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Of Mice and Men: Studying Innate and Adaptive Immunity Against the Epstein-Barr Virus

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STUDYING INNATE AND ADAPTIVE IMMUNITY AGAINST THE

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Of Mice and Men: Studying innate and adaptive immunity against the Epstein-Barr virus

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The Epstein-Barr virus (EBV) is a lymphotropic γ-herpes virus infecting over 90% of the human adult population. A striking feature that the virus shares with other γ-herpes viruses is its oncogenic potential. This transforming property can be observed as B cell transformation *in vitro* and lymphomas as well as epithelial cancers *in vivo*, but most immunocompetent individuals control EBV infection successfully without the occurrence of disease.

Cells of the innate immune system act in synergy to provide a first line of defense against pathogens. Here we describe that dendritic cells (DCs), matured with viral products or mimics thereof, activated natural killer (NK) cells more efficiently than other mature DC preparations. CD56^{bright}CD16$^-$ NK cells, which are enriched in human secondary lymphoid tissues, responded primarily to this DC activation. In fact, 100-fold less tonsilar than peripheral blood NK cells were required to achieve the same protection against EBV-mediated B cell transformation *in vitro,* indicating that innate immune control of EBV by NK cells is most efficient at this primary site of EBV infection.

The lack of an animal model of EBV infection prevents assignment of a protective value to immune subsets *in vivo*. We generated a small animal model that can be infected with

EBV by reconstituting NOD-scid $\gamma_c^{-/-}$ mice with CD34$^+$ hematopoietic stem cells. We demonstrated that primary T cell responses in these humanized mice control infection with EBV. These T cell responses were HLA restricted and partially specific for EBV derived peptides. In HLA-A2 transgenic animals T cell responses against lytic EBV antigens dominated over recognition of latent EBV antigens during early phases of infection similarly to human EBV carriers. This mouse model recapitulates features of symptomatic primary EBV infection, and generates T cell mediated immune control that resists oncogenic transformation. We were also able to demonstrate that humanized mice develop functional human NK cells, this will allow us now to study the contributions of NK cells to innate immune control of EBV *in vivo* in the future.

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1.1.1   History of the Epstein-Barr virus

The Epstein-Barr virus (EBV) was discovered in 1964 by Anthony Epstein, Yvonne Barr, and Bert Achong when they analyzed cell lines from Burkitt's lymphoma patients (Epstein et al., 1964). By studying pictures taken by electron microscopy they found that these cells were infected with a virus, which did not react with antisera that had been established against the so far known herpesviruses. From then on, EBV has served as a fascinating and useful model for virologists, immunologists, and clinical researchers (Young and Rickinson, 2004). First, virologists have been particularly interested in EBV as it was the first human virus that had been discovered to be associated with tumors. Later, it was found that EBV encoded proteins contribute to the oncogenesis of tissues of very different developmental origins such as lymphocytes and epithelial cells. Immunologists, on the other side, studied the cellular immune response to a genetically stable, persistent human virus. During the lytic and latent infection of EBV different sets of proteins are expressed. It has been a center of attention to study the induction and regulation of qualitatively different cellular immune responses to these different sets of antigens. These efforts revealed interesting insights into mechanisms regarding immunodominance hierarchies and the biological effectiveness of different immune responses. EBV has also received significant attention by clinical researchers, because soon after its discovery, it was shown that EBV is the causative agent of infectious mononucleosis and that a high frequency of Burkitt's and Hodgkin's lymphoma cell lines are also EBV positive. More recently, EBV has been implicated as a potential

environmental trigger for complex autoimmune diseases such as multiple sclerosis and systemic lupus erythematosus. In conclusion, these results demonstrate that EBV provides a valuable system to advance the understanding of the development of protective immune responses against persistent viruses in humans in general.

1.1.2 Basic biology of herpes virus infections

EBV belongs to the family of herpes viruses and is one of eight known members infecting humans (Thorley-Lawson and Allday, 2008). Upon transmission to a naive host, the herpes viruses first amplify the viral load through replicative (lytic) infection in a permissive cell type - in the case of EBV the infection occurs in a B cell (Young and Rickinson, 2004). Then the virus persists for the life of the host as an asymptomatic latent infection in the same or a different second cell type. Occasionally, the virus reactivates into the lytic cycle and produces infectious virions that are transmissible to a new host. For all herpes viruses the lytic cycle is characterized by the sequential expression of immediate early genes (IE), followed by early genes (E) and late genes (L). In contrast, the establishment of the latent cycle is different between the three sub-families of herpes viruses. While α- and β-herpes viruses immediately shut down the viral protein expression after entering their target cell, γ-herpes viruses express a set of proteins encoded by latent cycle genes that appear to be crucial for the establishment of the latent state. The transient expression of all or part of these genes is associated with a phase of cell proliferation that serves to amplify the latently infected cell pool. Thereafter, the expression is suppressed to give a stable reservoir of virus-infected cells that do not express viral proteins.

The gamma viruses themselves are divided into two genera, γ-1 and γ-2, with different sets of latent cycle genes and apparently different molecular strategies for the induction

of latent cell proliferation. The Karposi's sarcoma herpes virus (KSHV) and its distant relative murine γ-herpesvirus 68 (MHV-68) are classical γ-2 viruses. They establish latency in B cells, but have no independent capacity to drive B cell growth. From the evidence of MHV-68, amplification of the latent antigen-expressing B cell pool occurs within germinal centers and requires T cell help. In contrast, the more recently evolved genera of gamma-1 viruses or lymphocrytoviruses, including EBV, are only found in primates and have acquired direct B cell growth-transforming ability.

1.1.3 Biology of the EBV infection

EBV is an almost ubiquitous virus infecting around 90 – 95 % of the human population. In many countries with poorer hygienic conditions, the EBV infection takes place already during infancy and is asymptomatic (Young and Rickinson, 2004). In countries with higher hygienic standards the primary infection can be delayed until the second decade or later. In up to 25 – 50 % of cases of delayed primary infection, the infection is not asymptomatic but can present itself as infectious mononucleosis (IM)(Crawford et al., 2006). IM is an acute but self-limiting illness characterized clinically by fever, sore throat, and swollen lymph nodes. Based on the assumption that IM is a magnified version of asymptomatic primary infection, studies with IM patients and *in vitro* models have been used to characterize the initial events during primary infection (Hislop et al., 2007).

According to this model, EBV replicates in a permissive cell type in the oropharynx after oral transmission (Figure 1) (Young and Rickinson, 2004). This lytic infection leads to high rates of virus shedding into the throat, which is only slowly brought under control by the immune system. At the same time, the virus infects mucosal B cells and initiates a latent growth-transforming infection, leading to the expansion of lymphoblastoid cell line (LCL)-like cells in the extrafollicular areas of tonsillar lymphoid tissues and the

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appearance of large numbers of infected cells in the blood (Thorley-Lawson, 2001). Although many of these proliferating cells that express high amounts of viral antigens are removed by the immune response, some survive by down-regulating latent antigen expression and entering a resting state as members of the long-lived memory B cell pool. Subsequently, these cells persist as a recirculating population, predominantly found in blood and pharyngeal lymphoid tissues and very likely are subject to the same physiologic constraints for cell survival and turnover as the memory B cell pool as a whole. Thus, physiological events, such as antigen stimulation and receipt of a plasma cell differentiation signal, might drive occasional reactivations into lytic cycle. During viral latency a set of eight proteins including Epstein-Barr nuclear antigen (EBNA) 1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA leader protein (EBNA-LP) and latent membrane proteins (LMP) 1, LMP2A/B is expressed (Thorley-Lawson, 2001). Different patterns of latent protein expression have been found in infected individuals. Latency I is characterized by the sole expression of EBNA1 and is *in vivo* found in dividing EBV positive memory cells as well as in most Burkitt's lymphoma cells. In latency II LMP1, LMP2 and EBNA1 are expressed and this profile is commonly found in Hodgkin's lymphoma, NK/T cell lymphomas, and uterine leiomyosarcoma. Notably, in gastric and nasopharyngeal carcinomas both latency I and latency II can be found. Finally, in latency III the full set of latent proteins including EBNA1, EBNA2, EBNA3A/B/C, EBNA-LP and LMP1/2 are expressed (Thorley-Lawson, 2001). Latency III is only found *in vitro* in LCLs and *in vivo* in lymphomas of immunosuppressed patients. Interestingly, during latency EBV expresses also a number of non-coding RNAs including the commonly expressed Epstein-Barr encoded RNAs (EBERs) and in addition at least twenty microRNAs. However, so far the expression patterns and functions of these microRNAs have not been fully determined.
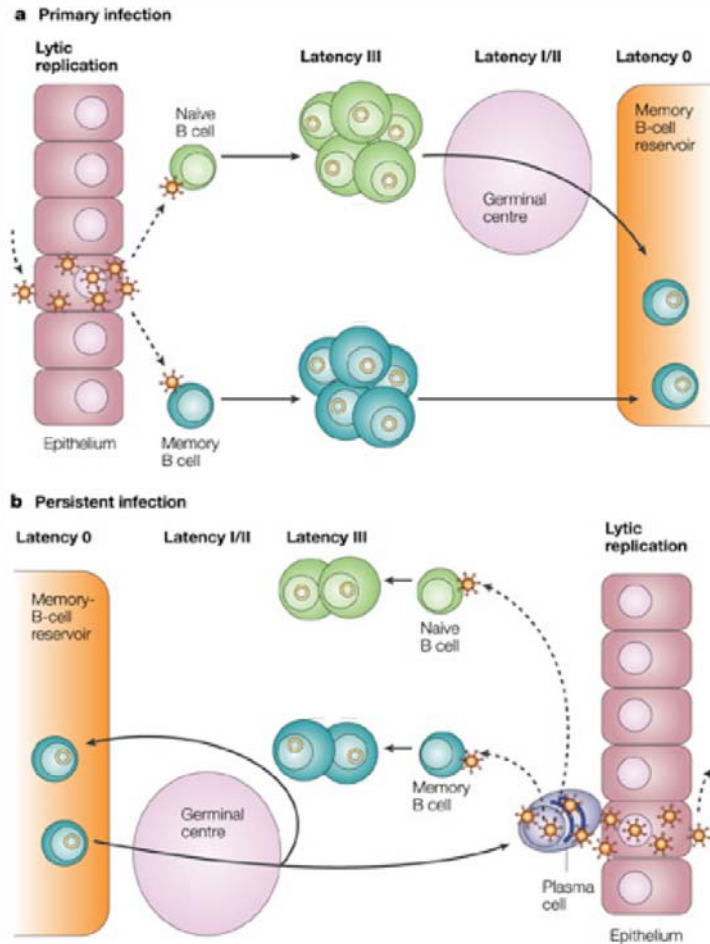
Figure 1: Life cycle of EBV. a) Primary infection. The incoming virus establishes a primary focus of lytic replication in the oropharynx after which the virus spreads throughout the lymphoid tissues as a latent (latency III) infection of B cells. Many of these proliferating cells are removed by the emerging immune response, but some escape by down-regulating antigen expression and establishing a stable reservoir of resting viral-genome-positive memory B cells, in which viral antigen expression is mostly suppressed (latency 0/I) b) Persistent infection. The reservoir of EBV-infected memory B cells becomes subject to the physiological controls governing memory-B-cell migration and differentiation as a whole. Occasionally, these EBV-infected cells might be recruited into germinal-centre reactions, entailing the activation of different latency programmes, after which they might either re-enter the reservoir as memory cells or commit to plasma-cell differentiation — possibly moving to mucosal sites in the oropharynx and, in the process, activating the viral lytic cycle. Virions produced at these sites might initiate foci of lytic replication allowing low-level shedding of infectious virus in the oropharynx, and might also initiate new latency III infections of naive and/or memory B cells; these new infections might possibly replenish the B-cell reservoir. (Adapted from Young and Rickinson, 2004)

1.2   Control of viral infections by the innate immune response

The immune system preserves the integrity of its host by recognizing and resisting invaders. Therefore, evolution has provided each species with the capacity to resist the

pathogenic challenges in its ecological niche. The innate and the adaptive immune system are the two arms of the immune system that provide indispensable protection of the host against pathogenic invaders. The innate immune system is the evolutionary older part of the immune system and consists of a number of different cell types and mechanisms that provide important non-specific restriction of infections (Medzhitov and Janeway, 1997). But unlike the adaptive immune system it is thought to provide no long-lasting immunity against secondary infections of the same pathogen. The most important functions of the innate immune system are the identification or sensing of an infection of the host, the recruitment of other immune cells to the site of insult through the production of chemokines and cytokines, the removal of foreign substances by phagocytosis, the early protection against microbial pathogens and finally the activation of adaptive immune cells by antigen-presentation (Medzhitov and Janeway, 1998).

