate and dicarboxylate metabolism	4	10	40.0000	0.00986	**
Thyroid cancer	6	21	28.5714	0.01017	*
Purine metabolism	18	108	16.6667	0.01037	*
Colorectal cancer	12	66	18.1818	0.01739	*
Glycan structures biosynthesis	7	30	23.3333	0.01780	*
Galactose metabolism	6	24	25.0000	0.01985	*
Ethylbenzene degradation	3	7	42.8571	0.02104	*
Phenylalanine tyrosine and tryptophan	3	7	42.8571	0.02278	*
SNARE interactions in vesicular transport	6	25	24.0000	0.02412	*
Fructose and mannose metabolism	7	32	21.8750	0.02503	*
TLR signaling pathway	11	62	17.7419	0.02628	*
ABC transporters	7	34	20.5882	0.03404	*
Leukocyte transendothelial migration	13	82	15.8537	0.03855	*

Table 6.1. Signaling pathways affected by differential gene expression in tumor infiltrating NK cells. To identify pathways that are likely to be affected by differential gene expression between blood and tumor infiltrating NK cells, an ORA approach using Fisher's exact test was performed [277]. Analyzed pathways were collected from the KEGG database (Kyoto Encyclopedia of Genes and Genomes), or manually generated. Number (Hits) and percentage (Hit percent) of differentially expressed signaling molecules of total molecules (Total) comprising the given pathways are shown.

Apart from the direct cytotoxicity, NK cells can exert anti-tumor functions through the production of cytokines and chemokines, which can act directly on tumor cells or can modulate the responses of other immune effectors. Transcripts of several chemokines were found to be elevated in TINs, such as CCL1, CXCL2 and CXCL9, as well as chemokine receptors CCR2, CCR7 and CXCR4, while CX3CR1 expression was reduced. Through the chemokine network established at the tumor site NK cells can attract other immune cells such as neutrophils, macrophages, DCs or T cells. In addition, those cells, including NK cells themselves, can further potentiate NK cell recruitment. Indeed, some of the given

chemokine-chemokine receptor interactions (CXCR3:CXCL9/10, CX3CR1:CX3CL1) have been previously shown to facilitate NK cell trafficking to the tumor [279-280].

Gene name	Gene symbol	day 10		day 17	
		p-value	Fold change	p-value	Fold change
chemokine (C-C motif) ligand 1	Ccl1	17.55	2.65	14.88	2.40
chemokine (C-X-C motif) ligand 2	Cxcl2	11.71	6.44	11.11	6.07
chemokine (C-X-C motif) ligand 9	Cxcl9	9.56	3.04	5.51	2.25
chemokine (C-C motif) receptor 2	Ccr2	13.91	1.87	15.75	1.97
chemokine (C-C motif) receptor 7	Ccr7	9.66	3.24	7.25	2.68
chemokine (C-X-C motif) receptor 4	Cxcr4	12.24	3.47	8.82	2.77
chemokine (C-X3-C) receptor 1	Cx3cr1	39.34	-2.60	40.89	-2.66

Table 6.2. Differential expression of chemokines and chemokine receptor by NK cells infiltrating the tumor tissue. Chemokine and chemokine receptors found to be differentially regulated on transcript level between blood and tumor infiltrating NK cells of tumor bearing animals are depicted. Fold change indicates the difference of tumor versus blood signal and is considered significant when p-value is ≥ 7.

Genes whose products are known to influence cell adhesion and motility, functions important for migration across the endothelium and tissue infiltration, were differentially regulated by TINs. For example, integrin α_2 (DX5) and α_6, CD9, cadherin 22, CD151, MCAM are some of the molecules that mediate binding to endothelium and different components of extracellular matrix (ECM), thereby might promote NK cell migration through the tumor tissue. However, they were found to be negatively regulated by NK cells isolated from the tumor (Figure 6.8A). On the other hand, enzymes that modulate ECM by cleaving its components (Adam8, Adam9, Plaur) showed elevated transcript levels. These modulations can have multiple consequences including the release of active cytokines and chemokines bound to the cell surface or matrix components, but also support of tumor cell invasion and metastasis formation. We confirmed reduced expression of DX5 by tumor infiltrating NK cells at the protein level (Figure 6.8B).

A.

Gene name	Gene symbol	day 10		day 17	
		p-value	Fold change	p-value	Fold change
integrin alpha 2	Itga2	2.50	-1.24	8.79	-1.59
integrin alpha 6	Itga6	5.91	-1.97	9.19	-2.43
CD9 antigen	Cd9	8.94	-2.04	11.42	-2.38
CD151 antigen	Cd151	2.22	-1.26	6.76	-1.55
cadherin 22	Cdh22	6.66	-1.82	7.75	-1.93
melanoma cell adhesion molecule	Mcam	15.55	-1.80	17.83	-1.90
a disintegrin and metalloprotease domain 8	Adam8	33.29	3.25	42.72	4.23
a disintegrin and metalloproteinase domain 9	Adam9	11.59	1.63	5.28	1.35
urokinase plasminogen activator receptor	Plaur	7.17	1.72	4.73	1.53

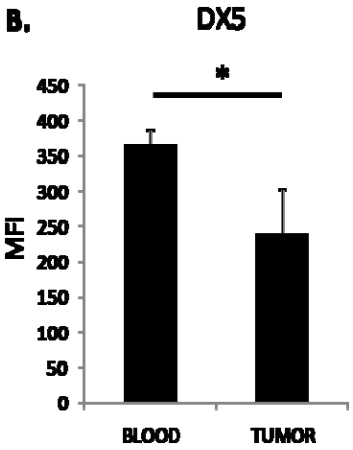


Figure 6.8. Differential regulation of several adhesion molecules and ECM modifying enzymes was detected in tumor infiltrating NK cells. (A) Adhesion molecules and enzymes specific for extracellular matrix (ECM) components found to be differentially regulated on the transcript level between blood and tumor infiltrating NK cells of tumor bearing mice are depicted. Fold change indicates the difference of tumor versus blood signal and is considered significant when p-value is ≥ 7. (B) Mice were inoculated subcutaneously with 10^6 RMA-S cells in 100 µl PBS. When tumor size exceeded 1 cm^2, mice were sacrificed and single cell suspensions were prepared from blood and tumor. Expression of integrin α_2 (DX5) on blood and tumor infiltrating NK cells, defined as $CD3^-NK1.1^+7AAD^-$, was analyzed by flow cytometry. Graph indicates mean±SD of the experimental group of three animals from one out of two experiments performed. *, $p<0.05$ by t-test

NK cells infiltrating the tumor showed increased transcript levels of cytokines, cytokine receptors and signaling molecules downstream of cytokine receptors (Table 6.1, Cytokine-cytokine receptor interaction and Jak/STAT signaling pathway). Lymphotoxin α and β, GM-CSF, TGFβ1, PDGFα and VEGFA transcripts were elevated in TINs when compared to blood NK cells (Figure 6.9A). Lymphotoxins and GM-CSF might support DC recruitment and activation [281-283], TGFβ can both suppress and promote tumor growth [284], while PDGFα and VEGFA promote angiogenesis [285]. Of note, NK cells showed elevated expression of many other molecules that facilitate angiogenesis (angiopoietin, arginase), indicating that they might play an active role in this process at the tumor site (Table 6.1, VEGF signaling pathway). IL-1R2, IL-2Rα, IL-4Rα, IL-7Rα, IL-15Rα, IFNγR1 and TGFβR2 are examples of increased regulation of cytokine receptors by TINs (Figure 6.9A). IL-7, IL-2 and IL-15 are shown to promote NK cell maturation, survival and activation [286]. Elevated expression of TGFβ receptor might render NK cell sesitive to TGFβ, which is known to suppress their ability to produce IFNγ [287]. As shown in Figure 6.9B, surface expression of IL-2Rα and IL-7Rα was increased on tumor infiltrating NK cells when compared to blood, indicating a positive correlation between transcript and protein level regulation. Protein levels of IL-4Rα and IFNγR1 were, however, unchanged.

Multiple genes regulating the cell cycle were upregulated by NK cells found in tumor tissue. Most of them (cyclin E, A, B, cyclin-dependent kinases 2, 25B and others) indicate that NK cells actively progress through the cell division. However, at the same time, elevated expression of GADD45B (growth arrest and DNA-damage-inducible 45 beta) suggests that cell division might be arrested due to the cellular stress [288]. Indeed, the tumor tissue is characterized by unfavorable conditions such as low oxygen levels, acidosis and high intestinal fluid pressure. Changes involving metabolic pathways support the adaptation to such biochemical environment [289-290]. For example, under hypoxia, cells cannot efficiently utilize oxidative phosphorylation and switch their energy production preferentially towards glycolysis. In tumor infiltrating NK cells, we have observed high upregulation of the transcription factor HIF1α, the main regulator of gene expression under low oxygen concentrations. In addition, enzymes that facilitate glucose uptake, phosphorylation and finally mediate its breakdown, which are also under the control of HIF1α, are

found to be positively regulated by NK cells in the tumor. Finally, pro-angiogenic function of NK cells can be also a consequence of hypoxia and HIF1α activation.

A.

Gene name	Gene symbol	day 10		day 17	
		p-value	Fold change	p-value	Fold change
interleukin 2 receptor, alpha chain	Il2ra	30.35	2.73	30.46	2.74
interleukin 7 receptor	Il7r	7.98	2.42	7.28	2.31
interleukin 1 receptor, type II	Il1r2	10.84	2.03	13.28	2.23
interleukin 4 receptor, alpha	Il4ra	13.67	1.37	20.24	1.49
interleukin 15 receptor, alpha chain	Il15ra	13.19	1.21	9.02	1.16
interferon gamma receptor 1	Ifngr1	10.09	1.81	6.37	1.57
transforming growth factor, beta 1	Tgfb1	7.70	1.33	4.05	1.21
transforming growth factor, beta receptor II	Tgfbr2	15.19	1.34	5.39	1.17
vascular endothelial growth factor A	Vegfa	30.22	2.48	14.91	1.81
platelet derived growth factor, alpha	Pdgfa	10.37	1.63	12.99	1.75
granulocyte-macrophage colony stimulating factor 2	Csf2	9.14	2.82	11.20	3.23

B.

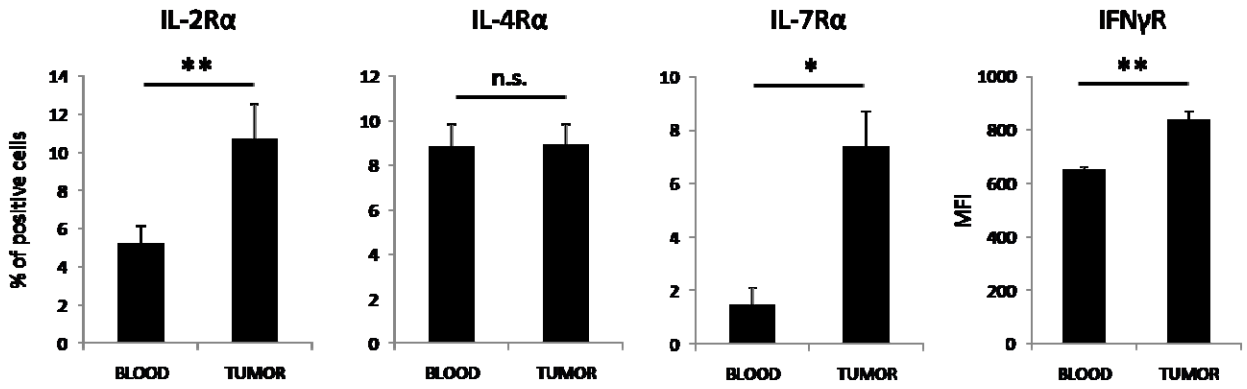


Figure 6.9. Multiple cytokines and cytokine receptors are differentially regulated between blood and tumor infiltrating NK cells. (A) Cytokines and cytokine receptors found to be differentially regulated on the transcript level between blood and tumor infiltrating NK cells of tumor bearing mice are depicted. Fold change indicates the difference of tumor versus blood signal and is considered significant when p-value is ≥ 7. (B) Mice were inoculated subcutaneously with 10^6 RMA-S cells in 100 µl PBS. When tumor size exceeded 1 cm^2, mice were sacrificed and single cell suspensions were prepared from blood and tumor. Expression of depicted cytokine receptors on blood and tumor infiltrating NK cells, defined as $CD3^-NK1.1^+$, was analyzed by flow cytometry. Graphs indicate mean±SD of experimental group of three animals. *, p<0.05; **, p<0.01 by t-test

In summary, our data reveal that tumor infiltrating NK cells showed strikingly different transcription profile compared to blood NK cells of tumor bearing animals. Differentially expressed genes are involved in multiple functions relevant for anti-tumor responses including target recognition and triggering through

activating NK receptors, vesicle trafficking and cytotoxicity, production and response to cytokines and chemokines, migration, angiogenesis and regulation of proliferation, survival and metabolic functions in response to hypoxia. We evaluated protein levels of selected differentially regulated genes and confirmed that changes on transcript levels in most of the cases correlated with protein expression. Detailed analysis of detected changes will provide a better understanding of NK cell effector response within the complex microenvironment of solid tumors and might allow for proper manipulation to achieve better immune control of tumor development and progression.

6.2.2. Tumor infiltrating NK cells downregulate expression of activating NK cell receptors

Around 570 genes were found to be negatively regulated on the transcript level when blood and tumor infiltrating NK cells are compared on both day 10 and day 17 of tumor growth. Among those genes, several NK cell activating receptors were detected (Table 6.2).

Gene name	Gene symbol	day 10		day 17	
		p-value	Fold change	p-value	Fold change
natural cytotoxicity triggering receptor 1	Ncr1/NKp46	6.03	-1.61	7.85	-1.75
killer cell lectin-like receptor subfamily B member 1C	Klrb1c/NKR-P1C/NK1.1	9.24	-1.55	7.18	-1.46
CD244 natural killer cell receptor 2B4	CD244/2B4	7.27	-1.49	4.03	-1.33
CD48 antigen	CD48	12.61	-1.47	16.41	-1.57
killer cell lectin-like receptor, subfamily A, member 1	Klra1/Ly49A	8.79	-1.23	3.07	-1.12
killer cell lectin-like receptor family E member 1	Klre1/NKG2I	12.15	-1.39	12.7	-1.4

Table 6.2. Transcriptional regulation of activating receptors by tumor infiltrating NK cells. NK cell receptors found to be differentially regulated on the transcript level between blood and tumor infiltrating NK cells of tumor bearing mice are depicted. Fold change indicates the difference of tumor versus blood signal and is considered significant when p-value is ≥ 7.

NKp46 belongs to the group of natural cytotoxicity receptors (NCRs). It was shown to recognize viral hemaglutinins as well as unknown ligand(s) on several tumor cell lines including RMA-S [291]. In addition, killing of different tumor cell lines can be partially or completely abrogated by blocking of NCRs [292-293]. NKp46 deficiency in mice was shown to hamper the control of lymphoma, however, the effect was dependent on the mouse strain and tumor cell line used [294]. NK1.1 belongs to the NKR-P1 family of receptors shown to recognize the Clr (Clec2, Ocil) family of ligands, both of which are C-type lectin-like molecules encoded within the same locus in the mouse genome [295-296]. Both stimulating and inhibitory isoforms of NKR-P1 receptors have been characterized and several ligands have been identified, which are either widely expressed or restricted to the certain cell types as macrophages, DCs or osteoclasts. The ligand for NKR-P1C, activating receptor recognized by PK136 (αNK1.1) mAb in BL6 mouse strain,

is still unknown. Both NKp46 and NKR-P1C (NK1.1) are coupled to the adaptor molecules CD3ζ and FcεRIγ, indicating that they could engage a similar signaling pathway leading to the similar outcome in response to different ligands. 2B4 is a receptor expressed on all NK cells, γδ T and $CD8^+$ memory T cells. It can mediate both activation and inhibition and mode of its action depends on the level of surface expression, degree of ligand engagement and nature of adaptor molecules it associates with [297]. Its ligand, CD48, is expressed on all hematopoiteic cells and it was proposed to deliver a signal upon binding to 2B4, probably through recruited adaptor proteins. A bidirectional 2B4-CD48 interaction has been shown to be important for the proliferation and generation of NK cell effector functions through the homotypic NK-NK interaction [298] as well as for NK cell mediated stimulation of T cell responses [299].

Microarray data analysis revealed that NKp46, NKR-P1C (NK1.1), 2B4 and CD48 transcript levels were reduced in TINs when compared to blood in tumor bearing animals (Table 6.2). We analyzed the surface expression of NKp46, 2B4 and CD48 receptors in TINs and confirmed that the negative regulation at mRNA levels correlated with decreased protein levels detected on the cell surface (Figure 6.10). In all performed experiments, NK cells were defined as $CD3^-NK1.1^+$ cells. We have observed a slight downregulation of NK1.1 expression on gated NK cells within the tumor, which was not always significant. In parallel, we confirmed that the surface expression of the activating NK cell receptor NKG2D, which was not regulated at the mRNA level, was unchanged in the tumor compared to blood.

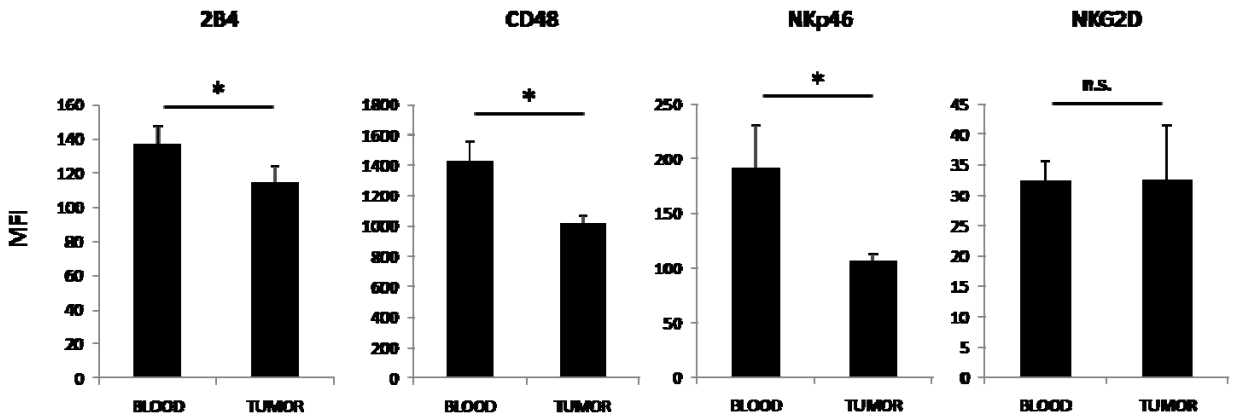


Figure 6.10. Tumor infiltrating NK cells downregulate expression of activating receptors NKp46, 2B4 and CD48. Mice were inoculated subcutaneously with 10^6 RMA-S cells in 100 µl PBS. When tumor size exceeded 1 cm^2, mice were sacrificed and single cell suspensions were prepared from blood and tumor. Expression of depicted receptors on blood and tumor infiltrating NK cells, defined as $CD3^-NK1.1^+$, was analyzed by flow cytometry. Graphs indicate mean±SD of experimental group of three animals and are representative of one out of two experiments performed. n.s., not significant; *, $p<0.05$ by t-test

Beside the depicted activating receptors, expression of the inhibitory receptor Ly49A was also negatively regulated at the transcript level in TINs compared to blood in tumor bearing animals (Table 6.2). Our attempts to compare expression of Ly49 receptors on blood and tumor infiltrating NK cells were unsuccessful due to their differential sensitivity to digestion enzymes that must be used for tumor tissue

dissociation. While Ly49G2 and Ly49C/I/F/H were completely sheded from the blood derived NK cells by collagenase or hyaluronidase treatment, their expression could still be detected on TINs at the levels comparable to the untreated blood (Figure 6.11). The inhibitory receptor Ly49A, which was negatively regulated at the transcript level, showed high surface expression on TINs, elevated compared to blood NK cells treated in an identical way as tumor tissue (Figure 6.11). However, it is not possible to say if tumor infiltrating NK cells increased the expression of Ly49A due to the fact that the receptor on blood NK cells was partially affected by the treatment. Since most of Ly49 receptors bind MHC I molecules and mediate NK cell inhibition, we compared the levels of their expression in RMA-S, MHC I deficient, and RMA, MHC I sufficient, tumors. NK cells found within RMA tumors showed a similar pattern of Ly49 receptor expression (data not shown) indicating that their regulation is not influenced by MHC I expression levels on tumor cells.

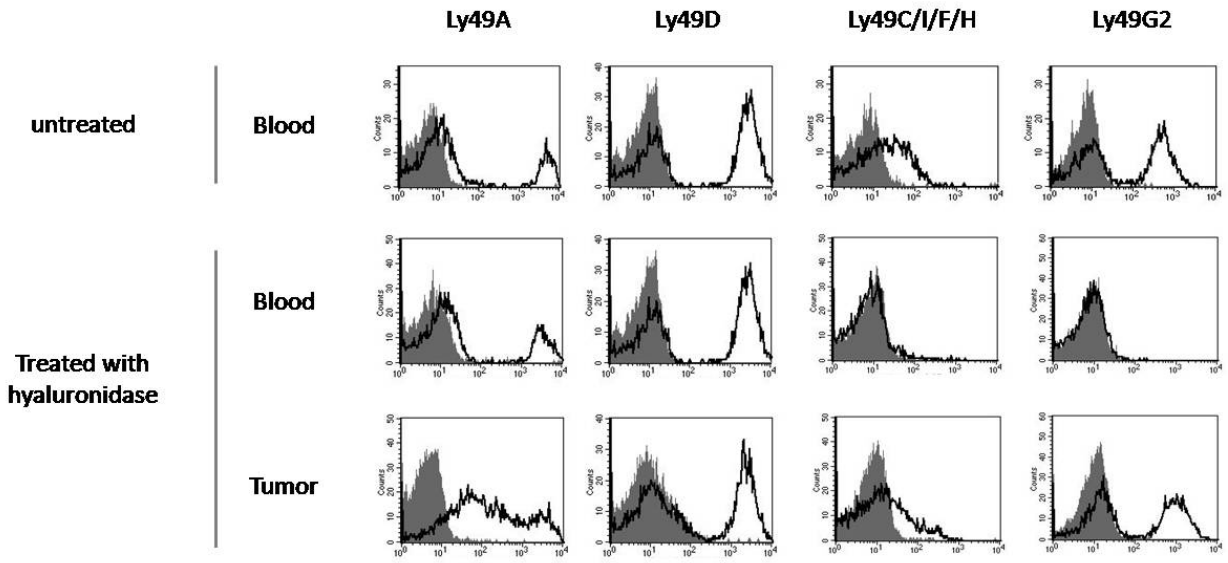


Figure 6.11. Differential regulation of Ly49 receptors by tumor infiltrating NK cells. Mice were inoculated subcutaneously with 10^6 RMA-S cells in 100 µl PBS. When tumor size exceeded 1 cm^2, mice were sacrificed and single cell suspensions were prepared from blood and tumor. Tumor tissue was digested with hyaluronidase as indicated in *Materials and Methods* section. Blood cells were treated with hyaluronidase or left untreated. Expression of depicted Ly49 receptors was analyzed by flow cytometry. Representative stainings show the expression of depicted Ly49 receptors on gated $CD3^-NK1.1^+$ cells.

In summary, our data showed that within tumor tissue NK cells negatively regulated expression of activating receptors NKp46, 2B4 and CD48 at both mRNA and protein level. Although NK1.1 was negatively regulated at the mRNA level, the surface protein was not found decreased in every experiment performed. However, this tendency was always observed. Expression of Ly49 receptors was rather regulated at the post-transcription and/or post-translation level in the manner that does not depend on the MHC I expression by tumor cells. Our data indicated that the surface expression of inhibitory Ly49 receptors might be stabilized within tumor tissue by unknown mechanisms.

6.2.3. Tumor infiltrating NK cells upregulate expression of B7 family members

Out of 1387 differentially expressed genes between NK cells from blood and tumor of tumor bearing mice, around 820 belong to the group of positively regulated transcripts. The subgroup of significantly upregulated genes was identified to belong to the B7 family of costimulatory molecules (Figure 6.12).

