Exosomal derived HLA-B-Associated Transcript-3 is a ligand for NKp30 and regulates Natural Killer Function in Innate and Adaptive Immune Responses

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vorgelegt von

Venkateswara Rao Simhadri

aus Andhra Pradesh, Indien

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1. Berichterstatter:

Prof. Dr. Jens Bruening

2. Berichterstatter:

Prof. Dr. R. Jürgen Dohmen

3. Berichterstatter:

Prof. Dr. E. Schierenberg

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1 Zusammenfassung

Natürliche Killerzellen sind wichtige Effektorzellen des angeborenen Immunsystems. Lange Zeit wurde angenommen, dass NK-Zellen virusinfizierte oder transformierte Zielzellen direkt und ohne vorherige aktivierung abtöten können. Aktuelle Befunde zeigen hingegen, dass die Aktivierung von NK Zellen ein komplexer Vorgang ist, der durch das Zusammenspiel von inhibitorischen und aktivierenden Signalen über korrespondierende NK-Rezeptoren gesteuert wird. Ihrerseits beeinflussen NK-Zellen die erworbene Immunantwort, indem sie mit dendritischen Zellen interagieren und diese stimulieren (reciprocal priming).

Unter den regulatorischen Oberflächen-Rezeptoren von NK-Zellen spielt der aktivierende Rezeptor NCR3 (NKp30) eine besondere Rolle, da NKp30 sowohl für die Lyse von Zielzellen als auch für die Interaktion mit unreifen dendritischen Zellen (iDCs) verantwortlich ist. Die NKp30-vermittelte Interaktion mit iDCs kann entweder zum Abtöten der iDCs oder zur gegenseitigen Aktivierung führen., Letztere bewirkt die Reifung der iDCs zu antigen-präsentierenden „mature DCs" (mDCs). Bis dato waren jedoch zelluläre Liganden des NKp30 Rezeptors unbekannt. Im Rahmen dieser Arbeit wurde gezeigt, dass HLA-B-associated transcript 3 (BAT3) ein funktioneller zellulärer Ligand für NKp30 ist, der von Tumorzellen und dendritischen Zellen exprimiert wird. BAT3 ist kein Oberflächenmolekül, sondern ein nukleärer Faktor, der jedoch nach Induktion durch Stresssignale wie Hitzeschock sekretiert wird. Die Sekretion erfolgt in Exosomen. BAT3 ist auf der Oberfläche der Exosomen exprimiert und die Bindung an NKp30 ist nachweisbar. Die Exosomen aktivieren NK-Zellen via BAT3/NKp30 und führen so zur Sekretion von IFN-γ, TNF-α und einer erhöhten Zytotoxizität. Diese neue Form der Regulation der Interaktion von Ligand und Rezeptor führt zu einem erweiterten Konzept der Zielzellerkennung durch NK-Zellen. In Anlehnung an die ‚missing-self' und ‚induced-self' Modelle, kann dieser Mechanismus als ‚danger induced recognition' bezeichnet werden. Dieses Modell beschreibt, dass NK-Zellen ihre Zielzellen (Tumorzellen, iDCs) ohne direkten Zell-Zellkontakt, über einen extrazellulären Faktor, der in Antwort auf Stresssignale abgegeben wird, erkennen.

2 Abstract

Natural Killer cells are key effector cells of the innate immune system. They were originally described as the first line of defense against infections and tumor cells that secrete cytokines and kill target cells without prior antigen stimulation.

Emerging evidence reveals that NK cell activation is a more complex process and includes priming of NK cells that in turn shape the adaptive immune response mainly by interacting with dendritic cells.

The activity of NK-cells is mainly mediated through their wide variety of receptors, activating and inhibitory in function. Among the versatile receptors present on NK cells, the activating receptor NCR3, NKp30 is a major receptor involved in both direct killing of target cells and mutual NK and dendritic cell activation. However, the cellular ligands on tumor-transformed cells and DCs have remained elusive. This study identifies HLA-B-associated transcript 3 (BAT3), as a tumor-associated cellular ligand for NKp30 and moreover it is shown that BAT3 is expressed in immature dendritic cells and released as exosomes into the extracellular environment. Further it demonstrates that BAT3 is expressed on the surface of exosomes either derived from tumor cells or iDCs activate NK cells resulting in a strong release of major cytokines (TNFα and IFNγ) and enhanced cytotoxicity. Moreover, BAT3 regulates the NK cell-mediated cytotoxicity against iDCs.

Thus, a novel concept is proposed for target cell recognition by NK cells beyond "missing-self" and "induced-self", mediated through extracellular factors. These factors may be derived from the target cells as well from the accessory cells. Moreover, these data also suggest that the reciprocal activation of NK cells and iDCs is partly due to BAT3 presenting exosomes independent from direct cell contacts.

3 INTRODUCTION

3.1 Natural Killer Cells and their function

Natural Killer (NK) cells arise from the same bone marrow progenitor cells as T cells and share two of their most important functions: (i) cytotoxicity and (ii) cytokine secretion (Biron, Nguyen et al. 1999). Unlike T cells, NK cells do not have variable receptors for antigens and are originally described to be directly cytotoxic without any prior stimulation of the antigen. They do not mature in the thymus but instead they migrate directly into the blood stream and provide an innate immune response as **natural cytotoxicity** (Smyth, Cretney et al. 2005). NK cells were originally discovered for their ability to target and kill primarily the tumor transformed and virus infected cells (Moretta and Moretta 2004; Lanier 2005). These cells have a unique function of discriminating between the normal and stressed cells that is regulated by their wide range of receptors.

NK cell effector functions such as cytotoxicity and cytokine secretion upon activation are of pathophysiological importance. These functions are exerted by various mechanisms: (i) release of cytoplasmic granules containing number of proteins like perforin and granzymes to lyse the target cells (Fig. 1), (ii) secretion of TNF family members to kill the targets and (iii) production of different cytokines like IFN-γ, that restrict tumor angiogenesis and stimulate the adaptive immunity (Smyth, Hayakawa et al. 2002; Smyth, Cretney et al. 2005). Apart from the first line of defense during inflammation, NK cells get activated by cytokines released from the accessory cells (Antigen Presenting Cells, APCs) and recruited to the secondary lymphoid organs like lymph nodes thus playing a major role in adaptive immunity to kill antibody- coated cells by antibody-dependent cellular cytotoxicity (ADCC) (Newman and Riley 2007). These mechanisms are maintained by a balance between the inhibitory and activatory signals provided by a variety of receptors on their surface (Cerwenka and Lanier 2001) which include inhibitory, co-stimulatory and activating receptors.
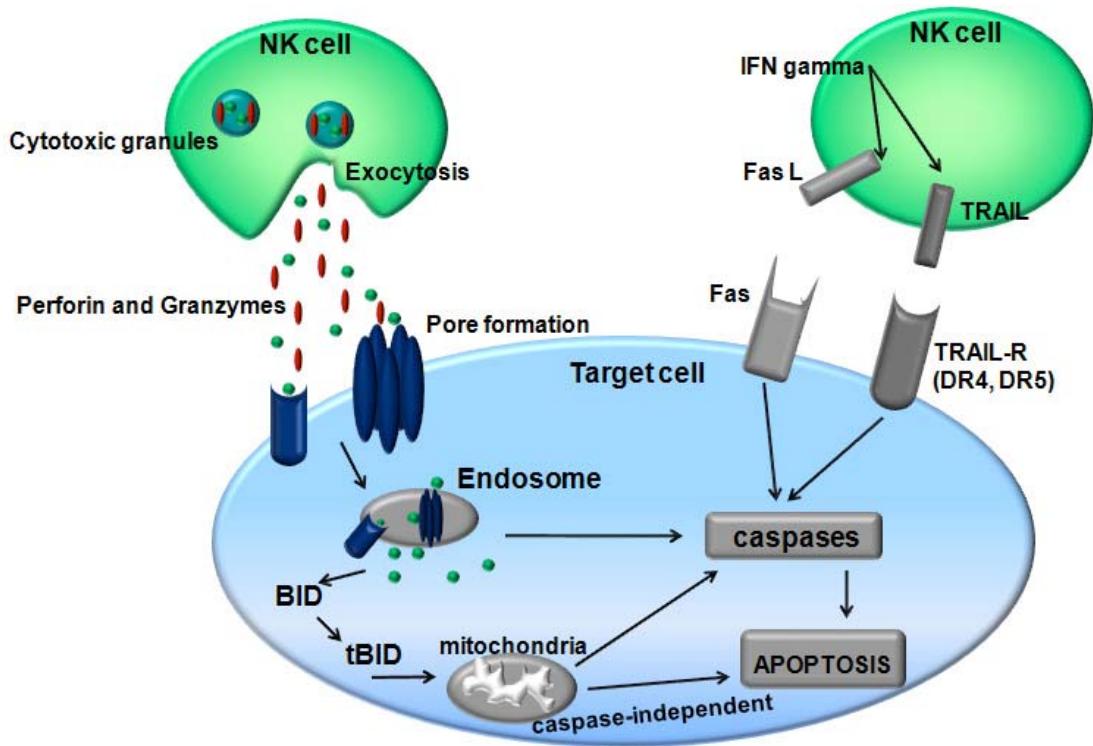
Figure. 1. NK-cell effector function

NK cells lyse the target cells mediated by different mechanisms (a) by the secretion of cytotoxic granules that contain perforin, granzymes and granulysin into the intercellularspace. These exocytic granules are endocytosed into the target cell by pore formation and these granule containing endosomes activate the caspases and mitochondrial proteins (caspase independent) to undergo apoptosis, and (b) the NK cell express the cognate ligands (Fas ligand and/or TRAIL) on their surface for the death receptors Fas (CD95) and TRAIL-R respectively present on the target cell surface. This is dependent on IFNγ. The signaling through the death receptors activate the caspases leading to appropriate apoptosis.

3.2 Regulation of Natural Killer Cytotoxicity

Regulation of NK cells and the recognition strategies are controlled by the dynamic balance between their different inhibitory and activating receptors and more importantly on the availability of their ligands on the target cells. The basic principle behind NK cell regulation is the protection of self cells (normal) and to destroy the non-self (transformed) cells (Fig. 2). Regulatory mechanisms include: (i) Recognition of activating ligands that are expressed on infected and transformed cells when the inhibitory ligands (self proteins) are down-regulated, termed as **"missing-self"** and (Raulet 2004) (ii) Recognition of self proteins (ligands) that are up-regulated in transformed or infected cells, termed as **"induced self"**.

3.2.1 Missing-self recognition

Initially, the activator mechanism of NK cell was discovered as missing-self recognition (Ljunggren and Karre 1985; Ljunggren and Karre 1990). Normal cells are protected by the presence of self antigens like MHC-class I molecules and their interaction with inhibitory receptors (KIRs) present on NK cells. The transformed cells lose the self antigens and thus the stimulatory ligands present on these cells are available for the activating receptors (NKG2D and NCRs) on NK cells thus, mediating the cytotoxicity of the target cells (Ljunggren and Karre 1985). Interestingly, T-cells are active when the MHC- Class I molecules are up-regulated whereas, the down-regulation of class I molecules activate NK-cell function. This missing of self antigens and recognition of activating ligands correspond to missing-self recognition (Fig. 3).

3.2.2 Induced-self recognition

Down-regulation of the self antigens (missing self) is often not sufficient for target cell recognition; instead the induction of ligands for triggering receptors (NKG2D and NCRs) results in activation. The ligands for NKG2D are up-regulated on the tumor transformed cells (Groh, Rhinehart et al. 1999) allowing recognition. The tumor ligands for the natural cytotoxicity receptors were not known but in general it is likely that the ligands for NCRs are up-regulated on transformed cells compared to the normal cells (Moretta, Bottino et al. 2001). Activating receptors that directly regulate NK-cell mediated cytotoxicity of tumor cells are termed as natural

cytotoxicity receptors (NCRs); NKp46, NKp30 (expressed on resting NK cells) and NKp44 (expressed only upon activation). All three receptors are associated with ITAM-containing signaling adapter molecules in the membrane (Moretta, Bottino et al. 2001).

Finally, in a scenario where the NK cells respond to pathogens is entirely different from the above two mechanisms. This mechanism requires the presence of accessory cells such as monocytes, macrophages and dendritic cells to secrete different soluble factors that activate NK cells. This is termed as accessory-cell-dependent activation and occurs in response to pathogens.

In this project the data provides an insight to a novel mechanism of regulation mediated by a soluble factor (a ligand for one of the activating receptors) released by target cells such as dendritic cells and tumor cells.
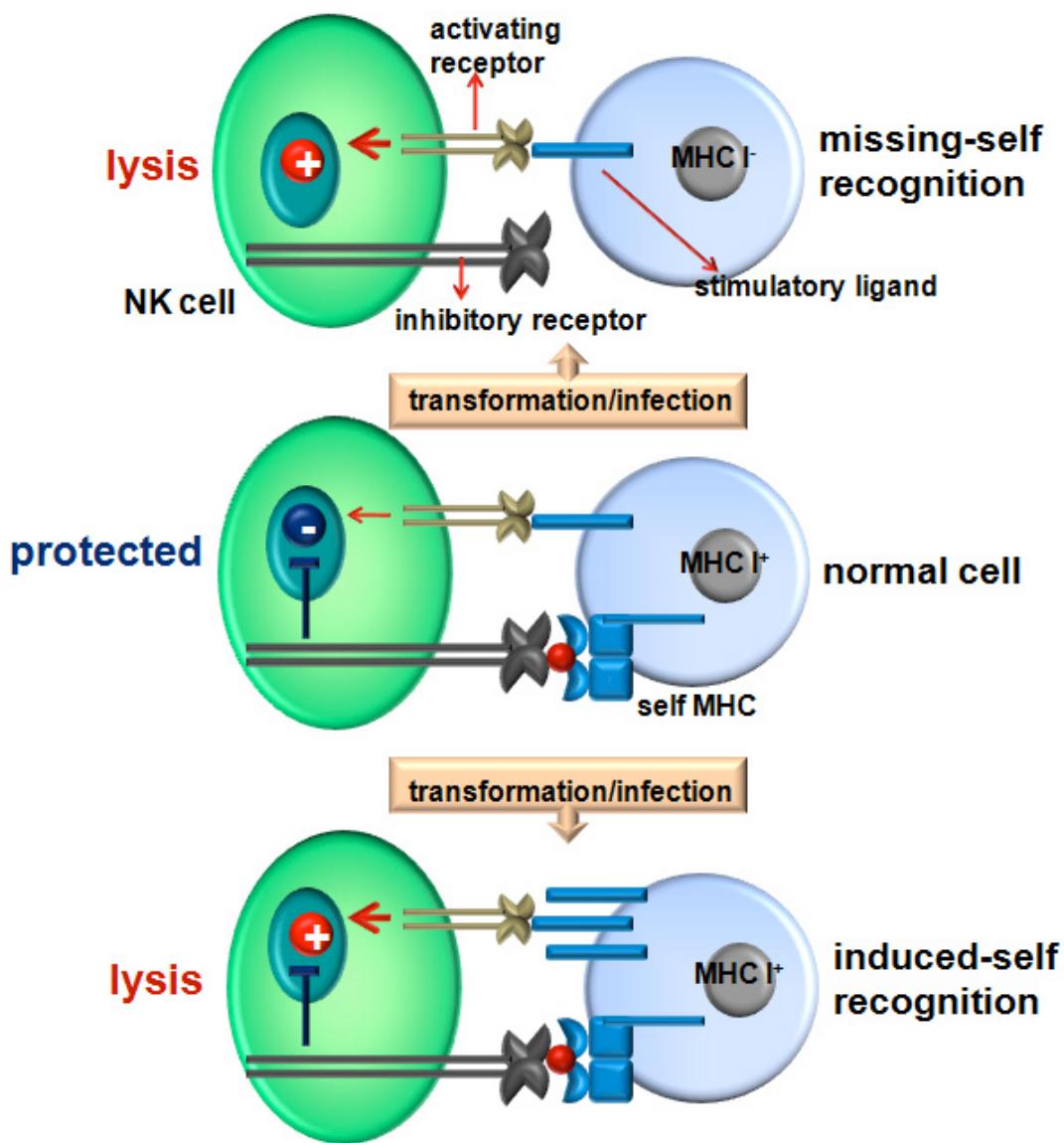
Figure. 2. NK-cell Recognition

The NK-cell regulation is entirely dependent on its receptor recognition to the specific ligands on the target cells. Normally, the unique feature of NK cells to protect the normal cells (self) is due to the interaction of inhibitory receptors on NK cells and to the self major-histo-compatibility (MHC) molecules present on normal cells (even when the activating receptors are engaged). But upon transformation or infection of normal cells, these cells undergo lysis by NK cells in two different mechanisms:-

   (a) **"Missing self"** The transformed cells lose their MHC molecules, thus loosing the inhibitory effect and therefore the engagement of activating receptors activate NK cells leading to natural cytotoxicity.

   (b) **"Induced self"** Under certain stimulation, normal cells are transformed exhibiting an induction of stimulatory ligands on their surface. Thus, the signals for the activating receptors to NK cells were enhanced and the inhibitory signal is masked. This induced recognition leads to killing of target cells.

3.3 NK-cell Receptors

3.3.1 Inhibitory Receptors

The human NK cell function is regulated by many inhibitory signals protecting the normal self cells from the NK-cell attack. The receptors responsible for this inhibitory function include the HLA class I specific killer immunoglobulin-like receptors (KIRs) and CD94/NKG2A considered as disulphide bonded heterodimers belonging to C-type lectin superfamily. These receptors recognize the polymorphic HLA class I molecules. They were well characterized by the presence of immune receptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic tails. Phosphorylation of the tyrosine residues lead to the recruitment of phosphatases such as SHP-1 and SHP-2 leading to the inhibition of NK-cell mediated cytotoxicity and cytokine secretion (Lanier 1998). Similar kind of receptors like CD94/NKG2C also recognize the HLA class I molecules but has an activating function due to the absence of ITIM motifs in their cytoplasmic tails. Their activating function is mediated by the association of KARAP/DAP12 transmembrane polypeptide-containing immune receptor tyrosine-based activating motifs (ITAM) (Olcese, Cambiaggi et al. 1997; Campbell, Cella et al. 1998; Lanier, Corliss et al. 1998).

3.3.2 Activating Receptors

Although the inhibitory receptors play a major role in regulating NK cell function, the stimulatory receptors are necessary for the initial activation of NK cell effector functions. These are termed as non-HLA specific receptors that include a low affinity Fc receptor FcγRIII (CD16); an antibody activating receptor, NKG2D; a triggering receptor and a subset of activating receptors (NKp46, NKp30 and NKp44) collectively termed as Natural Cytotoxicity Receptors (NCRs).

NK cells mediate antibody-dependent cellular cytotoxicity (ADCC) by expressing a low affinity Fc receptor **FcγRIII (CD16)**. This receptor possesses ITAM motifs and upon ligation, activated src-family tyrosine kinases (eg. Lck) bind and phosphorylate tyrosine residues contained within the ITAM motifs (Vivier, Morin et al. 1991; Wirthmueller, Kurosaki et al. 1992). This signal activates NK cells resulting

in secretion of cytokines, and antibody-dependent cellular cytotoxicity (ADCC) leading to apoptosis as a consequence of Fas-ligand induced cell death (Cassatella, Anegon et al. 1989; Azzoni, Anegon et al. 1995; Ortaldo, Mason et al. 1995; Eischen, Schilling et al. 1996).

Another well characterized NK-cell activating receptor, **NKG2D**, is a type II disulphide-linked dimer with a lectin like extracellular domain. NKG2D is also expressed on T-cells. The human cellular ligands for NKG2D are: (i) MHC class I chain –related molecules MICA and MICB are induced upon heat shock and oxidative stress (Bauer, Groh et al. 1999) and (ii) UL-16 binding proteins ULBP 1, 2 and 3 are induced upon tumor transformation (Cosman, Mullberg et al. 2001). This receptor signaling is associated with DAP10 and might also require secondary signals from the other activating receptors such as NCRs before they respond against the tumors (Pende, Cantoni et al. 2001).

More recently, three non-HLA-specific receptors have been identified as responsible molecules for the spontaneous cytotoxicity of NK cells. These are termed as Natural Cytotoxicity Receptors (NCRs): (i) NCR1, NKp46 (ii) NCR2, NKp44 and (iii) NCR3, NKp30. NKp46 and NKp30 are selectively expressed on resting NK cells, whereas the expression of NKp44 is restricted to activated NK cells (Sivori, Vitale et al. 1997; Vitale, Bottino et al. 1998; Pende, Parolini et al. 1999). NCRs are characterized by two Ig-C2 (NKp46) (Pessino, Sivori et al. 1998) or one Ig-V (NKp30 and NKp44) (Cantoni, Bottino et al. 1999; Pende, Parolini et al. 1999) domains in the extracellular portion. Their short cytoplasmic tail lacks the typical tyrosine-based activating motifs but instead the trans-membrane regions contain positively charged amino acids that allow association of ITAM- bearing polypeptides, CD3ζ and FcεRIγ for NKp46 and NKp30, while NKp44 associates with DAP12 (Moretta, Biassoni et al. 2000; Moretta, Bottino et al. 2001). Apart from their own signals, the NCRs have the ability to activate themselves, even if anyone of the receptors is activated, suggesting that a functional cross-talk specifically occurs among the different NCRs resulting in the amplification of the activating signals (Augugliaro, Parolini et al. 2003).

The activation of NK cells against tumor cells was also stimulated by a set of co-stimulatory molecules. In addition to the effects of activating and inhibitory signals the co-stimulatory receptor-ligand effects also play a role in NK-cell regulation.

Figure. 3. NK-cell Receptor-ligand pairs

The receptors of natural killer cells have versatile functions that regulate NK-cell activity. The receptors that perform the inhibitory function signal through ITIM (immune receptor tyrosine based inhibitory motifs) in the signaling domain. On the other hand, the activating receptors possess ITAM motifs that have activating functions. The search for cellular ligands for natural cytotoxicity receptors (NCRs) was still undercover, but the viral ligands were discovered very recently. In this project, the study identifies BAT3 (HLA-B-Associated Transcript-3) as a cellular ligand for NKp30.

The natural cytotoxicity of human NK cells is mediated by the activation of triggering receptors such as NCRs with their specific cellular ligands. The specific cytotoxic effects of the NK cells were exclusively characterized using monoclonal antibodies against NCRs. Since the identity of the NCR cellular ligands is still unknown to date. However, several viral ligands for the NCRs were recently identified. Hemagglutinin proteins of different viruses bind and engage to NKp46 and NKp44, thus activating NK cells (Arnon, Lev et al. 2001; Mandelboim, Lieberman et al. 2001). The main tegument protein of human cytomegalovirus pp65 binds to NKp30 and inhibits the NK cell function (Arnon, Achdout et al. 2005). Contribution of NK cells in adaptive immunity has been the hot topic. Among the NCRs, NKp30 plays a major role in direct killing of malignant cells and NK-dendritic cell activation

thus enhancing the adaptive immunity. Therefore, it is of great importance to identify the cellular ligands involved, for a better understanding of the NK cell biological mechanisms and probably for therapeutic values. Identification of the cellular ligands for NKp30, involved in both innate and adaptive responses, will be the topic of this project.

3.4 Dendritic Cells

Dendritic cells (DCs) are classically defined as the sentinels of the immune system. They play a major role in bridging the innate and adaptive immune responses (Moretta 2002). In brief, their main functions are; to uptake the foreign antigens, process and present the peptides to T-cells leading and initiating the adaptive immune response. During this process, DCs and their precursors migrate to the site of inflammation and transform into immature dendritic cells (iDCs) (Sallusto, Cella et al. 1995; Randolph, Beaulieu et al. 1998; Sallusto and Lanzavecchia 1999). These cells have the primary function of antigen up-take (Sallusto, Cella et al. 1995) and recognize a wide variety of pathogens by toll-like receptors (TLRs) present on their surface. With response to different cytokines substances like lipopolysaccharides (LPS) iDCs transform into mature dendritic cells (mDCs), a process called **"maturation"**. Reciprocal activation between DCs and NK-cells also result in maturation of iDCs mediated by the release of TNFα (Vitale, Della Chiesa et al. 2005). Mature DCs migrate to the secondary lymphoid organs (lymph nodes) and mediate T-cell priming.