1.2.1 Sensing the viral infection by immune and non-immune cells

The recognition of foreign invaders is crucial to mount any immune response by the host and the question how the immune system achieves this difficult task has been debated extensively. Competing theories have been proposed by Charles Janeway and Polly Matzinger around 15 years ago suggesting that the immune system recognizes either so-called pathogen-associated molecular patterns (PAMPs) or endogenous danger signals, respectively (Janeway, 1992; Matzinger, 1994). However, both of these theories are now widely accepted and the identity of a number of receptor systems to recognize danger signals or PAMPs has been described. According to Matzinger's theory, danger signals could be either active signs or passive signs of distress that are released actively or passively, respectively, when cells are stressed, infected with a pathogen or when tissue is damaged. One of these danger signals is the protein high-mobility group B1

(HMGB1), a nuclear protein well characterized in terms of its ability to modify DNA access for transcriptional proteins (Erlandsson Harris and Andersson, 2004). It also interacts with high-affinity receptors for advanced glycation end products (RAGE) and Toll-like receptor (TLR) 2 on dendritic cells (DCs). Other signals are crystalline uric acid or ATP that are released from dying cells and which activate the NALP3 inflammasome, an innate immune complex that controls inflammatory caspases and IL-1 activation (Petrilli et al., 2007). But, the immune system is also able to directly sense the presence of pathogens via the recognition of molecules or molecular structures that are not commonly found in the host such as unmethylated DNA, double stranded RNA (dsRNA) or lipopolysaccharides (LPS). In particular, phagocytes are equipped with several cell-surface receptors that recognize pathogen surfaces directly. In addition to triggering phagocytosis, binding of pathogens by dendritic cells and macrophages can also trigger induced responses of the innate immunity, and responses that eventually lead to the induction of the adaptive immunity (Iwasaki and Medzhitov, 2004). The best-defined activation pathway of this type is triggered through a family of evolutionarily conserved transmembrane receptors, called TLRs. They contain an ectodomain of leucine-rich repeats (LRR), a transmembrane domain and a cytoplasmatic domain known as Toll/IL-1 receptor (TIR) domain. These receptors were first described in adult flies where the Toll signaling pathway induces the production of several antimicrobial peptides that contribute to the fly's defense against infection (Lemaitre et al., 1996). In contrast to the fly where Toll serves as a regulator, the 10 TLRs in humans facilitate the direct recognition of microorganisms via the variable LRR region that interacts with such diverse structures such as LPS (recognized by TLR4), bacterial flagellin (TLR5), or dsRNA (TLR3). TLR activation by their cognate agonists leads to the recruitment of cellular adaptor molecules that contain TIR domains and the formation of multi-

component signal transduction complexes in the cytoplasm (Takeda et al., 2003). Signaling downstream of TLRs initiates the transcription of genes encoding cytokines, chemokines, and co-stimulatory molecules. Besides TLRs there are at least two other families of pathogen recognition receptors (PRRs), the family of RIG-I-like receptors (RLRs) recognizing viral infection and dsRNA and the family of Nod-like receptors (NLRs) recognizing bacterial infection (Meylan et al., 2006). It is thought that the interplay between these families ensures the efficient coordination of innate immune responses, through either synergistic or cooperative signaling. With respect to herpes virus infections, it was demonstrated that indeed in addition to TLR2, TLR3, and TLR9, RIG-I also synergizes with TLR receptors in the induction of interferon production after sensing of murine herpes simplex virus (Lund et al., 2003; Rasmussen et al., 2009; Sato et al., 2006; Tabeta et al., 2004). In the case of EBV, it was shown that EBV is recognized via TLR2 in monocytes resulting in the production of inflammatory cytokines such as MCP-1 (Gaudreault et al., 2007). In addition, EBV induces plasmacytoid DC to produce IFN-α in a TLR9-dependent manner (Lim et al., 2006). Therefore, PRRs contribute to the recognition of EBV by the immune system enabling the activation and priming of immune responses that allows control of acute and persistent infection by this human tumorvirus.

1.2.2 Basic biology of dendritic cells

Paul Langerhans first described morphologically a subset of dendritic cells in the skin in the late nineteenth century, which were later called Langerhans cells and were initially thought to be part of the nervous system. However, it was Ralph Steinman and Zanvil Cohen who identified dendritic cells (DC) in 1973 as a functionally distinct subset of immune cells (Steinman and Cohn, 1973). DCs serve as sentinels for the immune

system, patrolling the periphery surveying their environment for signs of danger or infection with pathogens (Figure 2). Upon activation which is called maturation, they change their phenotype and become potent activators of the innate and adaptive immune system (Banchereau and Steinman, 1998). DCs arise in the steady state from a common progenitor within the bone marrow, which is Lin$^-$Flt3$^+$M-CSFR$^+$ in mice (Naik et al., 2007; Onai et al., 2007). DCs can be divided morphologically and functionally into two types: conventional DCs (cDCs), and plasmacytoid DCs (pDCs). Conventional DCs consist of several subsets that are distinguished according to surface markers such as Langerin, CD11c in addition to CD11b and CD8a in mice and BDCA1, BDCA3, and CD16 in humans, respectively (Shortman and Liu, 2002). Importantly, these subsets can also be distinguished by functional aspects such as antigen processing and presentation (Dudziak et al., 2007; Soares et al., 2007). The precursor of cDCs emerges from the bone marrow to migrate in the blood to peripheral tissues to develop into different subsets of immature DCs. In contrast, plasmacytoid DCs are thought to be a relative homogenous population that develops completely in the bone marrow and can later be characterized by their ability to secrete large amounts of type I interferons (IFNs) in response to viruses and/or TLR7/9 ligands (Gilliet et al., 2008). Under inflammatory conditions, monocytes can also differentiate into so-called monocyte-derived DCs (moDCs)(Auffray et al., 2009). Notably, because of the low frequency of human DCs in the peripheral blood (~ 0.5 %) *in vitro* derived human moDCs have served as valuable model for human DCs.

In the steady state and during an immune response, DCs are specialized to capture and process antigens *in vivo*, converting proteins to peptides that are presented on major histocompatibility complex (MHC) molecules and recognized by T cells (Banchereau and Steinman, 1998).
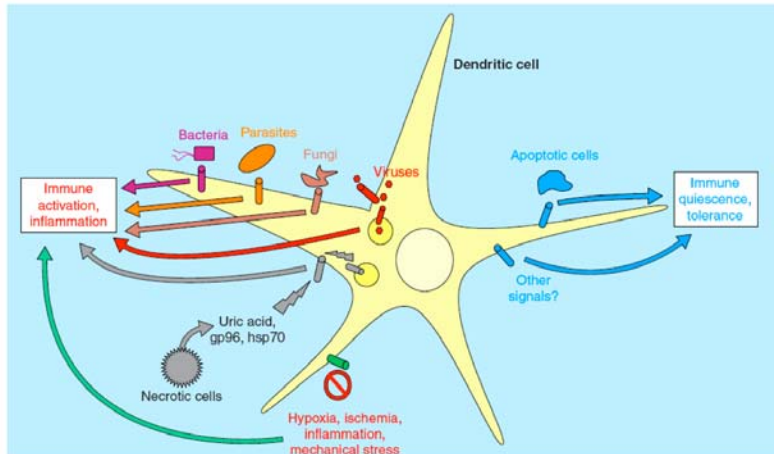
Figure 2: DCs as immune sensors of life, death and danger. Dendritic cells express many different receptors, including TLRs, which enable them to sense life (bacteria, viruses, parasites, fungi etc) and death (uric acid crystals and gp96 and hsp70 from necrotic cells, apoptotic bodies, etc). Many signals of life and death can harm the integrity of the local tissues and of the host itself — thus these constitute clear danger signals for the host. DCs rapidly become activated in response to such signals, and result in immune activation and inflammation. In contrast, 'quietly' dying apoptotic cells or other undefined signals that represent no harm to the integrity of the host may not activate dendritic cells or immune responses and may lead to immune quiescence or tolerance. (From Pulendran 2008)

While DCs or subsets of DCs slowly, but continuously traffic into secondary lymphoid organs to present antigens, the migration of DCs to lymph nodes (LNs) is pivotal for the establishment of an immune response. To enter LNs from the peripheral tissues DCs pass through the afferent lymphatic pathway. In the LNs, T cells scan the MHC/peptide complexes on DCs and after recognition of cognate antigens on DCs, T cells may undergo extensive expansion with devision rates of as high as 2–3 cell cycles a day with the appropriate costimulation provided (Steinman and Banchereau, 2007). However, clones of lymphocytes are also subject to silencing or tolerance by tolerogenic DCs, which either eliminate or suppress T cells. cDCs are recruited to inflamed tissues in response to inflammatory chemokines and then remobilized to regional LNs. In contrast, pDCs directly transmigrate to regional LNs via high endothelial venules. In addition to their important function as uniquely powerful antigen-presenting cells, DCs secrete a number of immunostimulatory cytokines such as IL-12 or IFN-α after stimulation that not

only influence the strength and quality of the adaptive immune responses, but also regulate other innate immune cells such as natural killer cells (NK cells) (Steinman and Banchereau, 2007). The ability of DCs to regulate both arms of the immune system makes them to important master regulators during both, steady state and inflammation.

1.2.3 Dendritic cells during viral infections

During a viral infection, DCs can be matured either through PAMPs, danger signals, or through cytokines secreted by other immune cells. In addition, viruses may also infect DCs by binding to surface molecules, or after being engulfed but not destroyed which can lead to the activation of the cell. In order to recognize viruses, DCs express TLRs recognizing either nucleic acids or structural components of viruses (Iwasaki and Medzhitov, 2004). In particular, pDCs express TLR7 and TLR9 enabling them to recognize ssRNA and DNA viruses, respectively, and produce rapidly large amounts of type I interferons (Gilliet et al., 2008). cDC express a different set of TLRs that are important during viral infections, namely TLR2, TLR3 and TLR8, that enable cDCs to recognize viral glycoproteins, dsRNA, or ssRNA, respectively and induce the production of type I interferons and proinflammatory cytokines such as IL-12 (Iwasaki and Medzhitov, 2004). These cytokines secreted by DCs fulfill several important functions, they i) activate immune cells, ii) induce an antiviral state in cells and iii) determine the type of adaptive immunity induced. In addition, after the uptake of viral antigens by phagocytosis or the direct synthesis of viral antigens in DCs, virally-derived peptides are expressed on the surface on both MHC class I and II molecules. This generates DCs that are able to activate naive CD8 T cells in order to generate cytotoxic CD8 effector T cells and also to activate CD4 T cells. Then, these CD4 T cells are important for helping CD8 T cells (Th1) and for the production of antibodies by B cells (Th2), but moreover

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can also have direct effector functions. Whether DCs stimulate Th1 or Th2 responses depend on the maturation stimulus, particularly on its molecular and physical nature (Iwasaki and Medzhitov, 2004).

Since DCs play such a crucial role in the induction of antiviral responses, many viruses have developed strategies to evade recognition and interfere with the functions of DCs. Numerous stages during the initiation of immunity by DCs have been targeted for inhibition by viruses and bacteria. These include antigen detection, DC maturation, DC migration, antigen presentation to lymphocytes by DCs, and effector cytokine release leading to a defect in immune function of DCs (Trifilo et al., 2006). For example, Toll-like receptors, acting as DC detector systems for viral and bacterial byproducts, have recently been shown to be targets for inhibition by viral infection (Kopp and Medzhitov, 2003). Specifically, vaccinia virus was found to block signaling of Toll-like receptors, resulting in the complete inhibition of DC maturation and immunosuppression of the host (Bowie et al., 2000). Another effective immune evasion strategy is the impairment of antigen presentation by DCs, which aborts T cell stimulation and either prevents activation of or nonproductively activates antigen-specific T cells. The ability to disrupt antigen presentation has evolved in many virus families, including adenoviruses, HIV, and herpesviruses (Rinaldo and Piazza, 2004).

A classical example for the generation of immune responses by DCs during viral infection is the beta-herpes virus murine cytomegalovirus (MCMV). During the early phases of infection with the MCMV, murine pDC and cDC collaborate to induce effective innate and adaptive immune control of the virus (Delale et al., 2005; Krug et al., 2004). After sensing the virus using multiple TLRs, pDCs and cDC are induced to produce type I interferons, TNF-α, and IL-12p70 that activate NK cells and polarize a Th1 adaptive immune response (Andoniou et al., 2005; Delale et al., 2005). In the case of MHV-68,

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DCs were found to produce IL-12, IFN-α and IL-6 *in vitro* in a TLR9-dependent manner. Finally, the recognition of EBV by pDCs was shown to activate NK cells and T cells to produce IFN-γ (Lim et al., 2006). Furthermore, moDCs were found to efficiently prime T cell responses in EBV sero-negative individuals *in vitro* (Bickham et al., 2003). The combination of these abilities of DCs to sense viral infections and then to activate innate and adaptive immunity accordingly makes DCs crucial players in anti-viral immune responses and their role during EBV infection needs to be further evaluated.