Gene name	Gene symbol/ Other name	day 10		day 17	
		p-value	Fold change	p-value	Fold change
cytotoxic T-lymphocyte-associated protein 4	Ctla4	33.79	6.84	45.3	11.57
programmed cell death 1 ligand 2	Pdcd1lg2/PD-L2/B7-DC	45.29	2.99	40.74	2.72
inducible T-cell co-stimulator	Icos	56.33	2.8	43.82	2.41
programmed cell death 1 ligand 1	Pdcd1lg1/PD-L1/B7-H1	33.81	2.5	26.55	2.15
programmed cell death 1	Pdcd1/PD-1	30.38	1.87	40.48	2.19
CD86 antigen	Cd86/B7-1	7.66	1.63	3.22	1.34
icos ligand	Icosl/ICOS-L	4.84	1.23	7.67	1.31

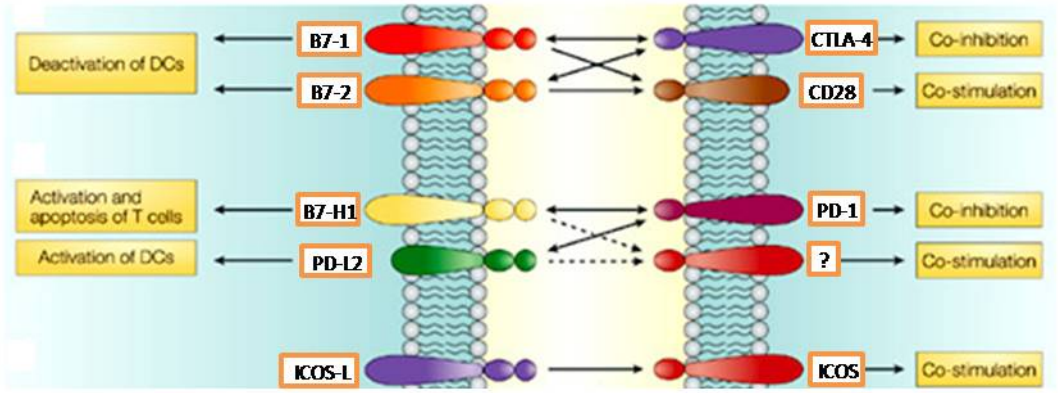


Figure 6.12. Positive regulation of B7 family members by tumor infiltrating NK cells. B7 family members found to be differentially regulated at the transcript level between blood and tumor infiltrating NK cells in tumor bearing mice are depicted in the table. Fold change indicates the difference of tumor versus blood signal and is considered significant when p-value is ≥ 7. Lower panel indicates the so far known interactions established between the members found in the table and their functional outcomes [300].

We analyzed the protein expression of B7 family members on NK cells from blood and tumor of RMA-S tumor bearing mice. As shown in Figure 6.13, B7 family receptors CD28, CTLA-4, PD-1 and ICOS were not detectable in blood of either naïve (data not shown) or tumor bearing animals. However, tumor infiltrating NK cells upregulated CD28, CTLA-4 and PD-1, while the expression of the activating receptor ICOS remained low/negative and unchanged at the protein level. These data indicate that PD-1 and CTLA-4 regulation at the transcript level correlates with the protein level expression, while CD28 expression is most probably regulated post-transcriptionally. Both CD28 and PD-1 expression levels were increased with tumor progression (data not shown) showing the highest expression at the high tumor load.

Interestingly, we did not detect CD28, CTLA-4 and/or PD-1 positive NK cells within the first 10 days of tumor progression. Typically, observed phenotypical changes were only detected in tumors bigger than 1 cm^2. In contrast to PD-1 and CD28, we did not measure substantial amounts of CTLA-4 on the cell surface of tumor infiltrating NK cells. Similarly to the expression pattern previously shown in T cells, CTLA-4 protein was stored intracellularly. However, we cannot exclude the possibility that small, yet undetectable amounts of the protein are transported to the cell surface and can have a functional effect. Indeed, short exposure on the cell surface followed by fast internalization has already been shown to take place in T cells and can have functional consequences due to the very high affinity of CTLA-4 for its ligands [190].

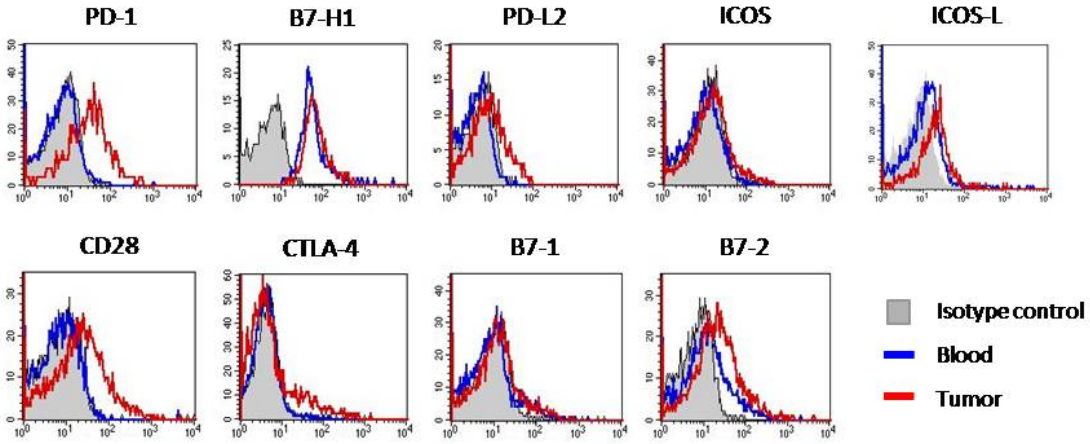


Figure 6.13. Expression of B7 family members on tumor infiltrating NK cells. Mice were inoculated subcutaneously with 10^6 RMA-S cells in 100 µl PBS. When tumor size exceeded 1 cm^2, mice were sacrificed and single cell suspensions were prepared from blood and tumor. Expression of depicted molecules on blood and tumor infiltrating NK cells, defined as $CD3^-NK1.1^+$, was analyzed by flow cytometry. Surface expression is depicted for all molecules except CTLA-4 whose expression is measured by intracellular staining of the protein. Representative stainings from one out of three experiments performed are shown.

Similar to the above mentioned receptors, PD-L2, the ligand of PD-1, was not expressed in blood, but induced on tumor infiltrating NK cells. ICOS-L already showed low expression in the blood and was slightly elevated in the tumor. In contrast to PD-L2, B7-H1, another PD-1 ligand, as well as binding partner of B7-1, is highly expressed on blood NK cells. We observed its elevated expression in the tumor, however, differences were not always significant. Expression of B7-1 and B7-2, shared ligands of CD28 and CTLA-4, was variable between experiments, which might be a consequence of their tight temporal regulation that can in addition be influenced by the tumor size. In general, the expression of B7-1 was low, while B7-2 was constitutively expressed by NK cells.

In summary, RMA-S infiltrating NK cells showed elevated expression of multiple B7 family members at both mRNA and protein level. For most of the analyzed molecules, a correlation between changes at transcript and protein level was observed. The main phenotypic features of tumor infiltrating NK cells were induced expression of CD28, CTLA-4, PD-1 and PD-L2 when compared to their blood counterparts.

6.2.3.1. Expression of B7 family members in NK cell depends on the tumor type

So far, we showed that NK cells that infiltrate different types of subcutaneous tumors had a very similar phenotype, as shown in Figure 6.1 and 6.2. We observed the induced expression of several B7 family members on NK cells that infiltrate RMA-S lymphomas, some of which were not reported to be expressed by NK cells before. Therefore, we analyzed the profile of several B7 family members on NK cells derived from the tumors of different origin. As shown in Figure 6.14, surface levels of the analyzed molecules are strongly dependent on the tumor type. While lymphomas (RMA, RMA-S) showed a tendency to highly upregulate PD-1 and CD28, melanoma (B16) and carcinoma (LL2) preferentially upregulated B7-H1. CD28 and PD-1 were expressed at very low levels in TINs of B16 and LL2 tumor bearing mice. However, in all tumor models analyzed, we observed expression of CTLA-4 (Figure 6.14), which seems to be a stable feature of NK cells infiltrating solid tumors.

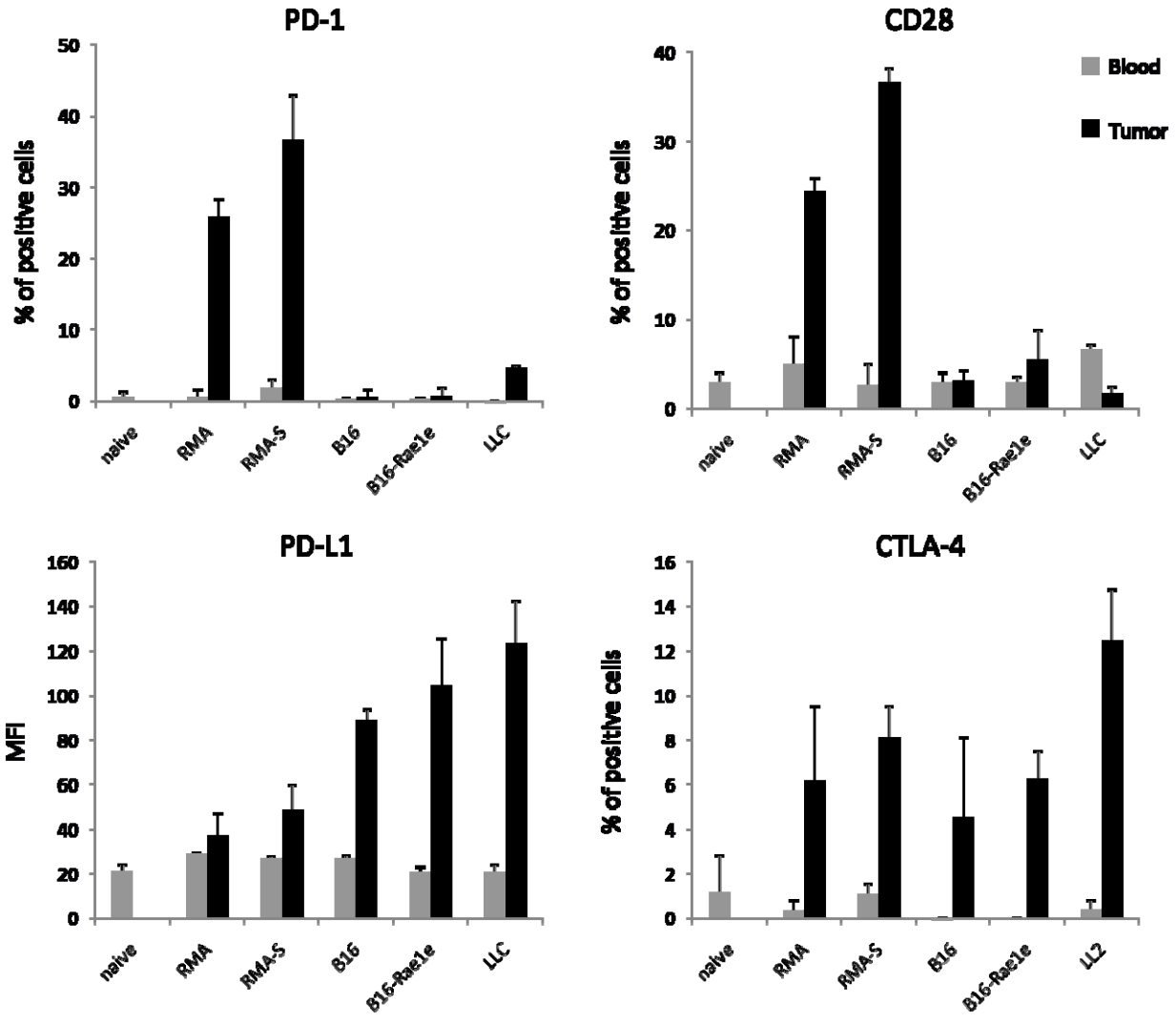


Figure 6.14. B7 family member expression in NK cells infiltrating tumors of different origin. Mice were inoculated subcutaneously with PBS (naïve controls) or 10^6 tumor cells in 100 µl PBS. When tumor size exceeded 1 cm^2, mice

were sacrificed and single cell suspensions were prepared from blood and tumor. Expression of depicted molecules on blood and tumor infiltrating NK cells, defined as $CD3^-NK1.1^+$, was analyzed by flow cytometry. Graphs indicate mean±SD of experimental group of 3 treated animals and are representative of one out of minimum two experiments performed.

6.2.3.2. Expression of B7 family members on tumor infiltrating T cells and macrophages

Several B7 family members were upregulated by NK cells found within the tumor tissue. It is possible that certain factors, both biological (e.g. cytokines) and chemical (pH, hypoxia) within the microenvironment were able to induce their expression. We analyzed if these molecules were similarly regulated on other immune cells found in RMA-S tumors. Figure 6.15 shows that both cells of the adaptive (T cells) and the innate (macrophages) immune system highly upregulated PD-1 in the tumor. In addition, T cells, both $CD4^+$ and $CD8^+$, showed elevated expression of B7-H1 and PD-L2, as well as high levels of CTLA-4 assesed by intracellular staining. CTLA-4^+ cells among the $CD4^+$ subset might, however, be composed of both activated T effector cells and T regulatory cells that constitutively express CTLA-4 [213] and were shown to accumulate in tumor [301].

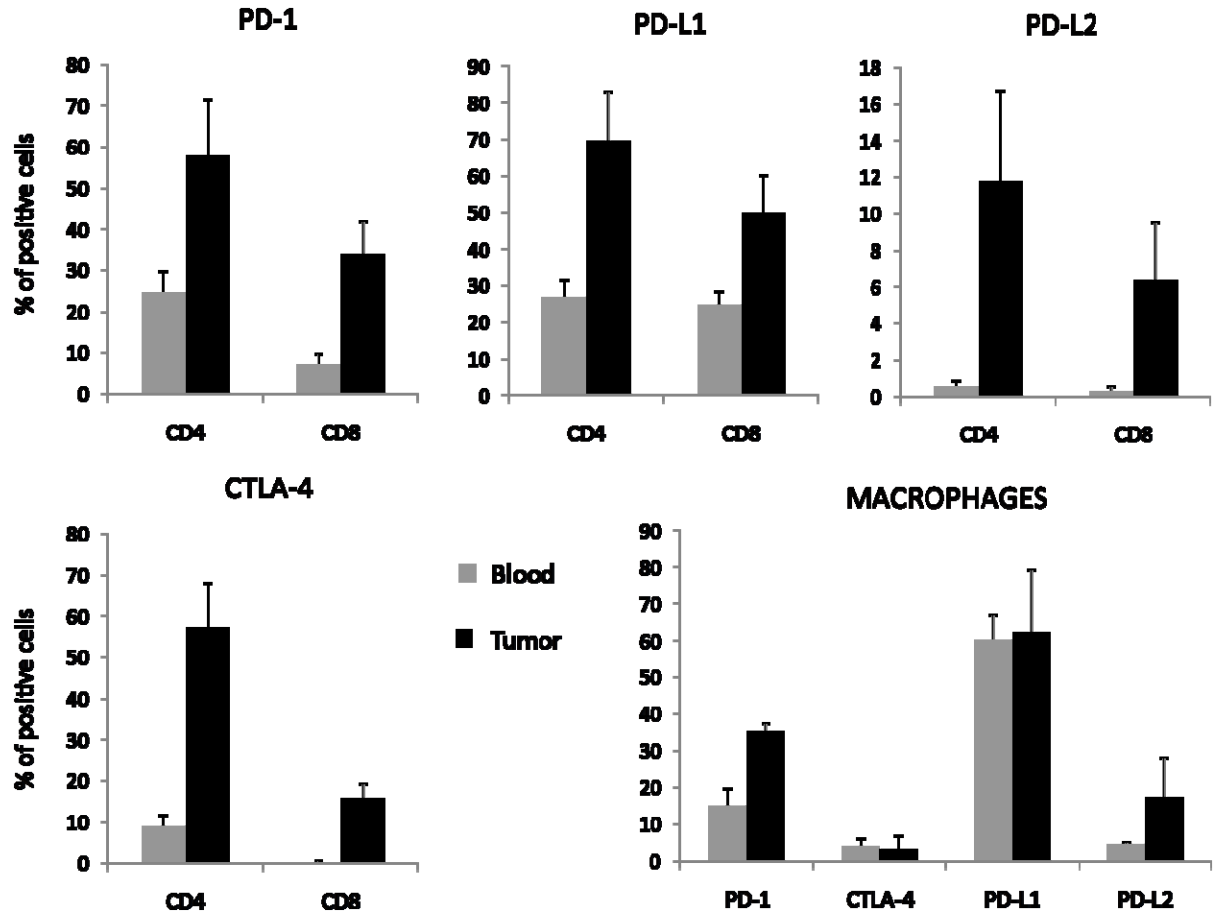


Figure 6.14. Expression of B7 family members by tumor infiltrating T cells and macrophages. Mice were inoculated subcutaneously with 10^6 RMA-S tumor cells in 100 µl PBS. When the tumor size exceeded 1 cm^2, mice were sacrificed and single cell suspensions were prepared from blood and tumor. Expression of the depicted molecules on blood and tumor infiltrating T cells ($CD3^+CD4^+$ and $CD3^+CD8^+$) and macrophages ($CD11b^+F4/80^+$) was analyzed by flow cytometry. Graphs indicate mean±SD of experimental groups of 3 treated animals.

Thus, expression of certain B7 family members, such as PD-1, might be induced by the conditions in the tumor microenvironment (hypoxia, cytokine/s present in high concentrations). Regulation of the expression of other molecules might in addition require more defined cell/cell intreractions and/or the presence of certain factors acting on the specific cell types.

6.2.3.3. Expression od PD-1 and CTLA-4 in RAG2 deficient mice

Expression of PD-1 and CTLA-4 on tumor infiltrating NK cells was typically observed in the tumors that exceeded the size of 1 cm^2 . This size corresponds to the phase of tumor progression, which is characterized by the established control of tumor growth and is followed by the final phase of progression that leads to animal death (Figure 6.4). The phase of control correlated to the time when the adaptive immune system is expected to be fully activated. To investigate if a factor/s derived from adaptive immune cells at that time might be responsible for PD-1 and CTLA-4 induction on NK cells in the tumor, we analyzed NK cells derived from RMA-S tumors established in RAG2 deficient mice. Tumors in RAG2 deficient mice grew with the similar kinetics within the first 7-10 days upon tumor cell inoculation, but progressed more aggressively in the late phase due to the absence of the adaptive immune response to tumor (data not shown). In the tumors dissected when the size of 1 cm^2 was reached in both WT and RAG2 deficient mice, we observed a similar expression of PD-1 (Figure 6.15). However, CTLA-4 expression was not induced in RAG2 deficient mice. These observations indicate that either a direct contact or/and factor/s derived from cells of adaptive immune system are necessary for the induction of CTLA-4 in NK cells. This putative interaction and/or the production of the factor/s is expected to take place only in the tumor since no CTLA-4 expression was observed in blood.

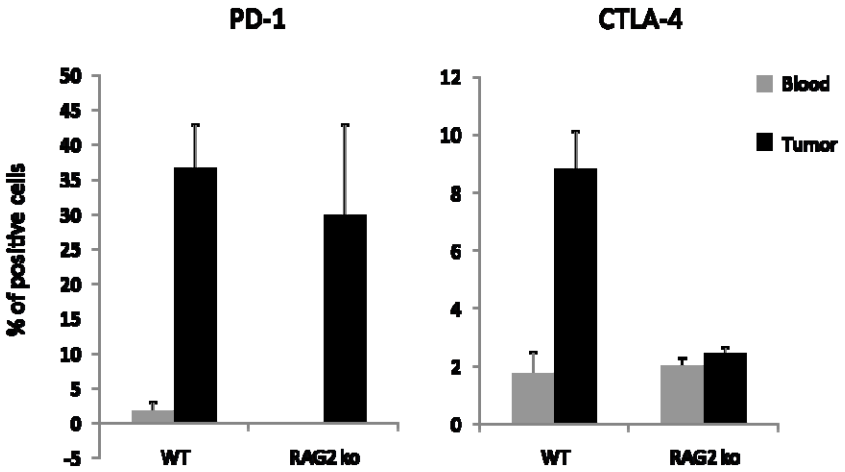


Figure 6.15. Induction of CTLA-4, but not PD-1, depends on the presence of the adaptive immune system. WT and RAG2 deficient mice were injected subcutaneously with 10^6 RMA-S cells in 100 µl PBS. When the tumor size exceeded 1 cm^2, mice were sacrificed and single cell suspensions were prepared from blood and tumor. Expression of PD-1 (surface) and CTLA-4 (intracellular) on blood and tumor infiltrating NK cells, defined as $CD3^-NK1.1^+$, was analyzed by flow cytometry. Graphs indicate mean±SD of experimental groups of 3 treated animals and are representative of one out of two experiments performed.

6.2.3.5. Expression od PD-1 and CTLA-4 in NK cell insensitive tumors

RMA-S is considered as a NK cell sensitive tumor cell line due to its sensitivity to NK cell lysis owing to the low expression of MHC I [23]. Thereby, when injected at low cell numbers into congenic mice, RMA-S cells are efficiently rejected in a NK cell dependent manner, while MHC I sufficient RMA cells lead to tumor formation [23]. However, when inoculated at high cell numbers, RMA-S form solid tumors, which we found to be infiltrated by PD-1 and CTLA-4 positive NK cells. We compared NK cells from MHC I deficient RMA-S and MHC I suficient RMA tumors of a similar size to see if efficient target recognition influences PD-1 and CTLA-4 upregulation. We observed no differences (Figure 6.16). Similar data were obtained when we compared PD-1 and CTLA-4 induction on NK cells infiltrating B16 and B16.Rae1ε solid tumors, where more efficient recognition of B16.Rae1ε cells is based on the expression of the ligand for the activating receptor NKG2D. Those data indicate that missing-self and induced-self recognition do not influence PD-1 and CTLA-4 expression in tumor infiltrating NK cells and that other factors might play a main role in their induction.

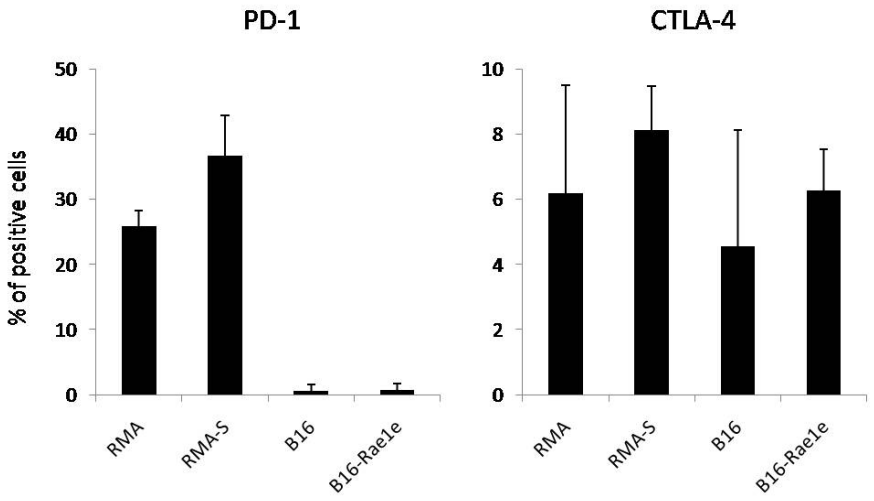


Figure 6.16. Missing-self and induced-self recognition do not influence PD-1 and CTLA-4 expression by tumor infiltrating NK cells. Mice were inoculated subcutaneuosly with 10^6 tumor cells in 100 µl PBS. When the tumor size exceeded 1 cm^2, mice were sacrificed and single cell suspensions were prepared. Expression of PD-1 (surface) and CTLA-4 (intracellular) on tumor infiltrating NK cells, defined as $CD3^-NK1.1^+$, was analyzed by flow cytometry. Graphs indicate mean±SD of experimental group of three animals and are representative of one out of two experiments performed.

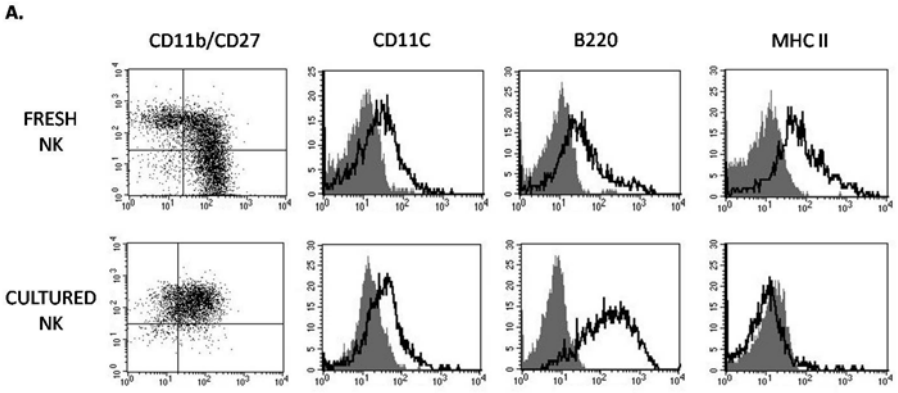
6.2.3.6. Expression of PD-1 and CTLA-4 in non-subcutaneous tumor models

Induction of inhibitory receptors PD-1 and CTLA-4 on NK cells within the tumor microenvironment has been observed to depend on the tumor size. In tumors smaller than 1 cm^2, expression was absent or very low. Bigger tumors are characterized by necrotic, and areas with low pH and low oxygen concentration. These conditions, and the factors produced by other cells in the microenvironment, can possibly favor the PD-1 and CTLA-4 upregulation. Metastases are usually smaller in size and also known to be better controlled by NK cells than large solid tumors. We aimed to analyse if NK cells found within the organs highly populated by metastases express PD-1 and CTLA-4. Two weeks after the intravenous injection of different tumor cell lines (RMA, RMA-S, B16, LL2) into congenic mice, we observed the metastasis formation in the lungs of inoculated animals. However, no PD-1 and CTLA-4 expression was detected on NK cells from either blood or lungs (data not shown). Similar data we obtained after tumor cell inoculation into peritoneum. Two weeks post-injection, no PD-1 or CTLA-4 positive NK cells were found in the blood or peritoneal lavage of inoculated mice (data not shown), indicating that the given phenotype of NK cells is feature of big solid tumors.