The final maturation of DCs is characterized by profound changes in the phenotype of the receptor expression and their functional behaviour. For instance, they lose the ability of antigen uptake by down-regulating the specific receptors responsible for the endocytosis. In contrast, maturation process up-regulate many other surface markers (CD40, CD58, CD80, CD86, CD83 and also MHC molecules) on mature dendritic cells. Most of the surface markers are involved in the subsequent interactions between DCs-T-cells and DCs-NK cells. A chemokine receptor, CCR7 is also induced and expressed in high levels during the maturation process and allows DCs to localize and migrate into secondary lymphoid organs (Saeki, Moore et al. 1999; Sallusto, Mackay et al. 2000).

In vitro, human immature DCs were experimentally generated from CD34$^+$ or adherent peripheral-blood mononuclear-cell (CD11C$^+$CD11B$^+$CD14$^+$) precursors

cultured in the presence of exogenous GM-CSF and IL-4. iDCs cultured in presence of TNFα and LPS, transform into mDCs.

3.5 Reciprocal Activation of Natural killer cells and dendritic cells

Recently there has been emerging evidence for the importance of the interaction between human NK cells and dendritic cells. The NK-DC interaction is a reciprocal activation that functions as an important control switch for amplifying or attenuating innate immune responses (Ferlazzo, Tsang et al. 2002; Gerosa, Baldani-Guerra et al. 2002; Zitvogel 2002). Thus, these two cell types can potentially activate each other in their maturation process; DCs activate NK cells during the process of priming the innate response and in turn the NK cells promote the DC maturation and cytokine production. This bi-directional signaling between NK-DC might take place at different stages of the innate and adaptive immune responses indicating that this cross-talk has an important role in controlling the links between innate and adaptive immunity (Moretta 2002).

The bi-directional cross-talk begins with the recruitment of these cells to the site of inflammation. Upon inflammation or infection there is a release of type I IFNs and chemokines (Biron, Nguyen et al. 1999). This stimulus recruits the NK cells from the blood stream. Both the resident and simultaneously recruited DCs are then able to promote NK-cell activation. IL-12 is released from DCs and induces NK cells to secrete IFNγ which is responsible for initiation of immediate anti-viral responses (Biron, Nguyen et al. 1999). After the process of antigen uptake, DCs burst a set of chemokines such as CCL3 and CXCL8 (IL-8) that act on NK cells further recruiting NK cells to the inflamed tissues (Fig. 4).

Once the NK cells reach the inflammatory site, there is a direct contact with iDCs resulting in maturation and activation of NK cells. Moreover, the activated NK cells acquire the potential cytotoxicity against the damaged or infected tissues and remarkably they also kill the surrounding iDCs. This mechanism might be a limiting factor for the recruitment of iDCs from the blood vessels. DC killing by NK cells is triggered by IL-2 that is released transiently from iDCs in the early time points (Granucci, Vizzardelli et al. 2001), IL-15 released by iDCs in response to type I IFNs (Mattei, Schiavoni et al. 2001) and (iii) the release of IL-12 induces the production of IFNγ from NK cells and also result in potential NK-mediated-cytotoxicity (Fig. 4).

The lethal effect of these activated NK cells on the surrounding iDCs is dependent on natural cytotoxicity receptor, NKp30 (Ferlazzo, Tsang et al. 2002).
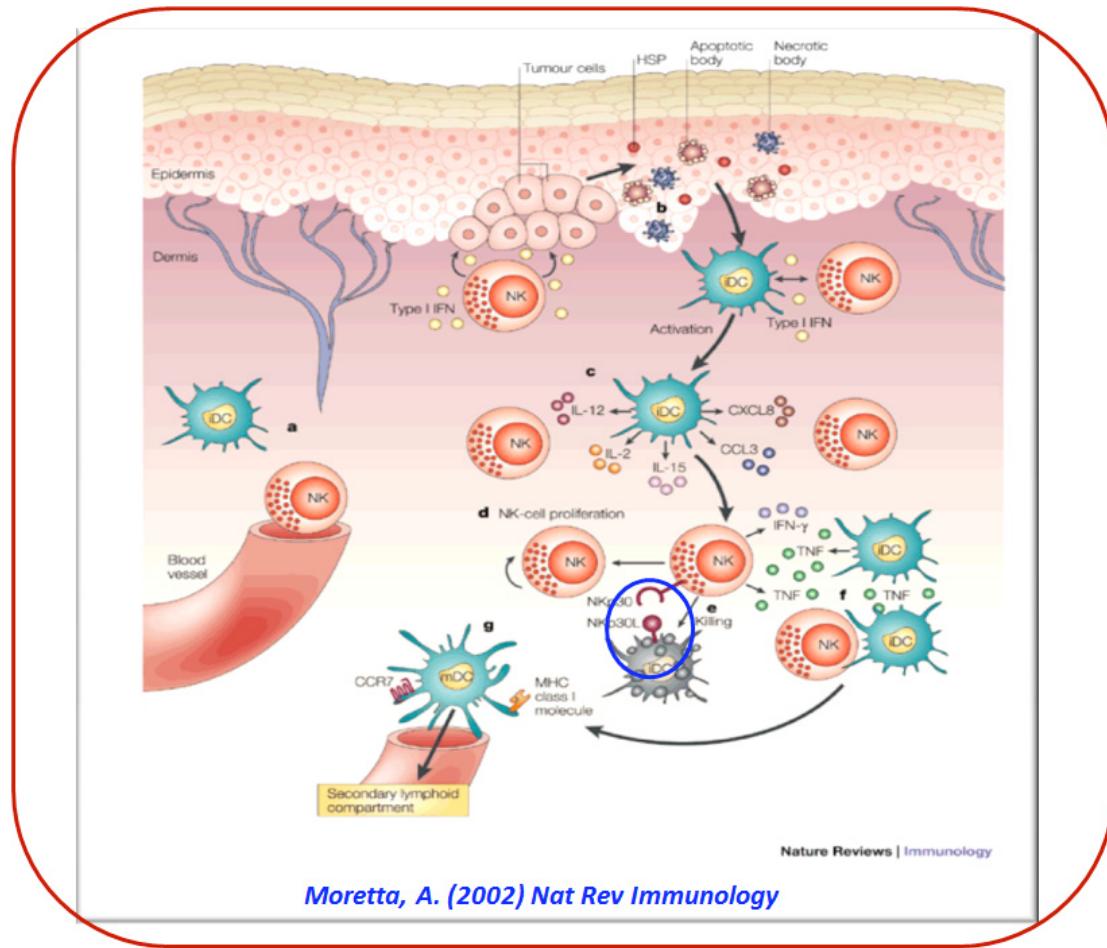
Figure. 4. Model for NK-DC reciprocal activation in the inflammatory region

The above model represents the recruitment of natural killer (NK) cells and dendritic cells (DCs) by the presence of tumor cells (susceptible to NK-mediated cytotoxicity) and might also occur during inflammatory responses to pathogens. (a) NK cells and iDCs were recruited from the blood stream to the injury site, and the NK cells get activated in response to chemokines secreted by endothelial cells and enhanced by the danger signals released by inflamed tissues. (b) Once the NK cells kill the tumor cells, these cells release a lot of heat shock proteins (HSPs), apoptotic and necrotic bodies that are taken up by dendritic cells. (c) Activation of iDCs by these tumor derived material enhances the release of different chemokines and cytokines. Moreover, the NK-DC interaction leads to further activation of NK cells. IL-12 secreted from iDCs enhances the cytotoxic effect of NK cells and the release of IFNγ. Activated iDCs also release IL-8 and recruit more NK cells from the blood stream. (d) Upon this mutual activation the NK cells proliferate in response to IL-15. (e) NKp30 on activated NK cells recognize the NKp30L on iDCs leading to the elimination of excess iDCs thus limiting the recruitment of DCs to the inflammatory region. (f) in additon to the release of IFNγ, NK cells also release TNFα that promote the maturation of iDCs to mature dendritic cells (mDCs). (g) mDCs release another set of chemokines like CCR7, especially important for these cells to migrate into the secondary lymphoid organs.

Very importantly, NK-cell-DC interaction not only promotes the maturation and cytotoxicity of NK cells, but also results in maturation of iDCs and lymphokine release. Depending on the ratio between NK cells and iDCs, the outcome of the interaction leads to either release of cytokines like TNFα or NK-cell-mediated cytotoxicity (Piccioli, Sbrana et al. 2002; Gerosa, Gobbi et al. 2005) and subsequently induce the iDC maturation rather than killing of DCs. At present the receptor-ligand pairs involved in priming phase of NK cells was not known but it has been shown that NKp30 on NK cells is involved in the effector phase of NK cells which helps in maturation of iDCs. Mutual activation of NK cells with other immune regulatory cells is mediated by several receptor-ligand interactions particularly NKp30 with its unknown ligand on DCs, NKG2D with its ligands ULBP1-3 and MICA/B, DNAM-1 with PVR and Nectin-2 and NKp80 with AICL(Newman and Riley 2007).

The other important aspect is the NK-cell-DC interactions in the secondary lymphoid organs (Fig. 5). The cross-talk of matured DCs and NK cells induces the NK cell proliferation. Importantly, this cross-talk does not induce the killing of mature DCs because of high expression of MHC class I molecules on the surface (Ferlazzo, Semino et al. 2001; Ferlazzo, Tsang et al. 2002). In the secondary lymphoid organs like lymph nodes NK cells play a major role in providing the high quality of mDCs that interact with T-cells. This putative function is exerted on the ability of NK cells to eliminate the mature DCs that express low levels of MHC class I molecules. In addition, there has been some undefined mechanisms, such as the release of IFNγ by migrating NK cells that might modulate the adaptive immunity during the course of NK-cell-DC interaction (Cooper, Fehniger et al. 2001; Robertson 2002).
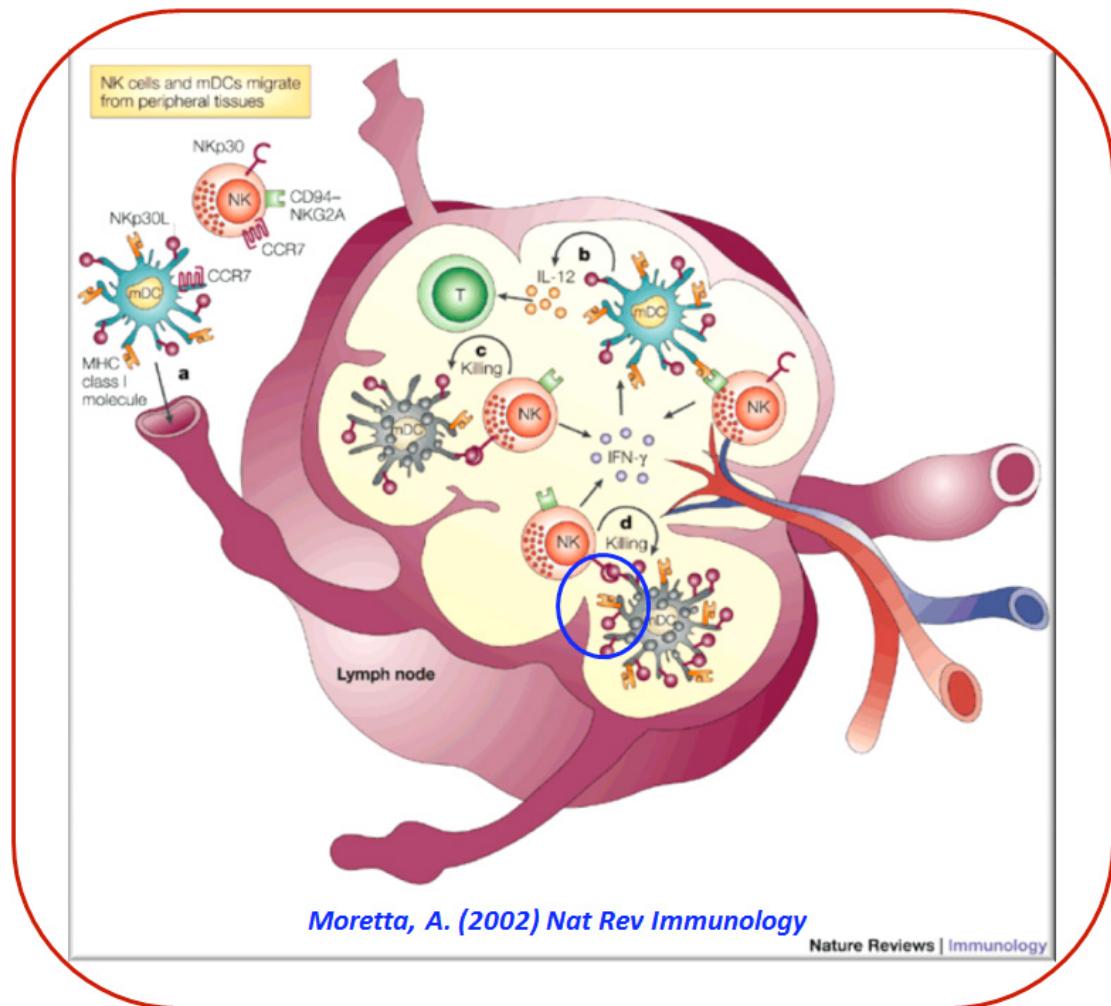
Figure. 5. Model of NK-DC crosstalk in the lymph node

Certain number of NK cells express CCR7 and high levels of L-selectin (Campbell, Qin et al. 2001; Cooper, Fehniger et al. 2001) and can migrate into lymph nodes. A possible functional role of NK cells at these sites could be the modulation of cytokine production by mDCs. (a) Matured NK cells and dendritic cells migrate into lymph nodes and might interact with each other. Strikingly, these NK cells are CD94-NKG2A$^+$ and possess the ability of secreting IFNγ (Cooper, Fehniger et al. 2001). The expression of ligands HLA-E on mDCs to NKG2A on NK cells will not result in killing but the IFNγ is produced induced by the cytokines like IL-2 or IL-15. (b) In turn, IFN promotes the production of IL-12 from mDCs, influencing the subsequent adaptive T-cell response. (c) However, if the mDCs do not express sufficient amount of MHC class molecules this would result in the NK-mediated killing of mDCs. To be noted is that activated NK cells also express NKp30 along with KIR-CD94-NKG2A. (d) Under pathological situations, the expression of NKp30 ligands are induced or overexpressed and the interaction NKp30-NKp30L overrides the inhibitory function of KIR-CD94-NKG2A receptor with HLA-E. Thus even under these matured conditions NK cells could kill mDCs through the activation of NKp30 receptor.

3.6 Exosomes---Ambassadors for the immune regulatory cells

A healthy immune regulation occurs with a balanced interplay among the cells in the immune system. For that, a proper communication between the cells is necessary and this is achieved with accurate signals and at correct time. The signaling is mediated through the receptor-ligand interactions. Besides the cell-cell contact dependent signals many soluble factors play important role in activation. In addition to the soluble ligands, cytokines, chemokines and interleukins; exosomes also play a major role in communicating within the immune cells.

Exosomes are small membrane vesicles that are secreted by various cell types as a result of fusion of multi-vesicular structures of late endosomes/lysosomes with the plasma membrane. The vesicles that are released into the extracellular space measure about 50-90 nm in diameter. They are termed as intraluminal vesicles (ILVs) in endosomes and termed as exosomes upon their release into the extracellular environment (Harding, Heuser et al. 1984; Pan, Teng et al. 1985; Johnstone, Adam et al. 1987).

In detail, the formation of multi-vesicular bodies (MVBs) is initiated at the early stages of endosomes as a consequence of the inward budding of the endosomal limiting membrane. During the endocytic process, many vesicles of diameter 60-80 nm accumulate in the lumen of late endosomes, hence, the name multivesicular bodies. Recently, there has been evidence to demonstrate that these luminal vesicles are truly free vesicles dissociated from the endosomal delimiting membrane (Murk, Humbel et al. 2003). Usually, these MVBs have three distinct phases:

(i) The pathway involved in targeting the incorporated proteins to lysosomes for degradation requires either direct fusion of MVB with lysosomes or a poorly understood complex maturation process (Futter, Pearse et al. 1996; Mullock, Bright et al. 1998). For example, epidermal growth factor receptor is sorted at MVB for proteolytic degradation after ligand induced endocytosis (Felder, Miller et al. 1990).

(ii) In the context of MVBs of immature dendritic cells where the antigen uptake is rapid, MVBs can also serve as the storage compartments for the MHC class molecules. Thus upon DC activation these luminal vesicles fuse with MVB limiting membrane and the transfer of MHC class II can occur (Kleijmeer, Ramm et al. 2001).

(iii) The third possible fate of the MVB occurs when the limiting membrane fuses with the plasma membrane resulting in the secretion of the vesicles into the extracellular milieu. These are now termed as exosomes (Stoorvogel, Kleijmeer et al. 2002; Thery, Zitvogel et al. 2002).

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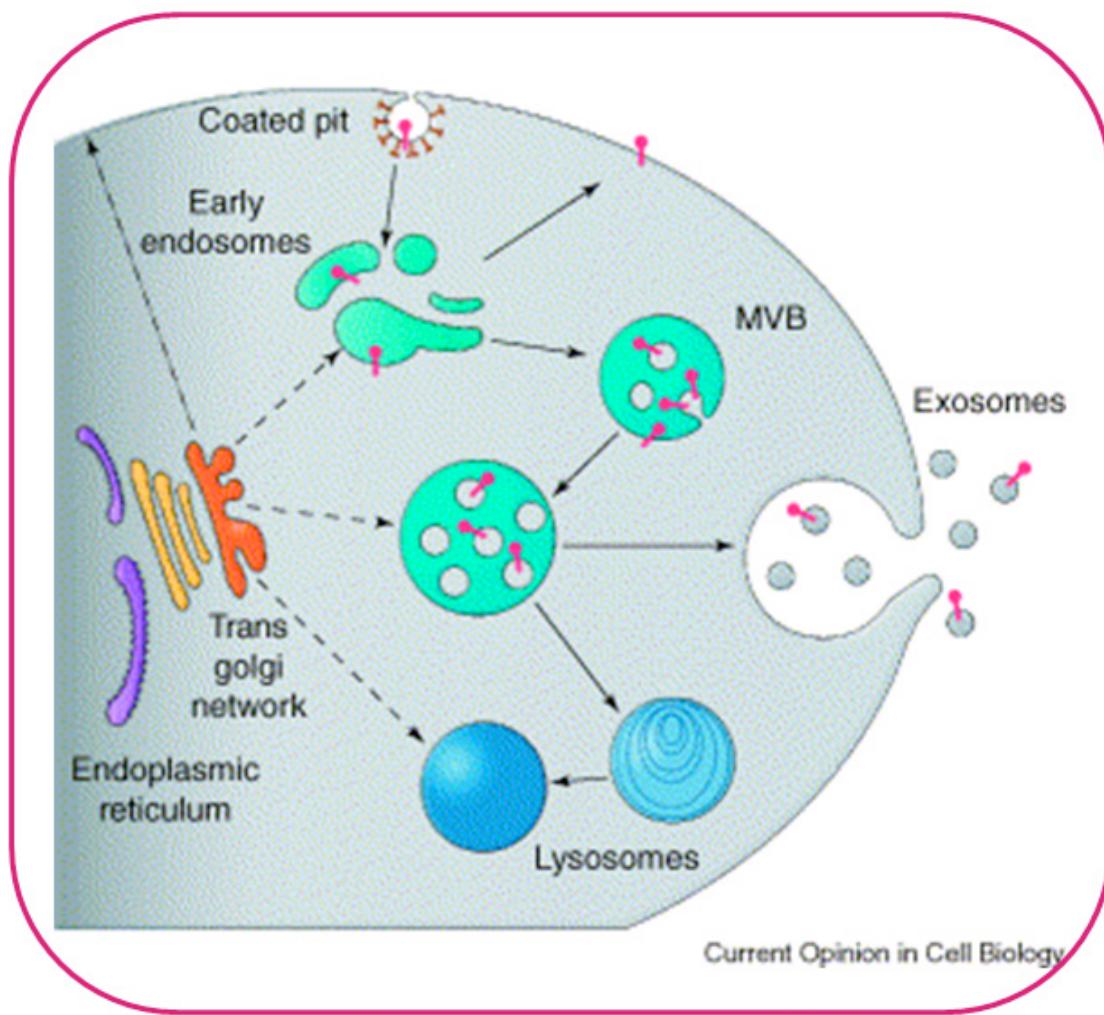
Fevrier. B and Raposo. G current opinion cell biol, 2004

Figure. 6. Schematic representation of the endocytotic pathway

Membrane proteins (in pink) were internalized through clathrin-mediated endocytosis and delivered through endosomes. In the early endosomes, the molecules are either recycled back to the plasma membrane or sequestered into the other vesicles inside the cell. Studies shows that these internal vesicles are of multivesicular bodies (MVBs) generated by budding from the limiting membrane into the lumen of endosomes (inter luminal vesicles, ILVs). Usually, these MVBs fuse with lysosomes and degrade and in several hematopoietic and non-hematopoietic cells the MVBs fuse with the plasma membrane and are releases into the extracellular environment as exosomes. Exosomes display the same features and orientation as the plasma membrane, with extracellular domains of proteins exposed on the surface and a droplet of cytoplasm enclosed.

Recently there has been much emerging evidence that exosomes derived from many cells could serve as immunotherapeutic vaccine against tumors and more importantly in communicating between immune regulatory cells. Although there is much information available for the crosstalk between exosomes and T-cell activation, very little is described with regard to NK cells.

3.7 Aim of the current study

Natural-killer (NK)-cells are lymphocytes that provide a link between innate and adaptive immunity through crosstalk with dendritic cells (DCs) (Munz, Steinman et al. 2005; Walzer, Dalod et al. 2005) and mediate T-cell activation (Hanna, Gonen-Gross et al. 2004; Adam, King et al. 2005; Laouar, Sutterwala et al. 2005). The Natural Cytotoxicity Receptors (NCR) including NKp30, NKp44 and NKp46 as well as NKG2D are triggering receptors responsible for NK cell-activation (Moretta and Moretta 2004; Lanier 2005). Until now, little is known about the molecular nature of the cellular ligands for NKp30, NKp44 and NKp46. These molecules are so far only identified indirectly by receptor-specific antibodies that inhibit the NK cell-cytotoxicity against target cells (Pende, Parolini et al. 1999; Bottino, Castriconi et al. 2005; Farag and Caligiuri 2006).

The orphan NKp30 receptor (NCR3, CD337) plays a special role as it is the only receptor involved in both, tumor cell lysis and lysis of normal self cells (Pende, Parolini et al. 1999; Ferlazzo, Tsang et al. 2002; Vitale, Della Chiesa et al. 2005). The 30-kD triggering receptor is selectively expressed on NK cells and associated with CD3ζ chains that become phosphorylated upon ligation (Pende, Parolini et al. 1999). Human dendritic cells (DCs) express the hitherto unknown NKp30 ligand that mediates the NK-DC interaction, resulting either in DC-activation or DC-killing, thus limiting the supply of dendritic cells (Pende, Parolini et al. 1999; Ferlazzo, Tsang et al. 2002; Vitale, Della Chiesa et al. 2005). Although the cellular ligands binding to the NCR receptors are not yet identified, functional data indicate that they are expressed on cells upon activation, proliferation or tumor transformation (Bottino, Castriconi et al. 2005). So far, it has been unclear why cell lines such as fibroblasts or lymphoma cells that are killed by NK cells in an NKp30-dependent manner, fail to bind to soluble NKp30-Ig fusion proteins (Pende, Parolini et al. 1999; Mandelboim, Lieberman et al. 2001; Arnon, Achdout et al. 2005; Bottino, Castriconi et al. 2005).