1.2.4   NK cells and NK cell subsets

NK cells were originally described as a homogenous population of innate lymphocytes characterized by their ability to spontaneously kill target cells (Herberman et al., 1975b; Kiessling et al., 1975; Trinchieri, 1989). However, already in the 1980s, it was proposed that human NK cells in peripheral blood can be divided into at least two subsets based on the expression of CD56 and CD16 (Table 1) (Lanier et al., 1986).The major subset of CD56^{dim}CD16^{+} NK cells constitutes around 90 % of total blood NK cells, kills target cells upon proper recognition and secretes only low levels of IFN-γ (Cooper et al., 2001). In contrast, CD56^{bright}CD16^{-} NK cells (<10% of total blood NK cells) produce large amounts of cytokines including IFN-γ, TNF and GM-CSF upon stimulation by pro-inflammatory cytokines, but acquire cytotoxicity only after prolonged activation. Further differences between the subsets have also been found with respect to expression of inhibitory and activating receptors. While CD56^{bright}CD16^{-} NK cells express high levels of the inhibitory CD94/NKG2A complex recognizing HLA-E, they do not express MHC class I allele-specific killer-inhibitory receptors (KIRs) that are in contrast expressed by CD56^{dim}CD16^{+} NK cells. Regarding the expression of activating receptors, both NK cell subsets in human peripheral blood express the activating receptors NKG2D and NKp30 as well as

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NKp46, whose ligands are induced in pathogen-infected or stressed cells. Yet a major discrepancy exists between the two subsets in the expression of the antibody dependent cellular cytotoxicity (ADCC) mediating CD16 (FcγRIII) receptor which is only present on the CD56^{dim} subset (Jacobs et al., 2001; Moretta et al., 2001). Finally, NK cell subsets also differ in the expression of chemokine receptors. Cytotoxic CD56^{dim}CD16$^+$ NK cells express CXCR1 and CX3CR1 and respond to IL-8 and fractalkine, the respective ligands for these receptors (Campbell et al., 2001). Interestingly, only CD56^{bright} NK cells express secondary lymphoid organ (SLO) homing markers such as CCR7, CD62L, and CXCR3, resulting in an enrichment of this subset in SLO and sites of inflammation, respectively (Campbell et al., 2001; Fehniger et al., 2002; Ferlazzo et al., 2004b). The question of whether or not the development of the human subsets interconnected has been under investigation for some time. Recently, a number of studies suggested that CD56^{bright}CD16$^-$ NK cells are able to differentiate into CD56^{dim}CD16$^+$ NK cells upon prolonged activation (Chan et al., 2007; Romagnani et al., 2007).

Murine NK cells share a lot of the properties of human NK cells, but since they do not express the homologue of CD56, it has proven to be difficult to identify functionally different NK cell subsets in mice. More recently however, several studies showed the presence of functionally different NK cell subsets in mice (Table 1) (Blasius et al., 2007; Hayakawa and Smyth, 2006; Vosshenrich et al., 2006). The first study suggested differentiating mature NK cells according to their expression of CD27 into Mac1^{high}CD27$^+$ and Mac1^{high}CD27$^-$ subsets (Hayakawa and Smyth, 2006). Although these two subsets differ in the expression of inhibitory and activating receptors as well as chemokine receptors, there are striking differences between human and mouse subsets. Most importantly, Mac1^{high}CD27$^+$ NK cells are superior to Mac1^{high}CD27$^-$ cells in both the production of cytokines as well as cytotoxicity, whereas in human NK cell subsets

CD56^{bright}CD16$^-$ NK cells are superior in IFN-γ production, but are not cytotoxic. In another study, a new developmental pathway for a distinct NK cell subset was described in the thymus (Vosshenrich et al., 2006). These cells are characterized by expression of the IL-7 receptor, CD127, and the transcription factor GATA-3. Interestingly, this subset resembles human CD56^{bright}CD16$^-$ NK cells in expression of CD127, lower expression of inhibitory molecules and cytotoxic molecules, yet higher cytokine production after IL-12 stimulation. Furthermore, this subset seems to be enriched in lymph nodes compared to other NK cells although to a lesser degree compared to humans (15-30 % vs. 75 %, respectively) (Ferlazzo et al., 2004b; Vosshenrich et al., 2006). It remains unclear so far which functions this NK cell subset has *in vivo*, and how closely its development reflects CD56^{bright}CD16$^-$ NK cell generation in humans, given the previously discussed experimental evidence for a linear instead of a separate development of the two functionally diverse NK cell subsets in humans. In the third study, NK1.1$^+$B220$^+$CD11c$^+$ NK cells were found to be enriched in secondary lymphoid tissues and to secrete higher levels of IFN-γ compared to other mouse NK cells (Blasius et al., 2007). However, NK cells of this subset also killed classical NK cell targets efficiently (Caminschi et al., 2007), and CD11c, B220 and MHC class II were upregulated on NK1.1 cells upon activation (Vosshenrich et al., 2007). Thus, NK1.1$^+$B220$^+$CD11c$^+$ cells might represent *in vivo* activated NK cells rather than the mouse equivalent of human CD56^{bright}CD16$^-$ NK cells. Nevertheless, the discovery of functionally different NK cell subsets in humans and mice has extended the field of NK cell research tremendously and yielded insight into important non-cytotoxic functions of NK cells.

Table 1: Differences between human and murine NK cells

Affected Aspect	Mouse	Human
Subsets:		
NK cell subsets enriched in SLO	Mac1^{high}CD27$^+$ (high/high)* CD127$^+$GATA-3$^+$ (low/high) CD11c$^+$B220$^+$ (high/high)	CD56^{bright}CD16$^-$ (low/high)
NK cell subsets enriched in blood	Mac1^{high}CD27$^-$ (low/low) CD127$^-$GATA-3$^-$ (high/low) CD11c$^-$B220$^-$ (low/low)	CD56^{dim}CD16$^+$ (high/low)
Frequency:		
LN	0.1 – 0.3%	1 – 5%
Spleen	2 - 3 %	7 – 50 %
Blood	2 - 3 %	7 – 25 %
Receptor/ligand interactions:		
Natural cytotoxicity receptors	NKp46	NKp30, p44, p46
Ligands for the activating NK cell receptor NKG2D	Rae1α–ϵ, H60, MULT-1	MICA/B, ULBP1–4, RAET1G
Inhibitory receptors for MHC class Ia molecules	Ly49	Killer cell Ig-like receptors

*: indicates cytotoxicity/IFN-γ secretion

1.2.5 Tissue distribution and trafficking of NK cells

In line with their role in innate immunity and immune surveillance, NK cells are widely distributed in mammals, yet, intriguingly, the distribution of NK cell subsets differs between distinct anatomical sites, suggesting a specialization of NK cell subsets (Ferlazzo and Munz, 2004; Ferlazzo et al., 2004b; Gregoire et al., 2007; Trinchieri, 1989). In humans, the CD56^{bright}CD16$^-$ subset is markedly enriched in SLO, making up to 75 % of NK cells in lymph nodes and 50 % in the spleen (Ferlazzo et al., 2004b). As lymph nodes are suggested to harbor 40% of all human lymphocytes, whereas probably only 2% of all lymphocyte circulate through peripheral blood at any given moment, CD56^{bright}CD16$^-$ NK cells in SLO constitute a remarkable pool of innate effector cells in humans. In mice, the distribution of NK cells subsets is also different as Mac1$^+$CD27^{high}, CD127$^+$ and B220$^+$CD11c$^+$ NK cells are all enriched in lymph nodes, whereas they

represent minor subsets in spleen and peripheral blood (Hayakawa and Smyth, 2006; Vosshenrich et al., 2006). In general, while mouse NK cells are mainly excluded from B and T cell areas in both spleen and lymph nodes in the steady state, human NK cells are present at significant levels of 1-5% of all mononuclear lymph node cells in perifollicular T cell zones in these secondary lymphoid organs (Table 1) (Fehniger et al., 2002; Ferlazzo et al., 2004a; Gregoire et al., 2007). Their localization in perifollicular regions in lymphoid organs potentially positions them to interact with incoming DCs that arrive through the afferent lymph. Notably, in human lymph nodes NK cells can be found in close proximity to resident DCs, furthermore intra-vital microscopy of mice revealed that NK cells were crawling and communicating with their environment in lymph nodes, forming contacts with DCs (Bajenoff et al., 2006; Ferlazzo et al., 2004a; Garrod et al., 2007). But, NK cells can not only be found in lymphoid tissues, also lung, liver and skin have been shown to harbor significant numbers of NK cells in both mice and humans. Finally, NK cell distribution is not static as NK cells can recirculate between organs in a subset-specific manner (Gregoire et al., 2007). In addition to their distribution and recirculation in the steady state, NK cells are recruited to sites of inflammation (Trinchieri, 1989). Detailed analysis of mouse NK cells showed that they can be recruited to lymph nodes, lung, liver, and central nervous system during infections. These NK cells apparently mainly originate from the spleen and the bone marrow as NK cell numbers decrease in these organs as they increase in other organs (Wald et al., 2006). Due to their expression of chemokine receptors such as CCR2, CCR5, CXCR3, and CX_3CR1, NK cells are able to respond to a large array of inflammatory cytokines (Colucci et al., 2003; Gregoire et al., 2007). As discussed above, NK cell subsets in both man and mouse differ in the expression of certain chemokine receptors. Notably, in humans mainly $CD56^{bright}CD16^-$ and only few $CD56^{dim}CD16^+$ NK cells were found in a number of

inflammatory sites suggesting that the CD56^{bright}CD16^{-} NK cell subset is specifically recruited to sites of infection, inflammation and tumorigenesis (Carrega et al., 2008; Dalbeth et al., 2004; Ottaviani et al., 2006). Finally, inflammation also causes a redistribution of mouse NK cells in spleen and lymph nodes into T cell zones into close proximity of DCs (Bajenoff et al., 2006). The CXCR3-dependent recruitment to the lymph node in the mouse might be very different from the situation in humans as human CD56^{bright}CD16^{-} NK cells express the lymph node-homing molecule CCR7 and are found in significant numbers also in non-inflamed lymph nodes (Fehniger et al., 2002; Ferlazzo et al., 2004b; Martin-Fontecha et al., 2004). Additionally, separate NK cell development to CD56^{bright}CD16^{-} NK cells in lymph nodes also contributes to the enrichment of this NK cell subset at these sites (Freud et al., 2005). Furthermore, in the non-inflamed human spleen, NK cells are found in close proximity to DCs in T cell zones, again demonstrating species-specific differences (Ferlazzo et al., 2004a). Altogether the analysis of the distribution and localization as well as the recruitment of NK cells and more recently of NK cell subsets in mouse and man has provided important insight into NK cell biology, highlighted differences between the species and led to the discovery of new functions of NK cell subsets.

1.2.6 Activation of NK cells during innate immune responses

Initially, NK cells were recognized for their ability to mediate spontaneous cytotoxicity against target cell lines, however, it was later discovered that additional signals were needed for NK cells to become fully activated. Numerous studies in humans and mouse models both *in vivo* and *in vitro* found that these signals can be provided by DCs (Ferlazzo et al., 2003; Ferlazzo et al., 2002; Fernandez et al., 1999; Gerosa et al., 2002; Piccioli et al., 2002; Yu et al., 2001). The activation of NK cells was demonstrated to be

mediated directly by cell-cell contact and indirectly via the secretion of cytokines such as type I interferons or monokines such as IL-12, IL-15, and IL-18 (Figure 3). Among the soluble factors, IL-12 has been repeatedly observed to induce IFN-γ secretion and proliferation and was thought to be the most pivotal signal enhancing factor for NK cell effector functions in humans and in mice (Ferlazzo et al., 2004a; Orange and Biron, 1996a; Orange and Biron, 1996b; Yu et al., 2001). Yet, a recent study suggests that IL-15 acts as an even earlier and more crucial regulator of NK cell differentiation and function at least in mice (Lucas et al., 2007). After *in vivo* stimulation with TLR ligands or bacterial and viral infection, it was shown that myeloid CD11c^{high} DCs need to prime NK cells via presentation of IL-15 to produce IFN-γ and become cytotoxic against a MHC class I low cell line expressing NKG2D ligands. Curiously in analogy to adaptive T cell responses, NK cells needed to enter the draining lymph node to receive the priming signal to subsequently perform effector functions in the periphery. In humans, we have recently shown that IL-15 receptor alpha colocalizes at the synapse between DCs and NK cells and contributes to NK cell survival (Brilot et al., 2007). Closer characterization of this synapse revealed that both activating signals such as mediated by IL-15 and inhibitory signals such as interactions of CD94/NKG2A and KIRs with MHC class I molecules are transmitted in spatially separated domains within the center of this synapse. The regulatory DC/NK cell synapse was formed very rapidly (1-5min), lasted for long time periods (≥10min) and was distinct from activating NK cell synapses formed with classical MHC class I[low] NK cell targets (Davis and Dustin, 2004; Vyas et al., 2002). Strikingly, DCs are protected from NK cell killing, although intracellular Ca^{2+} levels rise in NK cells upon interaction which have been correlated with killing of target cells (Brilot et al., 2007; Vyas et al., 2002). Notably, at later timepoints (>20 min) DCs seem to polarize

preformed vesicles of IL12 to the synapse providing further activation signals to conjugated NK cells (Borg et al., 2004). These studies suggest that efficient NK cell activation and maintenance requires synapse formation with DCs.