6.3. Expression of B7 family receptors and ligands by in vitro expanded NK cells

6.3.1. In vitro induction of B7 family members

Gene expression profiling of tumor infiltrating NK cells revealed that within the tumor tissue several B7 family members were induced on NK cells. For the most of selected candidates, the data obtained on the mRNA level were confirmed to correlate with the protein expression. High surface levels of PD-1 and substantional intracellular levels of CTLA-4 were detected in tumor infiltrating, but not blood NK cells (Figure 6.12). In contrast, the activating receptor CD28 was not found to be differentially regulated on the transcript level between blood and tumor infiltrating NK cells. However, while blood NK cells were CD28 negative, the receptor was induced on NK cells from the tumor tissue (Figure 6.12). Next, we aimed to analyze the biological functions of different B7 family members in NK cells to better understand possible consequences of their induction in the tumor. As a first step, we measured the expression of several B7 molecules on *in vitro* expanded NK cells. NK cells isolated from spleen and cultured for 7-10 days in the presence of 1700 U/ml rhIL-2, are preferentialy composed of the $CD27^{high}$ NK cell subset (Figure 6.17A), similar to the NK cells found in the tumor (Figure 6.1). They express high levels of B220, while MHC II expression was reduced compared to the freshly isolated NK cells (Figure 6.17A). Analysis of the B7 family expression revealed that expanded NK cells expressed CD28, CTLA-4 (intracellular), low levels of B7-1 and B7-2 and high levels of B7-H1, while PD-1 and PD-L2 were absent (Figure 6.17B). Compared to fresh NK cells, expansion in IL-2 did not change the surface levels of B7-1, B7-2 and B7-H1. These observations indicate that, although the culture in IL-2 did not precisely reproduce expression of B7 family members seen on NK cells in the tumor, it was capable of inducing CD28 and CTLA-4. Thus, *in vitro* expanded NK cells can be used for analysis of CD28 and CTLA-4 functional role in the regulation of NK cell effector responses.



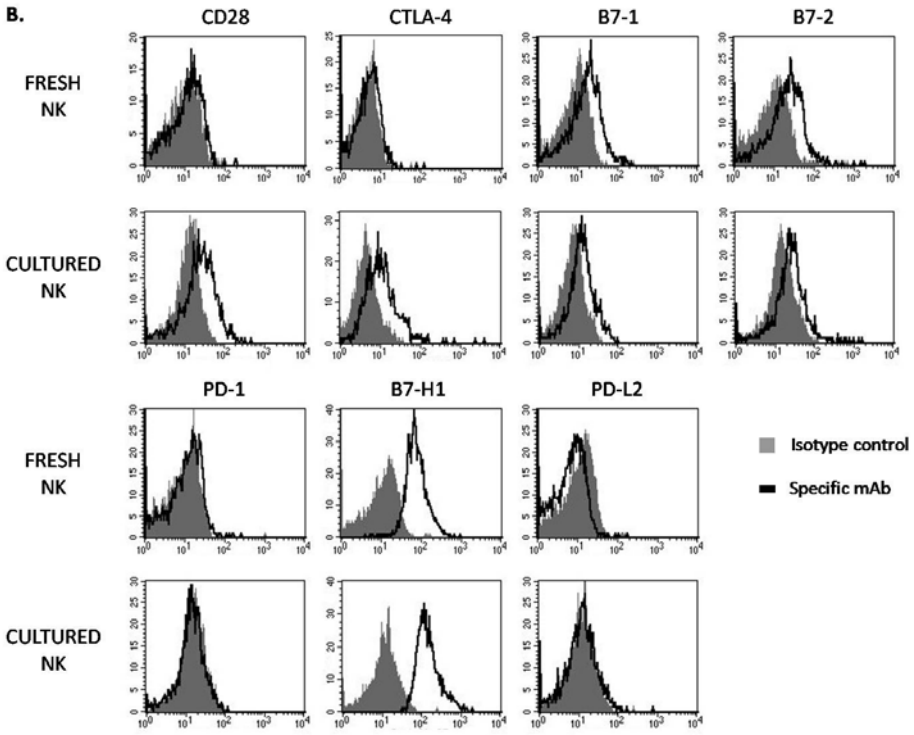


Figure 6.17. Phenotype of *in vitro* expanded NK cells. NK cells were isolated from spleens by magnetic cell sorting using $CD3^+$ cell depletion followed by DX5 positive selection. After one week of culture in the presence of 1700 U/ml rhIL-2 cells were analyzed by flow cytometry for (A) subset composition (CD11b/CD27 co-expression), IKDC markers (CD11c, B220, MHC II) and (B) expression of depicted B7 family members. Cultured cells were compared with freshly isolated splenocytes. Representative histograms show the expression of depicted molecules on gated $CD3^-NK1.1^+$ cells.

6.3.2. In vitro induction of PD-1

Since the expansion of NK cells in IL-2 led to CD28 and CTLA-4, but not PD-1 induction, we aimed to investigate if the addition of other stimuli would potentiate its expression. Single cytokines or their combinations, triggering of different NK cell receptors, incubation with tumor cells or the induction of transcription factor HIF-1α, the main regulator of hypoxia induced responses, were some of the conditions that failed to induce PD-1 expression on NK cells (Table 6.3). Since freshly isolated NK cells cannot survive without IL-2 for more than several hours, these stimuli were used for the short term stimulation of freshly isolated NK cells (4-8 h) or long term stimulation of IL-2 expanded NK cells (24-48 h). Therefore, we cannot exclude the possibility that some of the stimuli would be able to induce PD-1 expression the whole splenocytes, since PD-1 might be induced indirectly, through the accessory cells responding to the primary stimulus. However, we did not observe PD-1 expression in any of the experimental conditions applied.

Stimulus	
Cytokines	IL-1β, IL-2, IL-4, IL-6, IL-9, IL-10, IL-12, IL-13, IL-15, IL-18, IFNα, IFNβ
Tumor cells	RMA, RMA-S, B16, B16.Rae1ε at diferent E/T ratios
NK receptor triggering	NK1.1, NKG2D, 2B4, Ly49D
TLR ligands	Poly I:C, CpG
PMA and Ionomycin	Activation of PKC and Ca^{2+} flux
Chronic stimulation	Plate bound Abs and irradiated tumor cells for 7 days
HIF-1α induction	DMOG - prolyl-4-hydroxylase inhibitor, prevents HIF-1α targeting for degradation
Combinations of stimuli	Cytokine combinations Cytokine + Tumor cells Cytokine + DMOG Tumor cells + DMOG

Table 6.3. Experimental conditions used for analysis of PD-1 induction *in vitro*. Freshly isolated or IL-2 expanded NK cells and whole splenocytes were stimulated as indicated for a short term (4-8 h) or a long term (24-48 h) period. PD-1 expression was analyzed by flow cytometry. No induction was observed for any of the given experimental setup.

6.3.3. Regulation of CTLA-4 expression by in vitro expanded NK cells

We showed that culture in IL-2 induces CTLA-4 expression by NK cells. In T cells, CTLA-4 is induced by the TCR and CD28 engagement and most of the protein is found intracellularly [190]. Treg constitutively express CTLA-4, which is stored within the cell, but is exposed on the cell surface upon activation. In both cases, surface CTLA-4 is internalized shortly after the exposure and either targeted for degradation or recycled back to the plasma membrane. To investigate if a similar cellular compartmentalization of CTLA-4 exists in NK cells and how it is regulated, we applied several staining techniques for its detection (Figure 6.18).

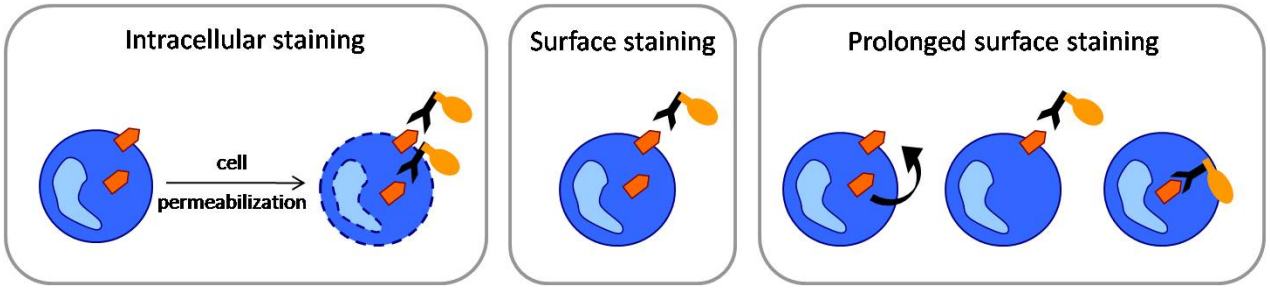


Figure 6.18. Staining strategies for CTLA-4 detection in NK cells. Intracellular staining protocol includes cell fixation and permeabilization of cell membranes, therefore both surface and intracellular protein is detected. Surface staining detects only the receptor exposed on the cell surface at the moment of staining. Prolonged surface staining detects any CTLA-4 molecule that reaches the surface within the last 4h of cell stimulation, which is the time period labeled αCTLA-4 mAb is added to the cell culture.

Intracellular staining detects the total protein content that can be distributed anywhere within the cell including the cell surface. Surface staining detects the receptor exposed on the cell surface in the moment of staining. Prolonged surface staining detects CTLA-4 that reaches the cell surface during a defined time period. In this case, fluorescently labeled αCTLA-4 mAb or isotype control was added to the culture for the last 4h of cell stimulation and can bind any CTLA-4 molecule that is expressed on the surface within a given time period.

By applying surface staining, we did not detect CTLA-4 expression on freshly isolated, cultured NK cells or cultured and then restimulated NK cells using different stimuli (data not shown). This means that the amount of the receptor on the surface could be too low to be detected by a classical flow cytometry staining technique. Of importance, even small hardly detectable amounts of CTLA-4 on the cell surface can have a significant functional role due to its high binding affinity for the B7-1 and B7-2 ligands [200]. Indeed, prolonged surface staining revealed that under certain conditions, CTLA-4 was exposed on the surface of NK cells where it could participate in the ligand binding and influenced NK cell effector responses.

6.3.3.1. Regulation of CTLA-4 surface expression by cytokines

IL-12 is a cytokine secreted by activated macrophages and DCs that plays an important role in NK cell activation [302]. Its main effect is the induction of IFNγ that further activates macrophages and DCs. IL-18 very efficiently synergizes with IL-12, which is the combination known to be one of the strongest inducer of IFNγ production by NK cells. In addition, IL-18 stimulates NK cell cytotoxicity [100]. We analyzed whether exposure to IL-12 or/and IL-18 might as well influence CTLA-4 expression by NK cells. As shown in Figure 6.19A, stimulation of *in vitro* expanded NK cells with a combination of IL-2, IL-12 and IL-18 for 24 hours strongly induced CTLA-4 surface expression as detected by prolonged surface staining (see Figure 6.18 for staining method). Single cytokines, IL-12 or IL-18, added to IL-2 did not have such an effect. In addition to the increased surface exposure, the triple cytokine combination (IL-2/12/18) elevated the levels of total CTLA-4 assessed by intracellular staining (6.19B).

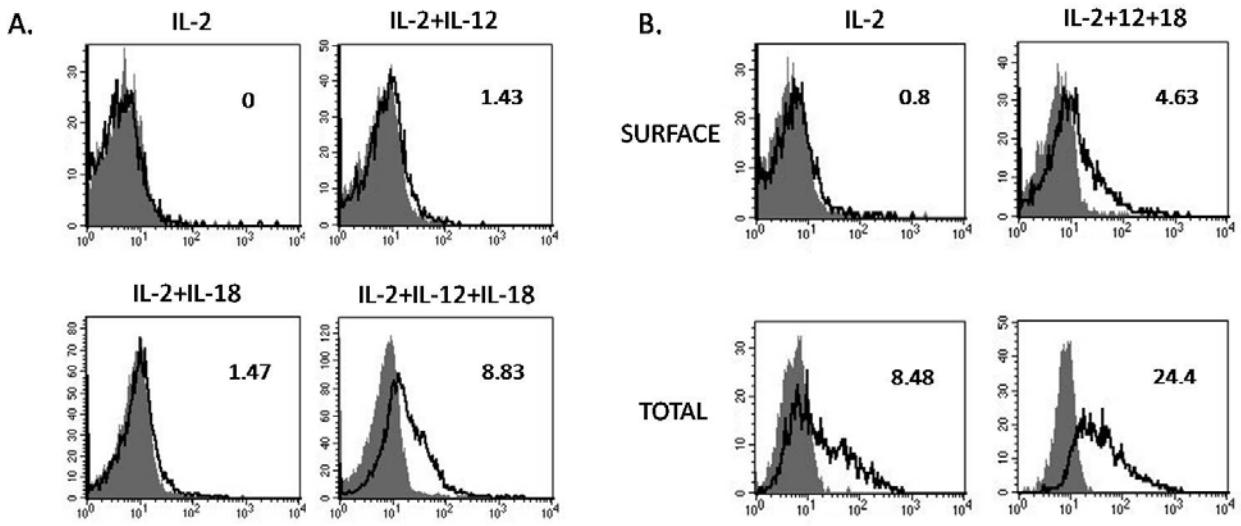


Figure 6.19. Regulation of CTLA-4 expression by cytokine stimulation. NK cells were isolated from spleens by magnetic cell sorting via DX5 positive selection (A) or $CD3^+$ cell depletion followed by DX5 positive selction (B). After one week of culture in the presence of 1700 U/ml rhIL-2, cells were stimulated with the indicated cytokines for 24h (IL-2 1700 U/ml, IL-12 5 ng/ml, IL-18 40 ng/ml). Prolonged surface staining (last 4h of stimulation) or intracelular (total) staining was used for the analysis of CTLA-4 expression on gated $CD3^-NK1.1^+$ cells. In the case of surface staining, dead cells were excluded by addition of 7-AAD. MFI (calculated as Geometric Mean of specific staining-Geometric Mean of isotype control) is depicted in each panel. Data are representative from one out of three experiments performed.

To better understand how the CTLA-4 synthesis and trafficking are regulated, we analyzed kinetics of CTLA-4 expression upon IL-2/12/18 treatment by prolonged surface staining. Interestingly, although substantial amounts of the protein existed in IL-2 expanded NK cells (Figure 6.19B), increased surface expression was not detected earlier than 24h upon IL-2/12/18 stimulation (Figure 6.20A and Figure 6.19B showing the expression after 24h). That indicates that cytokines probably did not directly mobilize the receptor from the intracellular pool, but that putative secondary factor/s induced by cytokines might do so. Therefore, we analyzed whether the CTLA-4 surface expression depends on *de novo* transcription and/or *de novo* translation. As shown in Figure 6.20B, NK cells treated with transcription inhibitor actinomycin D (ActD) showed partial reduction, and cell treated with translation inhibitor cyclohexamide (CHX) complete absence of surface CTLA-4 expression. These data show that CTLA-4 surface exposure partially depended on *de novo* transcription and completely on *de novo* protein synthesis.

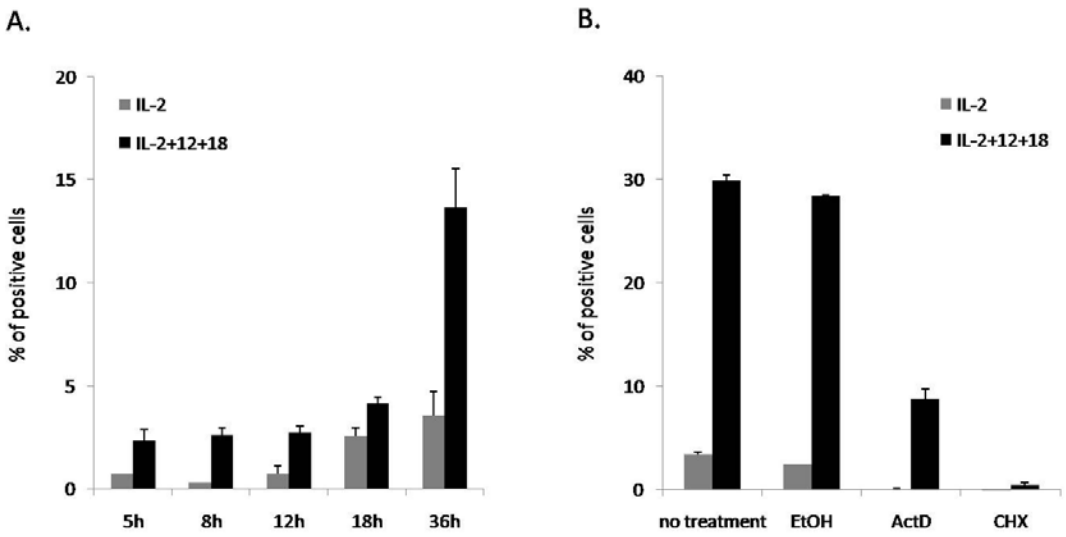


Figure 6.20. Requirements for CTLA-4 surface expression by *in vitro* expanded NK cells. NK cells were obtained by culture of non-adherent RAG2 deficient splenocytes in the presence of 1700 U/ml rhIL-2. After one week of culture NK cells were stimulated with the indicated cytokines (IL-2 1700 U/ml, IL-12 1 ng/ml, IL-18 10 ng/ml). (A) CTLA-4 expression was measured by prolonged surface staining (last 4h of stimulation) at different time points after beginning of stimulation. Total stimulation time is depicted on the x-axis. (B) NK cells were left untreated or treated with 5 µg/ml actinomycin D (ActD), 10 µg/ml cyclohexamide (CHX) or adequate volume of absolute ethanol (EtOH) as a solvent control. CTLA-4 surface expression was measured by prolonged surface staining 24h after the beginning of stimulation. Graphs indicate mean±SD of triplicate culture. Graphs indicate mean±SD of triplicate cultures.

6.3.3.2. Regulation of CTLA-4 surface expression by receptor triggering

In addition to the stimulation by cytokines from the microenvironment, NK cells can be activated in a direct contact with other cells by recognition of the ligands for activating NK receptors. Thus, we analyzed if triggering of NK cell receptors can modulate CTLA-4 expression. When IL-2 expanded NK cells were stimulated with plate-bound mAbs directed against different NK receptors, no change in the total or surface expression of CTLA-4 was observed (Figure 6.21), although triggering of some receptors, as NK1.1, showed the tendency to induce CTLA-4. When receptor triggering was combined with the triple cytokine stimulation, synergy was obtained between IL-2/12/18 and NK1.1 engagement in enhancing CTLA-4 surface exposure, detected by the prolonged surface staining.

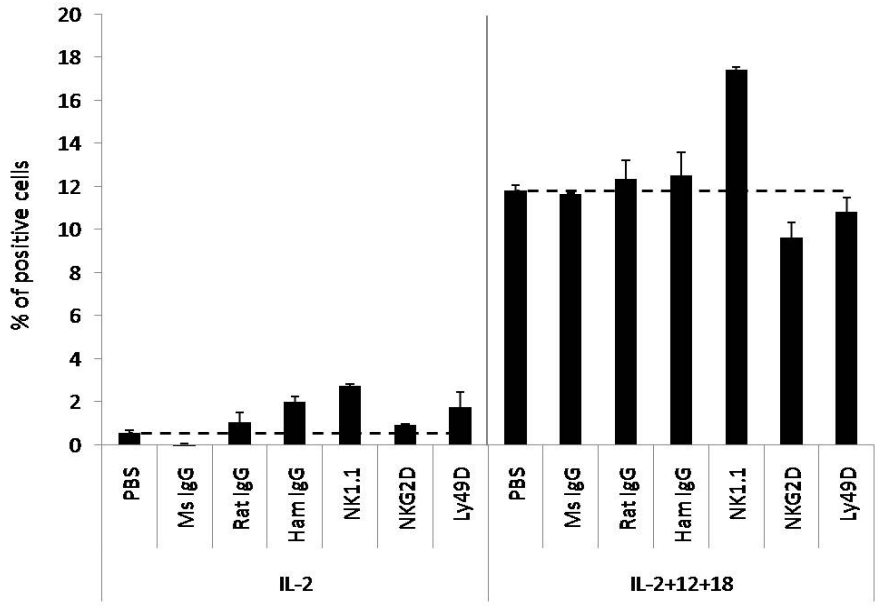


Figure 6.21. Regulation of CTLA-4 expression by receptor triggering. NK cells were obtained by culture of non-adherent RAG2 deficient splenocytes in the presence of 1700 U/ml rhIL-2. After one week of culture harvested NK cells were first incubated with αCD16/CD32 mAbs to block Fc receptors and subsequently stimulated with plate-bound Abs (10 μg/ml) for 24h in the presence of indicated cytokines (IL-2 1700 U/ml, IL-12 1 ng/ml, IL-18 10 ng/ml). CTLA-4 expression was measured by prolonged surface staining (last 4h of stimulation). Graphs indicate mean±SD of triplicate culture. Graphs indicate mean±SD of triplicate ciltures. Ms – mouse; Ham - Hamster

Interestingly, short exposure to cytokines, not sufficient to induce surface expression itself, can render NK cells sensitive to the NK1.1 mediated surface induction of CTLA-4. NK1.1 signals via the adaptor molecules CD3ζ and FcεRIγ [295]. On the other hand, Ly49D and NKG2D, that did not have any effect on CTLA-4 expression, are coupled to DAP12 and DAP12 or DAP10, respectively [303]. Therefore, we looked if another activating NK receptor that utilizes CD3ζ and FcεRIγ for signal transduction had similar effect as NK1.1. Indeed, short term NK cell stimulation with cytokines (IL-2/12/18) followed by triggering of NKp46, enhanced CTLA-4 surface expression similar to the levels obtained by NK1.1 engagement (Figure 6.22).

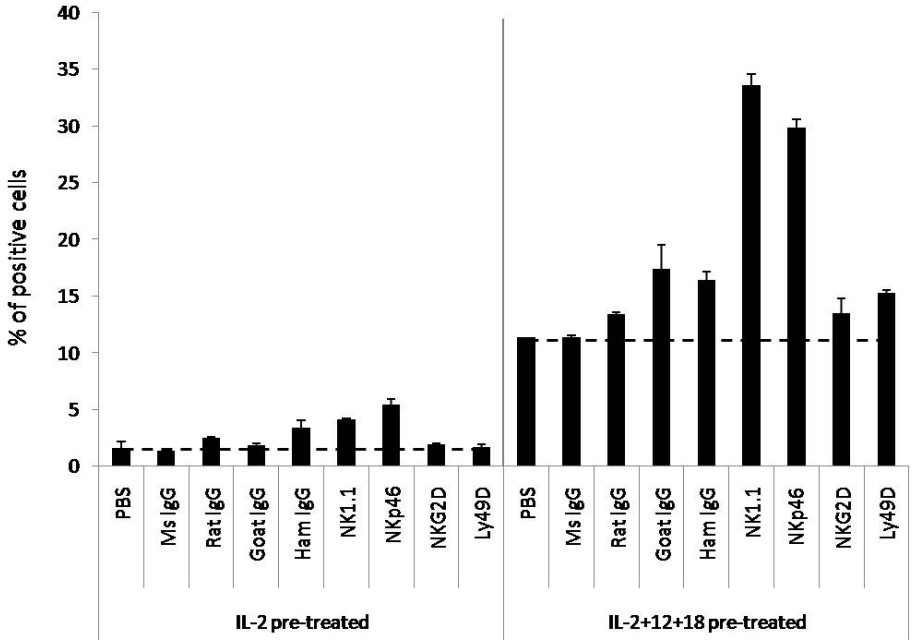


Figure 6.22. Regulation of CTLA-4 expression by CD3ζ and FcεRIγ coupled NK cell receptors. NK cells were obtained by culture of non-adherent RAG2 deficient splenocytes in the presence of 1700 U/ml rhIL-2. After one week of culture harvested NK cells were pre-treated with the indicated cytokines (IL-2 1700 U/ml, IL-12 1 ng/ml, IL-18 10 ng/ml) for 12h. After removal of the cytokines by washing, cells were first incubated with αCD16/CD32 mAbs to block Fc receptors and subsequently stimulated with plate-bound Abs (10 μg/ml) for additional 12h in the presence of IL-2. CTLA-4 expression was measured by prolonged surface staining (last 4h of stimulation). Graphs indicate mean±SD of triplicate culture. Graphs indicate mean±SD of triplicate ciltures. Ms – mouse; Ham - Hamster

In summary, our data indicate that cytokines commonly present in the inflammed tissues, namely IL-12 and IL-18, synergized with IL-2 in the upregulation of CTLA-4 expression and potentiated its exposure on the cell surface. CD3ζ and FcεRIγ coupled receptors, NK1.1 and NKp46, further increased CTLA-4 surface expression of IL-2/12/18 exposed NK cells. Thus,the recognition of cellular ligands in combination with cytokines from the microenvironment can modulate the expression of CTLA-4 receptor on the surface of activated NK cells.