Aim of the current study is to analyse whether there any intracellular ligands for NKp30 and if so how do they become accessible for the NKp30 surface receptor. The second aim is to elucidate, whether these intracellular factors are involved in the NK/DC cross/talk or exclusively responsible for the recognition of tumor and virus/infected cells. HLA-B-Associated Transcript-3 (BAT3) is an intracellular tumor

associated binding partner for NKp30 that was isolated in a yeast-two-hybrid system. The following questions will be addressed:

- (i) Is it possible to formally prove that BAT3 is a cellular ligand for NKp30, expressed on tumor cells and dendritic cells? ,
- (ii) How does BAT3 become accessible to the surface receptor? ,
- (iii) Does BAT3 have a functional role in activating natural killer cells? ,
- (iv) Is BAT3 involved in both killing of target cells and cross talk with DCs? and
- (v) Does BAT3/NKp30 interaction describe a novel model for target cell recognition?

4 Materials and Methods

4.1 Materials

Cell Lines

293T	Human Fibroblast kidney cell line
K562	Human Chrinic Myeloid Leukemia
LS174T	Human Colon Carcinoma cell line
RAJI	Human Burkitt's Lymphoma
HeLa	Human Cervical Cancer cells
RPMI8226	Human Multiple Myeloma cell line
NKL	Human Natural Killer cell lymphoma (Robertson et al., 1996)

Mammalian Expression Vectors

Vector	Fusion	Selection marker (bacteria)	Selection marker (mammalian cells)
pcDNA3.1	No tag	Ampicilin	Zeocin
pcDNA3.1 His/Myc A	His/Myc	Ampicilin	Geneticin
pcDNA3.1 His/Myc B	His/Myc	Ampicilin	Geneticin
pFuse 2	Human IgG1	Zeocin	Zeocin
pIG (pcDM8 background)	Human IgG1	Ampicilin	No resistance
pEGFP C1	EGFP	Kanamycin	Neomycin

Yeast ExpressionVectors

Vector	Fusion	Selection marker	Promoter
pGBT9	*DNA/bait*	TRP	ADH1
pACT2	*AD/library*	LEU	ADH1
pGADT7	*AD/library*	LEU	ADH1

Yeast strain used for two-hybrid assay

Strain	genotype
AH109	MATa or MATα *trp1-901, leu2-3,ura3-52, his3-200, gal4d, gal80d, LYS2 : : GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3 : : MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ*

Bacterial Strains used for cloning

Different bacterial strains Escherichia *coli (E.coli)* used for molecular biology: DH5α, XL-1 blue, TG-1, BL-21 and MC-1061

Antibodies

Antigen	Fluoro-chrome	Company	against species	from species	isotyp	clone
CD3	purified	BD Biosciences	human	mouse	IgG2a	HIT3a
CD3	FITC	BD Biosciences	human	mouse		
CD3	PE	Immuno Tools	human	mouse	IgG1	UCHT1
CD3	APC	Immuno Tools	human	mouse	IgG2a	MEM57
CD9	purified	BD Biosciences	human			
CD14	FITC	BD Biosciences	human			
CD14	PE	BD Biosciences	human	mouse		
CD16	purified	BD Biosciences	human			
CD16	PE	BD Biosciences	human	mouse		
CD30	purified	Laboratory of Immune therapy	human	mouse		5F11
CD30	PE	BD Biosciences	human	mouse		
CD56	purified	BD Biosciences	human			
CD56	FITC	Immuno Tools		mouse	IgG2a	MEM-188
CD56	PE	BD Biosciences	human	mouse		My31
CD56	APC	BD Biosciences	human	mouse		
CD1a	PE	BD Biosciences	human	mouse		
CD1a	APC	Bio legends	human	mouse		
CD83	FITC	BD Biosciences	human	mouse		
CD80	FITC	BD Biosciences	human	mouse		
CD80	PE	Bio Legends	human	mouse		
CD86	purified	Immuno Tools	human			
CD86	FITC	BD Biosciences	human	mouse		
CD86	PE	BD Biosciences	human	mouse		
NKp30	purified	R&D Systems	human		IgG2a	210845
NKp30	PE	Beckman Coulter	human	mouse	IgG1	Z25
NKp44	PE	Beckman Coulter	human	mouse	IgG1	
NKp46	purified	BD Pharmingen	human	mouse		9E/2
NKp46	PE	Beckman Coulter	human	mouse	IgG1	BAB281
NKp46	PE	R&D systems	human	mouse	IgG2b	
hNKG2D	PE	BD Biosciences	human	mouse	IgG1	
NKG2D	Fitc	Abcam	human	mouse	IgG1	1D11
hNKG2A	PE	BD Biosciences	human	mouse	IgG2a	
KIR(NKB1)	BD	BD Biosciences	human	mouse		
CD107a	PE	BD Biosciences	human	mouse		
GranzymeB	FITC	BD Biosciences	human	mouse		

Nkp30	purified	Santa Cruz	human	goat	poly IgG	G-19
NKp46	purified	Sanata Cruz	human	goat	poly IgG	C-20
HLA-ABC	purified	BD Biosciences	human	mouse	IgG1	G46-2.6
HLA-DR	purified	BD Biosciences	human	mouse		
HLA-A,B,C	PE	BD Biosciences	human	mouse		
HLA-DR	FITC	BD Biosciences	human	mouse		
CD63	PE	BD Biosciences	human	mouse		
LAMP2b	purified	Abcam	human	rabbit	IgG	
HSP 70	purified	StressGen		mouse		
p53	purified	BD Biosciences	human	mouse	IgG2b	PAb122
MICA/B	purified	BD Biosciences	human	mouse	IgG2a	6D4
BAT3	purified	Abcam		chicken	IgY	
RGS-HIS	purified	Qiagen		mouse	IgG1	
c-myc	purified	Dianova	human	mouse	IgG1	9E10.3(Ab-2)
IgG Fc	purified	Dianova	human	goat	F(ab)2	
IgG	purified	Dianova		goat	IgG	
IgG H+L	purified	Dianova	mouse	goat	F(ab)2	
IgG	PE	BD Biosciences	human	mouse		
IgG (H+L) human	POD	Dianova	rabbit	donkey		
IgG1	PE	BD Biosciences	mouse	goat		
IgG	APC	BD Biosciences	human	mouse		
7-AAD	7-AAD	BD Biosciences				
Annexin V	PE	BD Biosciences				
Chicken	PE	Dianova	chicken	donkey	IgG (H+L)	
anti-mouse	FITC	BD Biosciences	mouse	goat	poly	
anti-mouse	PE	Dako	mouse	goat	poly	
Cholera Toxin B	Alexa 594	Invitrogen				
Cholera Toxin B	FITC	Sigma				

All the laboratory chemicals and reagents were purchased from Roth Chemicals and Sigma Life Sciences.

All the oligo-nucleotides used for amplifying the desired gene products were obtained from MWG-BIOTECH.

4.2 Methods

All the standard molecular biological and biochemical techniques were performed by following the protocols designed in the book, "Molecular Cloning----A Laboratory Manual" written by Sambrook, J *et. Al.*

The yeast two-hybrid and the other basic protocols concerning to yeast were performed according to the manufacture's protocol handbook by Clontech (BD Biosciences).

Yeast two-hybrid system-3

Basic Principle

In Matchmaker System 3, when bait gene (fused to GAL4 DNA-binding domain) interacts with library gene (fused to GAL4 activation domain), there is a transcriptional activation of four reporter genes (ADE2, HIS3, MEL1 and/or *lac*Z. The sensitivity of this system is primarily attributable to high-fold amplification of positive signals in vivo (i.e. transcriptionally, translational and enzymatic) and moreover, it also allows for the proteins to interact in their native confirmations.

On the otherhand, the reduction of false positives is also an important factor to be considered in two-hybrid assay. This system 3 features the yeast strain AH109, which virtually eliminates the false positives by using three different reporter genes simultaneously—ADE2, HIS3, and MEL1 (*lac*Z) under the control of distinct GAL4 upstream activating sequences UASs and TATA boxes (allows to reduce the false positives that occur due to non-specific binding of proteins to GAL4 and TATA boxes). ADE2 provides the strong nutritional selection and HIS3 provides the stringency needed for eliminating the false positives. Furthermore, MEL1 and lacZ genes encode for enzymes like α-galactosidase and β-galactosidase respectively, can be used in assaying the interactions directly on X-α-Gal indicator plates, which employ blue/white screeing.
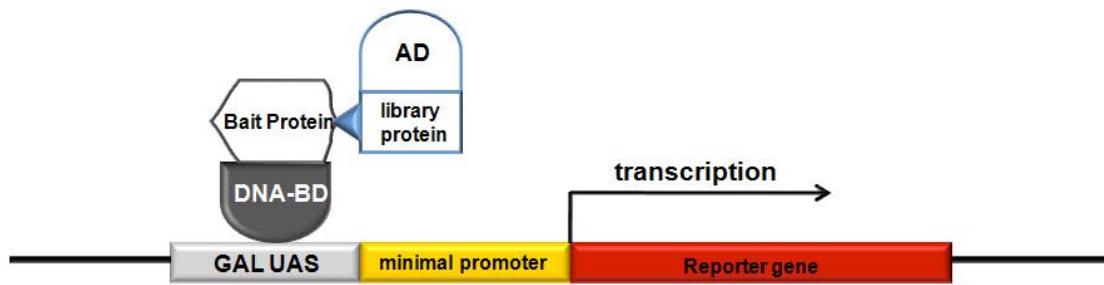
Figure. 7. Yeast two hybrid principle

The DNA-BD of the yeast GAL4 protein binds to the GAL-UAS upstream of the reporter genes. The activating domain (AD) of the GAL4 protein binds to the DNA-BD and this interaction functions as a transcriptional activator.

Cloning of Bait:

The extra-cellular domain including the leader sequence (1-110aa) of Nkp30 was PCR amplified using the cDNA purified from NKL cells (Qiagen RT-PCR kit) using the primers 5'-CG**GAATTC**CCGGGGATGGCCTGGATGCTGTT-3' and 3'-TGC-ACTGCACAACCA**ATGCAT**TGGACACGTAG-5'. The PCR product was digested with the enzymes EcoR I and Pst I and sub-cloned into the yeast expressing vector (bait plasmid) pGBT9 in frame with the Gal4 DNA binding domain. This clone pGBT9 Nkp30 was used for screening Human Erythroleukemia (K562 cell line) cDNA library cloned into the vector pACT2 (BD clontech). The cloning sites were EcoRI and XhoI, where the EcoRI site is from an adaptor sequence 5' **AATTC**GCGGCCGCGTCGAC 3'. This sequence also have Not I and Sal I sites which were used for further cloning. All the other control plasmids and the yeast host strains were provided in Matchmaker Two-Hybrid System 3 kit from clontech (BD Biosciences).

Yeast two-hybrid screening:

The yeast strain supplied with the kit and used in this study was AH109 *Saccharomyces cerevisiae* (GAL1, GAL2, and MEL1), a reporter host strain carrying three wildtype genes (HIS3, ADE2, and *lacZ*) under the control of GAL4 promoter. Yeast cells were grown or maintained in YPD (2% bacto peptone, 1% yeast extract, 2% glucose) or Synthetic Dropout (SD) medium lacking the appropriate nutrients to maintain the selection. Yeast cells were transformed using PEG/Lithium acetate method, as described elsewhere (Gyuris et al., 1993). Using PEG/lithium acetate

method competent cells were made and co-transformed with biat plasmid (pGBT9-NKp30) and with K562 cDNA library cloned in pACT2 along with salmon sperm DNA. The co-transformants were selected and maintained on SD/-Leu/-Tryp plates. The positive clones were screened on SD/-His/-Leu/-Tryp plates. The stringency for the positive clones was increased by plating the co-transformants on SD/-Ade/-His/-Leu/-Tryp plates. Positive clones obtained were further narrowed in number by control transformations and final clones were examined for *lacZ* reporter gene expression using a colony lift filter assay.

From the library screening the positive clones were rescued by transforming the plasmid DNA into E.*coli.* The positive clones were sequenced and re-tested for the interaction with Nkp30. Using NCBI blast the identity of the sequences revealed that it corresponds to the C-terminal half of the HLA-B associated transcript-3, BAT3- (555-1132). This clone do not have the amino acid sequence from 1053-1101, which was considered to be the BAG domain of BAT3-human Scythe. (Kenneth Thress *et.al*). Therefore, the missing aminoacids were amplified from K562 cDNA library using the primers 5' CAATGGC-C**GAATTC**GTCGTATGT 3' and 3' CCG**CTCGAG**CTAAGGATCATCAGCAAAG 5'. The amplified product (234 amino acids) contains the BAG domain was cloned into pACT2 vector using EcoR I and Xho I sites. Then the EcoR I fragment from the initial clone was incorporated into the single EcoR I site in frame with the Gal4 activating domain. The clone was confirmed using restriction analysis and sequencing. And this clone containing the BAG domain was also tested for the interaction with Nkp30.

Nkp46 (1-287aa) was PCR amplified using the cDNA purified from NKL cells (Qiagen RT-PCR kit) using the primers 5'-CG**GAATTC**CGATGTCTTCCACACT -3' and 3'-CG**GGATCC**CGCTCGCTCTCTAGTC -5'. The PCR product was digested with the enzymes EcoR I and BamH I and sub-cloned into the yeast expressing vector (bait plasmid) pGBT9 in frame with the Gal4 DNA binding domain. This construct was used as a control in the two hybrids.

Deletion Analysis of the C-terminal fragment of BAT3:

To generate different set of truncations and some over-lapping sequences, the restriction sites within the sequence and a PCR were used. The BamH I site at 735th position within the BAT3 sequence and the BamH I from the vector was used to clone A-(amino acids 555-735) into CIAP (calf-intestine alkaline phosphatase) treated pGADT7 vector cut with BamH I. The clone B-(amino acids 733-908) was PCR amplified from pACT2 BAT3 c-terminus using the primers 5'-GGAATTCCAT**ATGGCT**GCCTTCATACAACGCCT-CAGTG- 3' and 3'-CCG**CTCGAG**GGGATTCACCCCACGAGACATACGAC and the product was cloned into pGADT7 digested with Nde I and Xho I. The EcoR I site at 898th position of BAT3 and the EcoR I site from the initial adapter sequence was used to digest and re-ligate to be in frame with the Gal4 activation domain to provide the clone C-(aminoacids 898-1132) without BAG domain. Using the restriction analysis, the overlapping clones D-(amino acids 555-908) and E-(amino acids 733-1132) without Bag domain were cloned into the activating vector pGADT7 (clontech).

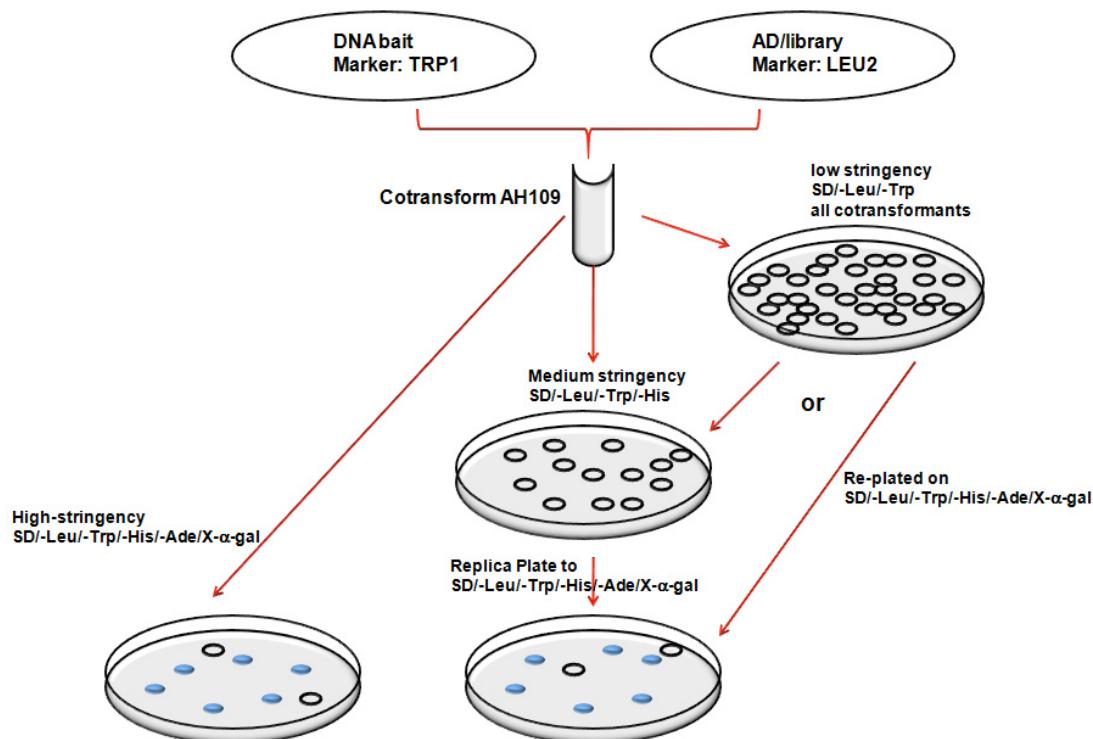**Figure. 8. Yeast two hybrid screening using strain AH109**

This schematic diagram represents the selection of the co-transformants under certain selection markers. The degree of stringency was also counted for the specificity of the interactions. Noticed was that the high stringency results in fewer colonies and reduce the number of false positives. Moreover, the weak interactions are also eliminated.

Cell biology, biochemical and Immunological Methods

Cultivation of Mammalian Cells

      All the tumor cells used in this study were cultivated in RPMI supplemented with 50 µg/mL penicillin, 50 µg/mL streptomycin, 2mM L-Glutamin and 10% fetal calf serum at 37°C with 5% CO_2. The viability and the cell count were determined by trypan blue staining. The transfection of DNA into the tumor cells (293T) was done by using lipofectamine 2000 (Invitrogen) according to the manufacture's protocol.

Purification of Natural Killer cells

      NK cells were separated from peripheral blood mononuclear cells (PBMCs) purified from healthy-donor buffy coats using Ficoll-Paque density gradient centrifugation with Leucosep columns from Greiner bio-one (Solingen, Germany). Non NK cells were depleted using the NK Cell Isolation Kit and VarioMACS (Miltenyi, Bergisch Gladbach, Germany). Separated polyclonal NK cells were cultivated in RPMI supplemented with 50 µg/mL penicillin, 50 µg/mL streptomycin, 10% fetal calf serum and 10U/mL recombinant human IL-2 (R&D Systems, Wiesbaden, Germany) at 37°C with 5% CO_2. When mentioned, the resting NK cells were without IL-2 and strongly activated NK cells were incubated with 100 U/ml IL-2. The purity of NK cells was nearly 95% and this was tested using flow cytometry and confirmed that the cells are $CD3^-CD56^+CD16^+NKp46^+NKp30^+NKG2D^+$.

Generation of Dendritic cells

      Monocyte derived dendritic cells were generated from PBMCs using standard protocols (engagement paper). PBMCs were isolated from the healthy donors by Ficoll Hypaque gradient centrifugation and subsequently allowed to adherent in 80cm² for 45 min at a denisity of 2×10^7 in RPMI 1640 meidum. After 45 minutes at 37°C, non-adherent cells were removed, washed thoroughly with medium and then cultured in Iscove's Modified Dulbecco's Media(IMDM) containing 10%FBS, GM-CSF(50ng/ml), IL-4(20ng/ml). After 5 days of culture, cells were analysed by flow cytometry with markers of $CD14^-CD86^+CD80^+CD1a^+$ and $CD83^-$. For maturation of the immature dendritic cells I have used TNFα at a concentration of 50 ng/ml or lipopolysaccharide (LPS) 1-2 µg/ml for 2 days. The enhanced expression of co-

stimulatory molecules was analysed using flow cytometry and determined as CD14$^-$, CD86$^+_{high}$, CD80$^+_{high}$, CD1a$^+$ and CD83$^+$.

Flow cytometry (FACS Analysis)

NK cells and dendritic cells were incubated with different fluorescent conjugated antibodies as mentioned for 30 minutes at 4°C and washed twice with FACS buffer (1x PBS, 2%BSA and 0.2% sodium azide). Binding of antibodies to viable cells was analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany). When specified, the supernatant derived from tumor cells was incubated with NK cells for 1 hour at 4°C to block the binding of NKp30 antibody (R&D Systems, mAb1849), which was later detected by goat anti-mouse FITC coupled secondary antibody by FACS analysis.

Transfection of siRNA into Hela cells

2 x 10^5 cells were plated (4 wells) in a 12-well and incubated for overnight prior to transfection. After acheieving 90% confluence the cells were transfected with 5µg of control (Qiagen) and BAT3 siRNAs (MWG-Biotech). The transfection was done using Fugene (Roche) as the protocol described. 20-24 hours post transfection; the cells were removed and were analyzed for the mRNA expression through RT-PCR and for protein expression through western blot analysis. The same cells were used for the NK cell specific cytotoxicity experiments. The sense and anti-sense sequences for the control and BAT3 siRNAs used in the experiment are

Control siRNA:-

Sense sequence UUCUCCGAACGUGUCACGUdTdT

Anti-sense sequence ACGUGACACGUUCGGAGAAdTdT

BAT3 siRNA:-

Sense sequence 5' – [CCUUCAAUCUUCCUAGUGA] RNA

Anti-sense sequence 5' – [UCACUAGGAAGAUUGAAGG] RNA

Transfection of siRNA into immature dendritic cells

1.5 x 10^6 cells (triplicates) were transfected with the siRNAs used for Hela cells following the AMAXA nucleofection protocol. And the cells were plated in a 12-well plate. 20-24 hours post transfection; the cells were removed and were analyzed for the mRNA expression through RT-PCR and for protein expression

through western blot analysis. The same cells were used for the NK cell specific cytotoxicity experiments.

Transfection of BAT3 siRNA into 293T cells

1.5 x 10^6 cells (triplicates) were transfected with control siRNA (Alexa 488 conjugated siRNA) and a specific siRNA targeting BAT3 from Qiagen. Quantifect transfection reagent method (Qiagen) was followed for 293T cells according to the manufacture's protocol. 24 hours post transfection the cells were removed and were analyzed for the mRNA expression through quantitative PCR using light cycler and for protein expression through western blot analysis. The heat shock supernatants obtained from these cells were subjected to exosome purification. The sense and anti-sense sequences for the control and BAT3 siRNAs used in the experiment are:

Control siRNA:-

Sense sequence UUC UCC GAA CGU GUC ACG UdT dT

Anti-sense sequence ACG UGA CAC GUU CGG AGA AdT dT

Product name: Negative Control siRNA Alexa Fluor 488

Catalog Number: 1022563 (Qiagen)

BAT3 siRNA:-

Sense sequence r (GCU CCG GUC UGA UAU ACA A)dTdT

Anti-sense sequence r (UUG UAU AUC AGA CCG GAG C)dTdG

Product name: Hs_BAT3_5_HP Validated siRNA

Catalog Number: NM_001098534 (Qiagen)

Construction and expression of BAT3 c-terminal fragment Δ BAG and BAT3 full length

The BAT3-CT Δ BAG was digested with Not1 and Xho1 from pACT2 BAT3-CT Δ BAG and cloned into mammalian expression vector pCDNA3.1 (Invitrogen). The BAT3-CT Δ BAG was digested with Sal 1 and Bgl II from pACT2 BAT3-CT Δ BAG and cloned into mammalian expression vector pEGFP C1 (Invitrogen) cut with Sal I and BamH I. And moreover, the c-terminal fragment was PCR amplified from pACT2 BAT3-CT Δ BAG using the primers 5'-**AAGCTT**GGTATGGCTCCACCGCCAG- 3' and 5'- CCGA-**CTCGAG**CGAGGATCATCAGCAAAGGCCCG- 3' and cloned into pCDNA3.1/myc-His B (Invitrogen) cut with the respective enzymes Hind III and Xho

I. The full-length BAT3 was cloned in two steps as, firstly, the peptide of amino acids 736-1132 was amplified from pACT2 BAT3-CT Δ BAG using the primers 5'-GGAATT-CCATATGGCTGCCTTCATACAACGCCTCAGTG- 3' and 3'-CCG**CTCGAG**AG-GATCATCAGCAAAGGCCCG- 5'. The product was digested with BamH I/ Xho I and cloned into pCDNA3.1/myc-His A (Invitrogen). Secondly, a large portion of BAT3 (amino acids 1- 735) was PCR amplified from the cDNA of Human Erythroleukemia (K562 cell line) using the primers 5'-GGA**AGATCT**TCATGGAGCCTAATGA-TAGTACCAGTACC-3' and 3'-GGA**AGATCT**ACTGAGGCGTTGTATGAAGGC-AGC- 5' and the product was digested with Bgl II on both the ends and cloned into the BamH I site of the first clone of the first step. The constructs, full length BAT3 and the c-terminal half were confirmed by restriction analysis and sequencing. The expression was checked by transfecting the DNA into 293T cells, lysed, followed by SDS-PAGE and western blot analysis using both myc (dianova) and His (qiagen) monoclonal antibodies.