Based on previous studies the different NK cell activating cytokines were believed to mediate different aspects of DC-induced NK cell stimulation and steer the innate immune response to distinct NK cell effector functions. While type I interferons, mainly secreted by plasmacytoid but also myeloid DCs are predominantly involved in the upregulation of NK cell-mediated cytotoxicity, IL-12 and IL-18 were found to influence IFN-γ production by NK cells (Andrews et al., 2003; Dalod et al., 2002; Nguyen et al., 2002; Orange and Biron, 1996a; Orange and Biron, 1996b). Finally, *in vitro* studies suggest that another pathway for the activation / coactivation of NK cells might be the direct recognition of microbial products via TLRs or of infected cells via NKG2D/NCRs (Becker et al., 2003; Girart et al., 2007; Moretta et al., 2000; Pende et al., 2002; Schmidt et al., 2004; Sivori et al., 2004). These distinct pathways of NK cell activation might converge to regulate and activate different arms of NK cell effector functions, and thereby tailor the NK cell response to the needs of the particular immune response.
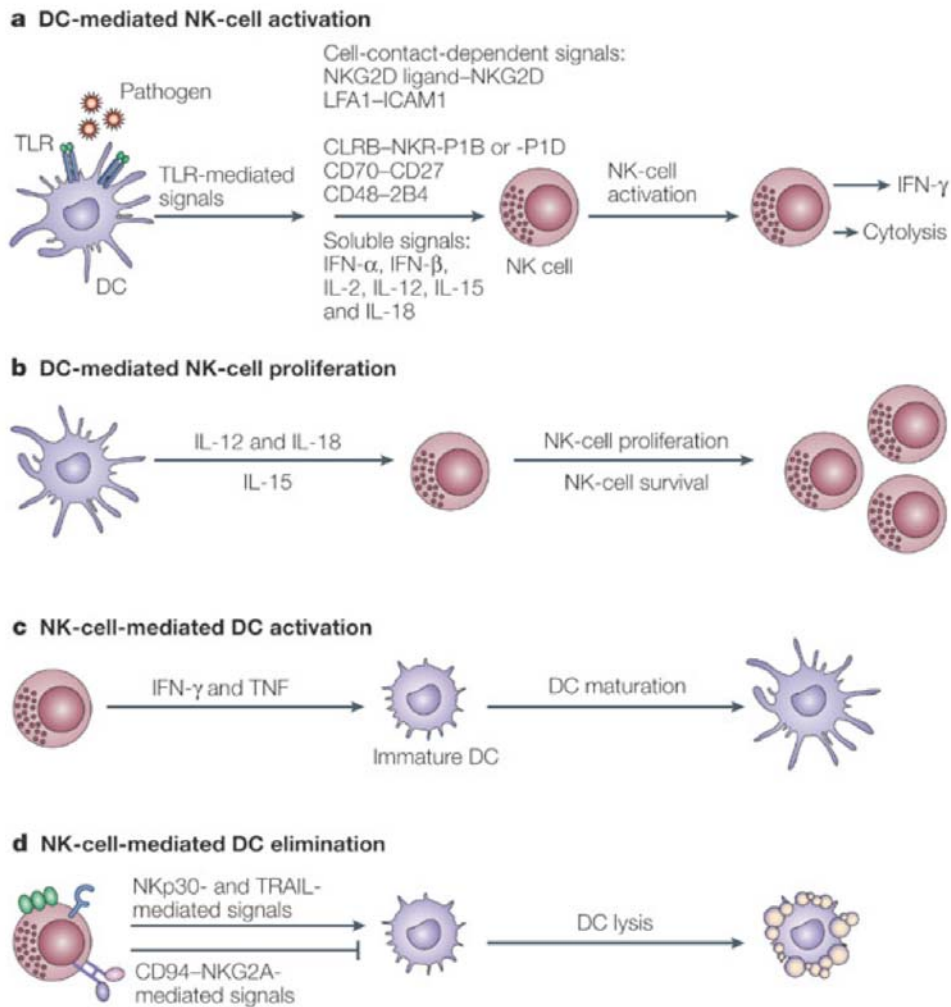
Figure 3: DCs can affect NK cell functions by inducing the activation and/or proliferation of NK cells. DC-mediated activation of NK cells, which results in increased NK-cell cytolytic activity and/or interferon- (IFN-) production, can be induced by both resting and activated DCs, although the latter are more potent activators. Both cell-contact-dependent interactions and soluble cytokine signals are involved. NKG2D (NK group 2, member D) ligands that are expressed by DCs in response to appropriate stimuli have been implicated. A role for adhesion molecules has also been indicated by the finding that LFA1 (lymphocyte function-associated antigen 1)–ICAM1 (intercellular adhesion molecule 1) interactions are important for DC-mediated activation of NK cells. The relevance of interactions mediated by CD70–CD27, CD48–2B4 and CLRB (C-type-lectin-related B)–NKR-P1B (NK-cell receptor protein 1B) or NKR-P1D requires assessment. Cytokine signals are essential for NK-cell activation, and several cytokines are involved (a). DC-derived cytokines have also been implicated in DC-mediated proliferation of NK cells (b). Reciprocally, NK cells can affect DC functions and lead to DC activation (c) or DC elimination (d). NK-cell-mediated activation of DCs seems to depend mainly on cytokines, principally tumour-necrosis factor (TNF) and IFN- (c), whereas cell-contact-dependent interactions are required for DC elimination (d). IL, interleukin; NKp30, NK-cell protein 30; TLR, Toll-like receptor; TRAIL, TNF-related apoptosis-inducing ligand. (Degli-Esposti et al., 2005)

1.2.7 The role of NK cells in viral immunity

As previously discussed, NK cells are readily recruited and activated at sites of viral infections. Traditionally, NK cells have been recognized for their protective role by directly killing infected cells. Intriguingly, the cytolytic activity of fully matured NK cells is controlled by the balance between inhibitory and activating signaling pathways (Moretta et al., 2001). To prevent killing of normal cells, most NK cells express an array of inhibitory receptors, many of which recognize major histocompatibility complex (MHC) Class I molecules, expressed by almost all nucleated cells (Kumar and McNerney, 2005). This observation led to the missing-self hypothesis, whereby the postulated role of NK cells is to destroy cells that express decreased levels of MHC Class I molecules(Ljunggren and Karre, 1990). Indeed, MHC class I molecules are often down-regulated in virally infected and cancer cells, supposedly a trait selected for avoiding cytotoxic T-lymphocyte (CTL) recognition (Tortorella et al., 2000). The binding of MHC class I complexes to KIRs or to the heterodimeric CD94/NKG2A receptor initiates inhibitory pathways that can override activation signals (Natarajan et al., 2002). On the other hand, reduced expression of MHC Class I is not the only requirement for NK cell activation, and overexpression of activating ligands on target cells can also trigger NK cell function. Moreover, absence of MHC class I only translates into NK cell recognition if activating NK cell receptors are also engaged by activating structures on the MHC class I low target cell. However, in addition to their cytolytic abilities, it has been established that cytokines such as IFN-γ and TNF-α, produced by NK cells, contribute to the control of multiple murine and human infections (Biron et al., 1999). The effects of IFN-γ in innate immunity are manifold, ranging from strengthening intrinsic immunity via the

induction of antiviral factors or degradative pathways in exposed cells, to the activation of other innate lymphocytes such as macrophages (Boehm et al., 1997).

The most prominent example of innate immune control of a herpesvirus by NK cells is the murine cytomegalovirus (MCMV), a β-herpesvirus. Innate resistance to this virus via cytotoxicity is mediated by NK cells that carry the Ly49H activating NK cell receptor (Daniels et al., 2001; Lee et al., 2001). Mouse strains susceptible to acute fatal MCMV infection lack Ly49H, and Ly49H transgene expression confers resistance to MCMV in these mice (Lee et al., 2003). Not unlike NKp46, this receptor selectively recognizes a viral surface protein on infected cells called m157 (Arase et al., 2002). In MCMV infection it was also further shown that DC-activated NK cells produce IFN-γ that directly contributes to control viral infection (Andrews et al., 2003; Orange et al., 1995). Moreover, it was demonstrated that NK cells can protect the host via secretion of IFN-γ, but not as efficiently as in combination with cytotoxicity (Loh et al., 2005). IFN-γ directly inhibits replication of MCMV, protecting against virus-induced pathogenesis and lethality (Presti et al., 1998; Scalzo et al., 2007). In addition, IFN-γ is also critical for efficient clearance of persistent viral replication and suppresses MCMV reactivation from latency (Presti et al., 1998). Notably, IFN-γ inhibits growth of MCMV in infected macrophages in a cell-type specific manner via suppression of immediate early protein 1 expression (Presti et al., 2001). With respect to human herpesviruses, there is evidence to support the role of NK cells in limiting the early infection of HCMV, a β-herpesvirus (Iversen et al., 2005). However, it remains unclear if NK cells also contribute to the immune control of gamma-herpes viruses such as EBV although several lines of evidence suggest a role for innate lymphocytes in the resistance against EBV-associated malignancies. Firstly, in male patients with X-linked lymphoproliferative disease (XLP), who frequently succumb

after primary EBV infection to EBV-induced lymphomas, a mutation in the SAP gene leads to defective recognition of EBV-transformed B cells by NK cells (Benoit et al., 2000; Morra et al., 2001; Parolini et al., 2000). Secondly, IL-2-activated peripheral blood NK cells have been shown to restrict EBV-induced B cell transformation *in vitro* (Kaplan and Shope, 1985; Masucci et al., 1983; Wilson and Morgan, 2002). Thirdly, NK cell depletion from PBMCs prior to adoptive transfer into *SCID* mice, rendered the animals more susceptible to tumor development after transfer of EBV-transformed B cells (Baiocchi et al., 2001). Fourthly, activated NK cells have been shown to lyse lytically EBV replicating B cells (Pappworth et al., 2007). Fifthly, a novel primary immunodeficiency with a specific NK cell defect was recently reported to be associated with EBV driven lymphoproliferative disease (Eidenschenk et al., 2006). Therefore, NK cells may be involved in the early phase of the EBV specific immune response.

1.3 Adaptive immune responses against herpes viruses

The innate immune system is effective in combating many pathogens, but it relies on germ-line encoded receptors for the recognition of conserved structures of the pathogen. As a consequence, many pathogens have developed strategies to evade recognition and phagocytosis by the innate immune system. The recognition mechanism used by the lymphocytes of the adaptive immune response has evolved to overcome the constraints faced by the innate immune system, and enables recognition of an almost infinite diversity of antigens, so that each different pathogen can be targeted specifically. This process of clonal selection and immunological memory has been the basis for the successful development of vaccines against infectious disease such as polio, influenza, diphtheria, and tetanus that save millions of lives every year.

1.3.1 T cell mediated control of herpes virus infection

Herpes viruses have evolved a number of strategies to evade the innate and adaptive immune response, most strikingly they have the ability to down-regulate viral gene expression and persist in a latent state in infected host cells (Young and Rickinson, 2004). By limiting viral protein production, they limit the amount of foreign antigen and infected cells evade recognition by the immune system. However, at the same time constantly a certain number of infected cells undergo lytic replication leading to the release of infectious virus and reinfection *in vivo* posing a constant challenge to the immune system. Indeed, pathological viral reactivation after immunosuppression of CMV and EBV sero-positive individuals demonstrate that constant immune surveillance is necessary to limit viral spread. In the case of EBV, it has been postulated that T cells constitute the decisive component of EBV specific immune control against virus associated malignancies, as adoptive transfer of EBV specific T cell lines can eradicate EBV associated post-transplant lymphomas (Gottschalk et al., 2005; Hislop et al., 2007). But, it remains unclear which T cell subset is the major contributor responsible for EBV immune control. Initial longitudinal studies focused on CD8$^+$ T cells since EBV specific CD8$^+$ T cells are expanded during IM with CD8 T cells specific for lytic or latent antigens making up to 40 % or 5 % of the CD8$^+$ T cell population, respectively (Callan et al., 1998)(Figure 4). In contrast, EBV specific CD4$^+$ T cells are not as drastically expanded as CD8$^+$ T cells, but elevated with frequencies up to 5 % (Amyes et al., 2003). Notably, as lytic responses decrease over time, LCL specific IFN-γ secretion by T cells is derived in equal parts from both CD4$^+$ and CD8$^+$ T cells during persistent infection (Bhaduri-McIntosh et al., 2008). In addition to the question about the roles of the T cell subsets, it is also unknown which T cell specificities are protective during lytic and latent infection. The CD8$^+$ T cell responses against lytic proteins are clearly focused on IE proteins

(BZLF1, BRLF1), and on few E proteins, while the CD4$^+$ T cell response seems to be broader (Adhikary et al., 2006; Precopio et al., 2003; Steven et al., 1997). In the case of latent infection, different latency patterns confer different degrees of immunogenicity for recognition by cytotoxic CD8$^+$ and helper CD4$^+$ T cells (Hislop et al., 2007; Khanna and Burrows, 2000). In healthy EBV carriers, the EBNA3 proteins are the dominant targets of CD8$^+$ T cell responses, while EBNA1, EBNA2, and EBNA3C are the most consistently recognized CD4$^+$ T cell. In contrast, the LMPs are subdominant T cell antigens antigens (Hislop et al., 2007).