6.4. In vitro responses of NK cells to B7-1

Our previous results showed that expression of CTLA-4 on NK cells can be modified by their exposure to cytokines (IL-2/12/18) and the engagement of activating receptors, such as NK1.1 and NKp46. These data indicate that situations that typically induce NK cell activation can in addition increase expression of inhibitory molecules, in this case the receptor CTLA-4. B7-1 and B7-2 are two described ligands of CTLA-4 [200]. Compared to B7-2, B7-1 binds CTLA-4 with a higher affinity. During the course of immune response, expression of the ligand, B7-1, and the receptor, CTLA-4, is induced at a similar time point. Thus, B7-1 is considered to be preferential and physiologically more important ligand for CTLA-4 [181].

Therefore, we analyzed how NK cell responses are controled by CTLA-4 upon recognition of B7-1. Of importance, B7-1 can be engaged by two other molecules, CD28 and B7-H1, that mediate activating or inhibitory effects, respectively [304]. In conditions where CTLA-4 expression has been observed on NK cells (tumor tissue, expansion in IL-2), CD28 and B7-H1 were also expressed (Figure 6.23). Thus, NK cell responses to B7-1 can be modulated through all three pathways.

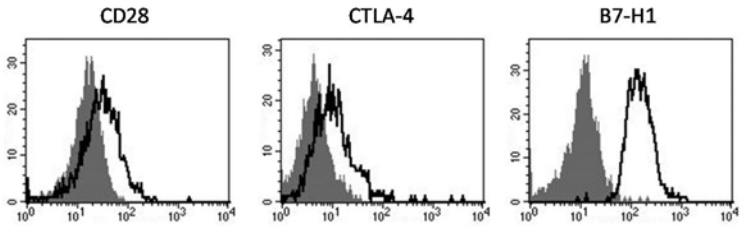


Figure 6.23. Expression of B7-1 binding receptors CD28, CTLA-4 and B7-H1 by IL-2 expanded NK cells. NK cells were isolated from spleens by magnetic cell sorting using $CD3^+$ cell depletion followed by DX5 positive selection. After one week of culture in the presence of 1700 U/ml rhIL-2 cells were analyzed for the expression of CD28 and B7-H1 by surface staining and CTLA-4 by intracellular staining.

6.4.1. Stimulation with B7-1 induces NK cell proliferation and IFNγ production

Next, we determined the functional impact of NK cell stimulation with B7-1. *In vitro* expanded NK cells, which we found to express B7-1 receptors CD28, CTLA-4 and B7-H1, were stimulated with plate bound B7-1 or control IgG fusion protein. As shown in Figure 6.24A, B7-1 induced IFNγ production in a dose dependent manner, which accummulated in the cell culture supernatant. Short pulse with BrdU, that allows the analysis of the frequency of proliferating cells, revealed that NK cells stimulated with B7-1 proliferated more extensively than controls (Figure 6.24B). At the same time, NK cells survival was analyzed by annexin V/7-AAD co-staining. B7-1 stimulated cells contained a slightly higher proportion of apoptotic cells (Figure 6.24C), but differences were much lower compared to differences in proliferation rates. Thus, in addition to inducing IFNγ, B7-1 stimulated NK cell proliferation and slightly increased apoptosis. This effect can lead to the increasement in the number of NK cells that can further respond to stimulation.

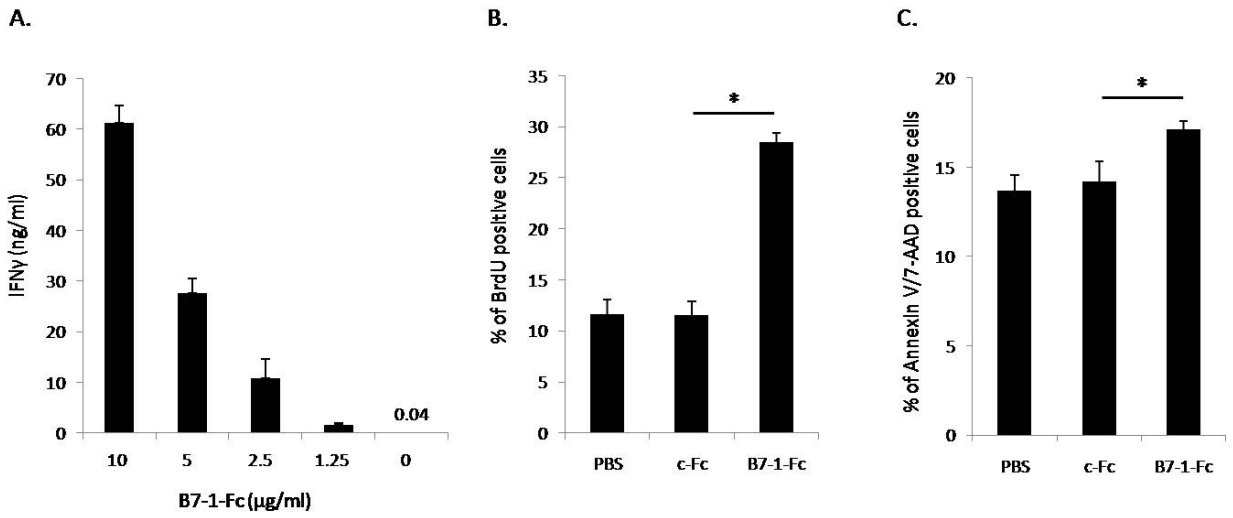


Figure 6.24. Stimulation with B7-1 induces IFNγ production and proliferation by *in vitro* expanded NK cells. NK cells were obtained by culture of non-adherent RAG2 deficient splenocytes in the presence of 1700 U/ml rhIL-2. After one week of culture harvested NK cells were pre-treated with αCD16/CD32 mAb to block Fc receptors and subsequently stimulated with plate-bound B7-1 (B7-1-Fc) or control (c-Fc) IgG fusion protein (2 μg/ml) in the presence of 1700 U/ml IL-2. (A) Increasing concentrations of plate bound B7-1-Fc were used for stimulation of NK cells. After 8h the culture supernatant was harvested and released IFNγ was measured by ELISA. (B). Last 45min of stimulation cells were pulsed with BrdU. The frequency of cells that have incorporated BrdU was measured by flow cytometry. (C) Cells were stimulated with B7-1-Fc for 24h. Proportion of apoptotic cells was measured by labelling with annexin V and 7-AAD. Graphs indicate mean±SD of triplicate cultures. *, $p<0.05$ by t-test; B7-1-Fc, B7-1 IgG fusion protein; c-Fc, control IgG fusion protein

6.4.2. B7-1 expression by tumor cells increases their susceptibility to NK cell lysis and potentiate IFNγ production

Many potential NK cell targets express B7 costimulatory ligands, such as some tumor cell of hematopoetic origin or dendritic cells [221, 305]. B7-1 expressed on tumor cells can engage both the activating, CD28, or the inhibitory, CTLA-4 and B7-H1 pathway. We measured the NK cell cytotoxic responses against tumor cell lines transduced with B7-1, namely RMA-S.B7-1 and B16.B7-1 (Figure 6.25).

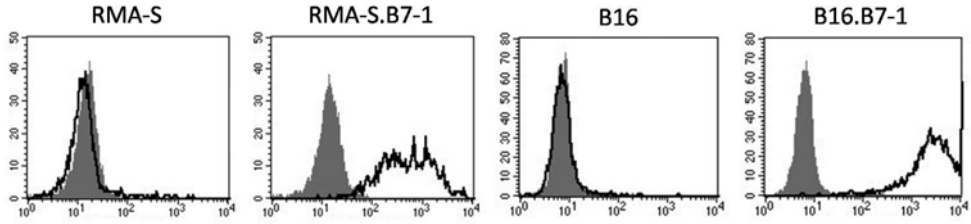


Figure 6.25. B7-1 expression on transduced RMA-S lymphoma and B16 melanoma cell lines. Cells were harvested in the exponential phase of growth after 7 days of culture. B7-1 expression was analyzed by flow cytometry.

B7-1 expression on MHC I deficient RMA-S lymphoma strongly increases their susceptibility to NK cell lysis (Figure 6.26A). In addition to the increased cytotoxic response, their recognition by NK cell effectors induced IFNγ production (Figure 6.26B). *In vivo*, subcutaneous injection of RMA-S.B7-1 into congenic mice led to the complete rejection of inoculated cells (Figure 6.26C). Importantly, both WT and RAG2 deficient mice were able to control high numbers of tumor cells (data not shown), indicating the central role of innate immune system in mediating the efficient rejection. Since *in vitro* studies showed that both cytotoxic and cytokine releasing effector responses are mobilized upon NK cell recognition of the B7-1 expressing lymphoma cells, both mechanisms might play a role in their efficient rejection *in vivo*.

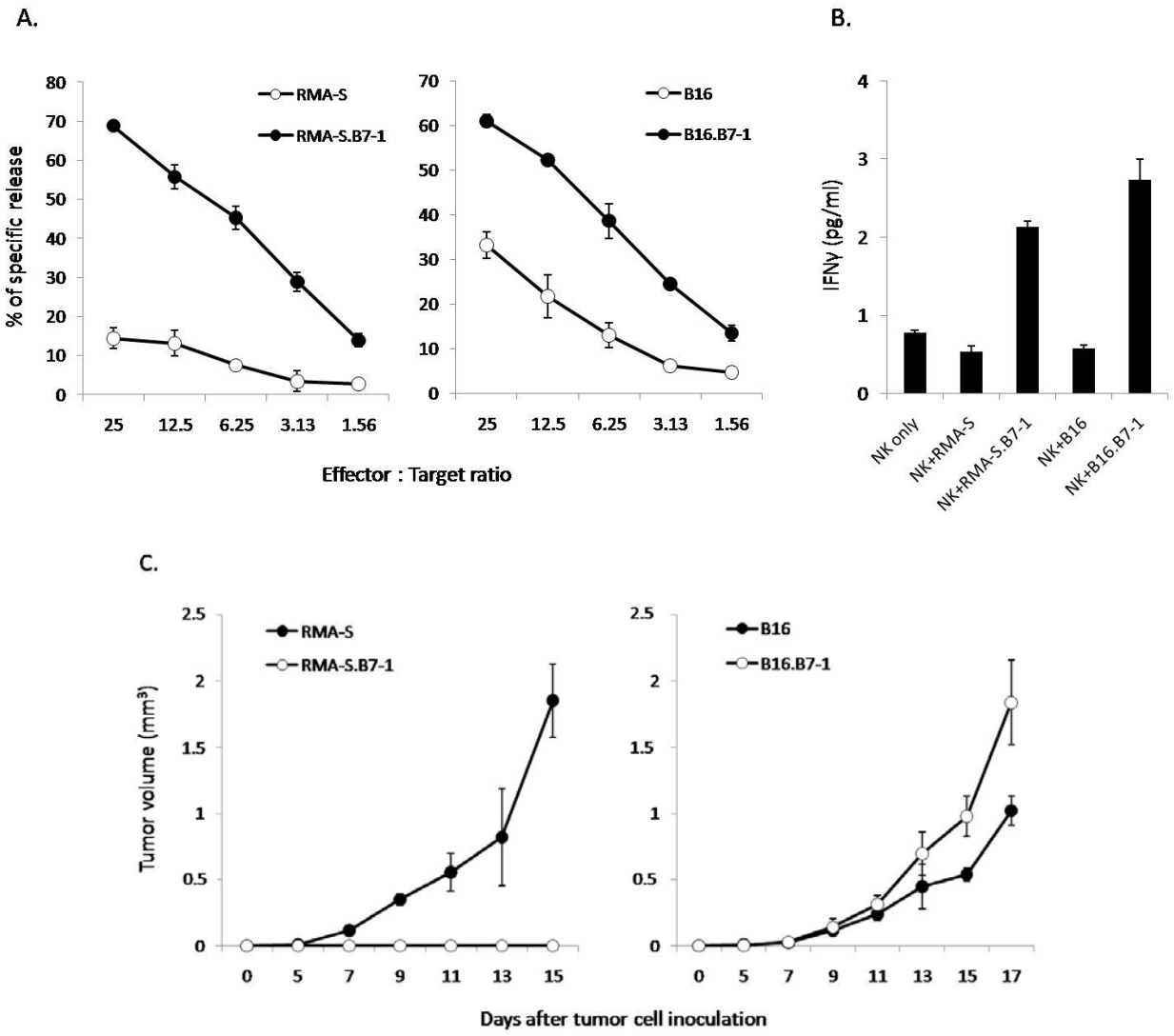


Figure 6.26. NK cell responses to B7-1 expressing tumor cells. NK cells were isolated from splenocytes by magnetic cell sorting using $CD3^+$ cell depletion followed by DX5 positive selection and further cultured for 7-10 days in the presence of 1700 U/ml rhIL-2. (A) NK cells were used as effectors in 4h ^{51}Cr release assay against RMA-S lymphoma and B16 melanoma $B7\text{-}1^+$ target cells. (B) NK cells were incubated alone or with the indicated tumor cells in a 1:2.5 ratio for 8h in the presence of IL-2. The amount of IFNγ released in the cell culture supernatant was measured by ELISA. Graphs indicate mean±SD of triplicate ciltures.

(C) RAG2 deficient mice were injected subcutaneously with 10^6 tumor cells (RMA-S, RMA-S.B7-1, B16, B16.B7-1) in 100 µl PBS. Tumor size was measured every 2 days starting from day 5 after tumor cell inoculation and expressed as product of three measured tumor dimensions. Graphs indicate mean±SD of triplicate culture (A and B) or mean±SD of experimental group of three animals (C).

We observed that the effect of B7-1 expression depended on the nature of tumor cell expressing it. In contrast to RMA-S lymphoma, B16 melanoma cells are killed by NK cells more efficiently *in vitro* (Figure 6.26A). Expression of B7-1 slightly increased specific lysis of B16 tumor cells. In addition, NK cells produced IFNγ in response to B7-1 expressing melanoma (Figure 6.26B). However, *in vivo* inoculation of both B16 and B16.B7-1 cells led to the progressive tumor growth in RAG2 deficient mice (Figure 6.26C). These data indicate that B7-1 expression by itself does not determine the NK cell responses *in vivo*, but it is rather integrated in the complex network of interactions established by NK cells, their targets and the microenvironment. As shown in Figure 6.13, the microenvironment formed by the tumor *in vivo* might differentially regulate the expression of receptors recognizing B7-1. NK cells found within RMA-S, but not B16 subcutaneous tumors, expressed the activating receptor CD28, that might mediate positive responses to B7-1 expressing lymphoma (Figure 6.14). On the other hand, high expression of the inhibitory receptor B7-H1 was detected on B16 infiltrating NK cells and could be responsible for a poor response and progressive growth of B7-1 expressing melanoma.

6.4.3. NK cell mediated trogocytosis of B7-1 from target cells

If a target cell is efficiently recognized by a NK cell, the stable contact is formed, which typically results in NK cell activation. The resulting outcome is target cell elimination and/or triggering of cytokine and chemokine production by NK cells. We observed that in addition to the activation of cytotoxicity and IFNγ production, NK cells cultured with B7-1 positive target cells become B7-1 positive themselves. This finding raised the question whether B7-1 was upregulated or acquired from the targets through the close synapse formed between two cells. The process of intercellular exchange of intact membrane patches, including membrane molecules, is termed trogocytosis and is considered to occur very frequently between interacting cells [306-307]. The main characteristic of the process is very fast kinetics since it occurs within minutes from the beginning of the interaction. The event of exchange is triggered by specific receptors and requires the signal transduction in recipient cell. Importantly, membrane proteins are transferred in full size and in the right inside-out orientation and therefore, they can potentially perform their functions.

We showed that co-culture with different B7-1 expressing tumor cell lines rendered NK cells B7-1 positive (Figure 6.27A). This process is very fast and already co-incubation with targets as short as 30 minutes resulted in a highly positive NK cell population (Figure 6.27B). The level of B7-1 expression correlated with the availability of B7-1 source, since increased numbers of tumor cells elevated the proportion of B7-1 positive NK cells (Figure 6.27B).

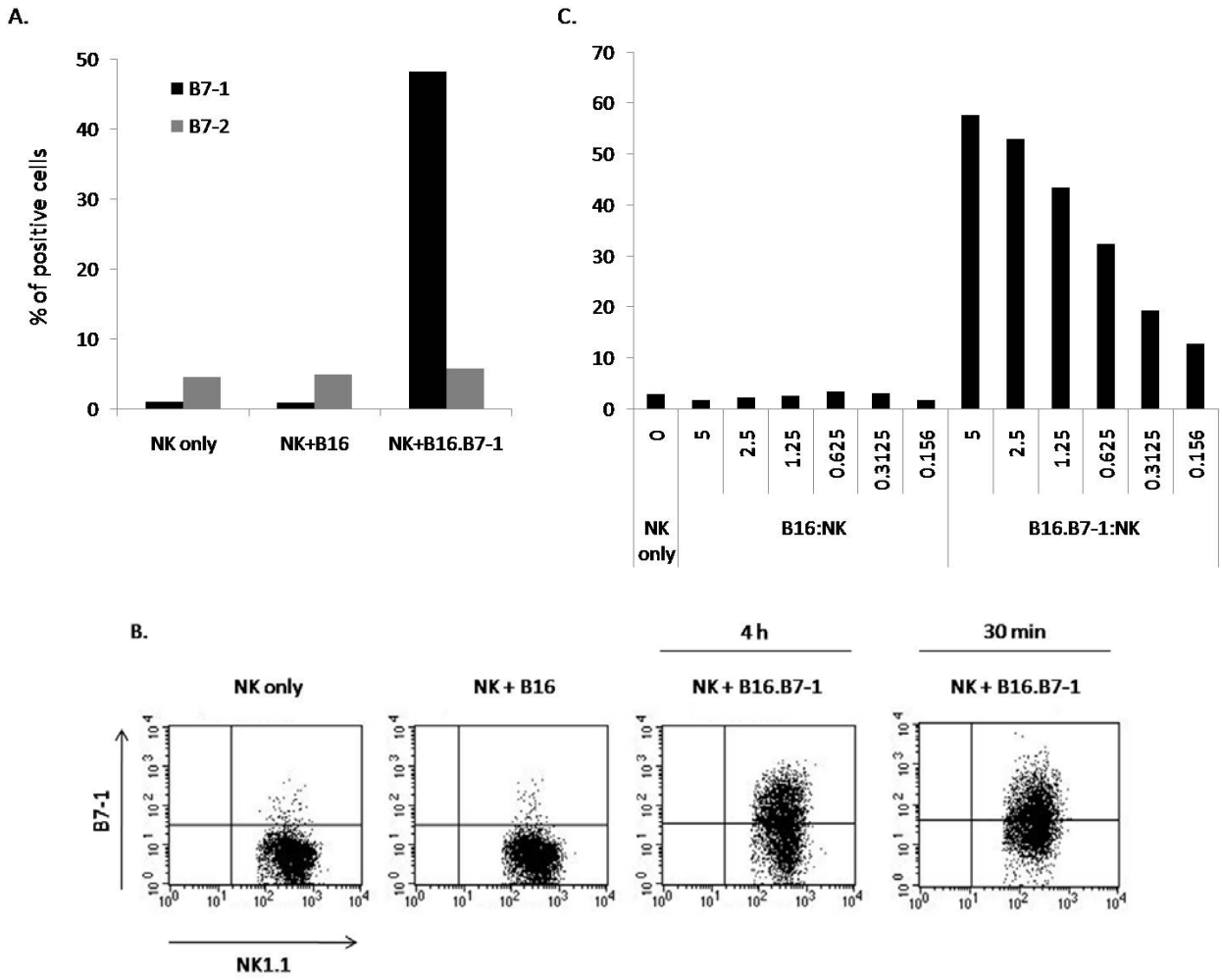


Figure 6.27. NK cells express B7-1 after co-culture with B7-1 expressing target cells. NK cells were obtained by culture of non-adherent RAG2 deficient splenocytes in the presence of 1700 U/ml rhIL-2. Cells were used after 7-10 days of expansion. (A) NK cells were cultured alone or with tumor cells in a 1:1 ratio in the presence of IL-2. After 4h, expression of B7-1 and B7-2 was measured by flow cytometry. (B) Representative dot plots show B7-1 staining after NK cell co-culture with B16 or B16.B7-1 tumor cells for 4h or 30min. (C) NK cells were cultured alone or with tumor cells at the indicated ratios. After 4h, expression of B7-1 was analyzed by flow cytometry.

Apart from B7-1 transduced tumor cells, B7-1 and B7-2 ligands are endogenously expressed by APCs. NK cells were shown to establish interactions with DCs that lead to either NK and DC activation or DC elimination if they are not mature [66]. Mature DCs express high levels of costimulatory molecules including B7-1 and B7-2. We showed that NK cells cultured with mature but not immature DCs displayed elevated surface expression of both B7-1 and B7-2 (Figure 6.28). Therefore, similar to tumor targets, increased availability of the ligands on DCs correlated with their higher expression by NK cells after co-culture.

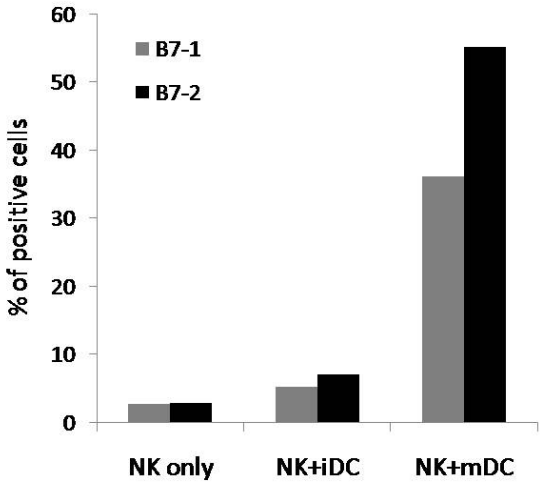
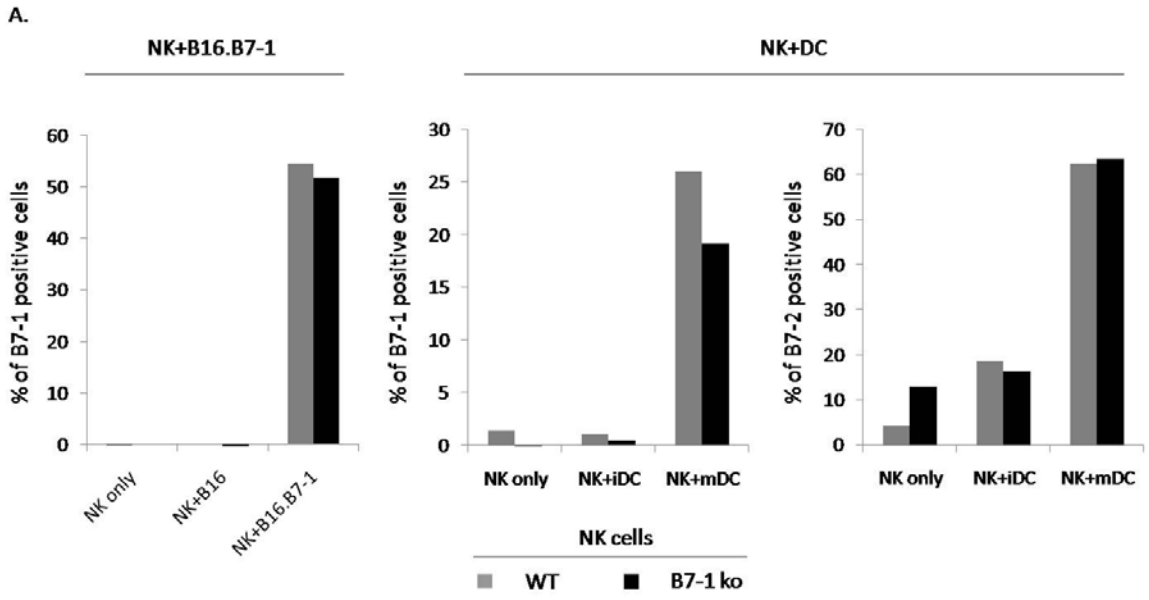


Figure 6.28. NK cells express B7-1 and B7-2 after co-culture with mature DCs. NK cells were obtained by 7 days culture of non-adherent RAG2 deficient splenocytes in the presence of 1700 U/ml rhIL-2. DCs were generated from bone marrow of WT mice by culture in the medium supplemented with GM-CSF. Cells were harvested on day 6 and treated with 100 ng/ml LPS (mature DC, mDC) or medium only (immature DC, iDC) for 12 hours.
NK cells were cultured alone or with iDC and mDC at a 1:1 ratio in the presence of IL-2 and GM-CSF (1/5 of amount used for expansion). After 24h of co-culture the expression of B7-1 and B7-2 on $CD3^{-}NK1.1^{+}$ cells was measured by flow cytometry. Dead cells were excluded by 7-AAD staining.