Expression and purification of the histidine tagged control protein DCoH is described elsewhere (Pogge von Strandmann et al., 2000).

Construction and expression of Nkp30Fc and preparation of partially stable cell line

The extra-cellular domain of Nkp30Fc was PCR amplified and cloned into a leader plasmid in frame with the leader sequence of human light chain kappa of IgG1. Using this cDNA as a template, the Nkp30 along with the leader sequence was amplified and cloned into the mammalian expression vector which was fused Fc portion of the human IgG1. It was cloned using Hind III and BamH I sites. The sequencing results identified that the extra-cellular domain of Nkp30 was in frame with the human Fc genomic DNA. 293T cells were transiently transfected and the supernatants were collected and incubated with protein-G sepharose beads (Amersham Biosciences). SDS-PAGE analysis revealed that the Nkp30-Fc fusion protein runs at 80 kDa approximately.

To obtain a partially stable cell line, the complementary DNA of the mammalian construct was digested with Hind III and Not I sites to obtain the extra-cellualr domain of Nkp30 and the Fc portion of human genomic IgG1. This was cloned into pCDNA3.1 (+Zeocin) purchased from invitrogen. The construct was sequenced and checked for its expression as described earlier. The cDNA was

transfected into a 24-well and then the transfected cells were put under the pressure of the antibiotic zeocin. After 24 hours the medium was changed with fresh medium containing zeocin. One of the living clones was picked and maintained in presence of the antibiotic zeocin. The increase in the expression was checked using SDS-PAGE analysis.

Co-Immunoprecipitation of BAT3 using NKp30Fc fusion protein

The mammalian expression constructs pcDNA 3.1 myc/His BAT3 and pcDNA 3.1 myc/His BAT3 CT were transfected into wild type and partially stable Nkp30Fc 293T cells. After 36-48 hours post transfection the cells were washed with cold PBS and lysed in lysis buffer (50mM Tris, 150mM Nacl, 0.1% Triton-X 100, 0.5% Sodium deoxcholate, 2mM EDTA and 0.1% SDS) containing cocktail protease inhibitors (Amersham Biosciences). For a better lysis, the lysates were freeze-thawed at -80°C for three times, sonicated and spun down at 10,000 *g for 15 minutes. The protein concentration of the lysates was confirmed using Bradford. Equal amount of lysate (200 µg) was incubated with 25µl of protein-G-sepharose beads slurry for one hour at 4 ^{o}C. The beads were washed with lysis buffer for three times and boiled for 5 minutes in SDS-PAGE sample buffer. The samples were subjected to SDS-PAGE on an 8% gel and further processed for the western transfer onto the nitrocellulose membrane. The membranes were blocked and blotted with anti-penta his (Qiagen) and anti-human Fc (Jackson Immuno Research) separately. Further, they were incubated with the respective secondary antibodies conjugated to horse-radish peroxidase and subjected to standard technique ECL (Amersham Biosciences).

Immunofluorescence

The transfected target cells were cultured on glass slides and incubated with NK cells (ratio 1:20) as indicated. The cells were fixed using acetone before staining with monoclonal his antibody (Qiagen), monoclonal CD30 antibody (Borchmann, Treml et al. 2003) or FITC-labelled choleratoxin (Sigma-Aldrich) followed by staining with labelled secondary antibodies. The antibodies were diluted in phosphate buffered saline with 10% serum. Fluorescence images were acquired by confocal laser scanning microscopy (Leica) with oil objective at pinhole size Airy 1. Crosstalk was minimized by serial acquisition of the fluorescence color channels. The digital images were merged by Leica LSM software without further processing. 100x objective lens,

NA 1,4, FITC and cy3 were excited by 488- and 543-nm laser light and emission was detected at 495-530 nm and 548-700 nm, respectively.

Preparation of tumor-cell derived supernatant and sub-cellular fractionation

The tumor cells were left untreated or exposed to a non-lethal heat shock. The cells (6×10^5) were incubated with 250µl of fresh opti-mem (serum-free) at 42ºC for different time points as indicated. After the heat shock the cells were spun and the supernatants were collected. The cells of each time point were fractionated to separate the proteins of different sub-cellular fractions mainly as total, nuclear, cytoplasmic and membrane fractions. All these fractions along with the supernatant obtained after heat shock were analysed for the expression of BAT3 through western blot analysis. The antibody used in this experiment was rabbit polyclonal serum (anti-scythe). Scythe is a homologue of BAT3 in Xenopus *levis.*

The sub-cellular fractionation was done using a protocol as previously described (Fazioli et al 1993). Breifly, 6×10^5 cells were washed twice with ice-cold PBS and lysed in a hyptonic lysis buffer, HBL [10mM Hepes (pH 7.4), 10mM NaCl, 3mM MgCl2, 1mM EDTA, 1mM EGTA, 1mM PMSF and cocktail preotease inhibitors (Roche)]. The cells were resuspended in 120 µl of lysis buffer, incubated on ice for 10 min, titurated through P2 tip 15-20 times and sonicated for 2-3 times by placing on ice in between. This is the total fraction (T). Remove 60 µl of lysate and centrifuge for 10 minutes at 375 g at 4ºC. The pellet is the nuclear fraction (N) and the supernatant is the post nuclear fraction (PNF). The nuclear pellet was washed for 3 times with 1ml of HBL containing 0.1% of NP-40 to remove membrane and/or cytoplasmic contamination. The PNF was taken into a fresh tube and spun at 375 g for 10 min once again to remove any nuclear contamination. The pellet was discarded and the cleared PNF was taken into a fresh tube and spun at 150,000 g for 30 minutes at 4ºC. The supernatant contains the cytosolic fraction (C) and the pellet is the membrane fraction (M). The membrane fraction was resupended in 250 µl of HBL and spun again for 30 min at 150,000 g at 4ºC to remove any cytosolic contamination. The supernatant was discarded and the pellet was resupended in 60 µl of HBL.

All the fractions were resupended in 5x SDS-PAGE sample buffer to 1x final concentration and loaded on 8% gel and further analyzed for the immunoblotting using rabbit polyclonal BAT3 antibody (anti-Scythe). The samples loaded in each

well correspond to 5 x 10^4 cells. As control, an anti-p53 blot was also performed to show the purity of the nuclear fraction and moreover to specify the point that the heat shock at different time points is non-lethal to cells.

The non-lethality of the heat shock cells were confirmed by trypan-blue staining and also by FACS analysis using Annexin V-FITC Apoptosis Detection kit I (BD Pharmingen).

mRNA Analysis and quantitative PCR

Total cellular RNA was isolated from heatshock treated and untreated 293T or immature dendritic cells by the Qiagen RNeasy Mini Kit according to the manufacturer's recommendations. Reverse transcription was carried out using the QuantiTect Rev. Transcription Kit (Qiagen). Quantification of the mRNA encoding for BAT3 was performed using LightCycler technology (Roche Diagnostics). Quantitative PCR was performed in a total reaction volume of 20μl using the QuantiTect SYBR Green PCR Kit. The following primers were used: BAT3-for: CTATTATCCAGCAGGACATTCAGAG; BAT3 rev: GCTAAGGATCATCAGCAAAGG and for the housekeeping gene c-abl, which served as internal control: c-abl-for: CTTCATCCACAGAGATCTTGCTG and c-abl-rev: ATACTCCAAATGCCCAGACG. Housekeeping gene and target gene were quantified simultaneously in duplicates in one LightCycler run, together with the appropriate non-template controls. The difference in RNA quality and quantity between samples was normalized as given by the ratio of the copy number of the target gene and the copy number of c-abl.

Immunoprecipitation of BAT3 and Hsp70 from the supernatants

293T cells were transfected with an expression construct encoding histidine-tagged BAT3 using lipofectamine (Invitrogen, Karlsruhe, Germany). 48 hours post transfection the cells were treated with a non-lethal heat shock at 42^oC for 30 minutes and recovered for 1-2 hrs at 37^oC. The heat shock supernatant was collected and incubated with rabbit polyclonal sera raised against BAT3 and Hsp70 monoclonal antibodies for one hour and followed by incubation with protein-A beads over night (buffering conditions were maintained using 10X Tris-Nacl). The beads were washed and subjected to immunoblotting for detection of BAT3.

Purification of Exosomes

Exosomes were purified as described elsewhere (Thery, Regnault et al. 1999), in brief 293T cells untransfected and transfected with BAT3 were exposed to a non-lethal heat shock at 42°C for 30 minutes followed by a recovery period at 37°C for two hours (BAT3-SN). Exosomes were purified from the supernatants by three successive centrifugations at 300 x g (5 min), 1200 x g (20 min) and 10 000 x g (30 min) to eliminate cells and debris, followed by centrifugation for 1 h at 100,000 x g. The exosomal pellet was washed once in a large volume of PBS, centrifuged at 100,000 x g for 1 h and re-suspended in PBS. Exosomes were confirmed by western blotting and FACS analysis. These were used for NK-cell activation assays.

Coupling of exosomes and FACS Analysis of exosome coated beads

Exosomes (30 μg) were incubated with 4.5 micron microsphere ploybead carboxylate latex beads (Polysciences) for 30 minutes at room temperature. The beads were washed once with PBS and blocked with 2% BSA in PBS for 40 minutes. Beads were washed again for 2 times with PBS and incubated with various human Ig fusion proteins and different antibodies as specified, and analysed on a Becton Dickinson FACS Calibur using Cell Quest Pro software. Beads alone were gated and isotype-matched antibodies were used as controls for the fluorescence analysis.

Immunofluorescence of dendritic cells

Monocyte-derived immature dendritic cells were stressed with heat-shock and were plated on poly-lysine coated cover-slips for overnight. The cells were washed with PBS and fixed with 4% para-formaldehyde for 15 min at 25 °C and also by ice-cold methanol for 10 min at -20°C. After the cells were washed with PBS, they were blocked with 10% bovine serum in PBS for 1 h at 25 °C and further incubated with optimally diluted primary antibodies for overnight at 4°C. Later, the cells were washed and the respective primary antibodies were detected using fluorescently labeled secondary antibodies, goat anti-mouse Alexa-flour 488 and goat anti-rabbit Alexa-flour 635 (Molecular probes, Invitrogen) diluted in blocking buffer. Hoechst 33342 was used to stain the DNA. After many washing steps, the cover-slips were mounted onto slides with mounting medium (aqua poly/mount – Polysciences, Inc). The slides were examined with FLUOVIEW FV1000 laser scanning microscope with an objective lens UPLSAPO 60X W NA:1.20. The images were obtained with the

respective wave-lengths (excitation-emission) of the dyes as; DAPI (405-461 nm), Alexa-fluor 488 (488-520 nm) and Alexa-fluor 635 (635-647 nm). The images were processed using softwares Image J and Adobe Photoshop.

ELISA binding assay of tumor cell and iDC supernatant to fusion proteins

Different human Ig fusion proteins (100ng/well) were coated on plates in duplicates and were incubated with histidine tagged purified protein (His BAT3), tumor cell and iDC supernatant derived from heat shock treated cells for 2 hours at room temperature and detected using rabbit polyclonal BAT3 specific antibody and the corresponding anti-rabbit secondary antibody.

BAT3 ELISA (Sandwich method)

For the determination of BAT3 in the supernatants released from the cells, a sandwich ELISA was established. A rabbit polyclonal antibody rose against the c-terminus of the BAT3 protein was coated on 96-well plates and incubated for overnight at 4°C. The plates were washed and blocked with 2%BSA in 1X PBS. After blocking, the supernatants obtained from cells treated differently were incubated for one hour at room temperature or 4°C overnight. Post incubation the plate was washed thoroughly and incubated with detection antibody (chicken polyclonal rose against n-terminus of BAT3 protein), followed by the respective anti-chicken secondary antibody.

Maturation of immature dendritic cells

The monocyte derived immature dendritic cells (iDCs) on 4th day were cocultured with activated NK cells (100 U/ml) at 5:1 ratio for 36-48 hours in medium (RPMI + 5% FCS + GM-CSF and IL-4) at 37°C and 5% Co_2. The cells were harvested and checked for the expression of dendritic cell markers that are specific for the maturation using flow cytometric analysis (FACS Analysis). Dendritic cells alone were used as the control for the co-culture experiments.

In another set of experiments the maturation was analyzed in presence of NK cell derived supernatants. NK cells were stimulated with exosomes derived from iDCs (allogenic) and the supernatants were collected. These were further subjected to cytokine release assay (to quantify TNFα and IFNγ secretion) and also used for the maturation of iDC (allogenic).

Cytokine release (IFNγ and TNFα) assays

$5x10^4$ primary NK cells were incubated for 48 hours with medium or exosomes derived from 293T cells either vector (mock) or BAT3 transfected (BAT3-SN) and with purified exosomes derived from dendritic cells. The NK cell derived supernatants were analyzed (final concentration 1:10 diluted) using IFNγ and TNFα -ELISA Detection Kits (R&D Systems, Wiesbaden Germany). The absorbance of the plates was measured using the ELISA-reader μ-Quant (Bio-Tek, Bad Friedrichshall, and Germany) in parallel with the measurement of the corresponding standards.

Cytotoxicity assays

The cytotoxicity was estimated in a standard 4h europium release assay (14 hrs assay when specifically mentioned) in a 96-well micro titer plate in a total volume of 200 μl with 5×10^3 target cells and at different effector: target ratios. When mentioned, NK cells (effector cells) were incubated with 30 % serum for 30 minutes at 4°C, washed and incubated with the monoclonal anti-NKp30 (R&D Systems, mAb1849) in the final concentration of 10 μg/ml for 1 hour at 4°C to block NKp30. NK cells were stimulated with immobilized or soluble recombinant BAT3 at a concentration of 1 μg/ml for 24-36 hours and then used as effector cells. The blocking anti-BAT3 serum (rabbit polyclonal) was used in a 1:1000 dilution. The MHC class I molecules on mature dendritic cells (preincubated with human serum) were blocked using HLA-A, B, C antibody (Mouse IgG1κ Clone: G46-2.6, BD Biosciences). In all the experiments, the spontaneous release did not exceed 25% of the maximum release of the target cells. The percentage of lysis of the target cells was calculated by the formula:

$$\text{Percentage of lysis} = \frac{\text{Experimental release} - \text{Spontaneous Release}}{\text{Maximal Release}} * 100$$

Statistics

The results of the NK cell-activation assays are indicated as means ± standard deviation. Significance was calculated with the GraphPadPrism software (San Diego, CA) using the student t test. Microsoft Excel was also used.

Bioinformatics

Primer design and analysis as well as elementary DNA and protein sequence analysis were performed with **DNA star.**

5 RESULTS

5.1 Interaction of BAT3 to NKp30

5.1.1 BAT3 is a direct binding partner for NKp30

To identify proteins interacting with NKp30, a fusion construct consisting of the GAL4-DNA-binding domain and the extracellular NKp30 sequence (pGBT9-NKp30) was used as bait. A K562-derived cDNA library was screened and a clone encoding the C-terminal sequence of BAT3 (BAT3-CT) was isolated. The cDNA fragment of 1.6 kb is characterized by a deletion of the highly conserved Bcl-2-associated athanogene (BAG) domain (Fig. 9A). This domain is responsible for the reported binding of BAT3 to heat shock protein 70 (Hsp70) (Takayama, Bimston et al. 1997; Thress, Song et al. 2001). Both isoforms, either including or lacking the BAG domain are expressed in tumor tissues, tumor cell lines and monocyte-derived dendritic cells (Fig. 9B). Analysis of BAT3 (full-length) and a panel of deletion constructs showed that the 1.6 kb C-terminal fragment was necessary and sufficient for the direct binding to NKp30, irrespective of the BAG domain. The NKp30-binding, however, may require a dimerization of BAT3 molecules, since a BAT3-BAT3 interaction was observed (Fig. 9A). Several control constructs which failed to interact with BAT3 or NKp30, respectively, were used to prove the specificity of the two hybrid results (as noted in the figure 9A, the extra cellular domain of NKp46).
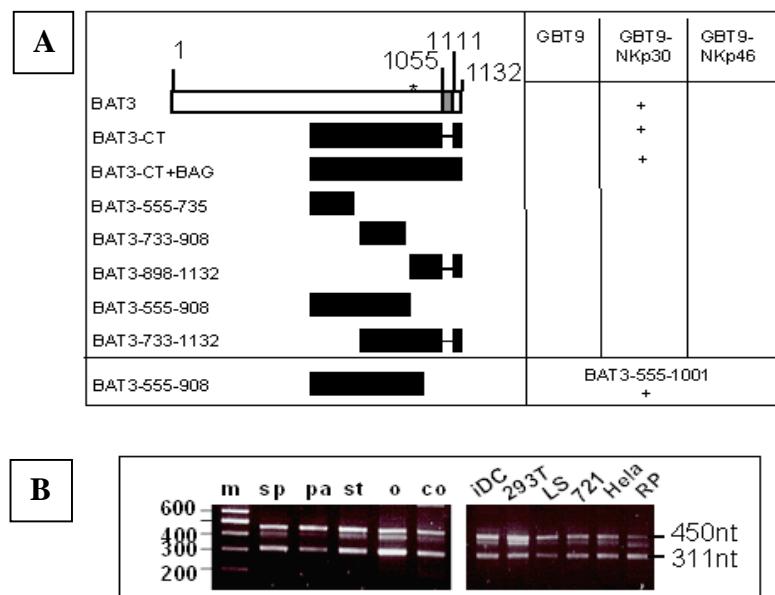
Figure. 9. Interaction between BAT3 and NKp30: Yeast two hybrid data

(A) The constructs tested for an interaction between BAT3 and BAT3-derived clones in the Yeast Two Hybrid System3 are listed. The 1.6 kb BAT3-CT fragment corresponds to amino acid 555 to 1132 of the BAT3 cDNA (NCBI accession number: gi:149158691). The caspase 3 cleavage site (*, pos.1001) and the BAG domain (shadowed region, pos. 1055-1111) are indicated. Interaction between the constructs is marked (+) and reflects activation of the GAL4-dependent reporter genes (clones growing on selection plates). No background interaction was detectable in the following control experiments: GBT9-NKp30 was replaced with the GBT9 vector or a GBT9-NKp46 construct. BAT3-derived clones were replaced with the pGADT7 vector or pGADT7 expressing DCoH (unrelated control cDNA, Pogge von Strandmann, 2000) (data not shown).

A specific interaction between BAT3 molecules was detectable using a BAT3-derived clone as bait (bottom line).

(B) RT-PCR to detect BAT3 transcripts in a panel of tumor tissues and primary iDCs obtained from peripheral blood monocytes. The primers to amplify the C-terminal fragment of the BAT3 cDNA hybridize up- and downstream from the BAG domain sequence, resulting in the amplification of a 311 bp (without BAG domain) or a 450 bp PCR product (including BAG domain). Both BAT3 isoforms and an intermediate transcript were detectable. No amplification products were detectable in the absence of reverse transcriptase or polymerase. The expression level of GAPDH estimated by RT-PCR was similar in all samples (not shown). M: marker, sp: spleen, pa: pancreas, st: stomach, o: ovary, co: colon, iDC: immature DCs, 293T, LS174T, 721.221, Hela, RPMI8226).

5.1.2 Human Leukocyte Antigen-B-Associated Transcript 3 (BAT3)

The gene encoding human BAT3 was originally identified within the inflammatory class III region of the human major histocompatibility locus on chromosome 6 (Banerji, Sands et al. 1990). The ability to distinguish self and non-self is mediated by major histocompatibility class I and class II molecules that are encoded towards telomeric and centromeric positions respectively on chromosome 6, a central interval of 1000 kilobases (kb) is termed as MHC class III region. BAT3 is thus clustered with many other immune regulatory genes such as genes encoding members of complement cascade, cytokines TNF factors α and β, heat shock protein HSP70 and the natural cytotoxicity receptor NKp30 (Fig. 10). Moreover, the previous literature suggests that the putative BAT3 promoter is very G + C rich and possess a heat shock element at position -125 (Bienz and Pelham 1987) and an another one within the first intron of the ubiquitin-like domain of BAT3. Thus BAT3 might be regulated by heat shock similar to the stress-response genes encoding HSP70 (Bienz and Pelham 1987; Banerji, Sands et al. 1990). The function of the heat shock elements in BAT3 is not clear so far.

BAT3 is described to be a nuclear protein and does not possess any of the qualities of a classical ligand such as; a trans-membrane domain or a signal peptide sequence. It is structurally characterized by C-terminal nuclear localization signals, an N-terminal ubiquitin-like region, a polyproline stretch and the conserved BAG (Bcl-associated anthogene) domain that interacts with HSP70 (Takayama, Bimston et al. 1997; Manchen and Hubberstey 2001; Thress, Song et al. 2001) (Fig. 10).