1.3.2   CD4$^+$ T cells in immunity against herpes viruses

CD4$^+$ T cells are classically thought to orchestrate adaptive immune responses and it is widely accepted that CD4$^+$ T cells provide helper functions for antigen-presenting cells and assist B cells in the production of antibodies. But, studies of MHV-68 have shown that CD4$^+$ T cells can also directly control infection and its malignant consequences *in vivo*, independent of CD8$^+$ T cells and B cells (Robertson et al., 2001). While the depletion of CD8$^+$ T cells alone resulted in higher virus loads in lung and spleen, the depletion of both CD4$^+$ and CD8$^+$ T cells leads to a fatal primary infection (Christensen et al., 1999). In the case of EBV, there is also *in vitro* evidence that CD4$^+$ T cells control EBV and EBV-associated malignancies. Depletion of CD4$^+$ T cells from *in vitro* assays abolishes immune control of EBV, and addition of CD4$^+$ T cells to T cell–depleted, EBV-infected PBMCs inhibited B cell transformation by EBV more efficiently than unsorted T cells. Furthermore, CD4$^+$ T cell clones specific for EBNA1 and EBNA2 were found to inhibit outgrowth of EBV-transformed B cells (Nikiforow et al., 2001; Omiya et al., 2002). B cell transformation by EBV can also be inhibited by EBV-specific CD4$^+$ T cells derived from the purified CD4$^+$ T cells of EBV seronegative donors that were primed *in vitro* by

DCs (Bickham et al., 2003). Together these findings show that EBV infection *in vitro* can be controlled by virus-specific CD4$^+$ T cells primed *in vitro* (i.e., a primary response) and by virus-specific CD4$^+$ memory cells primed *in vivo* (i.e., a secondary response). It appears that CD4$^+$ T cells can exert this control directly, independent of CD8$^+$ effector cells, which suggests that CD4$^+$ T cells can mediate virus-specific immune control as effectors on their own.

The protective effect CD4$^+$ T cells was classically thought to be mediated via the secretion of cytokines such as IFN-γ. IFN-γ is necessary for the protection of specific organs in mice against MCMV by CD4$^+$ T cells *in vivo*, but also for the immune control in MHV68 (Lucin et al., 1992; Presti et al., 1998). Furthermore, EBV-specific CD4 T cells have been found to consistently produce IFN-γ. In addition, it was recently also found that virus-specific CD4$^+$ T cells can directly kill infected cells. Notably, EBV-specific cells were among the first CD4$^+$ cytolytic T lymphocytes (CTLs) to be isolated, using LCLs as targets. These CD4$^+$ CTLs recognized a variety of different EBV proteins expressed in both latent—EBNA1 (Paludan et al., 2002), EBNA2 (Long et al., 2005), EBNA3C (Long et al., 2005), and LMP2 (Su et al., 2001)—and lytic—BHRF1 (Landais et al., 2004), BALF4, and BLLF1 (Adhikary et al., 2006)—stages of EBV infection. Apoptosis induced by the ligand of the death receptor Fas (FasL) (Nikiforow et al., 2001; Paludan et al., 2002) and killing by the death effector molecule perforin (Su et al., 2001) were both identified as cytotoxic mechanisms of EBV-specific CD4$^+$ CTLs. Interestingly, the EBNA1-specific CD4$^+$ CTLs as well as BLLF1- and BALF4-specific CD4$^+$ CTLs were found to target Burkitt's lymphoma, an EBV-associated B cell lymphoma which resists recognition by CD8$^+$ T cells due to down-regulation of the MHC class I antigen processing machinery. Therefore, particularly in humans where nearly all tissues can

express MHC class II molecules after immune activation, virus-specific CD4$^+$ CTL could fill the gap left by viral immune escape from CD8$^+$ CTL recognition.

The presence of CD4$^+$ CTL is neither rare nor exclusive to EBV immunity and to *in vitro*– cultured T cell lines. For example, in mice infected with the murine lymphocytic choriomeningitis virus (LCMV), killing by CD4$^+$ CTLs was observed *in vivo* after injection of fluorescent-labeled target cells coated with viral peptides (Jellison et al., 2005). This LCMV-specific CD4$^+$ T cell cytotoxicity was at least in part due to FasL-induced apoptosis. In addition, human peripheral blood contains CD4$^+$ T cells that are positive for the cytotoxic effector molecules perforin and granzyme (Appay et al., 2002) and some are specific for HCMV. However, while cytotoxic CD4$^+$ T cells have now been reported *in vitro* and *in vivo*, their functional relevance *in vivo* remains unclear and remains intensely debated due to it's potentially important impact on vaccine development.
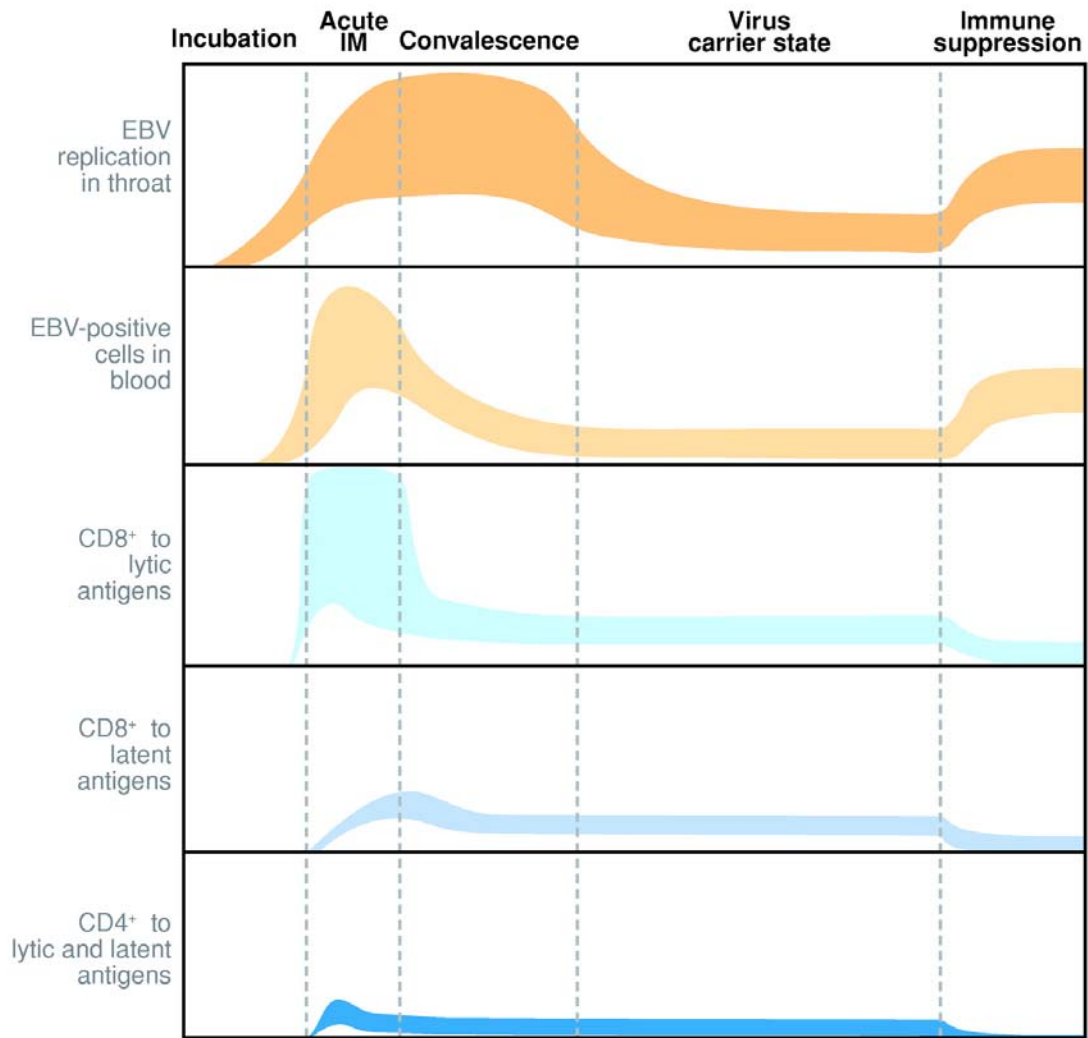
Figure 4: Correlation between viral titers and EBV specific immune responses. Diagrammatic representation of changes over time in virus replication in the throat and in the load of latently infected B cells in the blood during acute and convalescent IM, and in the long-term carrier state. Changes in the magnitude of T cell responses are shown over the same time scale. The effects of T cell suppression on these parameters of the virus-host balance, as seen in post-transplant patients, are also shown (From Hislop et al, 2007).

1.4 Animal models to study human-trophic viruses

The use of animal models in the study of human diseases has had obvious advantages.

Fundamental properties of the disease can be investigated more invasively and

thoroughly, while drug and vaccine toxicity and efficacy in animal models can provide

blueprints for ensuing trials in human subjects, limiting the risk, time and cost of trials in humans (Ambrose et al., 2007). Hence, researchers have made a considerable effort to generate animal models for human diseases, and mice are a preferred species for many avenues of immunological research *in vivo*. Since the evolutionary divergence of mouse and man 65 million years ago, however, these two species have inhabited different ecological niches and have been challenged with minimally overlapping groups of pathogens. The human and mouse immune systems, evolving to meet these challenges, have therefore accumulated many differences, making genes related to immunity, together with genes involved in reproduction and olfaction, the most divergent between the two species (Mestas and Hughes, 2004; Waterston et al., 2002). Finally and most importantly, a number of clinically important viruses such as human immunodeficiency virus (HIV), hepatitis C virus (HCV) and EBV do not infect mice and most other animal species tested. In the case of HIV, researchers took advantage of the observation that even though most nonhuman primates are resistant to HIV-1 infection, they do harbor relatives of the human virus. In fact, the majority of African monkey species are hosts for various simian immunodeficiency viruses (SIVs). However, in contrast to humans, most primates can easily live with high virus burdens and they do not usually develop disease. The host–virus relationship seems to be quite different in these animals, so they cannot be used as classical animal models of human HIV-1 infection (Ambrose et al., 2007). In order to overcome the limited availability and high cost of non-human primates alternative small animal models are desirable to carry out *in vivo* research without putting individuals at risk. Humanized mice, or mouse–human chimaeras, have been developed during the last decades to overcome these constraints and are now an important research tool for the *in vivo* study of human cells and tissues (Shultz et al., 2007).

1.4.1 Humanized mice to study human-trophic virus infection *in vivo*

Humanized mice can be defined as immunodeficient mice engrafted with human haematopoietic cells or tissues, or mice that transgenically express human genes. The development of mice that are 'humanized' by engraftment of human tissues, haematopoietic stem cells (HSCs) or peripheral-blood mononuclear cells (PBMCs) provides an opportunity to study human biological processes *in vivo* that would otherwise not be possible. Advances in the ability to generate humanized mice have depended on a systematic progression of genetic modifications to develop immunodeficient host mice (Shultz et al., 2007).