To investigate if B7-1 was indeed acquired from target cells or upregulated by NK cells, we used NK cells derived from B7-1 deficient mice as effector cells in the co-culture experiments. B7-1 ko NK cells had a comparable phenotype to WT NK cells (data not shown) both when analyzed fresh or after expansion in IL-2 for the expression of maturation markers (CD11b, CD27), activation markers (CD11c, B220, CD69, CD25) and B7 family members (CD28, CTLA-4, PD-1, ICOS, B7-H1, PD-L2, B7-2). After co-culture with B7-1 expressing tumor cells, both WT and B7-1 deficient NK cells displayed similar levels of B7-1 on the surface (Figure 6.29A left), which proved that B7-1 was acquired from tumor cells and not synthetized by NK cells themselves. After co-culture with DCs, both B7-1 and B7-2 were expressed at the comparable levels by WT and B7-1 ko NK cells (Figure 6.29A right), which proved that DCs are source of B7-1. As an additional confirmation of the B7-1 transfer is the observation that only B7-2 expression was detected on WT NK cells after co-culture with DCs derived from B7-1 deficient mice, while both B7-1 and B7-2 were expressed after incubation with WT DCs (Figure 6.29B).



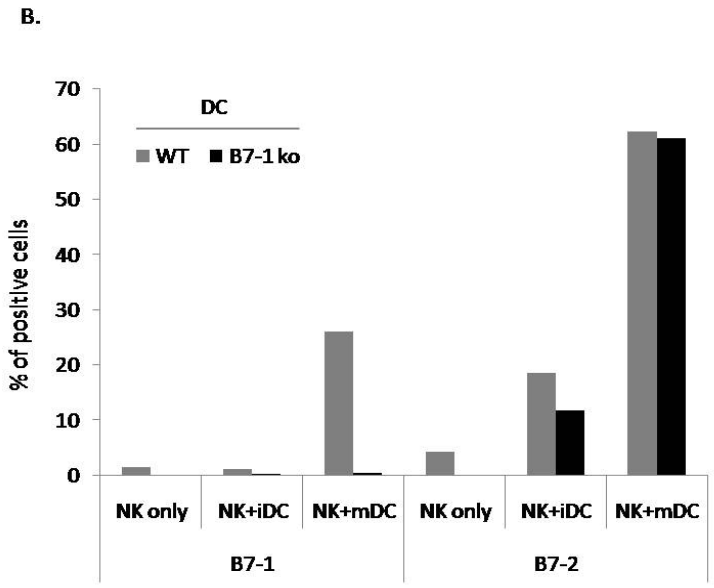


Figure 6.29. B7-1 is transferred from target cells to NK cells. NK cells were isolated from spleens of WT and B7-1 deficient (B7-1 ko) mice by magnetic cell sorting using $CD3^+$ cell depletion followed by DX5 positive selection and further cultured for 7-10 days in the presence of 1700 U/ml rhIL-2. DCs were derived from bone marrow of WT and B7-1 ko mice by culture in the medium supplemented with GM-CSF. Cells were harvested on day 6 and treated with LPS (mature DC, mDC) or medium only (immature DC, iDC) for 12 hours. (A) WT or B7-1 ko NK cells were cultured alone or with either B16 and B16.B7-1 tumor cells (left) or WT iDC and mDC (right) at a 1:1 ratio. NK/tumor cell co-cultures were supplemented with IL-2 (1700 U/ml) and NK/DC co-cultures with IL-2 and GM-CSF (1/5 of amount used for expansion). After 4h of co-culture with tumor cells or 24h with DCs, expression of B7-1 and B7-2 on $CD3^-NK1.1^+$ cells was measured by flow cytometry. Dead cells were excluded by 7-AAD staining. (B) WT NK cells were co-cultured with WT or B7-1 ko iDCs and mDCs as indicated for panel A. NK cell B7-1 and B7-2 expression was measured by flow cytometry as in A.

In summary, NK cells were capable of trogocytosis of B7-1 ligand from the cells they interact with. In our *in vivo* experiments we found indications, that this process could take place in the tumor tissue as well. NK cells within B7-1 positive melanoma expressed higher levels of B7-1 compared to the NK cells infiltrating B7-1 negative tumors (Figure 6.30). Elevated levels of the B7 costimulatory ligands on the surface of NK cells can have multiple functional outcomes that include both positive (e.g. T cell costimulation through CD28) and negative effects (e.g. Treg support through CD28, effector T cell inhibition through CTLA-4 or induction of IDO).

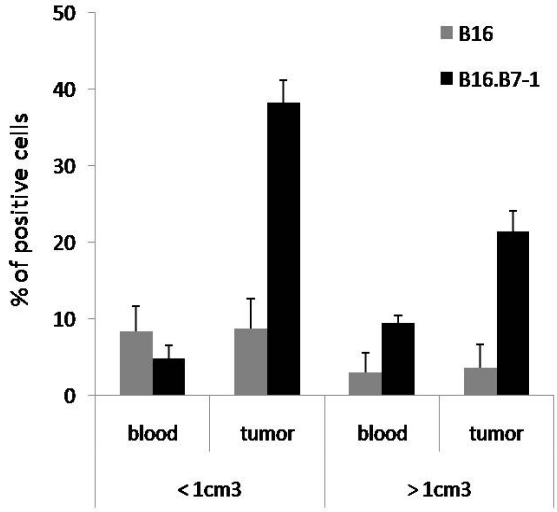


Figure 6.30. NK cells infiltrating B7-1 positive melanoma tumor express elevated levels of B7-1. Mice were injected subcutaneously with 10^6 tumor cells (B16 or B16.B7-1) in 100 µl PBS. The first group of mice were sacrified before and the second group after tumor exceeded 1 cm in diameter. Single cell suspensions were prepared from blood and tumor and expression of B7-1 and B7-2 on $CD3^-NK1.1^+$ cells was analyzed by flow cytometry. Graphs indicate mean±SD of experimental group of three animals.

6.5. Regulation of B7-1 induced responses by CD28, CTLA-4 and B7-H1

So far, we showed that *in vitro* expanded NK cells, similar to tumor infiltrating NK cells, expressed the B7 family receptors CD28, CTLA-4 and B7-H1, that all bind the same ligand, B7-1. In response to B7-1, NK cells proliferated and produced high amounts of IFNγ. Ectopic expression of B7-1 by RMA-S lymphoma and B16 melanoma tumor cells enhanced their sensitivity to NK cell lysis and stimulated NK cell IFNγ production. In T cells, that constitutively express CD28 and B7-H1, and upregulate CTLA-4 when activated, CD28 delivers positive, and B7-H1 and CTLA-4 negative costimulatory signals in the context of the TCR activation [59, 304]. We analyzed how B7-1 induced responses of NK cells are regulated by the given receptors. For this purpose, we used NK cells deficient in CD28, CTLA-4 or B7-H1, which were derived from CD28 ko, OT-I transgenic (OT-I-Tg) CTLA-4 ko and B7-H1 ko mice, respectively. CTLA-4 ko mice develop severe autoimmune phenotype characterized by the activation and expansion of self-reactive T cells, which results in animal death at the age of 3-4 weeks [203]. OT-I-Tg mice contain only $CD8^+$ T cells specific for ovalbumin peptide presented in the context H2-K^b. Although the signs of T cell activation are visible in OT-I-Tg CTLA-4 ko mice, disease progression is significantly slower [308], which allowed us to use NK cells derived from those mice at the age when NK cells are fully mature and the signs of immune activation are minimal (around age of 6 weeks). Freshly isolated or *in vitro* expanded NK cells from gene deficient mice had a phenotype comparable to the WT NK cells and expanded in IL-2 with similar kinetics. No differences in the expression of maturation markers (CD11b, CD27), activation markers (CD11c, B220, CD69, CD25) and B7 family members (CD28, CTLA-4, PD-1, ICOS, B7-H1, PD-L2, B7-2) were observed (data not sown).

6.5.1. IFNγ production is positively regulated by CD28 and negatively by CTLA-4 and B7-H1

When stimulated with the plate-bound B7-1 IgG fusion protein (B7-1-Fc), *in vitro* expanded NK cells produced IFNγ (Figure 6.25A). To investigate which of the receptors known to recognize B7-1 is responsible for this response, we used NK cells deficient for CD28, CTLA-4 or B7-H1 and measured their activation by B7-1-Fc. In response to plate-bound B7-1-Fc, CD28 deficient NK cells failed to produce IFNγ (Figure 6.31A), indicating that the positive response to B7-1 is completely CD28 dependent.

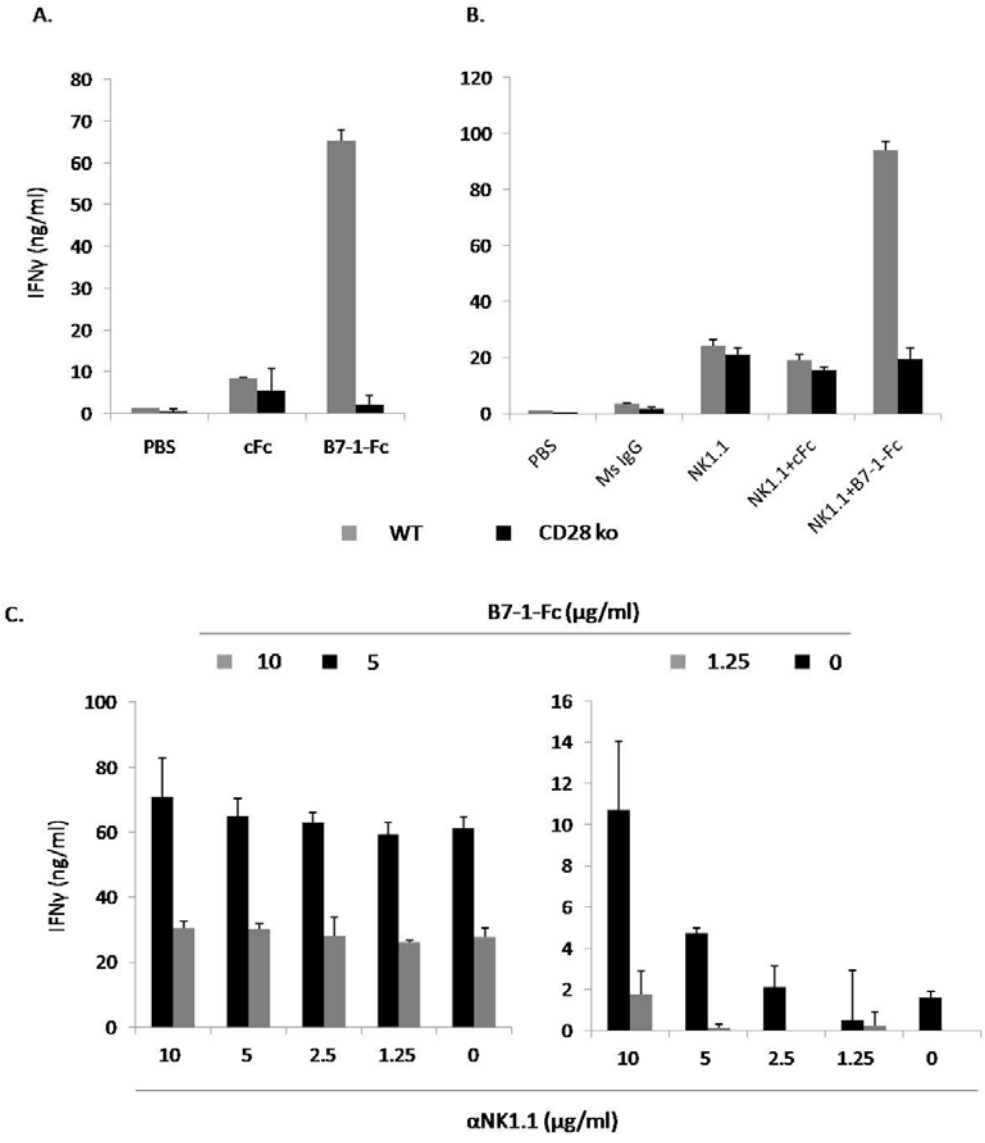
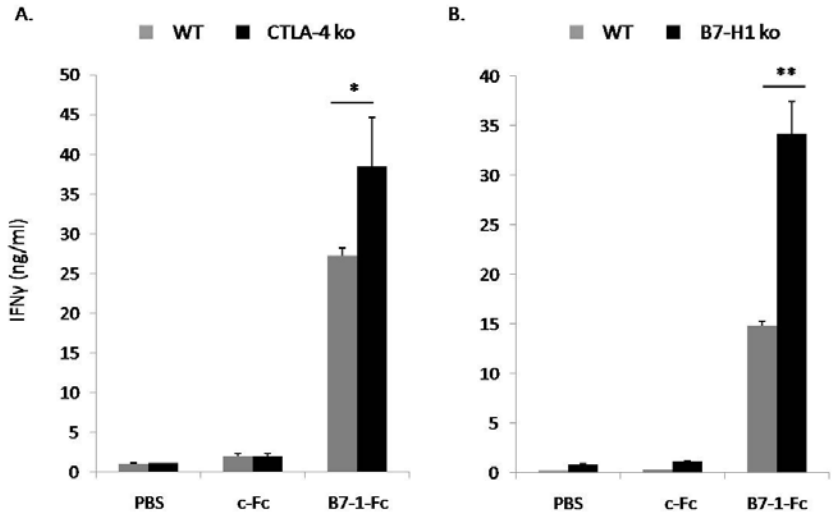


Figure 6.31. CD28 stimulates NK cell IFNγ production in response to B7-1. (A,B) NK cells were isolated from spleens of WT and CD28 deficient (CD28 ko) mice by $CD3^+$ cell depletion followed by DX5 positive selection. After one week of culture in the presence of 1700 U/ml rhIL-2, cells were incubated with 10 μg/ml αCD16/CD32 to block Fc receptors and then stimulated with the plate-bound IgG fusion proteins (2 μg/ml) or indicated mAbs (10 μg/ml). (C) NK cells were obtained by one week culture of non-adherent RAG2 splenocytes in the presence of 1700 U/ml rhIL-2. Cells were treated as in panel A and stimulated with the increasing amounts of B7-1-Fc and αNK1.1. After 8h of stimulation,

supernatants were harvested and amount of released IFNγ was measured by ELISA. Graphs indicate mean±SD of triplicate culture. Graphs indicate mean±SD of triplicate ciltures. B7-1-Fc, B7-1 IgG fusion protein; c-Fc, control IgG fusion protein

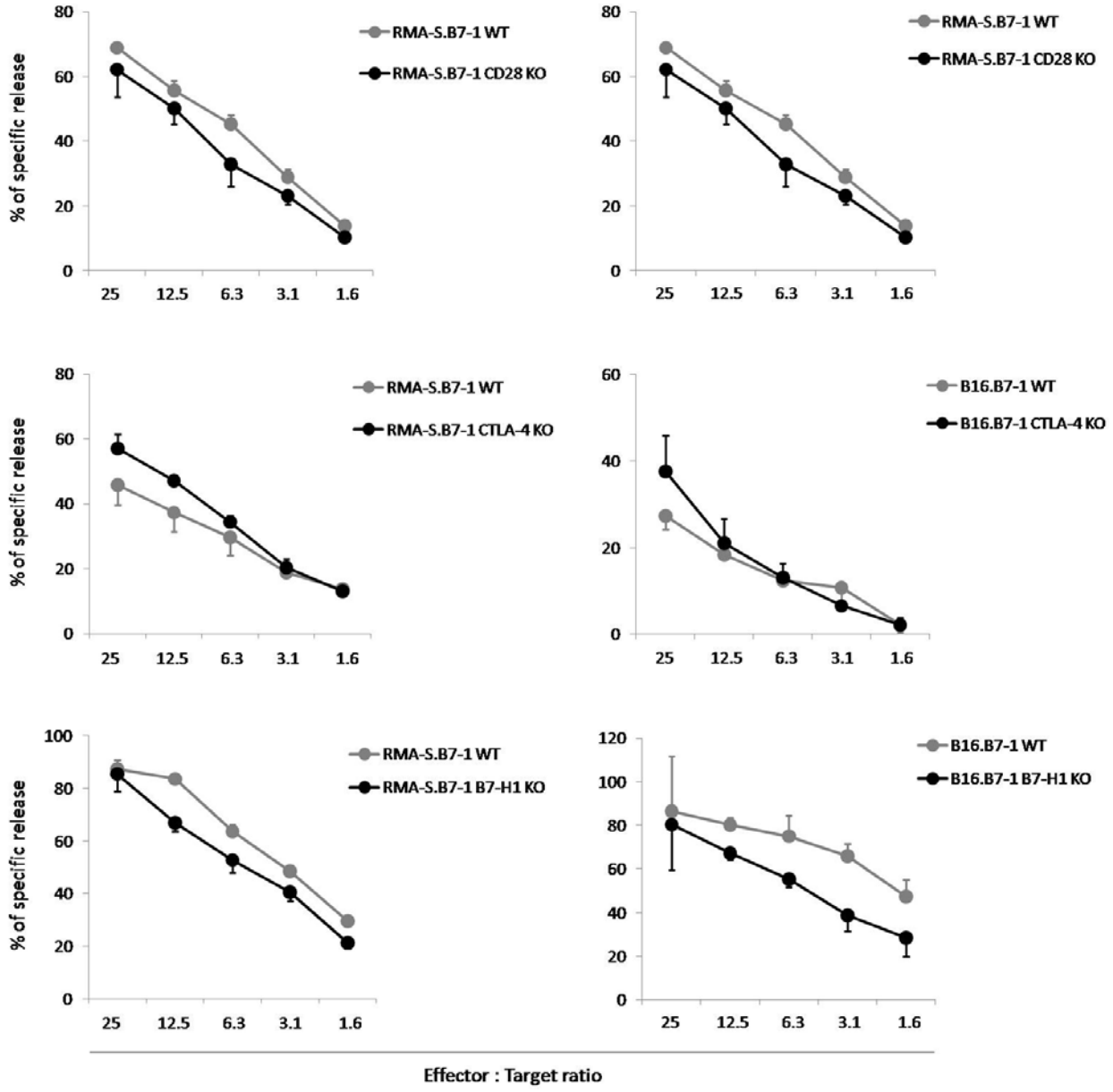
Several other activating NK receptors were shown to induce IFNγ, among them NK1.1. When both the B7-1-Fc and αNK1.1 mAb were used for NK cell stimulation, the effects on IFNγ production seemed to be additive, as shown by CD28 ko NK cells responding to αNK1.1 and αNK1.1/B7-1-Fc combination (Figure 6.31B). We further extended these findings by analysis of WT NK cell response to the combination of these two stimuli. When concentration of the B7-1 fusion protein was high, B7-1 delivered stimulus dominated and addition of αNK1.1 did not further increase IFNγ production (Figure 6.31C left). However, when the B7-1-Fc concentration was low and therefore the amount of stimulus limited, NK1.1 triggering contributed significantly to the response (Figure 6.31C right). As depicted in Figure 6.32, CTLA-4 and B7-H1 deficient NK cells showed elevated cytokine production in response to B7-1-Fc compared to WT NK cells. Therefore, similar to their function in T cells, CD28 positively, and CTLA-4 and B7-H1 negatively regulate NK cell IFNγ production.



6.32. Negative regulation of NK cell IFNγ production by CTLA-4 and B7-H1. NK cells were isolated from spleens of WT and OT-I transgenic CTLA-4 deficient (CTLA-4 ko, panel A) or B7-H1 deficient (B7-H1 ko, panel B) mice by $CD3^+$ cell depletion followed by DX5 positive selection. After one week of culture in the presence of 1700 U/ml rhIL-2, cells were incubated with 10 μg/ml αCD16/CD32 to block Fc receptors and then stimulated with plate-bound IgG fusion proteins (2 μg/ml). After 8h of stimulation, supernatants were harvested and the amount of released IFNγ measured by ELISA. Graphs indicate mean±SD of triplicate culture. *, $p<0.05$; **, $p<0.01$ by t-test; B7-1-Fc, B7-1 IgG fusion protein; c-Fc, control IgG fusion protein

6.5.2. Cytotoxicity is not regulated by CD28, CTLA-4 and B7-H1

We observed that B7-1 positive RMA-S lymphoma and B16 melanoma tumor cell lines were more sensitive to NK cell lysis than their negative counterparts. We further analyzed the regulation of cytotoxic response by CD28, CTLA-4 and B7-H1 by using receptor deficient NK cells. Suprisingly, CD28, CTLA-4 and B7-H1 deficient NK cells killed B7-1 transduced tumor cells equally efficient as WT NK cells (Figure 6.33).



6.33. Cytotoxic response of CD28, CTLA-4 or B7-H1 deficient NK cells. NK cells were isolated from spleens of WT and CD28 deficient (CD28 ko, upper panel), OT-I transgenic CTLA-4 deficient (CTLA-4 ko, middle panel) or B7-H1 deficient (B7-H1 ko, lower panel) mice by $CD3^+$ cell depletion followed by DX5 positive selection. After one week of

culture in the presence of 1700 U/ml rhIL-2, cells were used as effectors in 4h ^{51}Cr release assay against RMA-S, RMA-S.B7-1, B16 and B16.B7-1 tumor cells. Graphs indicate mean±SD of triplicate cultures.

6.5.3. Trogocytosis of B7-1 partially depends on CD28 expression

As we previously showed (Figures 6.27-29), in the interaction with the B7-1 expressing target cells, B7-1 ligand is transfered from the targets to NK cells. A previously described process of intercellular exchange of intact membrane patches including surface proteins is termed trogocytosis. Since trogocytosis is triggered by the receptor mediated recognition of the donor cell and requires the signal transduction in the acceptor cell [306-307], we analyzed if any of B7-1 recognition receptors is responsible for its transfer from the targets to NK cells. As an alternative, other receptor-ligand pairs might initiate the process and B7 ligands might be exchanged as bystander molecules belonging to the tranfered membrane patch.

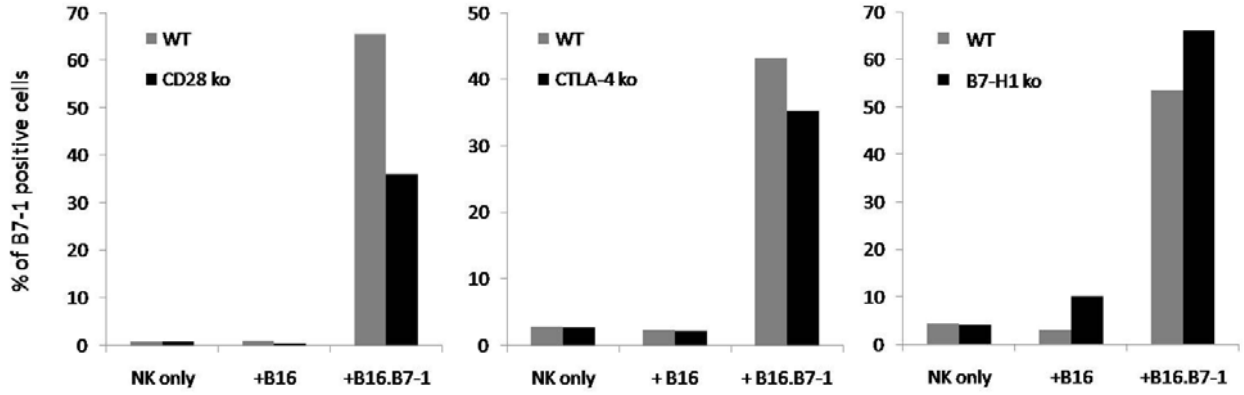


Figure 6.34. Regulation of B7-1intercellular transfer by CD28. NK cells were isolated from spleens of WT and CD28 deficient (CD28 ko, left panel), OT-I transgenic CTLA-4 deficient (CTLA-4 ko, middle panel) or B7-H1 deficient (B7-H1 ko, right panel) mice by $CD3^+$ cell depletion followed by DX5 positive selection. After one week of culture in the presence of 1700 U/ml rhIL-2, cells were co-cultured with B16 or B16.B7-1 tumor cell at 1:1 ratio. After 4h of incubation, expression of B7-1 on NK cells was analyzed by flow cytometry. Dead cells were excluded by 7-AAD staining.