A role for BAT3 in regulating both proliferation and cell death has been discussed. BAT3 seems to trigger ricin-induced apoptosis (Wu et al., 2004) and BAT3-deficient cells are more resistant to apoptosis in response to agents affecting the calcium flux in the endoplasmatic reticulum (Desmots, Russell et al. 2005). Recently a gene depleting approach was used to show the precise function: Nuclear BAT3 has an essential role in controlling the acetylation of p53, which is required for the cellular DNA damage response (Sasaki et al., 2007).
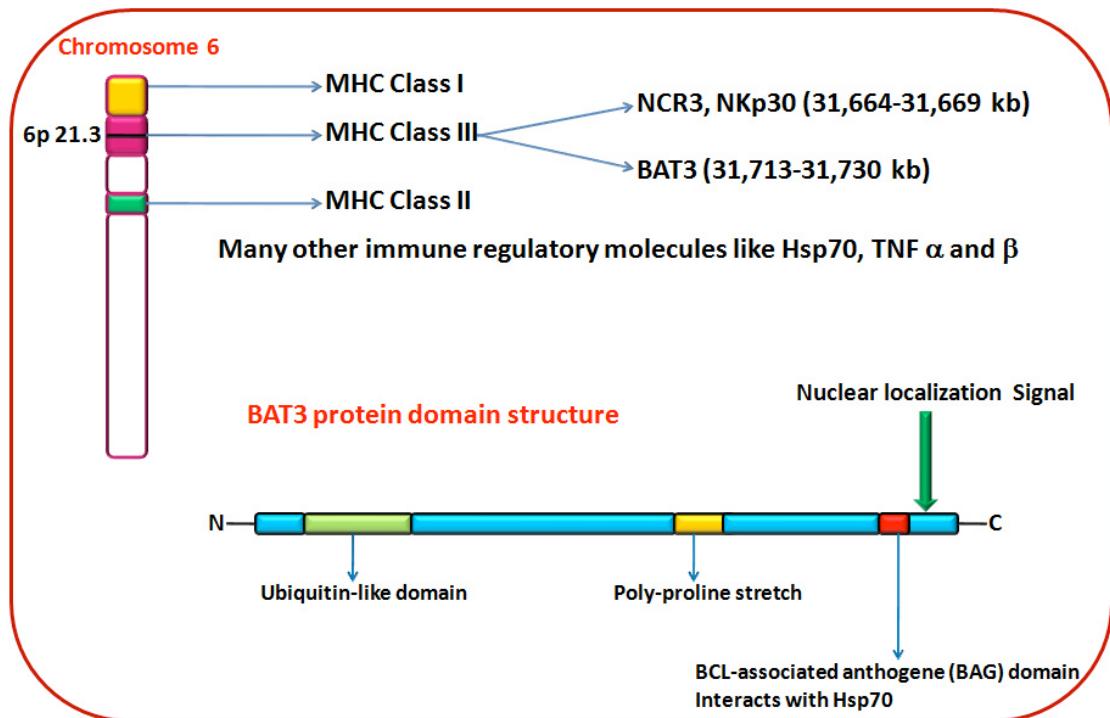
Figure. 10. HLA-B-Associated Transcript-3

5.1.3 BAT3 is released from tumor cells into extracellular environment

Since a nuclear factor is not accessible for the interaction with the surface receptor NKp30 it was tested directly, whether BAT3 was released from tumor cells. 293T cells were exposed to a non-lethal heat shock and the BAT3 distribution was monitored in sub cellular fractions and the cell supernatant (Fig. 11A). Endogenous BAT3 was predominantly expressed in the nuclear fraction. However, it was also detectable in the membrane fractions and cell supernatants in response to a non-lethal heat shock (Fig. 11A top). Overexpressed BAT3 was, similarly to the endogenous protein, detectable in the nuclei. Upon heat shock, BAT3 was found in the membrane and released from the cells (Fig. 11A middle). No changes were observed for the distribution of the nuclear protein p53 (Fig. 11A bottom). The number of apoptotic or necrotic cells was not increased by heat shock (data not shown), thus excluding the possibility that apoptotic or necrotic cells were the source for BAT3.
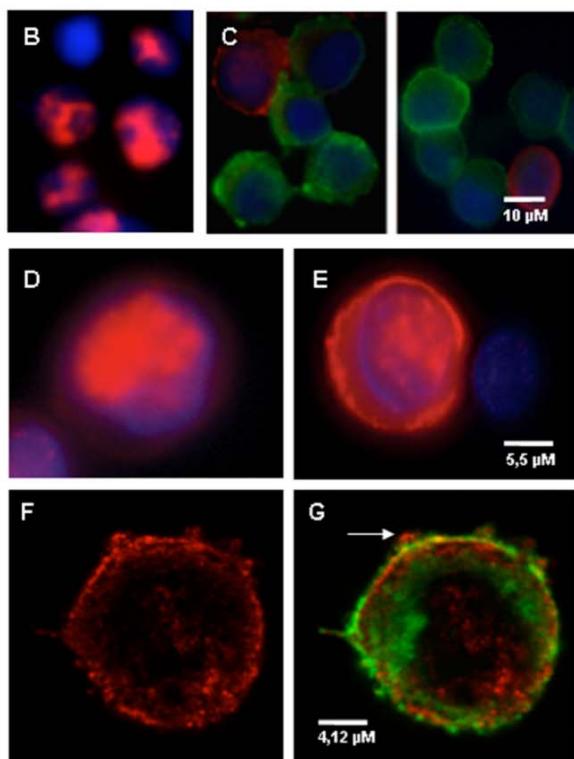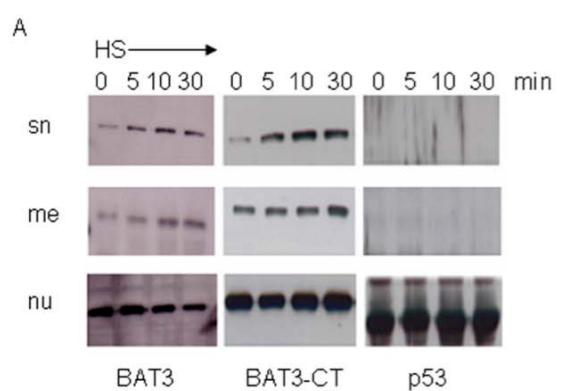
Next the expression pattern of BAT3 upon contact with NK cells or heat shock was analyzed. As expected a transfected histidine-tagged BAT3 protein ((His)$_6$ BAT3)

was mainly localized in the nuclei of 293T cells (Fig. 11B and D, red staining). Upon co-incubation with the human NK cell line NKL (Fig. 11C, green staining) or primary NK cells (Fig. 11E), this sub-cellular expression pattern changed dramatically. The nuclear BAT3 staining disappeared and instead staining of the cell membrane became evident. The surface expression and/or release of BAT3 are a prerequisite for a direct interaction with NKp30 and can thus be regarded as a part of tumor cells-NK cell interaction that leads to NK cell activation and tumor cell lysis. There is growing evidence that NK cell activation is a two-stage process and it was recently demonstrated that NK cell/tumor cell co-cultivation is a crucial step for the activation ("priming") of natural cytotoxicity (North, Bakhsh et al. 2007).

To confirm membrane expression, we stained the plasma membrane with FITC-labelled choleratoxin (FITC-CtxB). FITC-CtxB specifically stains the lipid ganglioside GM1 predominantly anchored in the outer leaflet of lipid raft domains (Harder, Scheiffele et al. 1998). Confocal microscopy revealed expression of BAT3 (Fig. 11F, red) in the plasma membrane compartment, since BAT3 and GM1 appeared partially co-localized (Fig. 11G, yellow). Taken together, these results provide evidence that BAT3 is released from living cells in an inducible manner. BAT3 is therefore at least transiently accessible for a direct interaction with NKp30 expressed on NK cells.

Figure. 11. Expression pattern of BAT3 in 293T cells

(A) Immunoblotting to detect BAT3 or p53 in proteins extracts (10 µg/lane) derived from supernatant

(sn), membrane (me) or nucleus (nu) of 293T cells, that were left untreated (0 min HS) or exposed to a heat shock for the indicated time (min HS: minutes heat shock). Left panel: endogenous BAT3, middle panel: transfected BAT3-CT), right panel: p53 blot. See supplemental data (S2A and S2B) for the BAT3 antiserum (rabbit).

(B-E) (His)₆BAT3 transfected 293T cells were grown on cover slips, left untreated (B, D) or incubated for two hours with NKL cells or primary NK cells (C, E) in the ratio of 1:20 and stained after acetone fixation with a monoclonal his antibody and a-mouse-cy3-labeled secondary antibody (red) (C) shows two examples for a co-staining of NKL cells with a CD30 antibody (anti-CD30-FITC, green), which specifically recognizes the CD30 surface receptor expressed on NK cells. Cell nuclei were stained with DAPI (blue).

Pictures were acquired with the digital Nikon Eclipse E800 microscope with the LuciaGF program (Nikon, Düsseldorf, Germany) using a 10x NA 0.17 (B,C) or a 60x NA 0.23 objective (D,E) and processed using Photoshop software (Adobe).

(F,G) Cytospins of (His)₆BAT3 transfected 293T were stained after acetone fixation with a-His-cy3 (a-his) to detect BAT3 (red, F) and with FITC-labeled choleratoxin (Ctx, green), which stains the plasma membrane of 293T cells (merge, G). Fluorescence images were acquired by confocal laser scanning microscopy (Leica) with oil objective at pinhole size Airy 1. Crosstalk was minimized by serial acquisition of the fluorescence color channels. The digital images were merged by Leica LSM software without further processing. 100x objective lens, NA 1,4, FITC and cy3 were excited by 488- and 543-nm laser light and emission was detected at 495-530 nm and 548-700 nm, respectively.

5.1.4 In vitro interactions of NKp30 with the tumor cell-derived and recombinant BAT3

Next experiments were performed to substantiate the claim that BAT3 is the natural ligand for NKp30. Firstly, the recombinant proteins NKp30-Ig and $His_{(6)}$ BAT3 were purified from the supernatants of 293T cells transfected with appropriate cDNA. The purification was further confirmed using western blot analysis (Fig. 12). Then a standard co-immunoprecipitation method was used to test the specific binding of BAT3 to NKp30. Wild-type 293T cells (WT) and a 293T cell line that constitutively expressed the extracellular domain of NKp30 fused to the Fc sequence of human IgG1 (NKp30-Ig) were transfected with BAT3-CT or BAT3 expressing constructs encoding histidine-tagged proteins. Both BAT3 constructs were co-precipitated by isolation of NKp30-Ig from the transfected cells using protein-G sepharose that binds to Fc sequence (Fig. 13). Unspecific precipitation was not detectable in the extracts of cells overexpressing the control protein hepatocyte nuclear factor 4 (HNF4) (left lane). These experiments proved the specificity of the BAT3-NKp30 interaction in mammalian cells and further supported the idea that BAT3 is a putative ligand for NKp30.
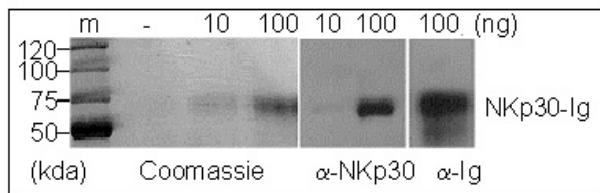

Figure. 12. Purification of NKp30 and co-immunoprecipitation of BAT3 and NKp30

NKp30-Ig protein was purified from the supernatant of 293T transfected cells for coomassie staining and immunoblotting. The purified NKp30-Ig protein (10ng or 100ng as indicated) was recognized by monoclonal NKp30 and by Ig antibodies.

Figure. 13. Co-immunoprecipitation of BAT3 and NKp30

NKp30-Ig protein was purified from the supernatant of 293T transfected cells for coomassie staining and immunoblotting. The purified NKp30-Ig protein (10ng or 100ng as indicated) was recognized by monoclonal NKp30 and by Ig antibodies.
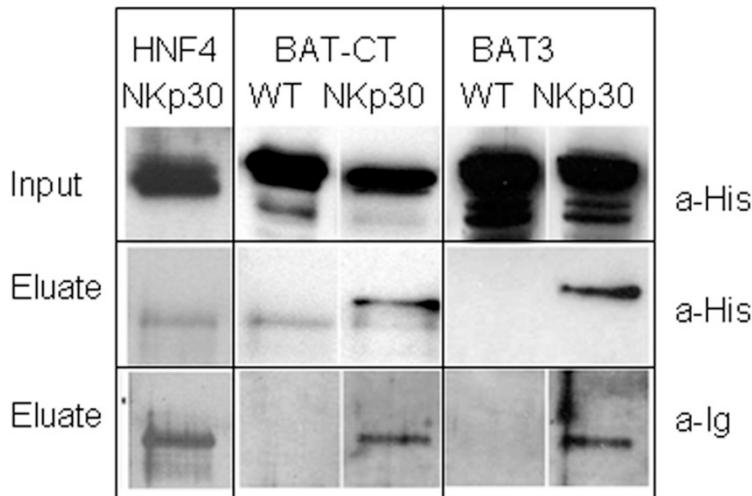
Wildtype 293T cells (WT) or NKp30-Ig expressing 293T cells (NKp30) were transfected with $(His)_6HNF4$ (control), $(His)_6BAT3$-CT (70 kda) or $(His)_6BAT3$ (>130 kda) and the lysates were analyzed for expression (a-His Blot, input). The lysates were used for immunoprecipitation with protein-G sepharose followed by immunoblotting of the eluates with monoclonal anti-his (a-His) or anti-Ig (a-Ig, eluate). In control experiments no protein precipitation was detectable with 293T cells, which constitutively expressed CD30-Ig or upon transfection of $(His)_6HNF4$ expression vector (left lane).

Since, a direct binding to NKp30, is a prerequisite for a possible biological function of released BAT3, the binding properties of BAT3-enriched supernatant and purified recombinant BAT3 protein were tested. The *in vitro* binding of BAT3 to NKp30 was demonstrated using a BAT3-specific ELISA. A specific binding to immobilized NKp30-Ig, but not to NKG2D-Ig and CD30-Ig, was observed for supernatant derived from BAT3 transfected cells (Fig. 14A, black bars) compared to control supernatant from wildtype cells (Fig. 14A, white bars). The ELISA revealed also a specific binding of purified BAT3 derived from BAT3-transfected cells (Fig. 14B, black bars) versus control purifications of wildtype cells (Fig. 14B, white bars) to NKp30-Ig. The weak binding of NKG2D-Ig to purified protein, anticipates that the

binding reflects unspecific protein interactions or might be attributed to traces of other soluble factors that were co-purified with BAT3.
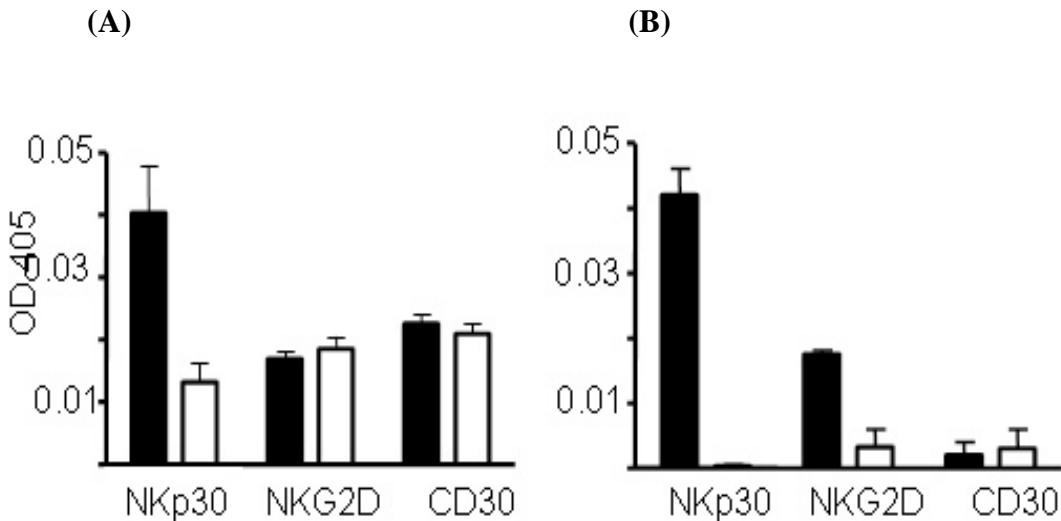
(A) **(B)**

Figure. 14. Binding Analysis by ELISA

ELISA plates were coated with recombinant NKp30-Ig, NKG2D-Ig or CD30-Ig (concentration 100 ng/ml) followed by incubation with 100 μl BAT3-enriched supernatant from BAT3-transfected 293T cells (black bars), control supernatant from wildtype cells (white bars) **(A)** or with 5 μg purified BAT3 protein (black bars) or with control purification from wildtype cells (white bars) **(B)** Binding was detected with anti-BAT3 serum and anti-rabbit-enzyme linked secondary antibody. Data represent absorbance at 405 nm after normalization to background of nonspecific binding to the plate. Error bars represent mean and standard deviation (SD) of triplicate samples.

Next it was tested, whether tumor-cell derived BAT3 (BAT3-enriched supernatant (BAT3-SN)) was able to bind to NKp30 on the surface of NK cells. Preincubation of the human NK-cell line NKL (Robertson, Cochran et al. 1996) cells with BAT3-SN, but not with a control supernatant (Fig. 15A, upper panel) blocked the binding of anti-NKp30 (the preincubation did not interfere with the binding of anti-NKG2D, not shown). The reproducible inhibition of NKp30 binding with BAT3-SN was even observed using fresh NK cells isolated from peripheral blood lymphocytes that also express NKp30 (Fig. 15A, lower panel).

Similar binding activity to NKp30 on NK cells was recently reported for pp65, the viral NKp30 ligand (Arnon, Achdout et al. 2005). In agreement, the inhibition of anti-NKp30 binding here presented for fresh NK cells was observed for BAT3-SN and pp65 (Fig. 15B). However, blocking of NKp30 using pp65 (kindly provided by Ofer Mandelboim) did not interfere with BAT3 binding to immobilized NKp30-Ig

(Fig. 15C) suggesting that BAT3 and pp65 bind to different, but not overlapping NKp30 domains. These binding studies demonstrated that tumor cell-derived BAT3 was able to bind to NKp30, and subsequently the functional consequences of this interaction were analyzed.
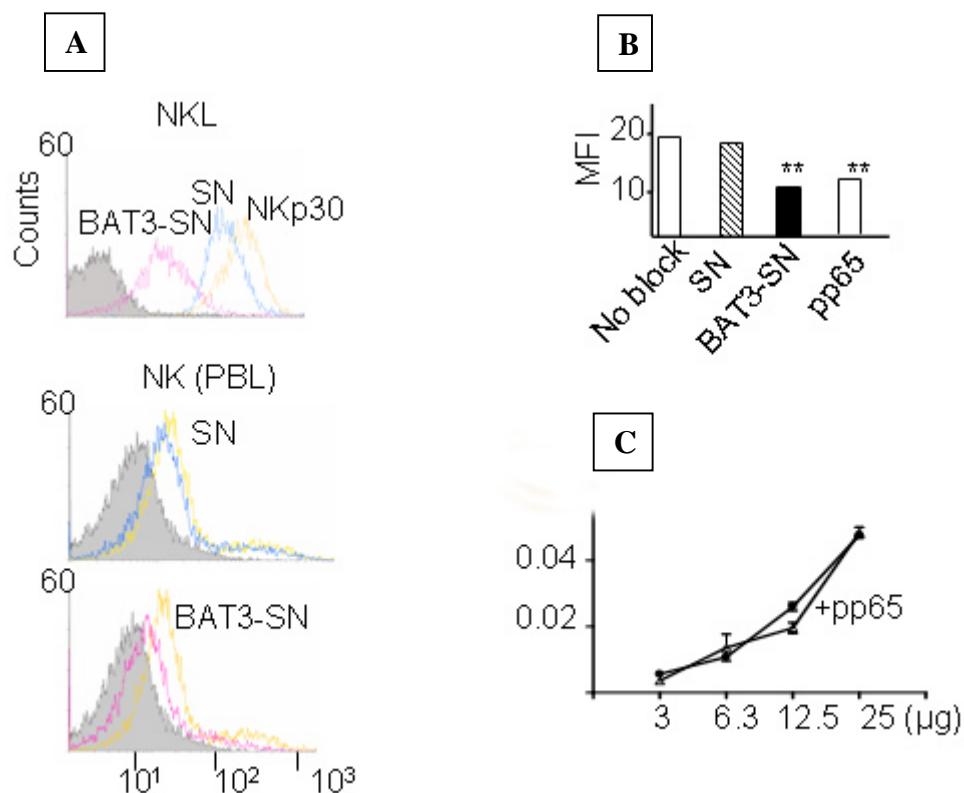

Figure. 15. Binding Analysis by Flow Cytometry

(A) FACS analysis to demonstrate the effect of BAT3-SN on NKp30 mAB binding to the human NK line NKL (upper histogram) and to fresh NK cells isolated from peripheral blood lymphocytes (NK (PBL)) (middle and bottom histogram). NKL cells and NK (PBL) were left untreated or preincubated with either control supernatant (SN) or supernatant of BAT3 transfected 293T cells (BAT3-SN) as indicated. Cells were stained with mAb to NKp30. Gray histograms: background secondary antibody staining; yellow: untreated NK cells, blue: cells blocked with SN, pink: cells blocked with BAT3-SN.

(B) The effect of BAT3-SN on anti-NKp30 binding to fresh NK cells bars indicate the mean fluorescence intensity (MFI). The BAT3-SN and pp65 mediated inhibition (presented for fresh NK cells) is significant (($P= 0.012;$ P= 0.042, respectively), unpaired t test, GraphPadPrism software).

(C) ELISA plates were coated with recombinant NKp30-Ig (concentration 100 ng/ml) followed by incubation with BSA (20 µg, filled triangle) or pp65 (20 µg, open triangle). All samples were incubated with recombinant BAT3 (3µg-25 µg) and binding of BAT3 was detected as described.

5.2 Regulation of NK-Cell Function by BAT3

5.2.1 Over-expression and down-regulation of BAT3 effects the Natural Killer Cytotoxicity

To investigate the direct functional properties of BAT3 on the prime activity of natural killer cells, loss and gain of function experiments were performed. A reduction of the BAT3 mRNA and protein amounts was achieved after transfection of Hela cells with the corresponding siRNA (Fig. 16A). The siRNA transfected cells were less efficiently lysed by peripheral blood derived NK cells than the control transfected cells (Fig. 16B). The decrease of NK cell-mediated lysis was reproducible using different donors, although the degree of inhibition varied. This reflects most likely the varying impact of triggering receptors and their ligands including NKp30, NKp46 and others depending on their expression profile. Interestingly, the blocking of NKp30-dependent lysis using a masking antibody was less efficient than BAT3 down regulation, particularly when the effector: target ratio was high (Fig. 16B). We speculate that this might be related to a direct or indirect BAT3-dependent engagement of triggering receptors distinct from NKp30. Vice versa, blocking using anti-NKp30 had still minor effects on NK cell mediated cytotoxicity of BAT3-siRNA Hela target cells (not shown). Remaining NKp30-mediated lysis upon BAT3 down regulation is not surprising, since siRNA down regulation of BAT3 is not complete (see Fig. 16A). In addition, it cannot be excluded that tumor cells express or co-express distinct ligands for NKp30, either on the cell surface or released that may interfere.

The overexpression of BAT3 in the colon carcinoma cell line LS174T induced an enhanced NK cell-mediated lysis (Fig. 16C). This effect was NKp30 mediated, because it was blocked by pre-incubating the target cells with the NKp30-Ig fusion protein in order to block NKp30 ligands. Collectively, these results suggest that BAT3 is an activating ligand for NKp30.
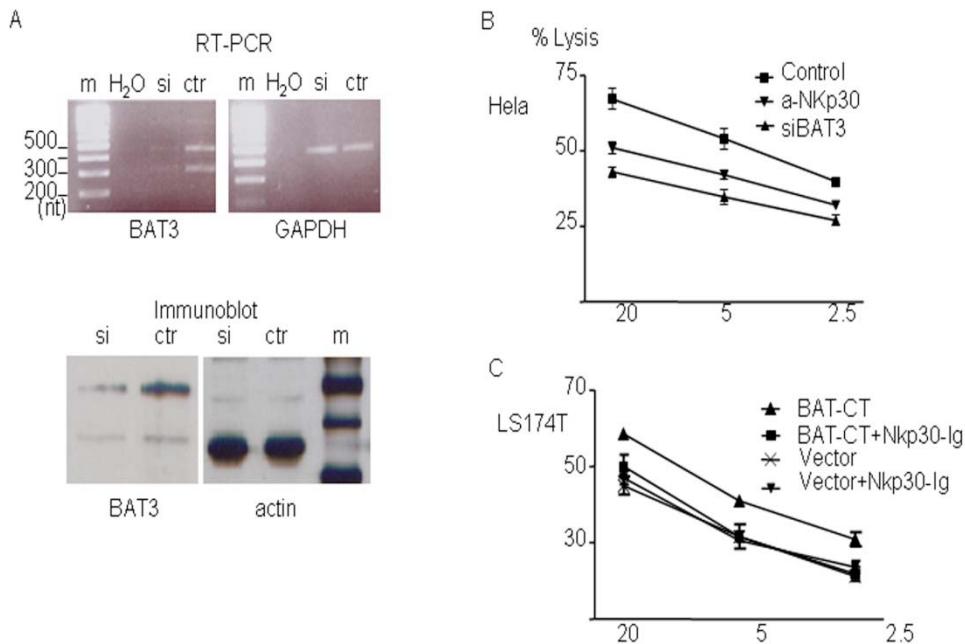
Figure. 16. BAT3-NKp30 mediated NK cell-cytotoxicity

(A) Down regulation of BAT3 expression in Hela cells. Hela cells were transfected with control siRNA or BAT3-siRNA and mRNA was isolated and analyzed for BAT3 and GAPDH expression by RT-PCR using gene specific primers. BAT3 specific primers flank the BAG domain and isoforms either lacking or including the BAG domain were detected (PCR products: 311nt and 450nt, respectively). Total protein lysates were prepared and analyzed for BAT3 and actin expression by specific immunoblotting. m: molecular weight marker; H_2O: control lacking cDNA, si: BAT3-siRNA, ctr: control.