Three main breakthroughs have occurred generating mouse models suitable to study longitudinal primary infection with pathogens such as HIV and EBV (Figure 5). First, the discovery of the *Prkdcscid* (protein kinase, DNA activated, catalytic polypeptide; severe combined immunodeficiency, abbreviated *scid*) mutation in CB17 mice was soon followed by the observation that human PBMCs, fetal hematopoietic tissues and HSCs could engraft in these mice (Bosma et al., 1983). However, engraftment occurred at only a very low level, and the engrafted human cells failed to generate a functional human immune system. Limitations impeding human-cell engraftment in CB17-*scid* mice include the spontaneous generation of mouse T and B cells during aging and high levels of host NK cell and other innate immune activity, which limit the engraftment of the human hematopoietic compartment[5]. The *scid* mutation also results in defective DNA repair and, consequently, an increase in radiosensitivity. Hence, targeted mutations at the recombination-activating gene 1(*Rag1*) and *Rag2* loci that prevent mature T- and B-cell development in the mice but have decreased radiosensitivity were developed (Shinkai et al., 1992). However, these mice retained high levels of NK-cell activity and had limited engraftment of human HSCs. The second breakthrough was the development of

immunodeficient non-obese diabetic (NOD)-*scid* mice (Shultz et al., 1995). Crossing the *scid* mutation onto different strain backgrounds led to the observation that NOD-*scid* mice supported higher levels of engraftment with human PBMCs than did any of the other strains that were tested. Furthermore, it was observed that NK-cell activity, which is one of the main impediments to the engraftment of human hematopoietic cells, was lower in NOD-*scid* mice than in CB17-*scid* mice. NOD-*scid* mice also have additional defects in innate immunity that allow higher levels of human PBMC and HSC engraftment. The third breakthrough was the targeted mutations of the interleukin-2 receptor (IL-2R) γ-chain locus (*Il2rγ*) (DiSanto et al., 1995). These mice support greatly increased engraftment of human tissue and especially HSCs as well as PBMCs compared with all previously developed immunodeficient humanized mouse models (Ishikawa et al., 2005; Ito et al., 2002; Traggiai et al., 2004). The absence of the IL-2R γ-chain leads to severe impairments in T- and B-cell development and function, and completely prevents NK-cell development. Based on these breakthroughs three different mouse models have been developed to study functional human immune responses *in vivo*. The first one uses BALB/c Rag2$^{-/-}$ $\gamma_c^{-/-}$ mice, which reconstitute macrophages, T, B, natural killer, and dendritic cells after neonatal intrahepatic HSC transfer (Gimeno et al., 2004; Traggiai et al., 2004). The second model reconstitutes NOD-*scid* $\gamma_c^{-/-}$ mice by intravenous injection of human HSCs (Ishikawa et al., 2005), which also leads to significant development of human myeloid and lymphoid cells. Finally, the most labor-intensive model is the BLT mouse, which requires implantation of human fetal liver and thymus pieces under the NOD-*scid* mouse kidney capsule in addition to intravenous HSC injection (Melkus et al., 2006). Immune compartment reconstitution in peripheral blood of BLT mice is very similar to human.
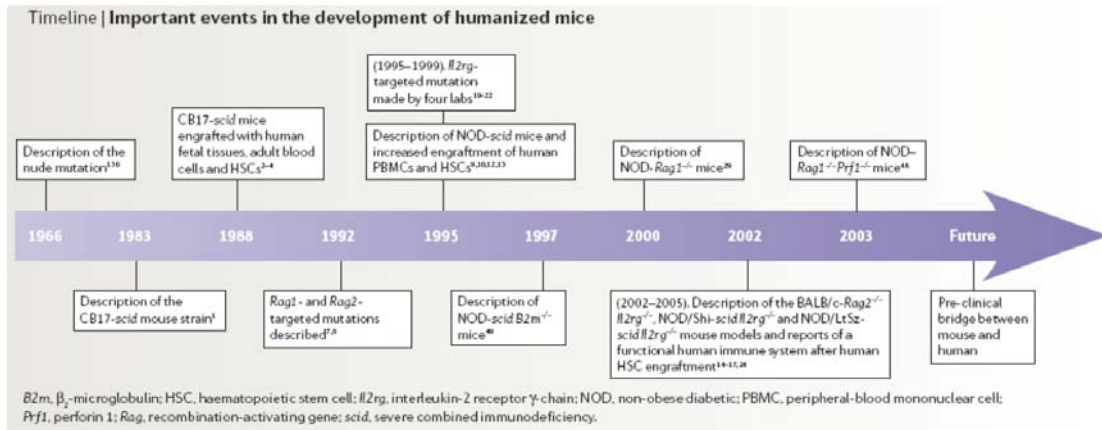
Figure 5: The advances in the ability to generate humanized mice have depended on a systematic progression of genetic modifications to develop immunodeficient host mice. Three main breakthroughs have occurred in this field. First, the discovery of the *Prkdc^scid* (protein kinase, DNA activated, catalytic polypeptide; severe combined immunodeficiency, abbreviated *scid*) mutation in CB17 mice. The second breakthrough was the development of immunodeficient non-obese diabetic (NOD)-*scid* mice. The third breakthrough was the humanization of immunodeficient mice homozygous for targeted mutations at the interleukin-2 receptor (IL-2R) -chain locus (from Shultz et al. 2007).

1.4.2   *In vivo* models for EBV

While no similarly oncogenic γ-herpesviruses of the EBV containing genus *lymphocryptoviridae* (LCVs) has been identified in rodents to date (Ehlers et al., 2008), priming of immune responses to human (EBV) or closely related monkey LCVs has been observed after infection in rhesus macaques (Fogg et al., 2006; Fogg et al., 2005), cottontop tamarins (Wilson et al., 1996). Cottontop tamarins were able to prime MHC class II restricted CD4+CD8+ T cells with cytotoxicity against EBV transformed B cells (Wilson et al., 1996). In rhesus macaques, strong cytotoxic and IFN-γ secreting T cell responses against the monkey virus homologues of the EBNA1 antigen and the immediate early lytic EBV antigen BZLF1 were consistently detected in infected animals (Fogg et al., 2006; Fogg et al., 2005). However, while these animals provide some insight into the relationship between EBV and the immune system, they are imperfect for a number of reasons. First, since these monkeys are not natural hosts of EBV, their

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infection and immune response potentially differ significantly from a human immune response. Second, research that involves monkeys always includes significant ethical and financial hurdles that make these model systems not suitable for large-scale studies. First generation mouse models to study EBV infection and EBV-associated malignancies based on the transfer of human PBMCs into mice carrying the scid mutation (Mosier et al., 1988). But, these models are limited in its utility due to the relatively low levels of engraftment and the anergic state of human cells engrafted in the mice (Hesselton et al., 1993). Injection of larger numbers of human cells to increase levels of cell engraftment leads to development of EBV-related human lymphoproliferative disorders (LPD) in the majority of animals (Rowe et al., 1991). Notably, transfer of EBV-specific T cell clones protected against LPD in this model (Lacerda et al., 1996). In order to improve these models, PBMCs or CD34+ HSCs were transferred into NOD-scid mice (Islas-Ohlmayer et al., 2004; Wagar et al., 2000). These early models served as a preclinical model for the transfer of EBV-specific T cell clones to treat lymphoproliferative diseases and also offered some insight into the contributions of immune cells to successful immune control of EBV. However they were not useful to study primary EBV infection and the development of EBV-specific immune responses. Hence, the lack of an appropriate animal model of EBV infection prevents assignment of a protective value to the known T cell specificities, which are required for the design and evaluation of vaccines against EBV associated tumors and symptomatic primary infection. Therefore, mouse models that partially reconstitute human immune system components after engraftment of HSC are of particular interest to study vaccine candidates and EBV specific immune responses *in vivo*. In this respect, three novel models of human immune system reconstitution have been recently described (Ishikawa et al., 2005; Melkus et al., 2006; Shultz et al., 2005; Traggiai et al., 2004). However, while signs of primary immune

responses were reported in all three of these current mouse models of human immune system reconstitution, the protective value of this immunocompetence and, thus, the potential of these *in vivo* systems as challenge models for vaccine development against pathogens with exclusive tropism for humans has not been evaluated.

2 Material and Methods

2.1 Reagents

2.1.1 Antibodies

Directly labeled monoclonal antibodies for flow cytometry were purchased from BD Bioscience, Caltag, Biolegend, eBioscience, and Immunotech. Unlabeled monoclonal antibodies for flow cytometry were purchased from R&D Systems. Secondary antibodies for flow cytometry were from Molecular Probes. The following monoclonal antibodies containing no azide / low endotoxin (NA/LE) were used for antibody-mediated blocking: anti-IL-12 (clone 24910), anti-IL15 (34593, both R&D Systems), anti-IL-18 (125-2H, MBL International), anti-type I interferon receptor (MMHAR-2, PBL Biomedical Laboratories), anti-HLA-A/B/C (W6/32, Biolegend), and anti-HLA-DR/DP/DQ(Tü39, BD Biosciences). IgG1, IgG2a, or IgG2b (NA/LE, Biolegend) were used as isotype control.

2.1.2 Primers

Table 2: Primers for semi-quantitative PCR

Target	Primer	Sequence
Actin	5'	CAAGAGATGGCCACGGCTGCT
	3'	TCCTTCTGCATCCTGTCGGCA
EBNA1	5'	GAGCGTTTGGGAGAGCTGAT
	3'	CATTTCCAGGTCCTGTACCT
EBNA2	5'	CATAGAAGAAGAAGAGGATGAAGA
	3'	GTAGGGATTCGAGGGAATTACTGA
EBER1	5'	AAAACATGCGGACCACCAGC
	3'	AGGACCTACGCTGGCCCTAGA
LMP1	5'	AGGTTGAAAACAAAGGAGGTGACCA
	3'	GGAACCAGAAGAACCCAAAAGCA

SybrGreen RealTime-PCR

Target	Primer	Sequence
GAPDH (transcript)	5'	AGCCACATCGCTCAGACAC
	3'	GCCCAATACGACCAAATCC
HLA-A2 (genomic)	5'	CGGCGCCCGCGGCTCCATCCT
	3'	GGGGAGCCCCGCTTCATCGCA

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HLA-DR1 (genomic)	5'	GGCCAGTTCTATCTGAATCC
	3'	CGTCTCCTTCTTTGCCATATCCA
LMP1 (transcript)	5'	AGGTTGAAAACAAAGGAGGTGACCA
	3'	GGAACCAGAAGAACCCAAAAGCA

Table 3: Primer for Taqman RealTime-PCR

Target	Primer	Sequence
EBV BamHI W fragment (genomic)	5'	GGACCACTGCCCCTGGTAA
	3'	TTTGTGTGGACTCCTGGGG
	Probe	TCCTGCAGCTATTTCTGGTCGCATCA
Bcl2 (genomic)	5'	CCTGCCCTCCTTCCGC
	3'	TGCATTTCAGGAAGACCCTGA
	Probe	CTTTCTCATGGCTGTCC
BZLF1 (transcript, B95.8))	5'	ACGCACACGGAAACCACA
	3'	CGCTTTATTTCTAGTTCAGAATCGC
	Probe	FAM-CAGCCAGAATCGCTGGAGGAA-BHQ-1
EBNA1 (transcript)	5'	GATCAGGGCCAAGACATAGAGATG
	3'	CCTTTGCAGCCAATGCAACT
	Probe	FAM-TGTCCGGAGACCCCAAAAACG-BHQ-1
EBNA2 (transcript)	5'	GGGATGCCTGGACACAAGAG
	3'	CATGCCCGACGTCATATCCT
	Probe	FAM-CATCACCTCTTGATAGGGATCCGC-BHQ-1
LMP1 (transcript)	5'	TGGAGCCCTTTGTATACTCCT
	3'	TGCCTGTCCGTGCAAATTC
	Probe	FAM-TGATCACCCTCCTGCTCATCGCTCT-BHQ-1
LMP2A$^+$B (transcript)	5'	TGCCTGGATTCTTACAGCAGG
	3'	GGCAGCATCTAATGACCCCA
	Probe	FAM-CCTGATTTTCCTCATTGGCTTTGCCCT-BHQ1
LMP2A (transcript)	5'	CGGGATGACTCATCTCAACACA
	3'	CAATTACAGGCAGGCATACTGG
	Probe	FAM-ATACGAAGAAGCGGGCAGAGGAAGTATGAA-BHQ1
LMP2B (transcript)	5'	GCAGTGTAATCTGCACAAA
	3'	CAATTACAGGCAGGCATACTGG
	Probe	FAM-TGGCGGCAGGCGGGAGGCCGTGCTTTA-BHQ1

2.2 Primary cells and cell lines

2.2.1 Human tonsils, lymph nodes and spleens

All tonsils, lymph nodes and spleens were obtained as part of Institutional Review

Board-approved protocols. Tonsils were collected immediately after surgery from

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patients undergoing tonsilectomy for chronic inflammation. Tonsils were not acutely inflamed at the time of removal. Spleens and lymph nodes were procured by the regional Organ Procurement Organization from brain-dead donors after obtaining informed consent from appropriate individuals. Soon after their removal, tissues were mechanically dissociated to obtain single cell suspensions and were then filtered through a 75-μm nylon cell strainer to exclude undissociated fragments. Debris and dead cells were eliminated by density-gradient centrifugation on Ficoll/Hypaque. Single cell suspensions were then extensively washed and cryopreserved.