Co-culture of WT NK cells with B7-1 expressing targets (B16.B7-1 tumor cells or mature DCs) led to transfer of B7 ligand from target cells to NK cells. If CD28 deficient NK cells were used, levels of transferred B7-1 were reduced (Figure 6.34 left). This observation indicates that CD28 is partially involved in B7-1 trogocytosis. It is possible that certain amounts of B7-1 ligand were acquired through an active process mediated by CD28, and the rest was transferred passively as the part of the exchanged membrane patch. When CTLA-4 and B7-H1 deficient NK cells were co-cultured with B7-1 expressing tumor cells or B7-1 positive DCs, levels of acquired B7-1 ligand were comparable to the levels measured on WT NK cells (Figure 6.34 middle and right). Therefore, these two receptors were not involved in the active B7-1 trogocytosis from target cells.

6.6. Manipulation of NK cell anti-tumor responses in vivo

Genetic expression analysis using whole genome microarrays, that we have performed, revealed that tumor infiltrating NK cells had a different transcription profile as compared to NK cells in the blood of tumor bearing animals. At the same time, differences observed between blood of naïve and tumor bearing animals were much less pronounced. We confirmed that most of the candidate genes we selected showed differential expression at the protein level too. Among those genes, B7 family receptors PD-1 and CTLA-4 were expressed by NK cells only in the tumor. CTLA-4, but not PD-1, could be induced by NK cell expansion in IL-2. Functional analysis using *in vitro* expanded NK cells derived from OT-I transgenic CTLA-4 deficient mice, showed that CTLA-4 negatively regulated NK cell IFNγ production in response to B7-1, but not cytotoxicity. CTLA-4 blockade *in vivo*, in combination with agents, such as GM-CSF or Flt3L producing tumor vaccines, has been shown to mediate potent anti-tumor responses. CTLA-4 blockade is shown to affect both the T cell effector and Treg compartment. Similar to CTLA-4, disruption of PD-1:B7-H1 interaction by application of mAbs *in vivo,* was shown to enhance anti-tumor immune responses. Since NK cells within the tumor tissue expressed PD-1 and CTLA-4, we were aimed to analyse how the receptor blockade could affect NK cells and tumor growth *in vivo*. To avoid a posible effect of αPD-1 and αCTLA-4 mAb on the T cell compartement, for our experiments we used RAG2 deficient mice that lack T and B cells.

6.6.1. αCTLA-4 mAb treatment does not improve the anti-tumor response in RAG2 deficient mice

We first investigated whether the application of blocking αCTLA-4 mAb will affect the progression of subcutaneously inoculated tumor cells in RAG2 deficient mice. Mice were injected with B16, B16.B7-1 or B16.Rae1ε.B7-1 melanoma, RMA-S lymphoma or LL2 carcinoma tumor cells. A comparison of tumor growth of PBS and αCTLA-4 injected animals in different tumor models did not reveal significant benefits for αCTLA-4 treated animals (Figure 6.35). Moreover, neither ectopic expression of B7-1, the ligand of the B7 family receptors CD28, CTLA-4 and B7-H1, nor Rae1ε, the ligand of the activating receptor NKG2D, on B16 melanoma tumor cells, influenced the outcome of the experiment.

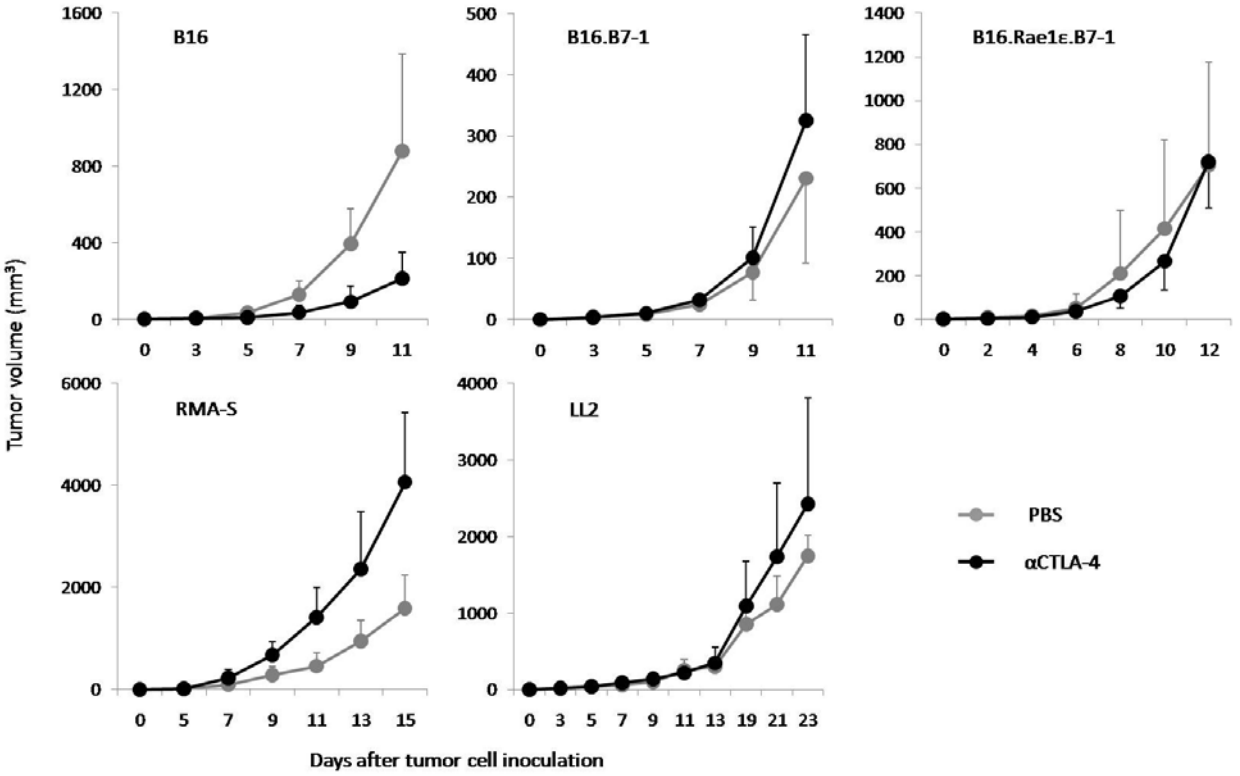


Figure 6.35. αCTLA-4 mAb treatment in RAG2 deficient mice. RAG2 deficient mice were injected with tumor cells subcutaneously (10^6 B16, B16.B7-1, RMA-S, LL2 or $5x10^6$ B16.Rae1ε.B7-1). Mice were treated with i.p. injections of PBS or 250 µg αCTLA-4 mAb every three days starting from day -1 respective to the tumor cell inoculation. Tumor size was measured every 2 days and expressed as the product of three tumor dimensions. Graphs indicate mean±SD of experimental groups of 4-6 treated animals.

6.6.2. Combination therapy of αCTLA-4 and IL-18 does not improve the anti-tumor response of RAG2 deficient mice

We showed previously that stimulation with a combination of IL-2, IL-12 and IL-18 *in vitro* could increase CTLA-4 expression by NK cells. Here, we tested whether the IL-18 application *in vivo* might have a similar effect. RAG2 deficient animals with established RMA-S subcutaneous tumors, that do not express CTLA-4 on NK cells withinn the tumor tissue, were treated systemically with IL-18 for two consecutive days. As depicted in Figure 6.36A, systemic injection of IL-18 did not induce CTLA-4 expression on blood NK cells, but elevated its expression in the tumor. Since IL-18 is known for its ability to enhance NK cell cytotoxicity and IFNγ production, we tested whether IL-18 application influences tumor growth control in RAG2 deficient mice. At the same time, we blocked CTLA-4 by application of αCTLA-4 mAb. As shown in Figure 6.35B, the therapeutic application of IL-18 or combination of IL-18 and αCTLA-4 did not influence RMA-S tumor growth in RAG2 deficient mice.

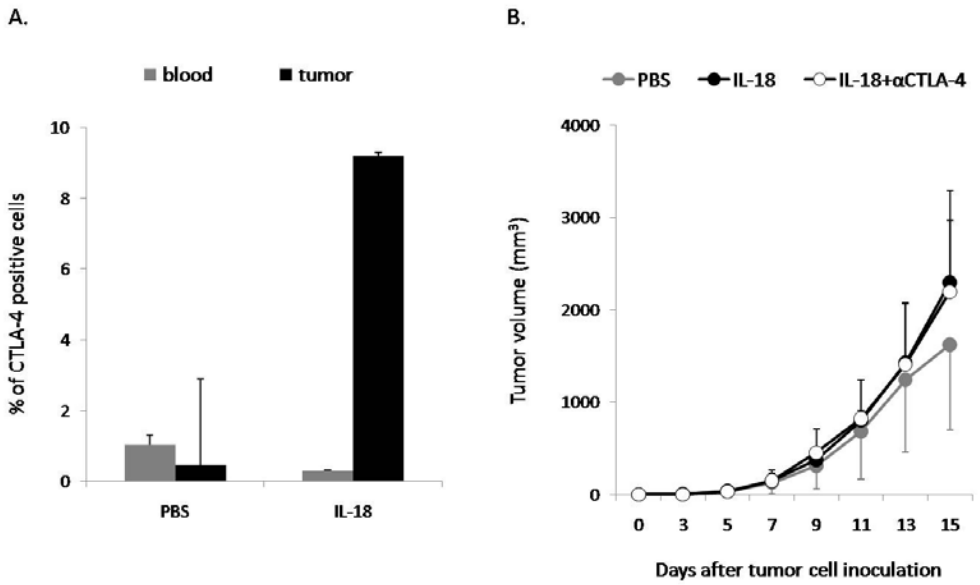


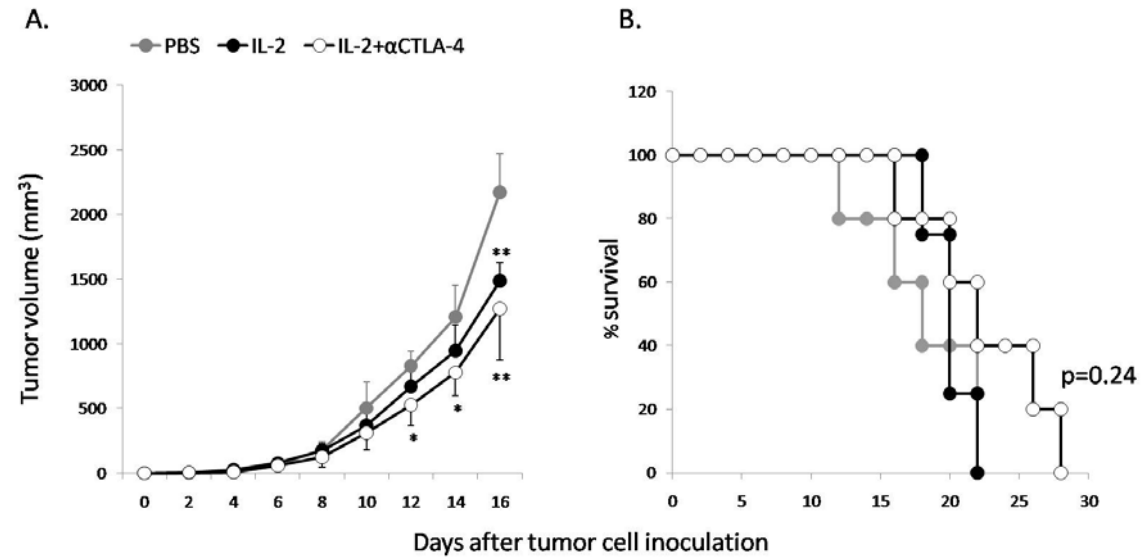
Figure 6.36. Combination therapy with αCTLA-4 and IL-18 in RAG2 deficient mice. RAG2 deficient mice were injected with 10^6 RMA-S cells subcutaneously. (A) When tumor size exceeded 1 cm^2, mice were treated with i.p. injections of PBS or 2 µg IL-18 for two consecutive days. On day 3 after the beginning of the treatment, mice were sacrificed and single cell suspension were prepared from blood and tumor. Expression of CTLA-4 in blood and tumor infiltrating NK cells was analyzed by flow cytometry after intracellular staining of the protein. (B) Mice were treated with i.p. injections of 2 µg IL-18 for 5 consecutive days starting from day 9 or/and 250 µg αCTLA-4 mAb every three days starting from day 8 respective to tumor cell inoculation. Tumor size was measured every 2 days and expressed as the product of three tumor dimensions. Graphs indicate mean±SD of experimental groups of 3 (A) and 5 (B) treated animals.

6.6.3. Combination treatment of αCTLA-4 and IL-2 reduces tumor progression in RAG2 deficient mice

NK cells expanded *in vivo* in the presence of IL-2 expressed CTLA-4 (Figure 6.17). IL-2 was shown to mediate NK cell activation and could enhance NK cell anti-tumor responses *in vivo* [102]. We tested whether IL-2 treatment combined with CTLA-4 blockade might further enhance control of tumor growth. We used B7-1 positive B16 melanoma tumor model and analyzed tumor growth and survival of treated animals. Figure 6.37A shows that IL-2 treatment alone slightly retained the tumor progression in RAG2 ko mice at the late stage of the tumor progression. Combination treatment of IL-2 and αCTLA-4 exert a similar effect, which was pronounced earlier compared to IL-2 treatment only. However, survival of mice treated with the IL-2/αCTLA-4 combination was not significantely prolonged compared to animals treated with IL-2 only (Figure 6.37B).

In contrast to the effects observed with B16.B7-1 tumor model, IL-2/αCTLA-4 treatment was not efficient when mice were inoculated with B16.Rae1ε.B7-1 tumor cells (Figure 6.37C). Since the Rae1ε expression

is already strong activating signal, NK cell effector functions might be not sensitive for further increasment with IL-2 and/or αCTLA-4 treatment in this model.



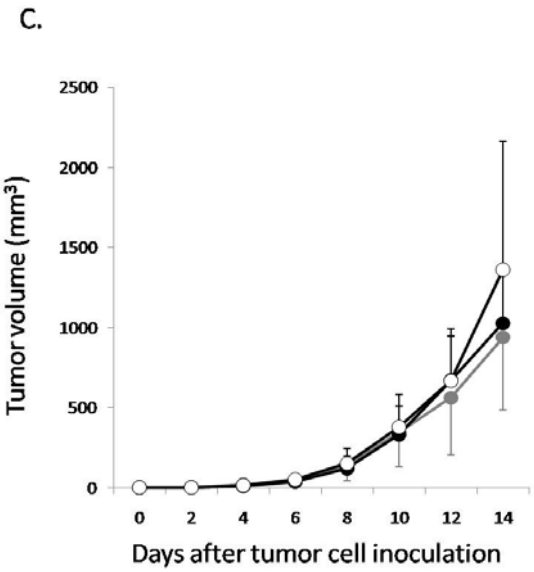


Figure 6.37. Combination treatment with αCTLA-4 and IL-2 in RAG2 deficient mice. RAG2 deficient mice were injected with tumor cells subcutaneously as follows: panel A - 10^6 B16.B7-1, panel B - $2.5x10^6$ B16.B7-1, panel C - $5x10^6$ B16.Rae1ε.B7-1. Mice were treated with i.p. injections of 10^5 U of rhIL-2 for five consecutive days starting from day 0 or/and 250 μg αCTLA-4 mAb every three days starting from day -1 respective to the tumor cell inoculation. Tumor size was measured every two days and expressed as the product of three tumor dimensions. Graphs indicate mean±SD of experimental groups of 4-6 treated animals. (A, C). Animals were sacrificed when tumor diameter exceeded 1.5 cm (B). *, $p<0.05$; **, $p<0.01$ compared to PBS treated group, by t-test. Log-rank test was used for p-vaule calculation of survival curves (p=0.24 compared to IL-2 group).

6.6.4. αPD-1 treatment does not improve the anti-tumor response in RAG2 deficient mice

Another B7 family receptor, PD-1, which we showed to be upregulated in the tumor infiltrating NK cells, is often expressed by many tumor cell lines, including RMA-S and B16 (data not shown). For testing the effect of PD-1 blockade in RAG2 deficient mice, we used LL2 carcinoma model. LL2 cells do not express PD-1, so the mAb treatment affected only the host cells. Although some mice receiving αPD-1 exibited the signs of possible immune activation at the tumor site, no reproducible differences were obtained between

control and αPD-1 treated animals. Representative tumor growth curves from one of the three experiments performed are shown in Figure 6.38.

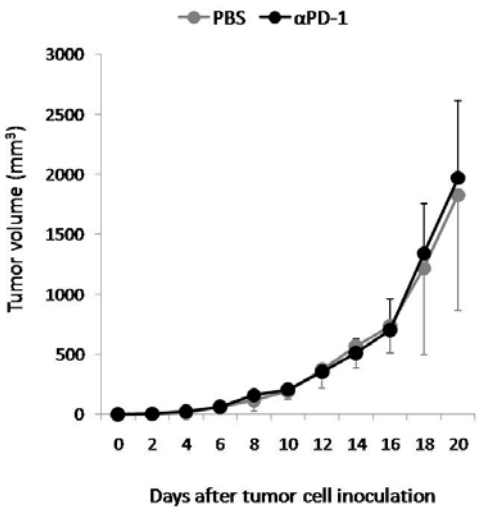


Figure 6.38. αPD-1 as a single reagent treatment in RAG2 deficient mice. RAG2 deficient mice were injected with 10^6 LL2 cells subcutaneously. Animals were treated with i.p. injections of PBS or 300 μg αPD-1 mAb every three days starting from day -1 respective to the tumor cell inoculation. Tumor size was measured every 2 days and expressed as the product of three tumor dimensions. Graph indicates mean±SD of experimental groups of 3-5 treated animals.

6.6.5. αPD-1 treatment combined with αCTLA-4 and IL-2 reduces survival of tumor bearing RAG2 deficient mice

So far, our results indicated that IL-2 treatment in combination with αCTLA-4 blockade slightly prolonged survival of mice injected with B16.B7-1 melanoma tumor cells. When inoculated with LL2 carcinoma, αPD-1 treated mice often exerted the signs of inflamation at the tumor site. We decided to combine the blockade of both receptors with IL-2 treatment in mice bearing LL2. LL2 cells expanded *in vitro* did expressed neither PD-1 nor CTLA-4, but expressed low levels B7-H1 and B7-1. In addition, Rae1, the ligand for activating receptor NKG2D, was also expressed by LL2 carcinoma cells (Figure 6.39).

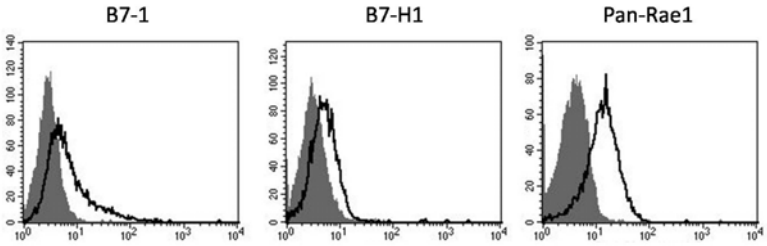


Figure 6.39. Phenotype of *in vitro* expanded LL2 carcinoma cells. Expression of B7-1, B7-H1 and Rae1 on LL2 carcinoma cells was analyzed by flow cytometry. Representative histogram plots show isotype control (grey histogram) compared to staining with specific mAb (black line).

As shown in the Figure 6.39, none of the applied treatments (IL-2, IL-2+αCTLA-4 and IL-2+αCTLA-4+αPD-1) significantly changed tumor growth kinetics in LL2 inoculated RAG2 deficient mice. In the previous experiments, we observed that the treatments with αPD-1 or αCTLA-4 had no effect on LL2 tumor progression (Figure 6.34 and 6.37). In contrast, animals treated with a combination of all three reagents, IL-2, αPD-1 and αCTLA-4, showed reduced survival (Figure 6.39 right). We again observed the signs of the immune system activation at the timor site of triple reagent treated mice. This might be a consequence of the strong induction of the innate immune system. This overamplification of the response could be responsible for the early death of treated animals.

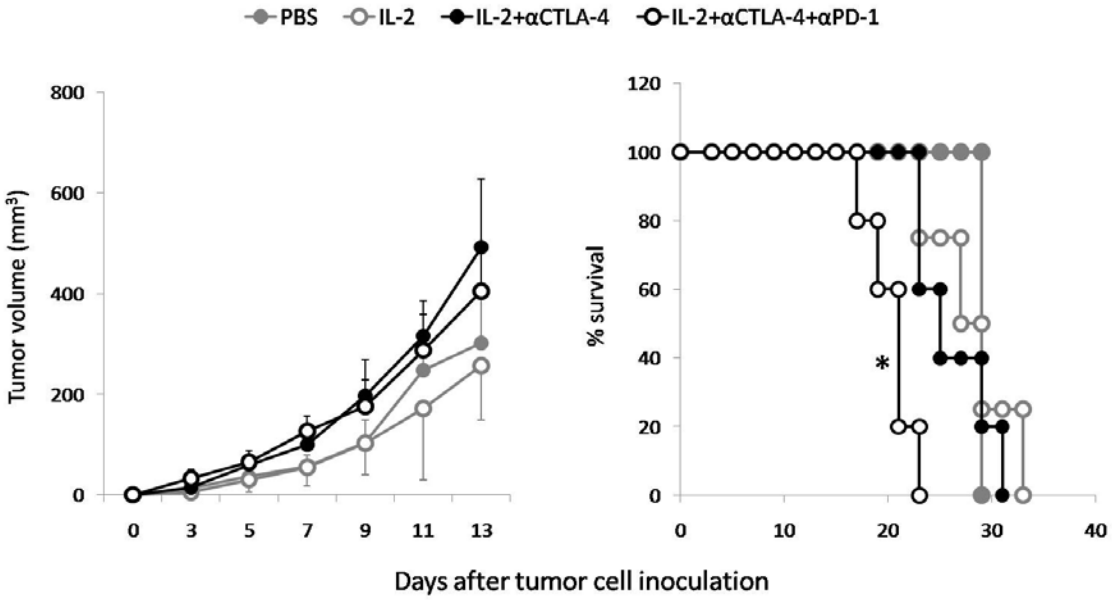


Figure 6.40. Combination therapy of αPD-1, αCTLA-4 and IL-2 in RAG2 deficient mice. RAG2 deficient mice were injected with 10^6 LL2 cells subcutaneously. Animals were treated with i.p. injections of PBS, 10^5 U of rhIL-2 for five consecutive days starting from day 0, 250 µg αCTLA-4 mAb and 300 µg of αPD-1 mAb every three days starting from day -1 respective to the tumor cell inoculation. Tumor size was measured every 2 days and expressed as the product of three tumor dimensions. Animals were sacrificed when tumor diameter exceeded 1.5 cm or when mice become moribund. Graph of tumor growth kinetics indicates mean±SD of experimental groups of 4-6 treated animals. Log-rank test was used for p-vaule calculation of survival curves. *, p=0.017 compared to IL-2+αCTLA-4 group

7. Discussion

7.1. Tumor infiltrating cells

NK cells were shown to infiltrate solid tumors in both mouse models and cancer patients [309]. A high number of NK cells found in the tumor tissue correlated with better prognosis in the cases of lung, gastric or colorectal cancer [274-275, 310-311]. However, the frequency of NK cells among total infiltrated immune cells is not high and therefore the ratio relative to tumor cells is very low. In addition, NK cells were often not located in the direct contact with tumor cells, but rather in the proximity of the blood vessels within the stroma [309]. Other immune cells within the tumor tissue are composed mainly of suppressive myeloid cells, immature DCs, macrophages polarized toward M2 phenotype or regulatory T cells [312]. Therefore, the number of real effectors, such as NK cells and $CD8^+$ T cells, that could eliminate tumor cells, is very low compared to the cells that rather support tumor progression. In addition to the tumor cells, certain immune cells were shown to be lysed by NK cells. Immature DCs or myeloid derived suppressor cells (MDSCs) can be eliminated by activated NK cells [66, 313]. Thus, within tumor tissue NK cells are confronted to multiple target cells whose elimination can be beneficial for the efficient control of tumor progression.

Solid tumors are characterized by specific microenvironment formed within tumor tissue. Apart from suppressive cells, which are either recruited or converted upon infiltration, several other factors do not favor effective immune response. Low pH and oxygen concentration were shown to hamper normal function of effector cells, including NK cells [314-315]. Various studies of anti-tumor immune responses, especially after the application of therapeutics, were conducted using peripheral blood mononuclear cells (PBMCs) sampled from the patients (Table 3.5). Although in many cases specific anti-tumor responses were detected *in vitro*, benefits for tumor control were often not observed. A possible explanation for the failure of *in vivo* responses included the absence of efficient tumor tissue infiltration and the presence of multiple immunosuppressive mechanism exerted by the tumor. First, *in vitro* studies are typically performed under optimal cell culture conditions (pH, O_2, humidity, etc), which are not met in the tumor tissue. Second, actual effector-to-target ratios in tumors are usually much lower than those used in the culture. And third, the microenvironment of the solid tumor is shaped by the presence of cytokines released by both tumor and infiltrated immune cells, which can differentially influence the studied responses and are not produced in *in vitro* systems. In addition, cells from peripheral blood might not precisely correspond to their tumor infiltrating counterparts. Indeed, several studies that analyzed effector functions of cells isolated from tumor tissue showed that their responses were impaired. Unresponsive tumor specific T cells were isolated from malignant melanomas [316], which recovered functional competence upon *ex vivo* stimulation, indicating that they were inhibited in the tumor microenvironment and that the process was reversible. Functional incompetence of tumor infiltrating T cells (TITs) was found

to directly correlate with the level of expression of the inhibitory B7 family receptor PD-1 [241]. A higher frequency of PD-1$^+$ T cells was detected in melanoma lesions as compared to blood or healthy tissue of patients. In addition, PD-1$^+$ TITs expressed another inhibitory B7 family receptor, CTLA-4. The possible mechanism underlying non-optimal T cell activation within the tumor tissue includes the alternation in the proximal TCR signaling machinery given by the decreased expression of CD3ζ or tyrosine kinases Lck and Fyn [317]. An aberrant activation of distal signaling molecules has been also reported: defects in the expression of NF-kB family proteins were correlated with the impairment in Th1 cytokine production and decreased CTL function [318]. Tumor infiltrating CD8$^+$ T cells showed a defective conjugate formation with tumor cells *ex vivo* due to the defective expression and activation of adhesion molecules [319].