(B) NK-mediated lysis of Hela cells. Hela cells either transfected with si control RNA or with BAT3-siRNA were incubated with primary NK cells at different effector:target ratios and the lysis of the target cells was determined in an europium release assay. An isotype and an NKp30 antibody were used to block lysis of the target cells and the NKp30-mab dependent lysis inhibition of BAT3-wildtype cells is indicated. The decrease of cell-lysis upon BAT3 down regulation is significant ($P=$ 0.0063*;* paired t test, one tailed, GraphPadPrism software).

(C) Primary NK cell-lysis of LS174T cells transfected with vector or BAT3-CT. LS174T cells were transfected with a control vector or a BAT3 expression vector. The target cells were pre-incubated with NKp30-Ig (15 µg ml^{-1}) prior lysis to block NKp30 ligands as indicated. Effector and target cells were co-incubated at different effector:target ratios. The increase of cell-lysis is significant for BAT3-CT transfected cells compared to vector transfected cells ($P=$ 0.0074), only if NKp30 ligands are not blocked with NKp30-Ig (paired t test, one tailed, GraphPadPrism software). Error bars represent mean $\pm$ s.d. of triplicate samples. CD30-Ig used as a control did not alter sensitivity of vector or BAT3-CT transfected cells. One representative experiment of three is shown (**B and C**).

5.2.2 BAT3 is crucial for NK cell mediated tumor lysis in vitro and in vivo

Moreover, the function of endogenous BAT3 was addressed *in vitro* and *in vivo*. BAT3 antibodies, that were able to effectively deplete BAT3 from the cell supernatant (Fig. 17A), were used to inhibit the NKp30-dependent lysis of Raji cells (Fig. 17B). Best results were obtained using an unusual long incubation period for the europium release assay (14 hours). This prolonged incubation period has also been described for the inhibition of NKp30 lysis by its viral ligand pp65 (Arnon et al., 2005).

Subsequently, the growth of RPMI8226-derived tumors was monitored in nude mice in the presence of human peripheral blood lymphocytes (PBL), either with a control antiserum (rabbit) or with blocking BAT3-specific antiserum (Fig. 17C). The rapid growth of subcutaneous tumors (8/10) could be suppressed completely by treatment with human PBLs (0/10). In contrast, the simultaneous injection of BAT3-specific antibodies resulted in a decreased tumor rejection (6/10), indicating that BAT3 is crucial for tumor cell recognition and killing in this model.
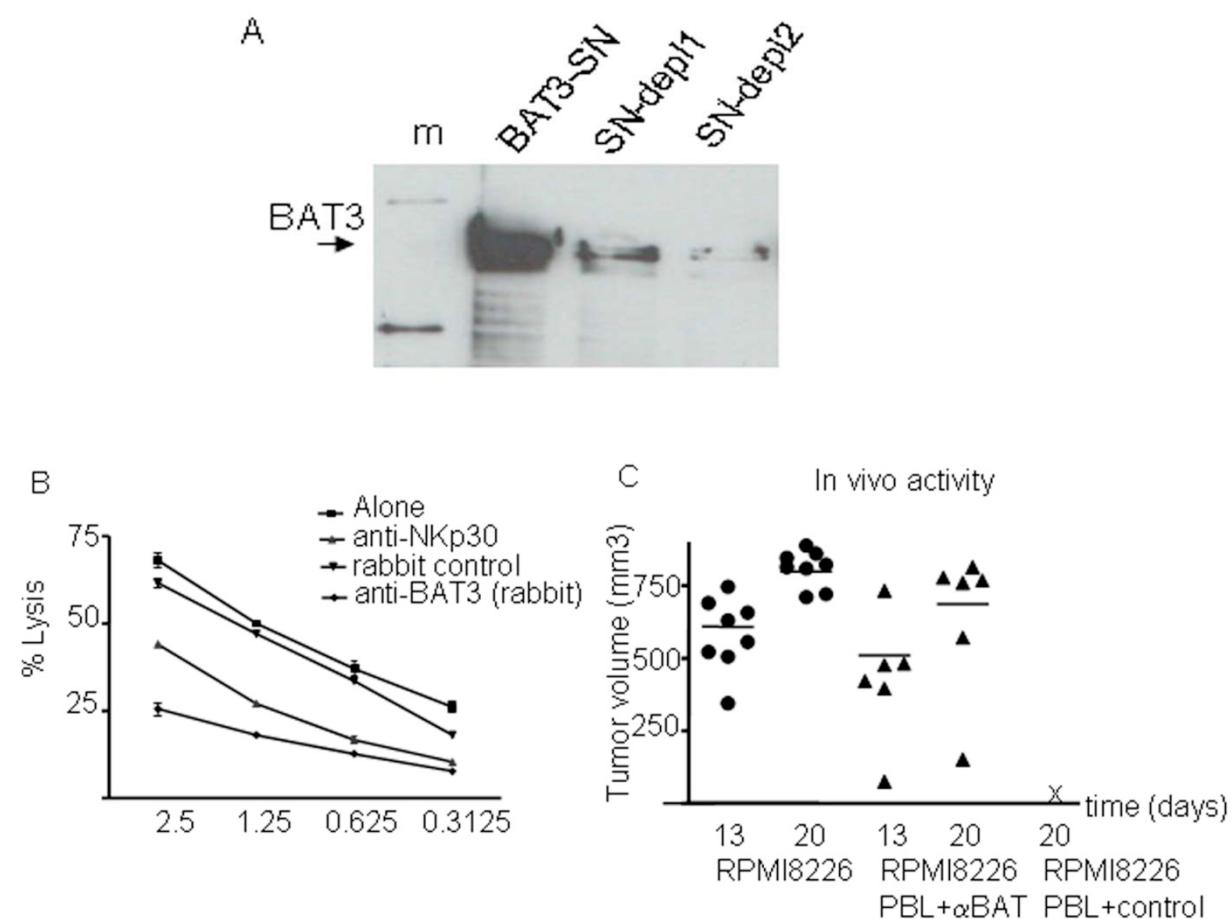
Figure. 17. In vivo activity of BAT3

(A) Two BAT3-specific rabbit antisera were used in a 1:5000 dilution to deplete BAT3 from 293T-derived supernatants. SN-depl2 was chosen for further experiments.

(B) Raji cells were incubated with primary NK cells for 14 hours at different effector:target ratios without competing antibodies (alone) or with an rabbit preimmune serum (rabbit control), anti-NKp30 and anti-BAT3. The % lysis was determined in a europium release assay.

(C) CD1 nude mice received $5x10^6$ RPMI8226 cells subcutanously alone (RPMI8226) or in combination with $5x10^6$ human peripheral blood lymphocytes that were either mixed 5:1 with 40µl control rabbit antiserum (RPMI8226+PBL+control Ab) or 40µl BAT3-specific antiserum (RPMI8226+PBL+anti-BAT3). The tumor volume at day 13 and 20 for each animal is indicated. Data from 2 independent experiments with 5 animals in each group are shown. The difference between RPMI8226+PBL+control versus RPMI8226+PBL+anti-BAT is significant (*P*=0.0168, day 20*)* (paired t test, one tailed, GraphPadPrism software).

5.2.3 BAT3-mediated cytokine release from NK cells

The secretion of IFNγ and TNFα from NK cells is crucial for the reciprocal activation of NK and dendritic cells and can be mediated through NKp30 (Pende, Parolini et al. 1999; Ferlazzo, Tsang et al. 2002; Vitale, Della Chiesa et al. 2005). Therefore we analyzed the influence of BAT3 on the NK cell-dependent cytokine release (IFNγ, TNFα). NK cells were incubated for 2 days with medium (med), control supernatant (mock) or supernatant containing BAT3 (BAT3-SN). The cytokine secretion was stimulated with BAT3-SN, and the stimulation could be blocked with NKp30 and BAT3-specific antibodies (Fig. 18A, left panels). The blocking was not always complete, particularly for the BAT3-antibodies, suggesting that factors distinct from BAT3 may also contribute to the NK cell-activation. Apparently paradoxically, the purified protein failed to induce any cytokine secretion per se, but rather revealed a signature of inhibitory effect. Here the co-incubation with BAT3-specific antibodies, which may alter the conformation or cross-link the purified recombinant BAT3, resulted in a strong cytokine release (Fig. 18A right panels). Control experiments with a purified soluble antigen and a specific rabbit antiserum revealed that the activation was not mediated via antigen-antibody complexes e.g. via CD16 (Fig. 18B). Thus, the BAT3 released from tumor cells activates the cytokine release, whereas the purified soluble protein inhibits the NK cell-dependent cytokine release.
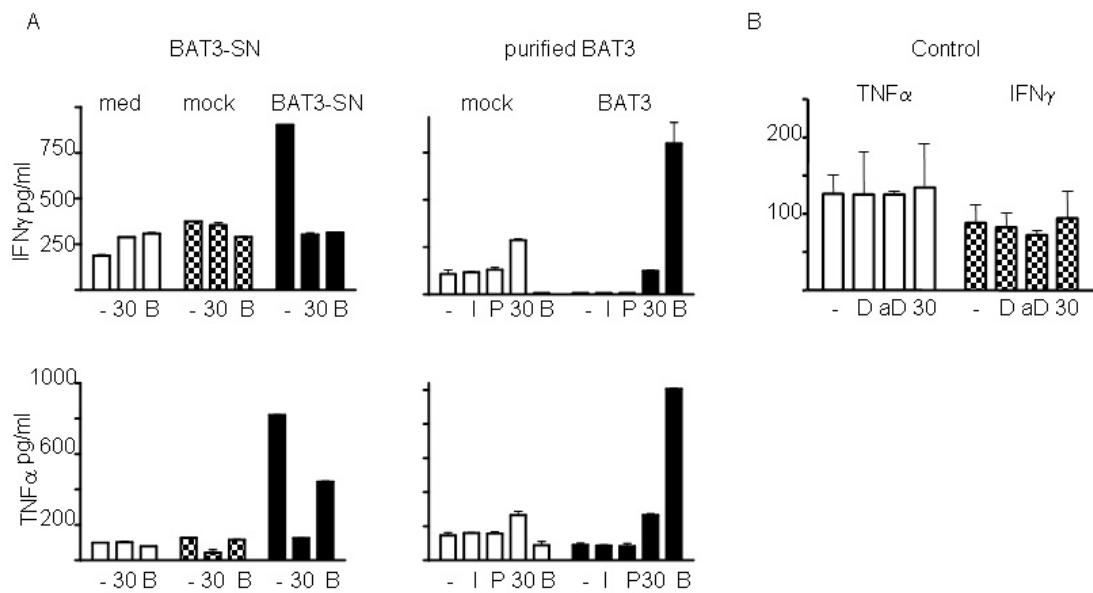
Figure. 18. NK cell-dependent cytokine release in response to BAT3

(A) Primary NK cells were incubated for 48h with medium (med), supernatant derived either from vector transfected cells (mock) or from BAT3-transfected 293T cells (BAT3-SN) (left panels) or with purified protein derived from mock or BAT3-SN, respectively (right panels). The following antibodies were added as indicated: 30: NKp30; B: BAT3 (left panels: chicken, right panels: rabbit); I: NKp30 isotype; P: rabbit pre-immune serum. The NK cell-derived supernatants were analyzed for IFN-γ and TNF-α content by specific ELISA. The means of triplicates and SD (pg/ml) are indicated. One representative experiment of three is shown.

(B) Primary NK cells were incubated with medium (-) or with a purified control protein DCoH (D) alone and in combination with a DCoH-specific antiserum (aD) or NKp30 (30) and the supernatants were analyzed for TNF-α and IFN-γ.

5.2.4 Effect of purified recombinant BAT3 on natural killer cytotoxicity

The above data demonstrated that recombinant purified soluble BAT3 inhibits and immobilized BAT3 activates NK cells with respect to cytokine release. So far, the influence of purified BAT3 on cytotoxicity has not been analyzed. To understand the direct effect of NK cells to lyse Raji cells, NK cells were purified freshly from isolated PBMCs of a buffy coat and stimulated with purified recombinant BAT3 as soluble and immobilized proteins. An unusual protocol of the europium-release assay with a prolonged incubation period of 16 hours revealed that NK-cell-dependent lysis

of Raji cells was inhibited when NK cells were stimulated with purified soluble protein HisBAT3 (Fig. 19A). The inhibitory function of His BAT3 was comparable to the Nkp30-antibody block and the control protein His BB4 did not alter any effect. A similar reduction in NK-cell cytotoxicity was also reported for the viral ligand pp65 (Arnon, Achdout et al. 2005). On the other hand, the immobilized BAT3 has an opposite effect by enhancing the cytotoxicity of NK cells against the target cells compared to the control protein HisBB4 (Fig. 19B). In line with the previous data, released BAT3 purified from cell supernatants enhanced the NK-cell cytotoxicity (Fig. 19B). Thus, let us hypothesize that BAT3 is released as a complex structure into the supernatant. Tumor-derived Hsp70-containing exosomes have the ability to regulate the migratory and cytolytic activity of NK cells (Gastpar, Gehrmann et al. 2005). Due to the interaction of Hsp70 and BAT3, it is tempting to speculate that BAT3 is secreted along with Hsp70. The exosomes are microvesicles with a complex structure that have the ability to enhance the activity and communicate among the cells in the immune system.

Moreover, it was described that the NK-DC crosstalk leads to the activation of NK cells and subsequently leading to the iDC maturation dependent on NKp30 (Vitale, Della Chiesa et al. 2005). Thus, we next asked, the role of soluble protein on the maturation of iDCs in NK-DC-co-culture experiments by analyzing the expression of the co-stimulatory molecule CD86. Co-cultivation of NK cells with monocyte derived iDCs leads to the upregulation of CD86. The soluble recombinant BAT3 has a great impact on the NK-cell-mediated adaptive immunity by inhibiting the maturation of iDCs, mediated by NKp30 (Fig. 19C).
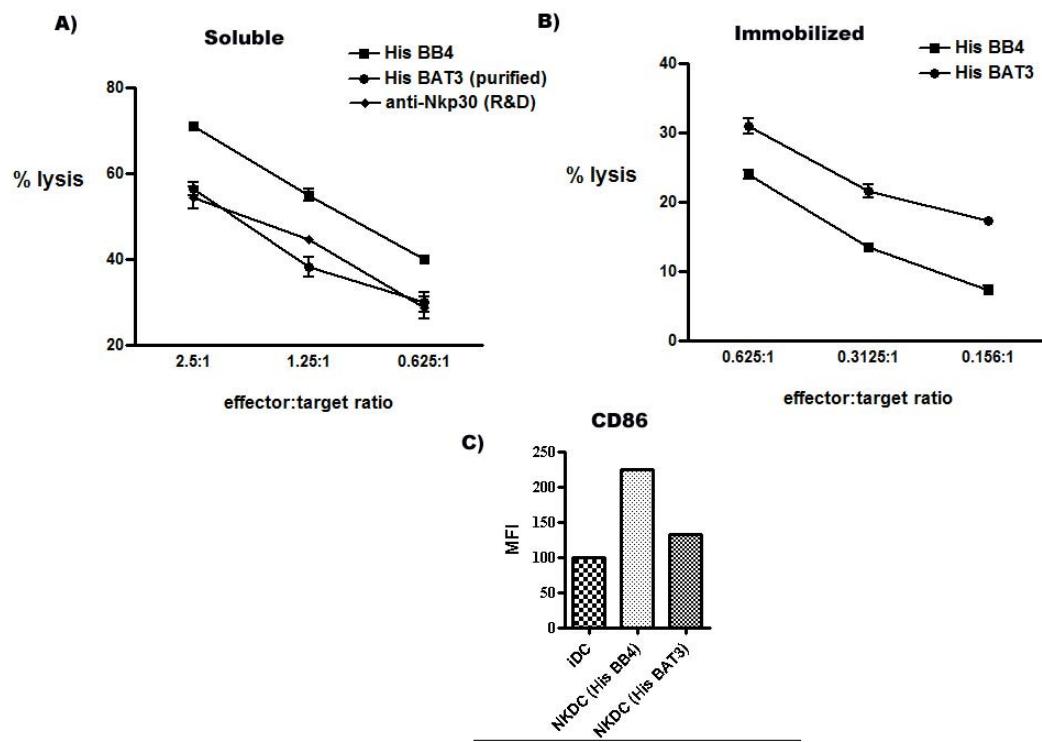
Figure. 19. Activity of recombinant purified BAT3

Purified histidine tagged proteins were used for this experiment. (a) NK-cell mediated lysis of Raji cells. Raji cells (target cells) were labeled with europium and incubated with 10U/ml activated primary NK cells at different effector:target ratios for an unusual period of 16 hours at 37^0C, and the lysis was determined by the europium release. An antibody to NKp30 was used to block the specific lysis of Raji cells and thus the NKp30 dependent lysis inhibition of purified BAT3 was indicated. His BB4 was used as the control and the decrease of the target lysis was significant with soluble purified BAT3. (b) The effector NK cells were prestimulated with immobilized control HisBB4 and purified BAT3 and the lysis of the Raji cells was observed by europium release assay. At different effector:target ratios immmbolized BAT3 altered an enhanced lysis with respect to the control HisBB4. (c) Maturation of iDC was observed in presence of purified proteins. As a read out the co-stimulatory molecule CD86 was analyzed using flow cytometric analysis on dendritic cells. Co-culture of iDCs with activated NK cells at 5:1 ratio promotes the maturation of iDCS as shown by the enhanced expression of CD86 and this effect is inhibited with the soluble purified BAT3. The CD86 expression is indicated by the mean fluorescence intensity (MFI) on the y-axis.

One representative experiment of three is shown.

5.3 Biochemical characterization of BAT3

5.3.1 BAT3 is secreted and present on the surface of the exosomes

The underlying mechanisms of BAT3 on NK-cell function as an inhibitor in soluble form and activating in immobilized form was directly tested whether the released BAT3 is present in the exosomal fractions. Exosomes were purified by ultracentrifugation of the supernatants obtained from the human fibroblast kidney-cell line 293T. The cells were stressed with heat shock to enhance the secretion of BAT3 into the supernatants. After ultracentrifugation, the exosomal pellet was washed with PBS once and finally resuspended in PBS. The western-blot analysis revealed that exosomes secreted from tumor cells contain BAT3, as well as Hsp70 and Lamp2 that were used as control proteins (Fig. 20A). Next, the expression of BAT3 on the surface of the exosomes was assayed using flow-cytometric analysis. Purified exosomes were incubated with latex beads of 4 µm in diameter and stained with different antibodies against BAT3, CD9, Hsp70, Lamp-2 and the ligands for NKG2D (Fig. 20B). The expression of BAT3 and CD-9 (a tetra spin) was clearly detectable on the surface of the exosomes, whereas the surface staining of Hsp70 and Lamp-2 was weak. At the same time, the ligands for NKG2D receptor were undetectable using NKG2D-Ig staining (not shown), suggesting that NKG2D activation is independent of exosomes derived from 293T cells. These experiments suggest that BAT3 in exosomes act as a membrane-associated molecule, that directly interacts with its specific receptor NKp30 resulting in NK cell activation. To verify the accessibility of exosomal BAT3 to NKp30 the binding of recombinant NKp30-Ig-fusion protein to BAT3 on exosomes was analyzed using flow cytometry. The recognition of NKp30-Ig to BAT3 exosomes was specific and no binding of the controls human-Ig and CD30-Ig (Fig. 20C) was observed. Interestingly, NKp46-Ig also binds to such an extent that it provides a hint to its ligands in the vesicles. The above data may explain the mechanisms involved in the crosstalk of NCRs-activating the NK cell function (Augugliaro, Parolini et al. 2003).

Until now, the functional importance of Hsp70 and BAT3 interaction was analyzed with regard to the regulation of apoptosis (Thress, Song et al. 2001). Due to the fact that both molecules are released into the cell supernatants and regulate NK-cell activity, their interaction was tested in tumor-cell-derived supernatant after applying a non-lethal heat shock to cells. As expected, there is an enhancement of

BAT3 secretion upon heat shock. BAT3 was precipitated with antibodies and detecting HSP70 (polyclonal BAT3 and monoclonal HSP70), suggesting that there is a BAT3/HSP70 complex (Fig. 20D). Gel filtration analysis on the heat-shock supernatant of 293T cells show the presence of Hsp70 and BAT3 in the same fractions of void volume (unpublished data). These data confirm that BAT3 along with Hsp70 is secreted into the extracellular environment as exosomes and that they may act in combination.

Figure. 20. Bio-chemical charachterization of the released BAT3

BAT3 was released from the heat shock treated cells and subjected for the purification of exosomes. (a) The exosomal fractions (30 μg) and the cell lysate (10 μg) were loaded on SDS-PAGE gel and
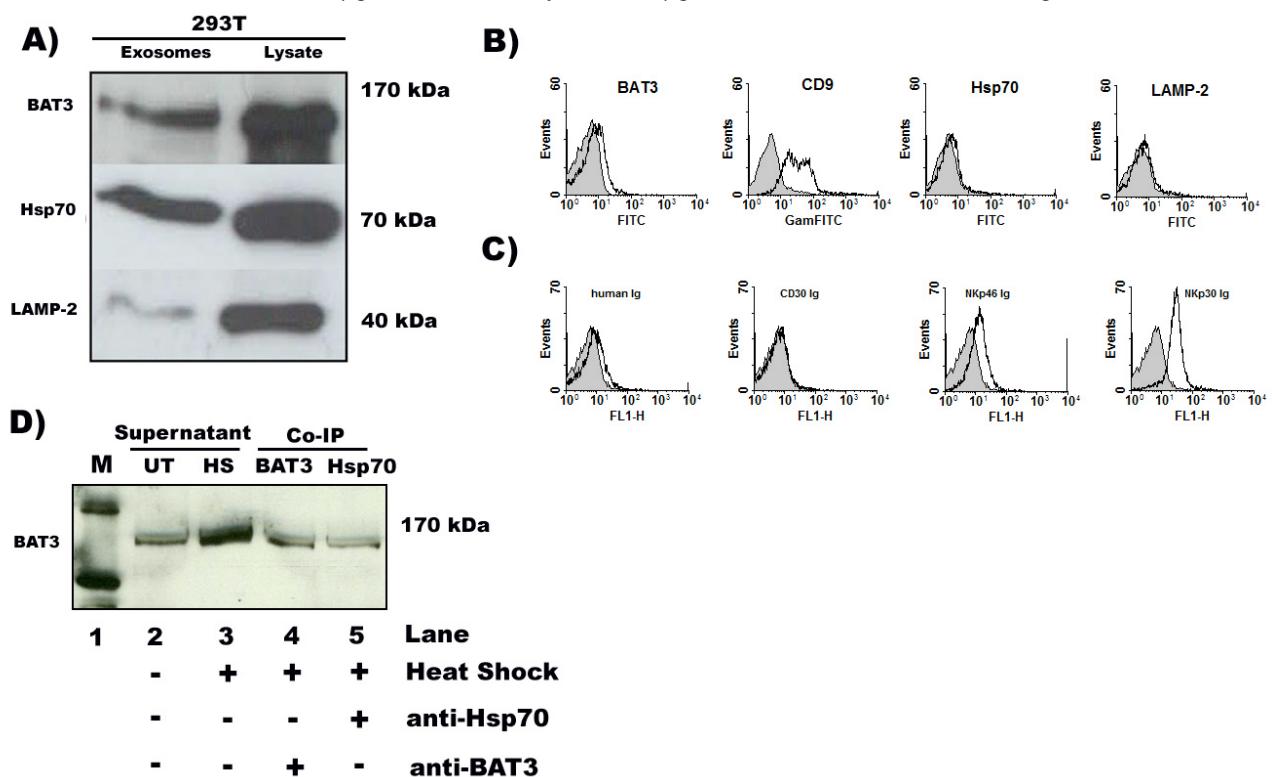
subsequent immunoblotting was done to detect the markers of exosomes Hsp70 and Lamp-2. Indeed, BAT3 was also detected in the exosomal fractions. (b) FACS analysis to demonstrate that BAT3 is expressed on the surface of the exosomes loaded on latex beads. As indicated, BAT3 and CD9 express on the surface and the other markers were very poor or absent. The grey back ground is the beads alone loaded with exosomes but no stained with antibodies. (C) FACS analysis to demonstrate the binding of NKp30-Ig to the surface expressed BAT3 on exosomes purified from BAT3 transfected cells. The background grey histograms indicate the beads alone coated with exosomes. (d) Western blot analysis demonstrates that the enhanced secretion of BAT3 into the supernatant obtained from tumor cells when treated with heat shock (HS, 3rd lane) or left untreated (UT, 2nd lane). The second half of the western blot demonstrates the co-immunoprecipitation of BAT3 by using polyclonal BAT3 antibody (4th lane) and also when a monoclonal ab against Hsp70 was used (5th lane).