2.2.2 Preparation of DCs

PBMCs were isolated from leukocyte concentrates (New York Blood Center) by density-gradient centrifugation on Ficoll/Hypaque. CD14$^+$ cells were isolated from PBMCs by positive magnetic cell separation (MACS, Miltenyi Biotec) and cultured for 5 days in RPMI1640 + 1% single donor plasma + IL-4 and GM-CSF according to standard protocols (Ferlazzo et al., 2004a). The CD14$^-$ cells were frozen for later isolation of B cells and NK cells. Splenic DCs were isolated as previously described by flow cytometric sorting using a BD FACSVantage SE cell sorter (Ferlazzo et al., 2004a). To isolate CD11c$^+$ cells from blood, PBMCs were overlayed with an Optiprep gradient (1.080 to 1.049) and centrifuged for 30 min at 700xg. Low-density fractions were collected and CD11c$^+$ DC were further enriched by depletion of CD14$^+$, CD3$^+$, CD8$^+$, and CD19$^+$ cells by MACS. CD11c$^+$ DCs were purified by flow cytometric sorting using a BD FACS Aria cell sorter by isolating lineage negative (CD3, CD14, CD19, and CD56), HLA-DR$^+$, and CD11c$^+$ cells. Purify after sorting was regularly higher than 99.5%. Monocyte-derived DCs were matured for 2d in medium with IL-4, GM-CSF, and i) 10 ng/ml IL-1β, 1,000 units/ml IL-6, 10 ng/ml TNF-α, and 1 μg/ml prostaglandin E$_2$ (cyt DC), ii) 25 μg/ml

polyinosine-polycytidylic acid (poly(I:C), Invivogen) (poly(I:C) DC), iii) 25 ng/ml IL-1β, 50 ng/ml TNF-α, 3,000 IU/ml IFN-α, 500 pg/ml IFN-γ, and 25 μg/ml poly(I:C) (DC1), iv) 250 ng/ml LPS (Sigma) (lps DC). CD11c$^+$ DCs were exposed to AGS-cell derived EBV at an MOI of 1. Maturation of DCs was monitored by flow cytometry using anti-CD25, anti-CD80, anti-CD83, anti-CD86, and anti-HLA-DR antibodies. Secretion of cytokines was quantified using IL-12p70 ELISA, IL-15 ELISA (both R&D Systems) and IL-18 ELISA (Bender Medsystems).

2.2.3 Isolation of B cells and NK cells

Frozen CD14$^-$ PBMCs were thawed, washed and B cells were isolated by positive selection using CD19-Microbeads (Miltenyi Biotec). NK cells were isolated from either CD19- or CD14- fractions by negative selection using the NK cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the isolated B cells and NK cells was higher than 90% and contained less than 5% contaminating T cells as determined by flow cytometry. For other experiments, B cells, NK cells, and NK cell subsets were isolated by flow cytometric sorting using a BD FACSVantage SE cell sorter.

2.2.4 Isolation of CD34$^+$ cells from fetal livers

Human fetal liver was obtained from Advanced Bioscience Resources as part of an Institutional Review Board-approved protocol. The tissue was minced and treated with 2 mg/ml collagenase D (Roche Diagnostics) in HBSS with $CaCl_2/MgCl_2$ for 30 minutes at RT followed by filtering through 70 μm nylon cell strainers (BD Biosciences). Mononuclear cells were isolated by density-gradient centrifugation on Ficoll/Hypaque. CD34$^+$ human hematopoietic stem cells (HSCs) were isolated using the Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec).

2.2.5 Isolation of mononuclear cells and CD34$^+$ cells from cord blood

Cord blood was obtained as part of Institutional Review Board-approved protocols from the New York Blood Center. Cord blood was processed within 16 h after birth to secure viability of cells. Cord Blood was diluted 1:4 with PBS and mononuclear cells (CBMCs) were isolated by density-gradient centrifugation on Ficoll/Hypaque. CD34$^+$ human hematopoietic stem cells (HSCs) were isolated using the Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec).

2.2.6 Cell lines

Table 4: Cell Lines

Name	Source	Medium
AGS	ATCC	F12 + 10 % FCS
AGS/EBV	L. Hutt-Fletcher	F12 + 10 % FCS + 500 mg G418
B95-8	ATCC	RPMI + 10 % FCS
CEM	ATCC	RPMI + 10 % FCS
Daudi	ATCC	RPMI + 10 % FCS
K562	ATCC	RPMI + 10 % FCS
LCL721.221	ATCC	RPMI + 10 % FCS
OKT4	ATCC	DMEM+ 10 % FCS
OKT8	ATCC	RPMI + 10 % FCS
Raji	ATCC	RPMI + 10 % FCS

2.3 Mouse Strains

Rag2$^{-/-}$γ$_c$$^{-/-}$ mice were kindly provided by Hergen Spits (UMC Amsterdam, Netherlands). NOD/LtSz-*scid IL2Rγ^{null}* (NSG) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). NOD/LtSz-*scid IL2Rγ^{null}* HLA-A2 transgenic (NSG-A2) mice were kindly provided by Gloria Koo (Memorial Sloan Kettering Cancer Center). All mice were raised under specific pathogen free conditions.

2.4 Experimental procedures

2.4.1 Generation of EBV-derived dsRNA

A 1.3 kB fragment spanning the coding region of LMP1 in one direction and part of the first intron of LMP2A in the other direction was cloned into pGEM (Promega) between the T7 and SP6 promoter. ssRNA was generated using linearized plasmid and the Riboprobe Combination System SP6/T7 (Promega). After verification of integrity of RNA by gel electrophoresis, ssRNA was purified using RNeasy Kit (Qiagen) and quantified by Nanodrop. Finally, equal amounts were annealed in siRNA buffer (Dharmacon) to generate dsRNA and successful annealing was confirmed by gel electrophoresis.

2.4.2 Quantification of gene expression

2.4.2.1 Quantification of gene expression by semi-quantitative one-step RT-PCR

RNA was isolated from non-treated and IFN-γ-treated B cells infected with EBV at indicated time point using the RNeasy Kit (Qiagen) according to the manufacturer's instructions. 10 ng of total RNA were used for semi-quantitative RT-PCR using the OneStep RT-PCR-Kit (Qiagen) and gene-specific primers using the following program: 50°C (30 min), 95°C (15 min), followed by an increasing number of cycles of 95°C (30 sec), 55°C (30 sec), and 72°C (30 sec). Primers sequences are detailed in section 2.1.2. The lowest cycle number for which a band was detected on an agarose gel was used to quantify the mRNA level.

2.4.2.2 Quantification of gene expression by semi-quantitative two-step RT-PCR

To generate cDNA for RealTime-PCR, total RNA was reverse transcribed using the TaqMan Reverse Transcription Reagent (Applied Biosystems), using the following program: 25°C (10 min), followed by 50°C (30 min) and 75°C (5 min sec). If SybrGreen was used to detect amplification of specific cDNA, amplification was performed using

cDNA from the reversed transcribed reaction, primer mixture (0.25 μM each of sense and antisense primers), and 1x SYBR Green Master Mix (Applied Biosystems). If gene-specific Taqman probes were used to detect amplification of specific cDNA, amplification was performed using cDNA from the reversed transcribed reaction, primer mixture (0.3 μM each of sense and antisense primers), gene-specific probe (0.25 μM) and 1x Taqman Master Mix (Applied Biosystems). PCR was performed in ABI 7900HT (Applied Biosystems) using following program: 50°C (2 min), 95°C (10 min), followed by 40 cycles of 95°C (15 sec), and 60°C (1 min). The final mRNA levels of EBV-encoded genes was normalized to GAPDH using the comparative C_T method. Primers sequences for RealTime-PCR are detailed in section 2.2.

2.4.3   Quantification of EBV viral loads by quantitative Real-time-PCR

Splenic EBV viral DNA load was quantified by Real-time PCR. DNA was extracted using the Tissue and Blood DNA kit (Qiagen) or the Wizard SV Genomic DNA purification system (Promega). following the manufacturer's protocol. A region from the BamHI W fragment of EBV and the human bcl-2 gene was amplified using primers detailed in section 2.1.2. Amplification was performed using 500 ng DNA, primer mixture (0.3 μM each of sense and antisense primers), gene-specific probe (0.25 μM) and 1x Taqman Master Mix (Applied Biosystems). PCR was performed in ABI 7900HT (Applied Biosystems) using following program: 50°C (2 min), 95°C (10 min), followed by 40 cycles of 95°C (15 sec), and 60°C (1 min). Plasmid DNA containing either region were diluted and used as standard. The EBV BamHI W fragment copy number per cell was calculated using the formula $N = 2 \times W/B$, where N is the EBV BamHI W copy number/cell, W is the EBV BamHI W copy number and B is the bcl-2 copy number. All samples were tested in triplicates.

2.4.4 Mouse genotyping by RealTime-PCR

DNA was isolated from ~3mm long pieces of tail. These pieces were digested in 200 ul 50 mM NaOH at 98 C for 1 hour, and the reaction was neutralized by adding 20 ul of 1M Tris HCl pH 8. Crude extracts were then spun at 4000 rpm for 3 minutes in a table top centrifuge and supernatants transferred to fresh tubes. 1 μl of DNA was used in a 25 μl reaction with the respective primer mixture (0.25 μM each of sense and antisense primers), and 1x SYBR Green Master Mix (Applied Biosystems). PCR was performed in ABI 7900HT (Applied Biosystems) using following program: 50°C (2 min), 95°C (10 min), followed by 40 cycles of 95°C (15 sec), and 60°C (1 min).

2.4.5 Tetramer production and purification

Tetrameric HLA-A2/peptide and HLA-B8/peptide complexes were generated as previously described (Busch et al., 1998). Recombinant proteins were generated separately from insoluble proteins trapped in inclusion bodies following induction with IPTG in *E. coli* strain BL21 (DE3). Inclusion bodies containing HLA-A2, HLA-B8 and β$_2$m, respectively, were dissolved in 8 M urea and stored at -80 C. For refolding, HLA-A2 or HLA-B8 was added to the respective peptide and protease inhibitors (1 µg/ml pepstatin, 1 µg/ml leupeptin) to stabilize monomeric HLA/peptide complexes. Subsequently, these were purified by gel filtration over a Superdex 200 HR column (GE Healthcare) and *in vitro* biotinylated for 12 hr at 20°C in the presence of 15 µg BirA (Avidity), 80 µM biotin, 10 mM ATP,10 mM MgOAc, 20 mM bicine, and 10 mM Tris-HCl (pH 8.3). To remove free biotin, monomeric complexes were again purified by gel filtration, and then tetramerized by addition of PE–labeled Extravidin (Sigma) at a molar ratio of 4:1.

2.4.6 Antibody production and purification

Antibody was produced in RMPI or DMEM, respectively, supplemented with 10% Ultra-low IgG FCS + gentamycin and purified using an Äktapurifier 10 and HiTrap Protein A HP columns. Antibodies were eluted using 0.1 M citrate buffer and the eluate was immediately neutralized with 1 M Tris /HCl, pH 8.0. Then, the antibody was desalted against PBS using HiTrap Desalting columns. The purity of the antibody was at least 95 % as determined by Coomassie stain. If necessary endotoxins were removed using Endotrap Blue columns and the final antibody preparations contained less than 0.1 EU/ml endotoxins as measured by LAL test (Cambrex Corporation).

2.4.7 Preparation of EBV

2.4.7.1 Preparation of EBV from B95.8 cells

The EBV$^+$ marmorset cell line B95-8 was seeded at 2x 10^5 cells / ml and cultured for 12 d in RPMI1640 + 10% FCS + gentamycin without refeeding. Virus-containing supernatant was centrifuged at 2000 rpm for 10 min and passed through a 0.45 μm filter.

2.4.7.2 Preparation of EBV from AGS cells

EBV$^+$ AGS cells were used to produce EBV as previously described (Borza and Hutt-Fletcher, 2002). Briefly, EBV lytic cycle was induced in EBV-positive AGS cells by addition of PMA and sodium butyrate. EBV was further purified by ultracentrifugation over a 25 % sucrose step gradient and subsequently titered on Raji cells. GFP$^+$ cells were counted 2 days later and titers calculated in Raji-infecting units. AGS-derived EBV preparations contained less than 0.1 EU/ml endotoxins as measured by LAL test (Cambrex Corporation).