In summary, studies that analyzed the tumor microenvironment and phenotypical and functional properties of T cells from blood and tumor of tumor bearing mice and cancer patients revealed a functional impairment of cells infiltrated into the tumor tissue. Since the failure of the numerous clinical trials was correlated with the unresponsiveness of the effector cells in the tumor, it is of importance to analyze the phenotype and function of the immune cells infiltrating the tumor tissue and their possible contribution to the outcome of novel treatment strategies.

7.2. Tumor infiltrating NK cells

While treatment strategies targeting T cells as the main anti-tumor effectors must rely on tumor antigen specificity, NK cells are considered as non-specific cytotoxic effectors that should be capable or recognizing a broad range of transformed cells. Of importance, data obtained in mouse models indicated that the activation of T cell anti-tumor responses to effectively reduce the tumor burden required an intact NK cell compartment [13, 69, 266]. Several clinical observations provided information about NK cells in tumor patients with regard to their numbers, activation state and functionality. An association of defective NK cell activity and severe cancer progression was reported. Decreased NK cell responses were detected in patients with lung, breast, colon and liver cancer as well as in the cases of melanoma, chronic myeloid (CML) and acute myeloid (AML) leukemia [148, 276, 320-322]. In several mouse tumor models NK cell progression to the last maturation stage in bone marrow was blocked at high tumor load, leading to the accumulation of immature NK cells [323]. At the same time, NK cells from spleen of tumor bearing mice did not show defects in cytotoxicity, while IFNγ production was reduced in response to PMA/Ionomycin but not IL-12 stimulation. Recently, Brenner and colleagues showed that in the transgenic mouse model of spontaneously arising lymphoma effector functions of tumor infiltrating NK cells became progressively paralyzed with increasing tumor load [324].

Due to the low NK cell numbers found within the tumor tissue and their functional impairment, studies aimed at the enhancement of anti-tumor responses of endogenous and/or adoptively transferred NK cells. As discussed in the section 3.2.5.2, those approaches included the facilitation of NK cell migration to the tumor site, their proliferation, cytotoxicity and cytokine production. Independently whether endogenous or

adoptively transferred NK cells are expected to directly or indirectly mediate tumor rejection, the influence of the tumor microenvironment on their activity must be taken into consideration in order to design the efficient strategy of their use in anti-tumor therapy.

7.2.1. Subset composition of tumor infiltrating NK cells

Our study aimed at the comprehensive analysis of the phenotype of tumor infiltrating NK cells including the expression of surface markers used to define their maturation stage, subset composition, activation status and functional competence. Importantly, we analyzed NK cells that infiltrated the tumor tissue without any exogenous manipulation. Our findings revealed that subcutaneous solid tumors of different origin are preferentially infiltrated with the $CD27^{high}$ NK cell subset (Figure 6.1). $CD27^{high}$ tumor infiltrating NK cells were composed of both the immature $CD11b^{low}$ and the more mature $CD11b^{high}$ population. CD27 expression on TINs directly correlated with the expression of c-Kit and CD127 (IL-7Rα), which are defined as markers of immature NK cells, and inversely correlated with the expression of markers for mature NK cells, KLRG1 and CD43. Fully mature $CD27^{low}CD11b^{high}$ NK cells were present in the tumor at a lower frequency compared to blood, where they represented the dominant subset. When NK cell subsets defined by CD27 expression were analyzed for their functional properties, it was found that fully mature $CD27^{high}$ NK cells were more competent to proliferate, lyse target cells, produce IFNγ and migrate toward certain chemokines [114]. In this respect, infiltration of tumor with $CD27^{high}$ NK cells can be seen as beneficial for successful anti-tumor responses.

Several mechanisms might be responsible for the prevalence of $CD27^{high}$ NK cell subset in the tumor. First, it is possible that all subpopulations equally infiltrate the tumor tissue where $CD27^{low}$ cells subsequently upregulate CD27. CD27 is a receptor of the TNF family shown to costimulate NK cells [68]. It is responsible for the potent response to tumor cells that express its ligand CD70 [69]. Since in our tumor model, tumor cells do not express CD70, CD27 expression by NK cells might be relevant for the interaction with other CD70 expressing cells. For example, CD70 expression by DCs contributes to T cell activation [67]. Since the NK/DC interaction is known to result in their reciprocal activation, engagement of CD70 on DC by the CD27 receptor on NK cells might contribute to this effect. Alternatively to the CD27 upregulation on $CD27^{low}$ cells, $CD27^{high}$ NK cells could be the subset preferentially recruited to the tumor. Co-expression of the maturation markers that correlate with CD27, as well as the previously reported enhanced migration abilities of $CD27^{high}$ subset compared to $CD27^{low}$ counterparts, favors this hypothesis. As an example, $CD27^{high}$ NK cells express chemokine receptor CXCR3, which is downregulated in the tumor [280]. Using gene deficient NK cells, authors showed that the migration of NK cells to the subcutaneous lymphoma *in vivo*, depended on CXCR3. Thus, CXCR3 downregulation on $CD27^{high}$ NK cell subset in the tumor might be the consequence of its engagement.

7.2.2. Relation of tumor infiltrating NK cells to IKDCs

Interferon producing killer dendritic cells (IKDCs) were initially defined as a cell population with the properties of both NK and DCs [131-132]. They were described to produce both IFNγ and exert cytotoxic responses, but unlike classical NK cells, they were capable of producing IL-12. Another characteristic considered to be the main feature of DCs, antigen presentation, was also performed by IKDCs. The presence of IKDCs within the tumor tissue might be beneficial, since they were shown to mediate potent anti-tumor responses [139]. Phenotypically, IKDCs were defined as a $CD11c^+B220^+NK1.1^+$ cell population that express MHC II upon activation. However, it is still controversial whether IKDCs have independent origin and biological futures from those of DCs and NK cells. IKDCs express NKp46, which is exclusively expressed by NK cells [325], as well as other NK cell receptors including NKG2D and Ly49 receptors [134-135]. Importantly, they depend on IL-15 and common γ_c chain for their development and homeostasis. Since there is no specific marker that can precisely separate them from the other cell types, it is not excluded that IKDCs could be composed of several different cell populations. Their high similarity to NK cells raised the hypothesis that IKDCs represent activated NK cells, which acquired APC functions. Indeed, detailed analysis of $NK1.1^+$ cells within $CD11c^+B220^+$ population in the studies of Vosshenrich *et al.* [135], revealed that both *in vitro* and *in vivo* activation of NK cells led to the upregulation of CD11c, B220, MHC II and costimulatory molecules, giving rise to IKDC-like cells with the ability to produce IFNγ and present antigens to T cells. In addition, among NK cells, defined as $CD19^-CD3^-NK1.1^+$, no bimodal expression of CD11c and B220 could be observed. $B220^{low}$ NK cells could produce similar amounts of IFNγ as $B220^{high}$ NK cells and could upregulate B220 upon stimulation induced proliferation.

Our analysis of tumor infiltrating NK cells, which we defined as $CD3^-NK1.1^+$, revealed an unchanged expression of CD11c compared to NK cells found in blood of tumor bearing animals (Figure 6.2). Expression of B220 was enhanced and, unlike blood NK cells, TINs were MHC II^+. In addition, NK cells within the tumor expressed costimulatory molecules B7-1 and B7-2 albeit at the low levels. Thus, similar to the cell population defined as IKDCs, the subpopulation of the tumor infiltrating NK cells could be considered as $NK1.1^+CD11c^+B220^{high}$MHC II^+. However, whether those cells represent IKDCs cannot be concluded, since IKDCs are primarily defined by their dual functional property of IFNγ/IL-12 production and performance of cytotoxicity and antigen presentation. Although MHC II expression indicated the ability for Ag presentation and high intracellular levels of perforin and granzyme B that we detected in TINs (Figure 6.3), cytotoxic potential, further functional studies are necessary to characterize the IKDC nature of this cell population.

Several studies reported that NK cells were able to present antigens and prime both $CD4^+$ and $CD8^+$ T cell responses [138]. NK cell potential for T cell priming was acquired upon recognition and elimination of sensitive targets. Similar to these observations, IKDCs can perform their APC functions only after the encounter of tumor or virus infected cells [137]. Since perforin deficient IKDCs cannot prime T cell responses, the elimination of the target is a prerequisite for antigen presentation. Triggering of specific activation pathways in NK cells represents the additional mechanism that could upregulate MHC II and

costimulatory molecules allowing the subsequent T cell activation by NK cells [138]. Cross-linking of NKp30, NKp46 and CD16, but not NKG2D, in human NK cells induced an APC-like phenotype. Thus, it is possible that a subpopulation of tumor infiltrating NK cells, activated by tumor cell recognition, or/and by other mechanisms within the tumor, acquired phenotypical and functional properties of APCs or IKDCs. In support for this hypothesis is an observation of Blasius and colleagues, who observed that $CD11c^{+}B220^{+}$ NK cells were enriched within the $CD27^{high}$ NK subset [134], which were also described as more efficient effectors than $CD27^{low}$ NK cells [120]. The enrichment of the $CD27^{high}$ NK cells in the tumor compared to blood might explain their higher B220 expression, while the upregulation of MHC II and costimulatory molecules might indicate that they are activated within the tumor tissue.

7.2.3. Transcription profile of tumor infiltrating NK cells

In addition to the phenotypical analysis of the markers that were previously described as the indicators of NK cell maturation and activation status, we took advantage of a more global approach and performed gene expression profiling of blood and tumor infiltrating NK cells (Figure 6.5). Some of the molecules that we have already shown to be expressed by $CD27^{high}$ NK cells in the tumor elevated compared to blood, such as c-Kit and CD127 (Figure 6.1), were also found to be differentially regulated at the mRNA level. This correlation indicated the high reliability of our microarray experimental data. The most striking observation of the experiment was that tumor infiltrating NK cells showed pronounced differences when compared with blood NK cells of tumor bearing animals (Figure 6.6). The differentially expressed genes between blood and tumor infiltrating NK cells comprised several groups functionally relevant for anti-tumor responses, as discussed in the section 6.2.1. Briefly, four main features were noticed. First, we observed that NK cells in tumor were activated, which was supported by the upregulation of cytokine receptors, several chemokines and chemokine receptors, some components of the cytotoxic pathway or regulatory molecules that drive cell division. Second, NK cells displayed responses to unfavorable environment within the tumor tissue. The examples are prevention of cell cycle progression or the metabolic changes as a consequence of hypoxia, which are driven primarily by the upregulation of transcription factor HIF-1α and its target genes [290]. The third group of the observed changes might be contributed to the direct actions of tumor microenvironment towards the downregulation of NK cell function. Several candidate genes from this group were chosen for validation and their differential regulation was confirmed to persist at the protein level too. Those include NK cell activating receptors and inhibitory molecules of the B7 protein family. Finally, NK cells exerted some features that might support rather than hamper tumor progression. Upregulation of VEGF, PDGF and angiopoietin are some of the examples of molecules that facilitate tumor angiogenesis. In addition, chemokines produced by NK cells might attract anti-tumor effectors but also suppressive cell types. Similarly, the enzymes that modulate extracellular matrix can support NK cell migration but also facilitate metastases formation by tumor cells. Thus, many functions of activated NK cells might be used by tumor and converted for the purposes of its own progression.

7.2.4. Activating receptors expressed by NK cells in the tumor

NK cells isolated from the tumor tissue negatively regulated expression of several activating NK cell receptors at both transcript and protein level. We confirmed that the surface expression of NKp46, 2B4 and CD48 was reduced on TINs compared to blood NK cells in RMA-S tumor bearing mice (Figure 6.9). Although we observed a constant tendency, the decrease in surface expression of NK1.1 was not always significant. Of importance, NKp46 and 2B4 can directly recognize RMA-S tumor cells. NKp46 recognizes viral hemaglutinins as well as unknown ligand(s) on several tumor cell lines including RMA-S [291]. In addition to MHC I deficiency that removes the inhibition and increases RMA-S sensitivity to NK cell lysis, the positive signal that triggers the cytotoxic pathway is deliverd through NKp46. Therefore, the reduction of NKp46 might be one of the mechanisms used by the tumor to evade the direct recognition and elimination by NK cells. It was already shown that tumors, which express ligands for the activating receptor NKG2D, use a similar strategy of evasion. Proteolytic shedding of MICA from the cell surface was reported to cause a systemic downregulation of NKG2D surface expression, thereby impairing the lysis of tumor cells [326]. In our model, expression of NKG2D was unchanged, indicating that the tumor could selectively target the receptor responsible for its recognition. The important difference between these two strategies is that the NKG2D expression was inhibited at the protein level, while NKp46 was regulated at the transcription level.

2B4 can mediate both activating and inhibitory functions [297]. It recognizes CD48, which is widely expressed on hematopoetic cells. RMA-S tumor cells express high levels of CD48 and can be directly recognized by 2B4 expressed on NK cells. 2B4 plays a very important role in the cross-talk of NK cells with other immune cells. Homotypic NK/NK interaction was shown as essential for the generation of NK cell effector function [298], while heterotypic NK/T cell interaction supports T cell activation [299]. Therefore, downregulation of 2B4 might impact anti-tumor response on multiple levels, including direct tumor recognition and support of NK and T cell effector responses.

NKp46 and NK1.1 are coupled to the adaptor molecules CD3ζ and FcεRIγ, that contain ITAM motifs in their cytoplasmic tails [36, 327]. Upon receptor engagement, tyrosine residues of ITAMs are phosphorylated and Syk and ZAP70 kinases are recruited. Dowstream of Syk/ZAP70, different signaling branches are activated including SLP76/LAT, PI3K, PLCγ, MAPK and release of Ca^{2+} (Figure 7.1). We observed that several signaling molecules involved in the depicted pathways were differentially expressed by tumor infiltrating NK cells, including most upstream kinases that phosphorylate ITAM motifs. This is similar to the previously observed modulation of proximal TCR signaling components in tumor infiltrating T cells [317]. In addition to the downregulation of receptors themselves, the downregulation of their signaling components can further contribute to the impairment of NK cell trigerring through ITAM coupled receptors. Of note, members of the MAPK pathway were rather upregulated in tumor infiltrating NK cells, which can be of importance for other receptors that utilize this signaling branch. One example is CD28, which did not appear to be regulated at the transcript level, but was upregulated at the protein level by TINs compared to blood NK cells.

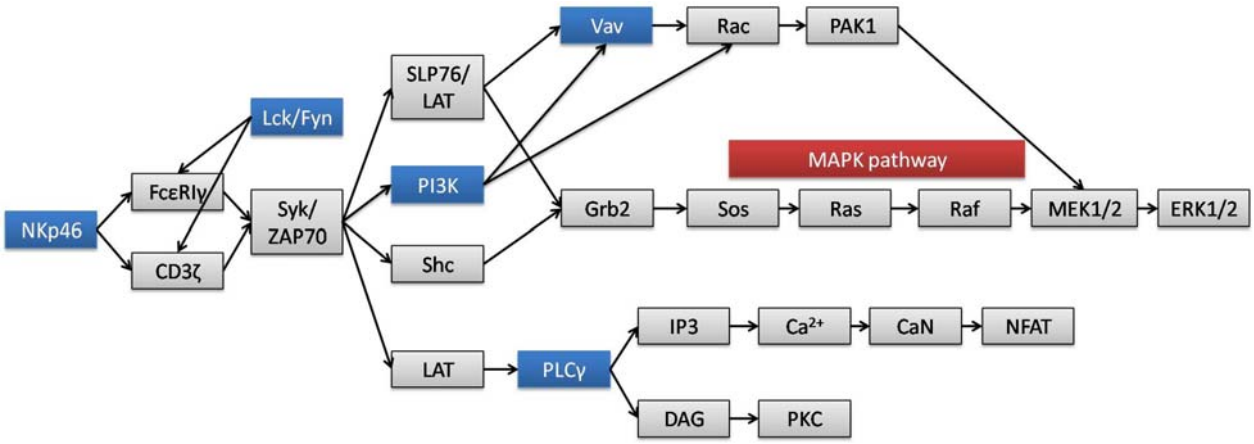


Figure 7.1. The signaling pathway downstream of the activating receptor NKp46 (obtained from KEGG database). NKp46 receptor is coupled to the ITAM bearing adaptor molecules CD3ζ and FcεRIγ, which are phosphorylated by Src kinases Lck and/or Fyn upon receptor engagement. Syk and ZAP70 are recruited to the phosphorylated ITAMs and initiate further signaling events. Molecules represented by blue boxes were negatively regulated at the transcript level in tumor infiltrating NK cells, while several members of the MAPK pathway were upregulated (red box).

7.2.5. Inhibitory receptors expressed by NK cells in tumor

The downregulation of the activating NK cell receptors in TINs was accompanied by an upregulation of inhibitory receptors. The main group of inhibitory receptors of NK cells recognize different MHC I molecules. In mice, they belong to the C-type lectin-like family of Ly49 receptors [328]. The BL6 mouse strain expresses several Ly49 members – Ly49A, Ly49C, Ly49D, Ly49G2, Ly49H and Ly49I. Ly49D and Ly49H belong to the subgroup of activating receptors. Only Ly49C and Ly49I bind to the H-2^b MHC I alleles and their cognate ligands are expressed by the BL6 strain. According to the results of our genetic expression profiling, Ly49A was downregulated in TINs and other Ly49 receptor family members were not regulated at the transcript level (Table 6.2). In contrast, TINs displayed enhanced surface protein levels of Ly49A when compared to the blood NK cells of tumor bearing animals (Figure 6.10). Ly49A mediates NK cell inhibition by the recruitment of protein and lipid phosphatases that dephosphorylate proximal signaling components downstream of activating receptors [328]. However, due to the absence of the cognate ligand, the functional significance of its increased expression in the tumor is not clear.

When we analyzed the cell surface expression of other Ly49 members, we observed that Ly49C/I or H (recognized by the same mAb) as well as Ly49D and Ly49G2 were sensitive to the enzymatic digestion with hyaluronidase and collagenase when expressed by blood or spleen, but not tumor infiltrating NK cells (Figure 6.10). We assume that those receptors could be protected from digestion by mechanisms that are functional in the tumor but not in other organs. It would be important to investigate whether the expression of Ly49 receptors is modified in a similar way in other conditions, such as viral and bacterial infections. Regulation of Ly49 receptor expression is mainly studied in respect to the acquisition of a specific Ly49

repertoire during development, which is regulated at the transcription level [329]. However, little is known about the regulation of the level of surface expression during the course of NK cell effector responses, including both transcriptional and post-transcriptional regulation. Cell intrinsic mechanisms could exist to preserve Ly49 receptor expression in the environment rich in the activity of ECM degrading enzymes, such as the tumor tissue. Cell surface expression of the H-2^b specific inhibitory molecules Ly49C/I might raise the threshold for NK cell activation and allow the protection of cells with low expression of MHC I on the cell surface. This mechanism would possibly lead to the decreased elimination of tumor cells with reduced MHC I expression or MHC I^{low} iDCs that accumulate in tumor beds and cause aberrant T cell activation.

7.2.6. Expression of B7 family members in tumor infiltrating NK cells

B7 family is a group of molecules that regulate T cell responses at multiple levels [59]. The interactions established between B7 family members determine the threshold for the activation through TCR, drive effector responses or mediate their shutdown. Interactions between family members are established at the T/APC interface and control priming of naïve T cells, or between T cells and peripheral tissues controlling their effector functions. Naïve T cell activation requires a minimum of two signals – triggering of TCR and costimulation delivered by the B7 family receptor, CD28. NK cell activation is considered similar as they require either the simultaneous absence of inhibition (e.g. low MHC I expression) and presence of stimulation (e.g. through NKp46) or stimulation through activating and cytokine receptors (e.g. NKG2D and IL-15R) [330]. There are also examples of costimulation of two activating receptors (e.g. NK1.1 and NKG2D) [331]. When T cells get activated, a number of molecules are engaged to control the level of activation including several B7 family members as ICOS, CTLA-4 or PD-1. So far, it is not known if such mechanisms, which regulate the activity once the responses are triggered, exist in NK cells. Several B7 family members are also expressed by NK cells, but their function is poorly investigated. It was previously shown that activated, but not resting NK cells express activating B7 receptor ICOS, which mediated the lysis of ICOS-L expressing target cells [72].

By analysis of transcript profiles of blood and tumor infiltrating NK cells we observed an upregulation of multiple members of the B7 family in TINs (Figure 6.11). Flow cytometric analysis confirmed the expression of CD28, PD-1, B7-H1, PD-L2, ICOS-L, B7-1 and B7-2 on the surface and CTLA-4 intracellularly (Figure 6.12). Of importance, CD28, PD-1 and CTLA-4 were expressed only in tumor infiltrating but not in blood NK cells of either naïve or tumor bearing mice. The expression of B7 molecules by TINs depended on the tumor type (Figure 6.13). TINs found in lymphomas were preferentially $CD28^{high}PD\text{-}1^{high}$, while NK cells in melanoma and carcinoma were $CD28^{low/-}PD\text{-}1^{-}B7\text{-}H1^{high}$. Importantly, in all tested tumor models NK cells within the tumor expressed CTLA-4. Differential expression of B7 molecules was observed only in the solid tumors that exceed the size of $1cm^2$ (data not shown). NK cells from lung and peritoneum of mice bearing lung metastases or tumor cells growing in the peritoneum did not express PD-1 and CTLA-4. In solid tumors of the given size, features that include

biochemical conditions, such as hypoxia and low pH, presence of suppressive cells and cytokine combinations secreted by both tumor and other infiltrating cells could contribute to the induction of specific TIN phenotype. In line with this hypothesis is the fact that the induction of PD-1 was observed only in the tumor and was unsuccessful *in vitro*, even when the combinations of multiple stimuli were used (Table 6.3). In addition, systemic application of IL-18 induced CTLA-4 expression by NK cells only in the tumor, but not in blood or spleen (Figure 6.35A). This indicates that additional factor/s in the tumor synergize with IL-18 to mediate CTLA-4 induction.

It appears that distinct pathways regulate PD-1 induction in different cell types. In T cells, PD-1 is induced upon the TCR triggering or cytokine stimulation through the common γ_c chain [332]. Simultaneous triggering of BCR with αCD40 induces PD-1 expression on B cells [221]. Macrophages display elevated PD-1 expression in mouse models and patients with sepsis [333], while PD-1 can be induced on DCs by Listeria infection or TLR2, TLR3, TLR4 and NOD engagement *in vitro* [334]. Recently, Brauner and colleagues showed that NK cells infiltrating the diabetic pancreas in NOD mice expressed PD-1 [335]. Furthermore, pancreatic NK cells were hyporesponsive compared to spleen NK cells and could be rescued by overnight culture in IL-15 and IL-18. PD-1$^+$ T cells from tumor or blood of chronically infected patients and mice, macrophages from humans and mice with sepsis and pancreatic NK cells from diabetic mice exerted hyporesponsiveness that correlated with PD-1 expression. We showed that NK cells in the tumor expressed PD-1 (Figure 6.12). The expression was detected on all NK cells and increased with the tumor size (data not shown). To investigate whether PD-1 might be a marker for nonfunctional tumor infiltrating NK cells further functional analyses have to be performed. Due to the low cell numbers that can be obtained from the tumor tissue, we could not conduct experiments that would test this hypothesis. We, however, observed that the culture in IL-2 or IL-15 after isolation from the tumor tissue results in downregulation of PD-1 (data not shown), indicating reversibility of the phenotype acquired in the tumor.