5.3.2 Exosomes activate NK cells in a BAT3 dependent manner

One of the important biological effects of NK-cell-mediated triggering is related to cytokine production. Next it was analyzed the role of tumor derived BAT3 exosomes for NK-cell activation. NK cells were purified from PBMCs of healthy donors and stimulated with exosomes. The supernatants were collected and subjected to ELISA. 293T-derived exosomes stimulated NK-cell activity with a strong release of cytokines TNFα (Fig. 21A) and IFNγ (Fig. 21B). To further prove that cytokine release was due to BAT3, 293T cells were overexpressed with BAT3 and the exosomes were purified. When compared to the wild type (untransfected), BAT3 release into the exosomes was enhanced by over expression as demonstrated by western blot (Fig. 21C) and flow cytometry (Fig. 21D). Similarly, cytokine release from NK cells was enhanced in the case of over-expressed exosomes (Fig. 21E and 21F). Thus, the activity correlates to the BAT3 expression level on the exosomal surface suggesting that BAT3 is one of the main factors of exosomes in the regulation of NK-cell activity.

In addition, the role of BAT3 was observed by loss of function. Studies suggested that down-regulation of BAT3 effects NK-mediated cytotoxicity (this work Fig. 16B). The reduction of BAT3 in protein levels and exosomes was achieved by using specific siRNA of BAT3 (commercially available from Qiagen) (Fig. 21G and 21H). The expression of Lamp-2 on these exosomal fractions remained unchanged (data not shown). Interestingly, NK cells that were treated with exosomes BAT3-depleted, failed to produce TNFα and IFNγ, whereas a robust release was normal with control exosomes (Fig. 21I and 21J). The reduction in cytokine release was reproducible with different NK-cell preparations of healthy donors; although the reduction varied from one experiment to another. Differences may depend on the degree of NKp30 expression and its ligand BAT3 on exosomes. In summary, the results imply that the exosomal BAT3 derived from tumor cells have a triggering function on NK-cell regulation.
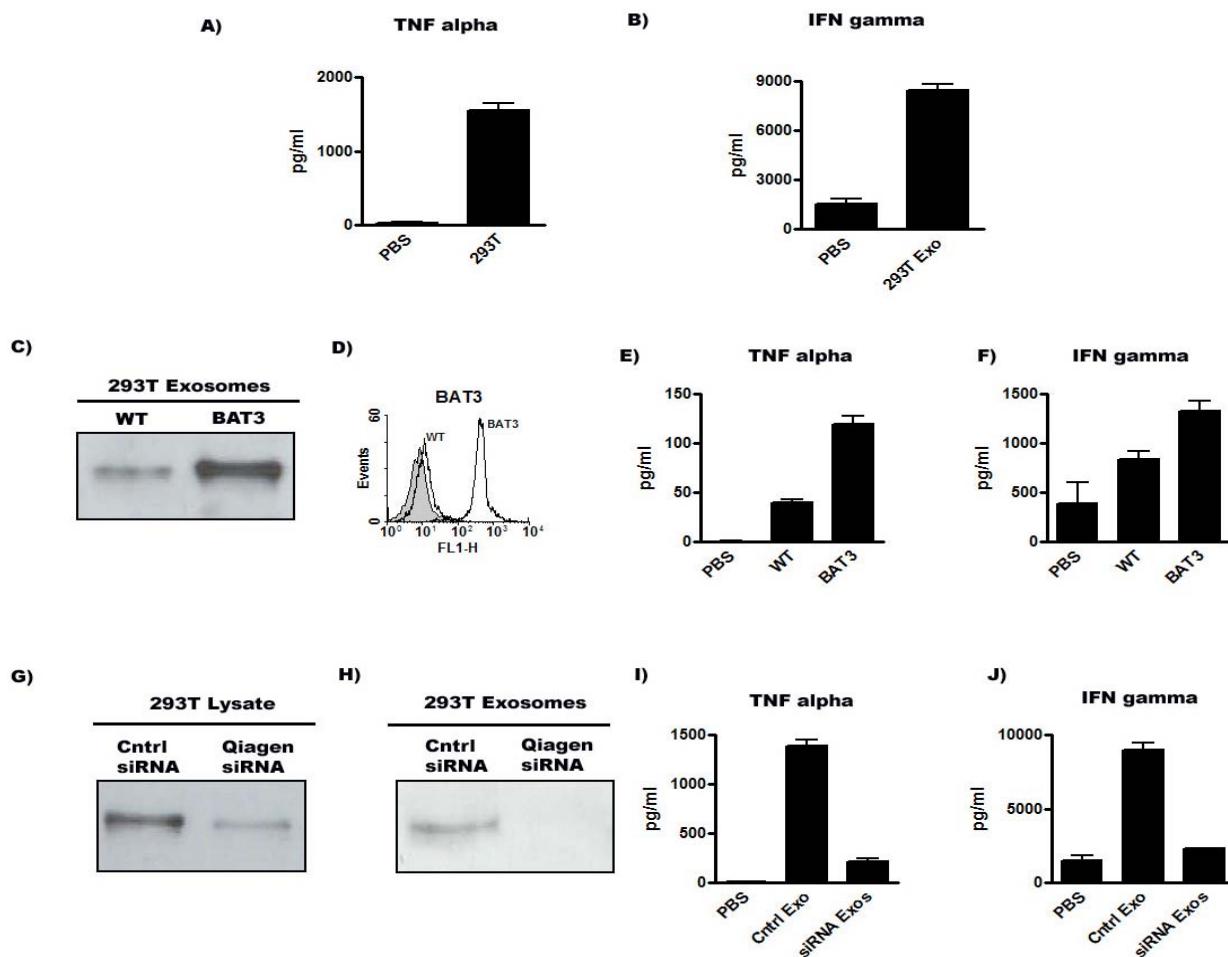
Figure. 21. Biological activity of tumor derived exosomes

Tumor derived exosomes activate NK cells. (a and b) ELISA detection of the cytokine release from NK cells, TNF-α (a) and IFN-γ (b). PBS and purified exosomes derived from tumor cells were incubated with 5×10^4 NK cells for 36-48 hours and the supernatants were collected and subjected to specific ELISA. The means of the duplicates and the concentration (pg/ml) were indicated. One representative experiment of five is shown. **Over expression of BAT3 in exosomes (c-f)** Exosomes were purified from untransfected (wt) and BAT3 transfected 293T cells (BAT3). As indicated by western analysis (c) and FACS analysis (d) there is an enhanced expression of BAT3 on the exosomal surface when over-expressed. The cytokine specific ELISA demonstrates the release of TNF-α (e) and IFN-γ (f) from the NK cells was enhanced with the respect to the over-expressed exosomes. **Depleted expression of BAT3 in exosomes (g-j)** Downregulation of BAT3 expression in 293T cells using siRNA technology was demonstrated by western blot analysis (g), western blot analysis shows that there is no release of BAT3 exosomes purified from the cells transfected with siRNA specific for BAT3 (h) The cytokine specific ELISA demonstrates the release of TNF-α (i) and IFN-γ (j) from the NK cells was inhibited when stimulated with depleted exosomes (siRNA Exos). The release was normal with untreated exosomes (Cntrl Exos). One representative experiment of three is shown.

5.3.3 BAT3 is expressed in immature dendritic cells and also released as exosomes

As a cellular ligand of NKp30, BAT3 has a major role in regulating NK cells. Previous studies describe that NKp30 plays a crucial role in triggering NK-mediated cytotoxicity and inducing maturation of iDCs by engaging undefined ligand(s) (Ferlazzo, Tsang et al. 2002; Vitale, Della Chiesa et al. 2005). In this context, we analyzed the expression of BAT3 in immature dendritic cells and its possible role. The iDCs were differentiated from monocytes derived from peripheral blood mononuclear lymphocytes (PBMCs). Importantly, we found that BAT3 is expressed as well in iDC lysate and released into the extracellular environment when stressed with a heat shock (Fig. 22A). To verify the recognition of released BAT3 by Nkp30, we examined the interaction using enzyme-linked immunosorbent assay (ELISA). The human immunoglobulin G1 (IgG1) fusion proteins NKp30-Ig and NKp46-Ig were immobilized and the supernatant was incubated and finally detected using anti-BAT3 antibody. As demonstrated, there is a specific interaction to NKp30-Ig with a ligand in the DC-derived supernatant. No binding of the supernatant was observed for NKp46-Ig as compared to the buffer control (Fig. 22B). This data supports the previous literature that NKp30 but not NKp46 is involved in the NK-DC crosstalk (Ferlazzo, Tsang et al. 2002).

Taking forward the concept of a specific interaction, we analyzed the exosomes derived from iDCs. On the 5th day, the cells were stressed with heat shock and then recovered for 1-2 hours at 37°C and the supernatants were processed for exosome purification. The western-blot analysis showed that BAT3 and the exosomal markers are detectable in the exosomal fractions (Fig. 22C). The flow cytometry of iDC-derived exosomes (Fig. 22D) demonstrates strong surface expression of BAT3, CD9, HLA- A, B & C, and HLA-DR and a weak or no surface expression of Hsp70 and Lamp-2. The co-stimulatory molecule CD86, expressed on iDCs, is also present on the surface of iDC-derived exosomes but not on tumor-derived exosomes (data not shown). The functional importance of iDC-derived exosomes has been elicited by potent T-cell-dependent antitumor immune responses *in vivo* quoting them as novel cell-free vaccines (Zitvogel, Regnault et al. 1998; Thery, Regnault et al. 1999; Chaput, Flament et al. 2006). Thus, one could postulate BAT3-containing exosomes derived from iDCs also regulate NK cells thereby enhancing the adaptive immunity.

Upon stress with heat shock, the sub-cellular fractionation of tumor cells described that BAT3 is present in both membrane and supernatant fractions (Fig 11). Results from laser scanning microscopy of immature dendritic cells treated with heat shock demonstrate that BAT3 is expressed predominantly in the cell nucleus. BAT3 is also detectable in the cell membrane. Interestingly, a co-localization with MHC class I molecules (HLA-A, B, and C) on the membrane was observed (Fig. 22E). Taken together, the expression profile of BAT3 in iDCs fits to a potential NKp30 ligand.
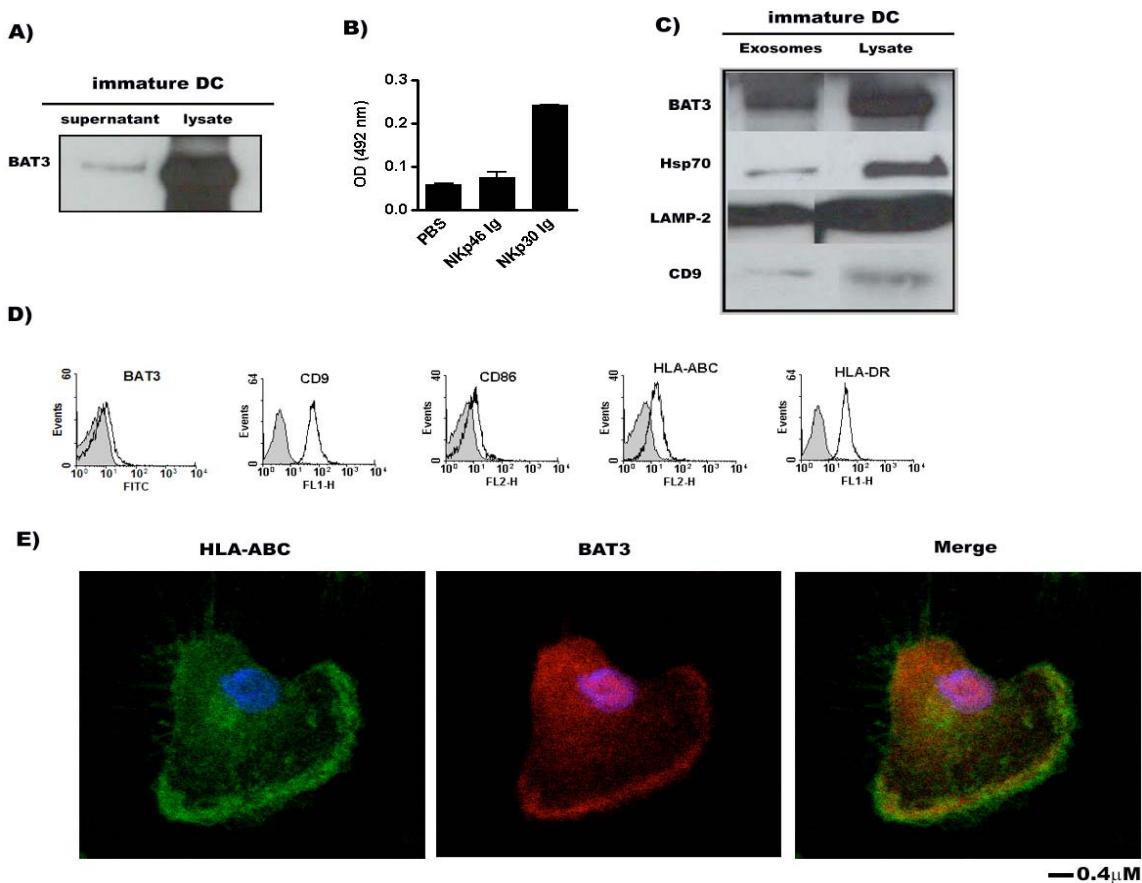
Figure. 22. Expression analysis of BAT3 on immature dendritic cells.

Expression of BAT3 **(a)** Total lysates and supernatant were prepared and analyzed for BAT3 by specific immunoblotting. **(b)** ELISA plates were coated with recombinant proteins, buffer control (Neg), NKp46-Ig and NKp30-Ig (concentration of 100 ng/ml) followed by incubation with 100 μl of concentrated supernatant obtained from heat shock treated iDCs. Binding was detected with anti-BAT3 serum followed by incubating with anti-rabbit-enzyme linked secondary antibody. Data represents absorbance at 492nm after normalization to background of nonspecific binding to the plate. Error bars indicate the standard deviation for the duplicate samples. One representative experiment of three is shown. **(c)** The exosomal fractions (30 μg) and the cell lysate (10 μg) were loaded on SDS-PAGE gel and subsequent immunoblotting was done to detect the markers of exosomes Hsp70, Lamp-2 and CD9. BAT3 was also detected in the exosomal fractions. **(d)** FACS analysis to demonstrate that BAT3 is expressed on the surface of iDC derived exosomes loaded on latex beads. As indicated, BAT3 is expressed on the surface and the other positive markers also expressed. The grey back ground is the beads alone loaded with exosomes stained with isotype control antibodies. **(e)** Laser Scanning Microscopy to demonstrate the co-localization of HLA- A, b, and C and BAT3 on dendritic cells. Cells were treated with heat shock and stained with antibodies; HLA-A, B, and C (left-green) and BAT3 (middle-red). The right image is the merge of the two molecules where the arrow indicates the co-localization on the membrane (yellow). The blue color indicates DAPI staining of nuclei and the pink indicates the co-localization of BAT3 in the nucleus.

5.3.4 BAT3 is up-regulated and DCs release functionally active BAT3 exosomes in response to heat-shock

BAT3 is clustered with many other immune regulatory genes such as genes encoding members of complement cascade, cytokines TNF factors α and β, heat-shock protein HSP70 and the natural cytotoxicity receptor NKp30. The putative BAT3 promoter is not analyzed functionally but characterized by a G + C rich bases and possess a heat shock element at position -125 (Bienz and Pelham 1987) and an another one within the first intron of the ubiquitin-like domain of BAT3. We next tested whether the induction of BAT3 protein in response to heat shock correlates with a transcriptional increase of BAT. Thus, we directly measured the expression of BAT3 in mRNA level using quantitative PCR analysis. Upon heat shock, both 293T and iDC reveal an increase of BAT3 mRNA (Fig. 23A and 23B respectively). The degree of enhanced mRNA expression in the case of dendritic cells varied from donor to donor.

In another set of experiments, we stressed the cells with a non-lethal heat shock and determined the release of BAT3 using a specific sandwich ELISA against BAT3. It is consistent with tumor data that BAT3 is released constitutively and it is enhanced by stress (Fig. 23C). The stimulation of NK cells with the respective exosomes causes an enhanced cytokine release in the case of heat shock exosomes. The enhanced release of TNFα (Fig. 23D) and IFNγ (Fig. 23E) due to the BAT3 enriched exosomes reflects to NK cells, that play a major role in the maturation and cytotoxicity of iDCs mediated through NKp30.

The bidirectional signaling of NK cells and iDCs is mediated by many factors involving direct cell contact or stimulation of the soluble cytokines. Among the receptor-ligand pairs involved in NK-DC crosstalk, NKp30-NKp30L pair plays a major role in the reciprocal activation (Ferlazzo, Tsang et al. 2002; Gerosa, Baldani-Guerra et al. 2002). Since we have demonstrated that iDCs secrete BAT3 (NKp30L) on surface of the exosomes, we directly tested whether iDC-derived exosomes have any regulatory function on NK cells. As demonstrated under both autologous and allogenic conditions, the exosomes purified from iDCs affected cytokine release (TNFα and IFNγ) from NK cells (Fig. 23F and 23G). One of the major outcomes due to the NK-DC crosstalk is the maturation of iDCs upon engagement of the NKp30 (Vitale, Della Chiesa et al. 2005). The maturing stimulus obtained by this activation is

due to the NK-cell mediated secretion of cytokines TNFα and to a certain extent IFNγ (Gerosa, Baldani-Guerra et al. 2002; Piccioli, Sbrana et al. 2002; Vitale, Della Chiesa et al. 2005). In order to explore this function, we collected the supernatants from NK cells stimulated with media control or the exosomes derived from iDCs. The expression of the co-stimulatory molecules CD80 and CD86 on dendritic cells was enhanced upon incubation with the supernatant obtained from non-stimulated NK cells. However, the stimulation was even stronger when the supernatant from NK cells pre-treated with exosomes was used (Fig. 23H and 23I).

However, as a direct proof of principle, we could not use the commercially available NKp30 antibody that could block NK cell activation, since it was found that the antibody alone binds to DCs non-specifically and enhances the activity (data not shown). However, these functional data demonstrate a novel role for exosomes, as soluble factors released from accessory cells to steer the regulation of NK cells on adaptive immunity.
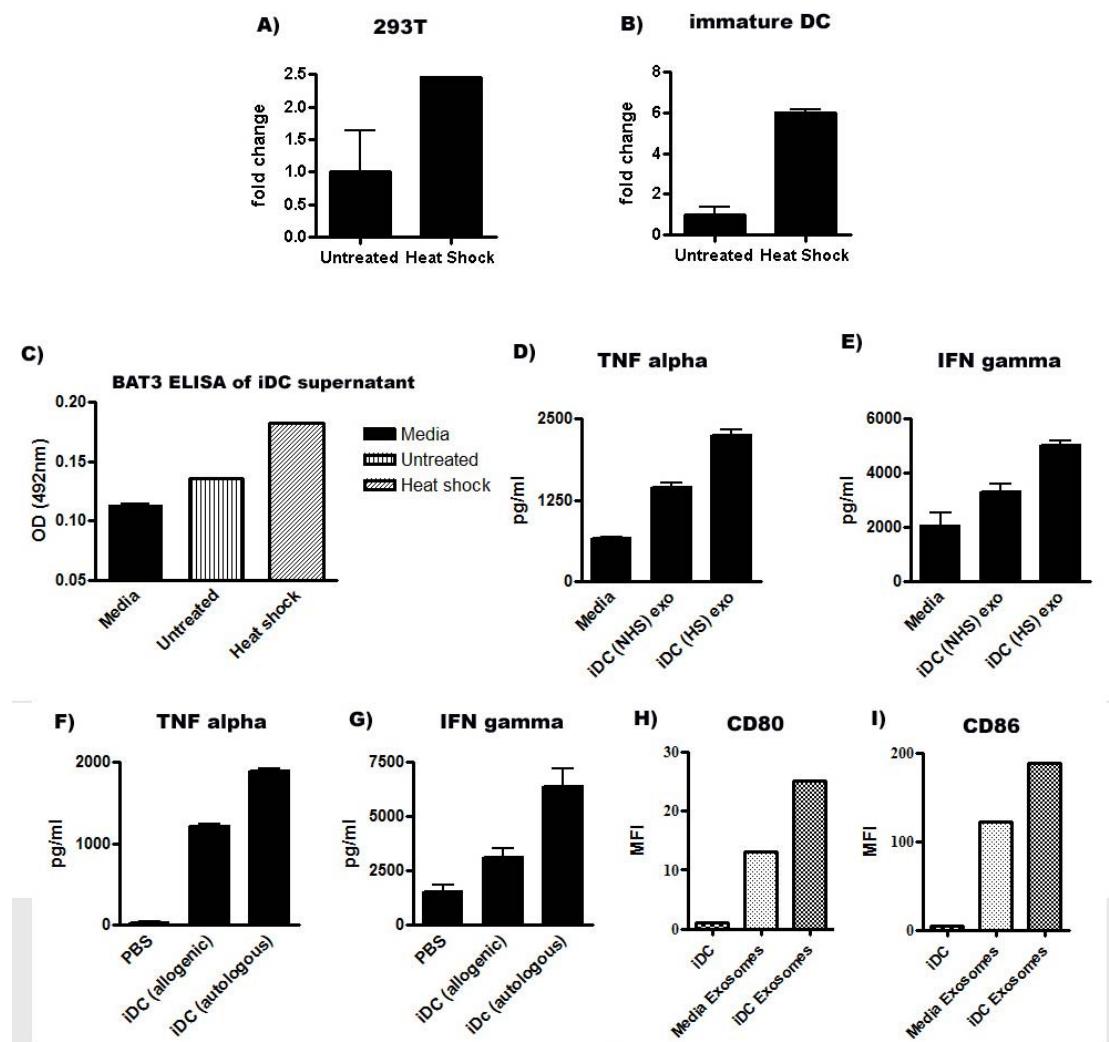
Figure 23. BAT3 is up-regulated

(a-b) BAT3 mRNA is up-regulated in 293T **(a)** and iDCs **(b)** analyzed in a real-time quantitative PCR. The Y-axis determines the fold change, where the untreated samples were normalized to factor 1 and the heat shock treated samples represent the enhanced fold change. **(c-e)** In another set of experiments, supernatants were collected from immature dendritic cells as alone (untreated) and treated with non-lethal heat shock (Heat shock) and the supernatants were subjected to BAT3 specific ELISA (sandwich method) to determine the amount of BAT3 in the supernatants **(c)** Error bars represent mean and standard deviation of duplicate samples. **(d and e)** ELISA detection of the cytokine release from NK cells, TNF-α **(d)** and IFN-γ **(e)**. Media, exosomes derived from untreated cells (iDC-NHS exosomes) and exosomes derived from treated cells (iDC-HS exosomes) were incubated with allogenic NK cells for 36-48 hours and the supernatants were collected and subjected to specific ELISA. The means of the duplicates and the concentration (pg/ml) were indicated. One representative experiment of three is shown.

iDC derived exosomes activate NK cells. (f and g) ELISA detection of the cytokine release from NK cells, TNF-α **(f)** and IFN-γ **(g)**. PBS and purified exosomes derived from iDCs were incubated with both allogenic and autologous NK cells for 36-48 hours and the supernatants were collected and subjected to specific ELISA. The means of the duplicates and the concentration (pg/ml) were indicated. One representative experiment of five is shown. **(h and i)** Here, the allogenic iDCs were incubated with nothing (iDC), NK-cell derived supernatant stimulated with exosomes derived from media (Media Exosomes) and NK-cell derived supernatant stimulated with exosomes derived from dendritic cells (iDC Exosomes). Maturation of iDCs was analyzed by FACS analysis detecting the enhanced expression of co-stimulatory molecules CD80 **(h)** and CD86 **(i)**. The bars indicate the mean fluorescence intensity (MFI). One representative experiment of three is shown.

5.3.5 BAT3 presented on exosomes triggers the lysis of immature dendritic cells

Besides the maturation of iDCs, the elimination of the excess iDCs is entirely dependent on activated NK cells driven by the NKp30 receptor (Ferlazzo, Tsang et al. 2002). It was already shown that tumor killing was inhibited specifically by BAT3-antibody block and depletion (Fig 17B and 16B). Stepping forward, it was directly tested the effect of BAT3 in killing iDCs by blocking and depleting BAT3. NK cells were purified from PBMCs and used as effector cells against monocyte-derived DCs as the target cells. Addition of anti-BAT3 (rabbit polyclonal) significantly inhibits NK-mediated cytotoxicity when compared to the cells alone (Fig. 24A). Moreover down-regulation of BAT3 in iDCs with siRNA reduced the NK-cell-cytotoxicity compared to the control siRNA (Fig. 24B). Maturation of DCs leads to high expression of MHC class-I molecules and thus protect from NK-cell-mediated cytotoxicity. It was demonstrated that the mature DCs are less susceptible to lysis compared to iDCs due to the high expression of MHC class I (Ferlazzo, Tsang et al. 2002). However, subsequent blocking of MHC-class-I molecules on mature DCs with HLA-A, B, C-specific mAb leads to efficient lysis of mDcs by NK cells and this effect is blocked by BAT3 antibody (Fig. 24C). This demonstrates that activity of NK cells on DCs is NKp30-BAT3 dependent. In summary, we suggest that BAT3 is

responsible for NK-cell recognition of tumor cells and dendritic cells. Further its mechanism is primarily regulated in the form of exosomes.
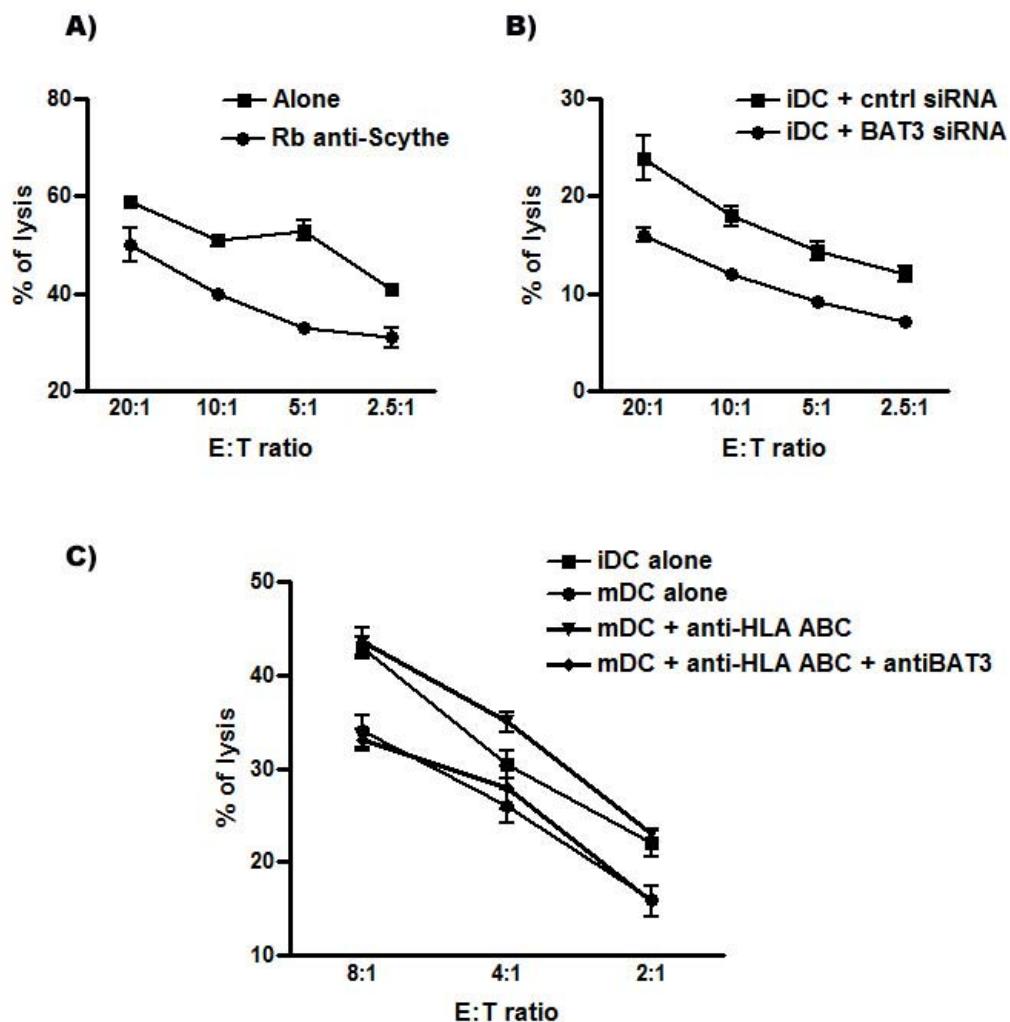

Figure. 24. Role of BAT3 in dendritic cell lysis

NK-cell mediated cytotoxicity on dendritic cells at different effector:target ratios. (a) The differences between the killing of control dendritic cells and cells incubated with BAT3 specific antibody after a 4 hour europium release assay were significant. Error bars represent the mean ± s.d. of three replicates. One representative experiment of four is shown. (b) Immature DCs transfected with either the control siRNA or with BAT3 siRNA were incubated with NK cells and the lysis was analyzed in a europium release assay. The decrease of iDC lysis upon BAT3 down regulation was significant. (c) Both immature and mature DCs were used for the NK-cell mediated lysis. The mature DCs became susceptible to NK cell lysis when they were pre-incubated with HLA A, B and C antibody. This enhanced lysis of mature DCs was blocked by BAT3 specific antibody. Immature DCs were used as the sensitive control for NK-cell lysis. The data shown here were obtained with a polyclonal NK cell population cultured for 18 days in presence of IL-2 (mean of triplicates). One representative experiment of three is shown.

6 Summary and Discussion

In the current study, HLA-B-Associated Transcript-3 (BAT3) is identified as a cellular ligand for a natural cytotoxicity receptor (NCR3, NKp30) on NK cells. NKp30 plays a major role in natural cytotoxicity in innate immune responses and is also involved in the NK-DC crosstalk in adaptive immune responses (Ferlazzo, Tsang et al. 2002; Smyth, Cretney et al. 2005). This study claims that BAT3 may serve as a cellular ligand for NKp30 as it is expressed in an inducible manner in tumor cells (i) BAT3 is released and triggers cytokine secretion of NK cells (TNFα, IFNγ), (ii) it binds directly to NKp30, (iii) loss and gain of function of BAT3 reduces or enhances NK cell-mediated killing, respectively, (iv) it mediates tumor rejection in a multiple myeloma xenograft model. In addition to the functional importance of BAT3 in the innate immune response, the study also demonstrates that BAT3 is expressed and released from dendritic cells and induces adaptive immune response with its involvement in the bidirectional signaling of natural killer cells and dendritic cells.

The study proposes a novel mechanism for receptor-ligand interaction in target-cell recognition beyond missing-self and induced-self hypotheses. This is the first example that an activating ligand is released into the extra-cellular environment in an inducible manner. Besides this aspect, the striking observation is that purified recombinant BAT3 inhibited the NK-cell function in its soluble form in contrast the immobilized BAT3 was able to activate NK cells, comparable to the endogenous protein derived from cell supernatant..

Finally, explaining these observations it was demonstrated that BAT3 is released as exosomes, binds to NKp30Ig and activates NK cells resulting in strong release of cytokines (TNFα and IFNγ). Moreover, NK-cell derived supernatants obtained upon activation with iDC-derived exosomes lead to the maturation of dendritic cells. Finally, BAT3 plays a crucial role in the lysis of immature dendritic cells and also mature dendritic cells when the MHC Class I molecules are blocked. Taken together the results show that the NKp30-BAT3 interaction is part of the innate and adaptive immunity.

6.1 Identification of BAT3 as a ligand for NKp30

For the last few years the identification of the cellular ligands for the Natural Cytotoxicity receptors has been the focus for the immunologists in NK cell biology. NK cell recognizes its target when there is a downregulation of MHC Class I molecules, but just the down-regulation does not lead to the target recognition. Instead the ligation of triggering surface receptors (NCRs) is necessary for their target cell recognition. So far, the function for the NCRs was defined by re-directed cytotoxicity assays using specific blocking antibodies. Initially, there was evidence that membrane-associated heparan sulphate proteoglycans might act as the ligands for NKp30 and NKp46 (Bloushtain, Qimron et al. 2004). But later, another study proved that heparan sulphate is not the cellular ligand (Warren, Jones et al. 2005). Recently, it was learnt that the discrepancy of binding studies is due to the altered glycosylation of NKp30. (Hershkovitz, Jarahian et al. 2008). It has also been difficult to identify the cellular ligands for all these years since, lack of binding was reported for NKp30-Ig and NKp46-Ig to 721.221 cells (Mandelboim, Lieberman et al. 2001) although their killing is directly mediated by these receptors (Pende, Parolini et al. 1999). Thus, the discovery of the cellular ligand HLA-B-Associated Transcript-3 (BAT3) for NKp30 is very important and might provide some hints about the missing cellular ligands for the other NCRs.

BAT3 is characterized to be a nuclear protein, which is not reminiscent of a classical ligand. Moreover, structurally, the protein also lacks the leader sequences which are one of the properties of secreted proteins. The presented data demonstrates that BAT3 is released and activates the NK cells. The possibility that NKp30 might have intra-cellular ligands was raised for the first time by Arnon et al. (2005). They recently identified the viral NKp30 ligand pp65 from the human cytomegalovirus (CMV). This ligand induces a general suppression of the NK cell activity through a specific and direct interaction with NKp30 (Arnon, Achdout et al. 2005). The viral factor pp65 has no structural homology to BAT3, but is like BAT3 localized in the cell nuclei (of infected cells). Therefore it is unclear where and when pp65 interacts with NKp30 in vivo and it has been speculated that soluble pp65 derived from the direct lysis of virus-infected cells or from apoptotic cells may bind to NKp30 (Arnon,

Achdout et al. 2005). Similarly, it was shown for another nuclear protein HMGB1 (High Mobility Group Box 1) to be acetylated and then shuttle from the nucleus to the cytoplasm (Bonaldi, Talamo et al. 2003). The BAT3-binding protein HSP70 is known as a potent stimulator that triggers the activity of NK cells, when expressed on the cell surface or upon secretion (Asea, Kraeft et al. 2000; Millar, Garza et al. 2003; Gastpar, Gehrmann et al. 2005; Gehrmann, Marienhagen et al. 2005).

NKp30 also plays a major role in regulation of dendritic cells by its reciprocal activation leading to maturation and killing of iDCs. BAT3, a tumor derived ligand for NKp30 is also expressed and released from dendritic cells into the extracellular space with regulatory function on NK cells. Dendritic cells are considered to be the sentinels of the immune system that can circulate through the blood activating the cells involved in both innate and adaptive immunity. During inflammation, NK-cell activation seems to be mediated by signals provided from accessory cells, such as monocytes, macrophages or dendritic cells (Haller, Serrant et al. 2002; Atochina and Harn 2005; Newman, Korbel et al. 2006; Welte, Kuttruff et al. 2006). The antigen-presenting accessory cells (APCs) activate by both contact dependent and soluble factors. The unique feature of NK cells and dendritic cells is their reciprocal activation mediated mainly by the triggering receptor NKp30 in maturation and elimination of the DCs (Ferlazzo, Tsang et al. 2002; Gerosa, Baldani-Guerra et al. 2002; Piccioli, Sbrana et al. 2002).

6.2 Characterization of BAT3 as an exosomal derived soluble factor

The data clearly demonstrated that BAT3 released into the extracellular environment activates NK cells. But strikingly, when BAT3 was used as purified recombinant protein in soluble fraction it has an inhibitory function on both cytokine release and cytotoxicity assays. Most interestingly, the immobilized purified protein has entirely an opposite effect (activating function). Taking together, this data introduces a new concept for the intracellular ligands that are released in a complex structure into the supernatant. This structure could also imply to the understanding and involvement of other ligands indicating the synergism of natural cytotoxicity

receptors (NCRs) on NK cells. In fact, a high molecular protein complex containing BAT3 and HSP70 was purified from 293T cell supernatants (data not shown).

Immobilized BAT3 activation of NK cells could be a mimic of surface molecule which engages the receptor, thus the mechanism of BAT3 release in exosomes could well suit for the receptor-ligand activation. Opposite biological effects for membrane expressed versus soluble factors is not an unusual observation for ligands engaging a triggering NK cell receptor. Surface expression of MICA (Major Histocompatibility Complex Class I Chain-related Gene A), a NKG2D-specific ligand, marks transformed cells for destruction by immune effector cells, whereas soluble ligands directly inhibit NKG2D-mediated activation and may promote tumor cell escape from immune-surveillance (Groh et al., 2002; Bottino et al., 2005). However, the impact of soluble factors has to be explained carefully as there are some factors which are released in complex structures as multimeric or exosomes, which are regarded as important immune regulators of the immune system. In fact, BAT3 as a result of shedding might also exist as pure soluble protein counter-acting the activating function of the complex structure. An intra-cellular viral protein, pp65 was reported as an inhibitory ligand for NKp30. It was hypothesized that the secreted pp65 from the lysed target cells engages NKp30 and inhibits the activating signal by dissociation of CD3-ζ chain. Taken together there are different mechanisms for inhibition effecting post-translational modification, signal transduction or internalization of the receptor molecules.

The knowledge of expression pattern of the NCR ligands is extensively limited and it has only been shown on a limited number of tumor cells (Arnon, Achdout et al. 2004; Nowbakht, Ionescu et al. 2005). Detection of the ligands using receptor-Ig constructs failed on several cell lines. Recently, a comprehensive analysis showed NKp30L expression on normal and neoplastic cells; this suggested that the ligands for NCRs were concentrated intra-cellularly in vesicular compartments. More specifically, NKp30-Ig revealed a similar pattern to the early endosomes marker (Byrd, Hoffmann et al. 2007). This is in agreement with the formation of exosomes and supports the conclusion that the cellular ligand BAT3 is localized in the endosomal vesicles and secreted as exosomes. The expression of BAT3 on the surface of exosomes and the specific binding of NKp30Ig suggests that NK cells could well interact with exosomes and regulate its function. The gain and loss of function of

exosomes on NK cells was explained by over-expression and depletion of BAT3. However, the composition and activity of the exosomes released from different cell types might have a different functional activity on natural-killer function.

Exosomes are small vesicles originally contained in endosomes, fuse with the cell surface and are released into the extracellular space. These small vesicles measure about 50-90 nm in diameter and are termed as intraluminal vesicles (ILVs) in endosomes; finally, they are termed as exosomes when they are released into the extracellular environment (Harding, Heuser et al. 1984; Pan, Teng et al. 1985; Johnstone, Adam et al. 1987). Recently, a lot of evidence has emerged that exosomes derived from many cells could serve as immunotherapeutic vaccine against tumors and more importantly in communicating between immune regulatory cells.

Cells can secrete these vesicles under various stress conditions and can stimulate the function of immune-regulatory cells (Stoorvogel, Kleijmeer et al. 2002; Thery, Zitvogel et al. 2002). Exosomes are secreted into many cell-culture supernatants and various biological fluids suggesting their role in multiple biological processes (Caby, Lankar et al. 2005; Thery, Amigorena et al. 2006). They have an indirect effect, where the dendritic cells were pulsed by exosomes *in vitro,* and are more immunogenic to T-cells (Andre, Schartz et al. 2002; Bard, Hegmans et al. 2004). The exosomes derived from DCs have a direct effect on T-cell activation; however, the mechanism of DC exosomes on NK-cell function has not been clearly explained. Here the data suggests that the NKp30-mediated bidirectional signaling of NK-DC is dependent on BAT3-containing exosomes. Maturation experiments of iDCs are directly dependent on the supernatants derived from NK cells stimulated by exosomes suggesting that the activation of DCs is mediated by exosomes leading to adaptive immunity. Even more, it is speculated that the NK-DC activation through the exosomes may cause the immune cells to migrate to their respective tissues which could act as a missing link.

For the last few years, the application of the exosomes as immunogenic molecules was studied *in vivo*, demonstrating that these can communicate between the cells within the immune system. Although cellular immune therapy has become the highlight in treating cancer, it still remains controversial in the aspects of presenting the antigens. In this regard, the implementation of exosomes could act as immune adjuvants and increase the efficiency of immunotherapy. The mechanisms of tumor-

derived exosomes are poorly understood, with reported activating (Gastpar, Gehrmann et al. 2005) and inhibitory effects (Liu, Yu et al. 2006; Clayton, Mitchell et al. 2007). However, the pathway of how BAT3 is subjected to exosomes is unclear expect for one report which gives rise to the speculation that secretory carrier membrane proteins (SCAMPs) aid in this transport by interacting with BAT3. It is well described that SCAMPs are expressed ubiquitously and are the components of vesicles shuttling to and from plasma membrane and other vesicles inside the cell (Fernandez-Chacon, Achiriloaie et al. 2000; Castle and Castle 2005).

6.3 Involvement of BAT3 in NK-DC crosstalk

In addition to the function of iDC derived exosomes in NK-DC crosstalk for the maturation of dendritic cells, the data also provides an insight that BAT3 plays an important role in mediating the cytotoxicity of dendritic cells. The elimination of excess iDCs is of utmost importance in acquiring most specific and high-quality adaptive immune response. Even if iDCs contain a substantial amount of MHC class I on the surface, lysis of iDCs is prone to NKp30-mediated cytotoxicity overcoming the inhibitory function of killer-inhibitory receptors (KIRs). This suggests that the activation of NKp30 is either due to high expression of its ligands or it is dependent on the accessibility of its ligands. Thus, we hypothesize that probably the ligand BAT3 activates NK cells through the exosomal pathway. Treating iDCs with BAT3 antibody and depleting BAT3 using siRNA technology hampers the lysis of iDCs by NK cells. Our data showed that the cytotoxicity of mature DCs is blocked by MHC class I antibody and can be reversed by BAT3 antibody; this confirms that BAT3 is a ligand for the activating receptor NKp30 on NK cells. Thus the data demonstrates the fact that mature DCs (self cells) are protected from NKp30-BAT3 mediated cytotoxicity by possessing high levels of MHC class-I expression (Ferlazzo, Semino et al. 2001; Ferlazzo, Tsang et al. 2002).

6.4 A novel recognition model for NK-cell mediated regulation

The classical mode of natural killer (NK) cell regulation is entirely governed by the signals produced from activating, inhibitory and co-stimulatory receptors (Smyth, Cretney et al. 2005). Down-regulation of MHC-class-I molecules reduces the strength of the inhibitory receptors and leads to the subsequent activation of NK-cell killing of damaged or transformed cells. This activation is mediated either by direct contact of activating receptors with the respective ligands or through soluble factors signaled by the accessory cells (Smyth, Hayakawa et al. 2002; Newman and Riley 2007). Moreover, a different mode of recognition including pathogen-encoding molecules was exemplified by the mouse Ly49H NK receptor (Vivier and Biron 2002). In humans NK receptors specific for pathogens are NKp46 and NKp44 receptors, which bind to hemagglutinin (Mandelboim, Lieberman et al. 2001).

Thus it is conceivable that such nuclear proteins, which exhibit cytokine function to alert the immune system, are sensors for DNA instability or damage of transformed cells. This model is supported by the exciting finding that BAT3 is crucial for the p53 acetylation (Sasaki et al. 2007), a central event for the cellular DNA damage response. A direct link of the DNA damage response to innate immunity and cancer was recently discovered by Gasser et al. (2005) demonstrating that the DNA damage pathway directly regulated the expression of ligands engaging the NKG2D receptor. Factors such as HSP70, HMGB1 and BAT3 are emerging as a class of immune regulatory proteins, which may be regarded as an intracellular subgroup of a larger set, the damage-associated molecular patterns (DAMPs) (recent review Bianchi (2007).

Our functional in vivo findings on BAT3 were reminiscent to heat shock proteins. HSP70, a multifunctional factor acting as a chaperone or cytokine, can induce tumor rejection when purified from the tumor (Millar, Garza et al. 2003; Calderwood, Theriault et al. 2005). Interestingly, it has been reported that nuclear protein complexes, that contain HSPc70 together with High Mobility Group (HMG) proteins B1 and B2 and with BAT3 and HSPc70, are involved in the cytotoxic response to DNA damage (Krynetski, Krynetskaia et al. 2003). HMGB1 is, so far, the best characterized nuclear factor, which exhibits cytokine activity upon release and this factor is mainly involved in NK-cell mediated maturation of dendritic cells (Lotze

and Calderwood, 2005; Semino et al., 2006). HMGB1 is thought to transduce its function through Toll-like receptors (TLR 2 and 4) and Receptor of advanced glycation end-products (RAGE), although other not yet identified receptors might be involved.

Thus, the data presented here shows for the first time that a released factor engages a triggering receptor on NK cells and thus support a model for a **"danger induced-recognition"** by factors released from tumor cells or accessory cells (dendritic cells, macrophages, monocytes and neutrophils etc).

In summary, it is demonstrated that BAT3 is released into the extracellular environment as exosomes from accessory cells (tumor cells and dendritic cells) during the immune response and activates NK-cell function. This study provides novel insights of how NK cells recognize their targets and communicate with DCs. Supporting this model it was recently observed that the factors released from sensitive target cells upon NK-lysis, activate NK cells and may induce lysis even of the resistant target cells (North, Bakhsh et al. 2007). It is proposed that soluble factors (exosomes) released from accessory cells activate NK cells to prime the innate immune response and shape the adaptive immune responses.

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Appendix

NK	Natural Killer
iDC	immature dendritic cells
mDC	mature dendritic cells
NCR	Natural Cytotoxicity Receptors
BSA	Bovine serum albumin
FBS	Fetal bovine serum
TNF	Tumor necrosis factor
IFN	Interferon
GM-CSF	Granulocyte Macrophage- Colony Stimulating Factor
IL-4	Interleukin-4
BAT3	HLA-B-Associated Transcript-3
BAG	Bcl-2 associated anthogene
kbp	kilo base pairs
cDNA	complementary DNA
kDa	Kilodalton
OD	Optical Density
PCR	Polymerase chain reaction
pH	Negative log of hydrogen ion concentration
RT	Room temperature
g	Relative centrifugal force
rpm	revolutions per minute
DNA	Deoxyribonucleic acid
RNA	Ribonucleicacid

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Eidesstattliche Erklaerung

Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt.

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Lebenslauf

Name: Venkateswara Rao, Simhadri

Geburtsdatum: 14.01.1978

Geburtsort: West Godavari, A.P, Indien

Staatsangehörigkeit: Indisch

1993-1994	Gymanasium (SSLC), Andhra Pradesh, Indien
1994-1996	Abitur (Intermediate), Andhra Pradesh, Indien
1997-2000	Bachelor of Science (B. Sc), Andhra University, Indien
2000-2002	(Diplom) Master of Science (M. Sc), Bharathidasan University, Indien.
2002-2004	Junior Research Fellow Dr. Rohit Mittal's Lab Tata Institute of Fundamental Research Mumbai, Indien
2004-2008	Doktorarbeit by PD. Dr. Elke Pogge von Strandmann am Laboratory of Immune therapy, Universität zu köln

Title: Exosomal derived HLA-B-Associated Transcript-3 is a ligand for NKp30 and regulates Natural Killer Function in Innate and Adaptive Immune Responses

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