Expression of the inhibitory B7 family receptor CTLA-4 was highly increased at the transcript level in TINs compared to blood NK cells in mice with tumors (Figure 6.11). The expression at the mRNA level correlated with the protein expression (Figure 6.12). Intracellular staining revealed that CTLA-4 was expressed only in tumor but not in blood and spleen of tumor bearing animals. In contrast to PD-1, CTLA-4 was inducible *in vitro* by NK cell expansion in IL-2 (Figure 6.17). Intracellular content of the protein could be further increased by stimulation with IL-12 and IL-18 (Figure 6.19). While short-term incubation with fluorescently labeled Ab failed to detect any CTLA-4 on the cell surface, prolonged incubation revealed that a certain amount of CTLA-4 reaches the NK cell surface (Figure 6.19). The surface expression is positively regulated by the stimulation with IL-12, IL-18 and αNK1.1 or αNKp46 (Figure 6.22). Of importance, all factors that we found to regulate CTLA-4 expression *in vitro*, can influence its expression in the tumor tissue as well. Still unidentified NKp46 ligand is expressed by RMA-S cells [291]. IL-18 and IL-12 are secreted by macrophages and dendritic cells that also infiltrate the tumor [336]. In fact, CD11b$^+$ cells, among them macrophages, comprise the main pool of hematopietic cells infiltrating the tumor. NK cell ability to respond to various signals from the microenvironment is beneficial for maximizing

the responses against infected or transformed cells. At the same time, the induction of negative regulators, such as CTLA-4, may serve as a mechanism to keep NK cell responses under control.

7.3. Regulation of NK cell effector functions by B7 family members

We showed that TINs expressed the B7 family receptors PD-1, CD28, CTLA-4 and B7-H1 and aimed at the analysis of their function in the context of NK cell responses. *In vitro* expanded NK cells in the presence of rhIL-2 expressed CD28, CTLA-4 and B7-H1 (Figure 6.17). Since all three molecules recognize the same ligand, B7-1, we determined their role in B7-1 induced responses of NK cells. Stimulation of *in vitro* expanded NK cells with the B7-1 IgG fusion protein induced NK cell proliferation and IFNγ production (Figure 6.24). By using NK cells derived from gene deficient mice, we showed that IFNγ production was triggered through CD28 and it was negatively regulated by CTLA-4 and B7-H1 (Figure 6.30-31). In T cells, CD28 engagement leads to the phosphorylation of the tyrosine residues in its cytoplasmic tail, which drives the recruitment of several signaling molecules [171]. CD28 dependent production of IL-2 and IFNγ in T cells is mediated via PI3K and Grb2 pathways, which are shown to associate with CD28. A similar activation pathway in NK cells is used by the NKG2D receptor that is coupled to the DAP10 adaptor molecule [303]. DAP10 and CD28 share the YMNM motif in their cytoplasmic domain known to recruit PI3K and Grb2. Therefore, CD28 on NK cells might use a similar signaling pathway described to operate in T cells leading to IFNγ production. Indeed, PI3K was already described as a key player in NK cell effector function including IFNγ production. Stimulation of activating NK receptors in the absence of the p110δ, PI3K catalytic subunit, results in a decreased IFNγ release [337]. Similarly, ICOS induced IFNγ is reduced in the presence of a PI3K inhibitor [72].

As already described in the section 3.3.2.1, several mechanisms are used by CTLA-4 to inhibit T cell responses (Figure 3.13) [196]. Among cell intrinsic mechanisms, delivering of negative signal and competition for the ligands with CD28 have been reported to contribute to CTLA-4 mediated inhibition. Both mechanisms might underlay CTLA-4 mediated inhibition of NK cells. Using CD28 deficient NK cells, we observed that B7-1 stimulation of NK cells that lead to IFNγ production, entirely depended on positive signals delivered by CD28 (Figure 6.30). The response is negatively regulated by CTLA-4 and B7-H1 indicating the direct inhibition of the CD28 mediated response (Figure 6.31). Both CTLA-4 and B7-H1 have a higher affinity for B7-1 compared to CD28 [304] and could efficiently sequester the ligand. In addition, CTLA-4 can recruit phosphatases that interfere with proximal CD28 signaling. Binding of PI3K and CD3ζ to the CTLA-4 cytoplasmic tail was also reported [195]. While these interactions might promote some of the positive effects mediated by CTLA-4, as increased survival, they can also sequester signaling molecules from other activating receptors. PI3K is recruited by CD28 and NKG2D, and CD3ζ by NK1.1 and NKp46, which are all potent inducers of IFNγ. Therefore, it would be of interest to investigate whether CTLA-4 co-engagement with the given receptors might influence their responses. We also showed that two receptors, namely CD28 and NK1.1, can synergize in the induction of IFNγ when the level of CD28

stimulation is low (Figure 6.30). In these situations, cooperation of two receptors might be crucial to induce response and, at the same time, more sensitive to inhibition. Therefore, the levels of CTLA-4 mediated suppression that we measured *in vitro* might be even more pronounced in *in vivo* conditions when the low levels of stimuli are provided by the microenvironment.

Among the cell extrinsic mechanisms underlying CTLA-4 inhibition of T cells, TGF-β mediated suppression was described. In activated T cells, simultaneous cross-linking of αCD3 and CTLA-4 leads to the production of TGF-β [338]. Importantly, we found increased transcript levels of TGF-β in TINs compared to blood NK cells in tumor bearing animals. Future investigation will be conducted to investigate whether CTLA-4 mediated this effect. TGF-β was shown to impede NK cell dependent IFNγ production [287]. Thus, it is possible that the induction of TGFβ represents additional mechanisms used by CTLA-4 to suppress NK cell IFNγ production.

B7-H1 has recently been reported to bind to B7-1 and inhibit T cell proliferation, cytokine production and expression of activation markers induced by CD3 cross-linking [304]. It is unknown how B7-H1 delivers the inhibitory signal. The intracellular domain of B7-H1 is short, but highly conserved in all so far investigated species [225], which indicates that it might be preserved due to the function it mediates. The evidence for B7-H1 signaling came from the studies of Kuipres *et al*, who showed that the incubation of DCs with soluble PD-1 IgG fusion protein led to the IL-10 and IDO induction [339]. Further investigation is necessary to show how B7-H1 inhibits CD28 driven IFNγ production and whether IL-10 and IDO might be induced upon its engagement. In the later case, a negative regulation mediated by B7-H1 can be indirect and can also affect other cells within the tumor tissue.

While all three molecules, CD28, CTLA-4 and B7-H1, regulated B7-1 driven IFNγ production, none of them influenced the cytotoxic responses against B7-1 expressing target cells (Figure 6.32). Similar to our studies, proliferation and IFNγ production induced by B7-1$^+$ tumor cell lines has been contributed to CD28 in studies of Kelly *et al.* [61]. However, the enhanced lysis of B7-1 expressing targets observed by Chambers and colleagues was not affected by CD28 and CTLA-4 deficiency of NK cell effectors [58]. The same group showed that the efficient elimination of B7-2 positive tumor cells both *in vivo* and *in vitro* did not depend on CD28 [56]. Our data revealed that the third receptor that recognizes B7-1, B7-H1, also did not mediate enhanced cytolysis. Of note, it is possible that intrinsic differences of B7-1 transduced cells might lead to their higher sensitivity to NK cell lysis compared to the control cells. Therefore, it is of importance to show that the expression of B7-1 is responsible for the better recognition of transduced cells, by using the B7-1 blockade or tet-inducible system. In the case of positive results, the existence of the fourth receptor for B7-1 can be postulated. However, it is also possible that the engagement of B7-1 by any of the so far known receptors induces reverse signaling already shown to take place in DCs [183], which might lead to cell death by unknown mechanisms. The use of NK cells deficient in all three known B7-1 receptors would be required to test presented hypotheses.

7.4. B7 family members on NK cells - possible implications for the anti-tumor responses

B7 family members were already exploited as the potential biomarkers for the activation status of anti-tumor effector cells and as the potential targets for anti-tumor immune therapy [269]. PD-1 is considered as a marker for functionally exhausted unresponsive effector T cells found in chronic viral infection of mice and human as well as in melanoma lesions of cancer patients [211, 241]. Disruption of PD-1:B7-H1 interaction with mAbs was shown to restore T cell responsiveness and mediate tumor rejection in the mouse tumor models [234, 243-244, 340]. Both αPD-1 and αB7-H1 mAbs target multiple cells (T effector cell, Treg, macrophages, tumor cells, etc) and multiple interactions (PD-1:B7-H1, PD-1:PD-L2, B7-H1:B7-1). Therefore, they can have diverse effects. As an example, in a sepsis model, when compared to WT, PD-1 deficient macrophages exert higher phagocytic capacity and produce higher levels of proinflammatory cytokines including IFNγ and IL-12 [333]. On the contrary, *Listeria* infection induces PD-1 expression on DCs, which reduces their ability to produce IL-12 and TNFα [334]. We showed that B7-H1 negatively regulated IFNγ production in NK cells. Thus, application of αPD-1 or αB7-H1 mAb could affect NK cell responses as well. Therefore, NK cells can contribute to the better outcome of αB7-H1 mAb treatment by increased IFNγ production.

Several studies revealed that αCTLA-4 treatment of tumor bearing mice had a potential to generate or enhance anti-tumor T cell responses leading to tumor regression [264, 341-342]. However, the efficacy of αCTLA-4 monotherapy strongly depended on immunogenicity of the tumor and the stage of the tumor growth when the treatment was applied. In the majority of the studies, CTLA-4 blockade was beneficial when combined with other regimens, such as peptide immunization [343], tumor cell vaccine [344] or peptide vaccine combined with DC stimulation via αCD40 [345]. So far, the expression of CTLA-4 by NK cells was not reported and the application of αCTLA-4 mAb as a component of an anti-tumor therapy was assumed to affect only the T cell compartment. The study of Peggs and colleagues, who combined the receptor blockade with GM-CSF producing tumor vaccine, showed that both the T effector and the Treg compartment responded to αCTLA-4 treatment to achieve tumor growth control [346]. Of importance, the efficacy of the treatment completely depended on NK cells, since their depletion abrogated the positive effects of the vaccine [266]. The authors speculated that NK cells were required to kill tumor cells at early time points of tumor progression providing the antigens that could be uptaken and processed by DCs that subsequently prime T cell responses. Another evidence of possible involvement of NK cells in the αCTLA-4 mediated tumor regression came from the studies conducted with B7-1 transduced prostate cancer cell line [342]. Although expressing no or low MHC I on the surface, those tumor cells were efficiently rejected upon CTLA-4 blockade *in vivo*. We demonstrated that NK cells expressed CTLA-4 in the tumor tissue and could be the additional direct targets of αCTLA-4 treatment. By use of CTLA-4 deficient NK cells, we showed that in the absence of CTLA-4, NK cells produced more IFNγ when stimulated with the B7-1 IgG fusion protein (Figure 6.31) or B7-1 expressing tumor cells (data not shown). In addition, B7-1 induced NK cell proliferation (Figure 6.24), which was previously shown to depend on CD28 [61]. Therefore, the functional blockade of CTLA-4 on NK cells is expected to result in their enhanced proliferation and IFNγ production.

We observed that repeatedly given αCTLA-4 mAb did not influence tumor progression in RAG2 deficient mice inoculated subcutaneously with different tumor cell lines. These data are in agreement with a previous study of van Elsas *et al.* showing that αCTLA-4 monotherapy did not affect the progression of poor immunogenic B16 melanoma in WT animals [266]. We also observed no beneficial effects when the combination therapy of IL-18 and αCTLA-4 was administrated to the RAG2 deficient RMA-S tumor bearing mice, although IL-18 was shown to be a potent anti-tumor agent mediating its effect via NK cells [347]. The reason for non-effectiveness of the treatment might be explained by the observations of the previous reports showing that IL-18 potentiated NK cell anti-tumor responses against FasL but not perforin sensitive tumors [92, 102]. Therefore, we cannot exclude the possibility that IL-18/αCTLA-4 therapy might be successful in different tumor models. We showed that IL-2 treatment *in vitro* positively regulated CTLA-4 expression (Figure 6.19). Therefore, simultaneous CTLA-4 blockade with IL-2 application might represent the desired strategy when NK cell responses are to be harnessed against tumor. When we combined αCTLA-4 treatment with IL-2 in the B7-1 expressing melanoma model, we observed a modest but significant delay in the tumor growth, which also resulted in the prolonged survival of treated mice. IL-2 can have multiple effects on NK cell anti-tumor responses including better infiltration of the tumor and enhanced proliferation and cytotoxicity. Since we administrated IL-2 during the first five days of tumor progression, we cannot exclude that repeated treatment or higher dosage would induce more potent responses to tumor. In addition, tumors other than melanoma might be more sensitive to the treatment.

We showed that the main effect of CTLA-4 in NK cells was the regulation of IFNγ production, while cytotoxic responses against B7-1 transduced tumor cells were not affected by the receptor. These observations implicate that CTLA-4 blockade *in vivo* might not affect the direct lysis of B7-1$^+$ target cells. On the other hand, multiple cells expressing B7-1/2 in the tumor might trigger the IFNγ production by NK cells. Since downregulation of B7-1/2 is one of the mechanisms used by tumors to evade T cell responses, they are rarely expressed by tumor cells. However, myeloid cells that represent the main population infiltrating the tumor, usually express the ligands. Importantly, IFNγ production is the most common result of the direct, cell contact dependent, or the indirect, cytokine mediated, activation of NK cells by myeloid cells, namely DCs and macrophages. Owing to the very important role of IFNγ to bridge innate and adaptive immune responses that finally results in the efficient activation of T cells, suppression of its production during an ongoing anti-tumor response is not desirable. NK cells are seen as the important cytotoxic effectors that lyse tumor cells at early time points and provide antigens for DCs to initiate T cell responses [348]. However, recognition of target cells and the interaction with DCs also induce IFNγ release that is necessary for efficient T cell priming. Therefore, increased IFNγ production provided by NK cells during an ongoing anti-tumor response is essential for an efficient T cell mediated tumor control [349]. Our *in vitro* data indicate that the induced expression of CTLA-4 on NK cells in all solid tumor tested and elevated B7-H1 expression detected in melanoma and carcinoma, might suppress B7-1 induced NK cell IFNγ production. Expression of CD28 in TINs further supports the hypothesis that IFNγ might be induced via the interaction with B7-1 expressing cells. In RAG2 deficient mice that we used for testing the efficiency of CTLA-4 blockade in NK cell mediated anti-tumor responses, only a modest improvement of

tumor growth control was seen with IL-2/αCTLA-4 combination. The beneficial effects might be a consequence of higher numbers of NK cells in the tumor and/or their higher cytotoxic capacity due to the IL-2 mediated activation. Indeed, a significant reduction in tumor size was achieved by IL-2 single treatment at the very late stages of tumor progression. Additional CTLA-4 blockade might further increase IFNγ release, which might act on several different aspects. IFNγ was shown to increase tumoricidal activity of various immune cells including innate effectors as macrophages and neutrophils [94]. It can directly act on the tumor cells by promoting anti-proliferative and anti-apoptotic effects or on tumor vasculature by preventing angiogenesis [350-351]. Some of those actions might be involved in tumor growth delay by the combined IL-2/αCTLA-4 treatment. However, the crucial effectors whose anti-tumor action is to be directly or indirectly potentiated by NK cell derived IFNγ, which are T cells, were missing in our system. Therefore, for the final prove that CTLA-4 blockade on NK cells plays the important role in the enhancement of anti-tumor responses, mice with targeted deficiency of CTLA-4 in NK cells should be created and analyzed.

So far, we tested the blockade of the inhibitory receptor CTLA-4 in the combination with agents that directly activate NK cells, namely IL-2 and IL-18. In addition, NK cells can be also activated by myeloid cells, macrophages and DCs, in a cell contact dependent manner or via various cytokines [66]. Therefore, agents that induce DC or macrophage activation can be also considered as a choice for priming NK cell responses. Indeed, CpG oligonucleotides, which bind TLR4, are proven to be potent activators of myeloid cells and initiators of events that prime potent anti-tumor responses [352]. Our preliminary results indicated that CpG could induce a significant tumor growth delay in T and B cell deficient mice. Further studies will be conducted to investigate whether the CpG treatment combined with CTLA-4 blockade in therapeutic settings could mediate the eradication of established tumors. Our future studies will be aimed at the optimization of therapeutic protocols that could lead to maximal NK cell activation against tumor and include the facilitation of NK cell responses in combination with prevention of their inhibition within the tumor tissue.

7.5. B7 family expression by NK cells - crucial issues for NK cell based anti-cancer therapy

Despite the knowledge about NK cell anti-tumor responses, the clinical efficacy of trials targeting NK cells, has been modest. The most effective use of NK cells is in situations when the tumor burden is relatively low, such as in patients with minimal residual disease, post surgery or following relapse. Studies in mice indicate that NK cells have a very limited capacity to affect established malignancies. The high percentage of human tumors show a deficient expression of MHC class I molecules and cannot be efficiently targeted by CTLs [273]. However, those tumors might be selected for NK cell based therapy. NK cell infiltration of solid tumors in human patients is variable. Although higher numbers of NK cells found within the tumor tissue correlated with a better prognosis, their level of activation is often not sufficient to efficiently control

the tumor growth [275-276]. Our studies shed some light on the possible mechanisms that might impede the effective NK cell response within established solid tumors. Global expression profile of tumor infiltrating NK cells provided the data for a detailed study of NK cell functional state within the tumor. We confirmed the downregulation of activating receptors and the upregulation of inhibitory receptors of the B7 family on NK cells in the tumor. Our data suggest that NK cell IFNγ production might be the main target of inhibition within the tumor tissue, with the potential to prevent effective T cell activation and T cell mediated tumor regression. In agreement with the emerging evidence from other studies, strategies based on the combination therapies are expected to be the most effective anti-tumor treatments. The knowledge about the phenotype and function of tumor infiltrating NK cells will be essential for their efficient implication in the novel treatment strategies.

CTLA-4 blockade in cancer patients was shown to be beneficial for late stage cases of melanoma and renal cell carcinoma [268, 353]. When CTLA-4 blockade was combined with peptide vaccine, no additional clinical benefits were observed. A combination of CTLA-4 blocking mAb with GM-CSF producing tumor vaccine induced large tumor necrosis and long-term freedom from progression after the resection of necrotic tumor masses in melanoma patients. However, the patients who showed clinical benefits usually developed immune-related adverse events, including rush, vitiligo, colitis, diarrhea, hypophysitis, hepatitis and nephritis due to the breakage of self-tolerance and activation of self-reactive T cells. Therefore, it would be important to investigate whether CTLA-4 blockade can be combined with NK cell activating agents in the dose and application regimen that would produce clinical benefits without adverse effects.

8. References

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9. Abbreviations

β_2m	β_2 microglobulin
µg	Microgram
µl	Microliter
µM	Micromolar
7-AAD	7-Aminoactinomycin D
Å	Angström
ACK	Ammonium chloride potassium phosphate
ADCC	Antibody dependent cell mediated cytotoxicity
Ag	Antigen
ALL	Acute lymphoid leukemia
AML	Acute myeloid leukemia
ANOVA	Analysis of variance
AP-1 and AP-2	Activator protein-1 and 2
APC	Allophycocyanin
APC(s)	Antigen presenting cell(s)
BC	Breast cancer
BCG	Bacillus Calmette-Guérin
BCR	B cell receptor
BM	Bone marrow
BMT	Bone marrow transplantation
BP	Binding protein
BrdU	Bromodeoxyuridine
BTNL2	Butyrophilin-like protein 2
CAECAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
CAM	Cell adhesion molecule(s)
CD	Cluster of differentiation
cDNA	complementary DNA
cFc	control fusion protein
CFSE	Carboxyfluorescein succinimidyl ester
CML	Chronic myeloid leukemia
CMV	cytomegalovirus
CNS	Central nervous system
CR	complete response
cRNA	complementary RNA
CTL(s)	Cytotoxic T lymphocyte(s)

CTLA-4	Cytotoxic T lymphocyte associated antigen-4
Cy	Cyanine
d	Day(s)
DAP10	DNAX-associated protein of 10 kDa
DAP12	DNAX-associated protein of 12 kDa
DC(s)	Dendritic cell(s)
ddH2O	Double distilled water
DEN	Diethylnitrosamine
DEPC	Diethylpyrocarbonate
DFS	Disease-free survival
DMBA	7,12-Dimethylbenz(a)anthracene
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
DMOG	Dimethyloxaloylglycine
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
dNTP	Deoxyribonucleotide triphosphate
E:T	effector to target ratio
EAE	Experimental autoimmune encephalomyelitis
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-signal-regulated kinase
FACS™	Fluorescence-activated cell sorting
Fas	Fibroblast associated
FasL	Fas ligand
FcR	Fc receptor
FCS	Fetal calf serum
FITC	Fluorescein-isothiocyanate
Flt3	FMS-like tyrosine kinase 3
FVAX	Flt3L expressing cancer vaccine
g	Gram(s)
GATA-3	GATA-binding protein-3
GM-CSF	Granulocyte-macrophage colony stimulating factor
Grb2	Growth factor receptor-bound protein 2
GVAX	GM-CSF producing cancer vaccine
Gzm	Granzyme
h	Hour(s)

HD	Hodgkin's lymphoma
HER-2	Human epidermal growth factor receptor-2
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HVEM	Herpesvirus entry mediator
i.p.	Intraperitoneal
i.v.	Intraveneous
ICAM	Intercellular adhesion molecule
ICOS	Inducible T cell costimulator
ICOS-L	Inducible T cell costimulator ligand
iDC	Immature dendritic cells
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IgC	Immunoglobulin constant
IGIF	IFNγ inducible factor
IGV	Immunoglobulin variable
IKDC(s)	Interferon producing killer dendritic cell(s)
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
Iono	Ionomycin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
JNK	c-Jun N-terminal kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
KIR(s)	Killer-cell immunoglobulin-like receptor(s)
KLRG1	Killer cell lectin-like receptor subfamily G member 1
KO	Knock out
L	Ligand
L	Liter
LAK(s)	Lymphokine activated killer cell(s)
Lin	Lineage
LIR	Leukocyte immunoglobulin-like
LN	Lymph node
LPS	Lipopolysaccharide
M	Molar concentration
mAb(s)	monoclonal antibody(ies)
MACS®	Magnetic cell sorting

MCA	methylcholanthrene
MCAM	Melanoma cell adhesion molecule
MCMV	Murine cytomegalovirus
MCP-1	Monocyte chemoattractant protein-1
mDC(s)	Mature dendritic cell(s)
MDSCs	Myeloid derived suppressor cell(s)
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIC	MHC class I chain related
min	Minute(s)
mg	Miligram
ml	Mililitre
mM	Milimolar
mRNA	Messenger RNA
MS	Multiple sclerosis
MULT1	Murine UL-16-binding protein-like transcript 1
NCAM	Neural cell adhesion molecule
NCR(s)	Natural cytotoxicity receptor(s)
NF	Nuclear factor
NFAT	Nuclear factor of activated T cells
NHL	Non-Hodgkin's lymphoma
NK	Natural killer
NKG2	NK group 2 member
NKP	NK cell precursor
NOD	Non-obese diabetic
NOS	Nitric oxide syntase
OR	Overall response
OS	Overall survival
OVA	Ovalbumin
ORA	Over-representation approach
PAMP	Pathogen-associated molecular patterns
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCM	Primary cell medium
PD-1	Programmed cell death-1
pDC	Plasmacytoid DC
PDGF	Platelet derived growth factor
PD-L	Prrogrammed cell death 1 ligand

PD-L1 and PD-L2	Programmed cell death-1 ligand-1 and 2
PE	Phycoerythrin
PerCP-Cy5.5	Peridinin-chlorophyll-protein-complex-cyanine 5.5
pH	Potential hydrogeni
PI3K	Phosphoinositide 3-kinase
PLC	Phospholipase
PMA	Phorbol 12-myristate 13-acetate
Poly I:C	Polyinosinic-polycytidylic acid
PP2A	Protein phosphatase 2
PR	Partial response
PRF	Perforin
PRR(s)	Pattern Recognition Receptor(s)
R	Receptor
RA	Rheumatoid arthritis
Rae-1	Retinoic acid early inducible-1
RAG	Recombination activation gene
RCC	Renal cell carcinoma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rounds per minute
RPMI	Roswell park memorial institute medium
RT	Room temperature
s.c.	Subcutaneous
SD	Stable disease
SD	Standard deviation
SH-2	Src homology-2
SHIP	SH2-containing inositol polyphosphate 5-phosphatase
SHP-1 and SHP-2	SH2-containing protein-tyrosine phosphatase-1 and 2
SLE	Systemic lupus erythematosus
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TF	Transcription Factor
TGFβ	Transforming growth factor β
TGN	Trans-Golgi network
Th	T helper
TIN(s)	Tumor infiltrating NK cell(s)
TIT(s)	Tumor infiltrating T cell(s)
TLR	Toll-like receptor
TNF	Tumor necrosis factor

TPA	12-O-tetradecanoylphorbol-13-acetate
TRAIL	TNF-related apoptosis inducing ligand
TRAMP	Transgenic adenocarcinoma mouse prostate
Treg	Regulatory T cell
TREM	Triggering receptor expressed on myeloid cells
TREML2	Triggering receptor expressed on myeloid cells-like 2
TRIM	T-cell receptor-interacting molecule
U	Unit
ULBP	UL16-binding protein
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
W/	With
W/O	Without
WT	Wild type

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