2.4.8   Proliferation assay

Isolated NK cells, or B cells were labeled with 0.5 μM CFSE in PBS for 10 min at 37°C. After washing twice with RPMI1640 + 5% human serum + gentamycin, cells were counted and used for culture. Typically, NK cells (2.5 x 10^5 cells in 96 well plate) were cultured with 500 IU/ml IL-2 or autologous DCs at a ratio of 5:1 for 6 days at 37°C in RPMI1640 + 5% human serum + gentamycin. In selected experiments, isotype control antibody (5 μg/ml) or blocking antibodies against IL-12, IL-15 (5 μg/ml each), and IL-18 (1 μg/ml) were added to the cultures at the beginning and on day 3 of culture. Where indicated, DCs (bottom) were separated from NK cells (top) by 0.4 μm pore membranes (Corning). CFSE fluorescence and CD16 staining was evaluated on CD3$^-$CD56$^+$ cells by flow cytometry. Where indicated, B cells were CFSE labeled as described above and proliferation of CD19$^+$CD20$^+$ cells was evaluated with and without 10,000 pg/ml IFN-γ addition after infection with EBV as CFSE dilution by flow cytometry. Live cell numbers were determined by trypan blue exclusion.

2.4.9   Detection of IFN-γ secretion by intra-cellular cytokine staining

For intracellular staining of IFN-γ, isolated NK cells from blood (2.5 x 10^5 cells in 96 well plate) were incubated with 500 IU/ml IL-2 or autologous DCs at a ratio of 1:2 at 37°C in RPMI1640 + 5% human serum + gentamycin. In selected experiments, isotype control antibody (5 μg/ml) or blocking antibodies against IL-12, IL-15 (5 μg/ml each), and IL-18 (1 μg/ml) were added. Brefeldin-A was added after 6 h of co-culture and additional 6 h later cells were harvested and stained with anti-CD3, anti-CD56 and anti-CD16. After fixing the cells with 2 % paraformaldehyde, they were permeabilized and stained with anti-IFN-γ.

2.4.10 Detection of IFN-γ secretion by ELISA

IFN-γ was detected in cell culture supernatants or mouse plasma using a commercial ELISA (Mabtech). For NK/DC co-culture experiments, NK cells and DCs were cultured direcly together at a ratio of 5:1 or DCs (bottom) were separated from NK cells (top) by 0.4 μm pore membranes and IFN-γ production was measured after 20h. To directly compare IFN-γ production, sorted NK cell subsets from blood, spleen, tonsil and lymph node (1 x 10^4 cells CD56^{bright}CD16$^-$, 1 x 10^5 CD56$^+$CD16$^+$) were then cultured with allogeneic or autologous DCs at a ratio of 1:10 or 1:1, respectively and after 20 h IFN-γ levels were determined using ELISA.

2.4.11 Analysis of EBV-specific T cell responses by IFN-γ ELISPOT

EBV-specific T cell responses were analyzed using an IFN-γ ELISPOT as previously described (Munz et al., 2000). Briefly, splenocytes were depleted of human CD19$^+$ cells and mouse CD45$^+$ using anti-CD19 and anti-CD45 microbeads (Miltenyi Biotec). The negative fraction after the depletion was stimulated with autologous LCLs at a ratio of 1:4 for 18 hrs. Spots were counted with an ELISPOT reader (Autoimmun Diagnostika GmbH, Germany). In blocking experiments, LCL recognition by T cells was blocked by preincubation of the target cells with 10 μg/ml anti-HLA-A/B/C, anti-HLA-DR/DP/DQ and combination thereof, respectively.

2.4.12 Degranulation assay

To characterize the multifunctionality of EBV-specific clones, T cell clones were stimulated at an effector to target ratio of 5:1 with autologous LCLs for 6 hrs in the presence of anti-CD107 antibody. Similarly, IFN-γ secretion and cytotoxicity of NK cells in bulk PBMCs, CBMNCs, or splenocytes was measured by coculturing them with target

cells at an effector to target ratio of 10:1. To detect spontaneous degranulation and cytokine production, a control without target cells was included. After 1 hrs, monensin (Sigma-Aldrich, 1μg/ml) was added to all samples. At the end of the incubation, cells were stained with antibodies against surface antigens, and then they were fixed, permeabilized, and stained with an anti-IFN-γ antibody. Samples were finally analyzed by flow cytometry.

2.4.13 Cytotoxicity assay

To evaluate the cytolytic activity of NK cells, following NK cell-sensitive cell lines were used as target cells: LCL721.221, K562, and CCRF-CEM. The cytolytic activity of T cell clones was measured against the autologous LCL. Cytotoxicity assays were performed, as previously described (Ferlazzo et al., 2004b). Briefly, target cells were labeled with PKH26 (Sigma-Aldrich, St. Louis, MO), and then incubated with effector cells at different effector cell/target ratios. After 6 h, cells were harvested; TO-PRO-3, a membrane-impermeable DNA stain, was added to each culture (1 μM final concentration); and cells were finally analyzed by flow cytometry. Background and maximum TO-PRO-3 staining were obtained by incubation of target cells with medium and detergent, respectively. The percent specific lysis was calculated as (% TO-PRO-3$^+$PKH26$^+$ cells in efector/target cell co-culture - % TO-PRO-3$^+$PKH26$^+$ cells in medium)/(% TO-PRO-3$^+$PKH26$^+$ cells in detergent - % TO-PRO-3$^+$PKH26$^+$ cells in medium) x 100%.

2.4.14 B cell transformation assay with peripheral blood, splenic and tonsillar mononuclear cells

Isolated B cells (1 x 10^5 cells in 48 well plate) were cultured in RPMI1640 + 5% human serum + gentamycin, infected with EBV and isolated NK cells were added at indicated numbers. In experiments including DCs, they were added at B cell to DC ratios of 1:1. In

other experiments, B cells and DCs (bottom) were separated from NK cells (top) by 0.4 μm porous membranes. After 12 d, numbers of transformed B cells were quantified by counting live cells via trypan blue exclusion and determining the ratio of CD19+CD21+CD23+ cells to total live cells by flow cytometry. Restriction of B cell transformation was calculated by comparing numbers of transformed B cells between respective samples with and without NK cells; % Restriction of B cell transformation = (1 – total transformed B cell number of sample with NK cells / total transformed B cell number of sample without NK cells) x 100.

2.4.15 B cell transformation assay with bulk tonsillar mononuclear cells

Cryopreserved tonsillar mononuclear cells were thawed, washed, and then stained with anti-CD3 and anti-CD56. Cells were then depleted i) of CD3+ cells (Tonsil -T) or ii) of CD3+ and CD56+ cells (Tonsil -NK-T) by flow cytometric sorting using a BD FACSVantage SE cell sorter. The number of sorted cells per condition was adjusted according to the ratio between sorted cells and input cell numbers (1 x 10^6 cells in 48 well plate). Then, the cells were infected with EBV and, where indicated, DCs were added at ratio of total cell to DC of 10:1. After 12 d, numbers of transformed B cells were quantified by counting live cells via trypan blue exclusion and determining the ratio of CD19+CD21+CD23+ cells to total live cells by flow cytometry. Restriction of B cell transformation was calculated by comparing numbers of transformed B cells between respective samples with and without NK cells; % Restriction of B cell transformation = (1 – total transformed B cell number of sample with NK cells / total transformed B cell number of sample without NK cells) x 100.

2.4.16 Cloning of antigen-specific T cells

Splenocytes or PBMCs were then labeled with 0.5 μM CFSE (Invitrogen, CA), and cultured in complete medium containing a single peptide (1 μM), libraries of EBV peptides (1 μM each) or autologous irradiated LCLs at a ratio of 5:1, respectively. On day 6, CFSE^{low} CD3$^+$ cells were sorted by flow cytometry and cloned by classical limiting dilution. Briefly, 10, 1, 0.3 cells were plated per well of a 96 well plate in RPMI + 8 % human serum + gentamycin containing 150 U/ml IL-2 and 1 μg/ml PHA. After initial expansion, individual clones were screened by IFN-γ-ELISPOT after restimulation with the cognate peptides or autologous irradiated LCLs, respectively. Individual clones that recognized one of the libraries were further tested against a matrix of peptide sub-pools to identify the specific peptides.

2.4.17 Preparation of humanized mice

2-5 days old NSG mice were irradiated with 100 cGy and injected intra-hepatically with 1-3 x 10^5 CD34$^+$ HSCs 6 hrs post irradiation. The mice were bled 10-12 weeks post engraftment and peripheral lymphocytes were analyzed by FACS to check for the reconstitution of the human immune system.

2.4.18 EBV infection of mice and *in vivo* depletion of T cells

Reconstituted mice were infected with EBV at different infectious doses ranging from 10^5 to 10^6 Raji-infecting units by intra-peritoneal injection. In selected experiments, human CD4$^+$ and CD8$^+$ T cells cells were depleted prior to EBV infection by intra-peritoneal injection of 100 μg OKT-4 and 100 μg OKT-8 on three consecutive days. In order to deplete T cells for the duration of the experiment, the same injection regimen was repeated two weeks later.

2.4.19 Microscopically analysis of tissues

Immunohistochemical stainings were performed on formalin-fixed, paraffin-embedded tissue sections by Amy Chadburn and Yi-Fang Liu from Weill Medical College. The antibodies used included anti-CD20 (clone L26), anti-CD8 (clone C8/144B), anti-CD68 (clone PGM1), anti-EBNA2 (clone PE2), anti-LMP1 (clone CS1-4; DAKO Cytomation, Denmark), anti-CD3 (clone SP7, Thermo Fisher Scientific, CA), anti-CD21 (clone 2G9) and anti-CD56 (ERIC-1; Novocastra, UK). In situ hybridization for Epstein Barr virus was performed using an EBER probe (Vision Biosystems, Australia). Double immunohistochemical staining and dual in situ hybridization with immunohistochemical staining was performed using the Bond Max Autostainer (Leica Microsystems). Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and endogenous peroxidase was inactivated. For the first antibody, antigen retrieval was performed using either the Bond Epitope Retrieval Solution 1 (ER1) or the Bond Epitope Retrieval Solution 2 (ER2) at 99-100°C for 20-30 minutes. Following retrieval, the sections were incubated sequentially with the primary antibody for 25 minutes, post-primary for 15 minutes and polymer for 25 minutes (Bond Polymer Detection System; Vision Biosystems) followed by colorimetric development with diaminobenzidine (DAB; Vision Biosystems). For the subsequent staining with the second antibody, the sections were heated in Bond Epitope Retrieval solution (either ER1 or ER2) at 99-100°C for 20-30 minutes followed by blocking of endogenous alkaline phosphatase using Dual Endogenous Enzyme Block (DAKO Cytomation). The sections were then sequentially incubated with the second primary antibody, biotinylated link and streptavidin-AP (LSAB 2 System-AP; DAKO Cytomation) for 25 minutes, 15 minutes and 30 minutes, respectively, followed by red chromagen development with permanent red (DAKO Cytomation). With respect to dual in situ hybridization – immunohistochemistry, in situ

hybridization was performed first according to the manufacturers instructions (Vision Biosystems) with colorimetric development using DAB followed by immunostaining as described for the second antibody. Single immunohistochemical staining was performed as described for the first primary antibody above.

2.4.20 Statistical analysis

Statistical analyses were performed with the paired two-tailed Student t-test or the Mann-Whitney test as indicated. The p value of significant differences is reported. Plotted data represent mean plus standard deviation (SD), unless otherwise stated.

3 Results

3.1 The role of NK cells during primary EBV infection

3.1.1 NK cell activation by differently matured DCs.

The outcome of the crosstalk between NK cells and DCs depends strongly on the activation status of both cell types. In order to define the optimal activation conditions for anti-viral NK cell responses, differently matured human monocyte-derived DCs were tested for their capacity to elicit proliferation and IFN-γ secretion by NK cells. DCs were matured using a standard mixture of proinflammatory cytokines (IL-1β, IL-6, TNF-α and PGE$_2$: cyt DC), the TLR3 and mda-5 ligand poly(I:C) (poly(I:C) DC), and the TLR4 ligand LPS (lps DC). In addition, DCs were matured with poly I:C supplemented with proinflammatory cytokines (IL-1β and TNF-α) and type I and II interferons (DC1) to generate DC1 cells according to Mailliard and colleagues (Mailliard et al., 2004). As expected, all of these maturation stimuli were able to significantly up-regulate MHC class II and costimulatory molecules such as CD80, CD83 and CD86 (Table 4). However, when we compared different DC preparations for their capacity to activate NK cells from peripheral blood, we found that DCs matured with poly(I:C) were far superior in NK cell activation compared to the other DC preparations (Figure 6). poly(I:C) DCs and DC1s induced strong proliferation with 40-75% of the NK cells cycling after 6 days. Further characterization showed that the number of NK cells producing IFN-γ increased significantly (4-fold to 10-fold), when they were activated by poly(I:C) DCs and DC1s compared to immature DCs (iDCs) or cyt DCs, respectively (Figure 6). In line with previous findings, the CD56^{bright}CD16$^-$ NK cells were preferentially stimulated by these DC preparations to proliferate and secrete IFN-γ (Figure 6). These data demonstrate that

DCs matured by poly(I:C) are efficient stimulators of CD56^{bright}C16$^-$ NK cells, the NK cell subset enriched in human secondary lymphoid tissues.
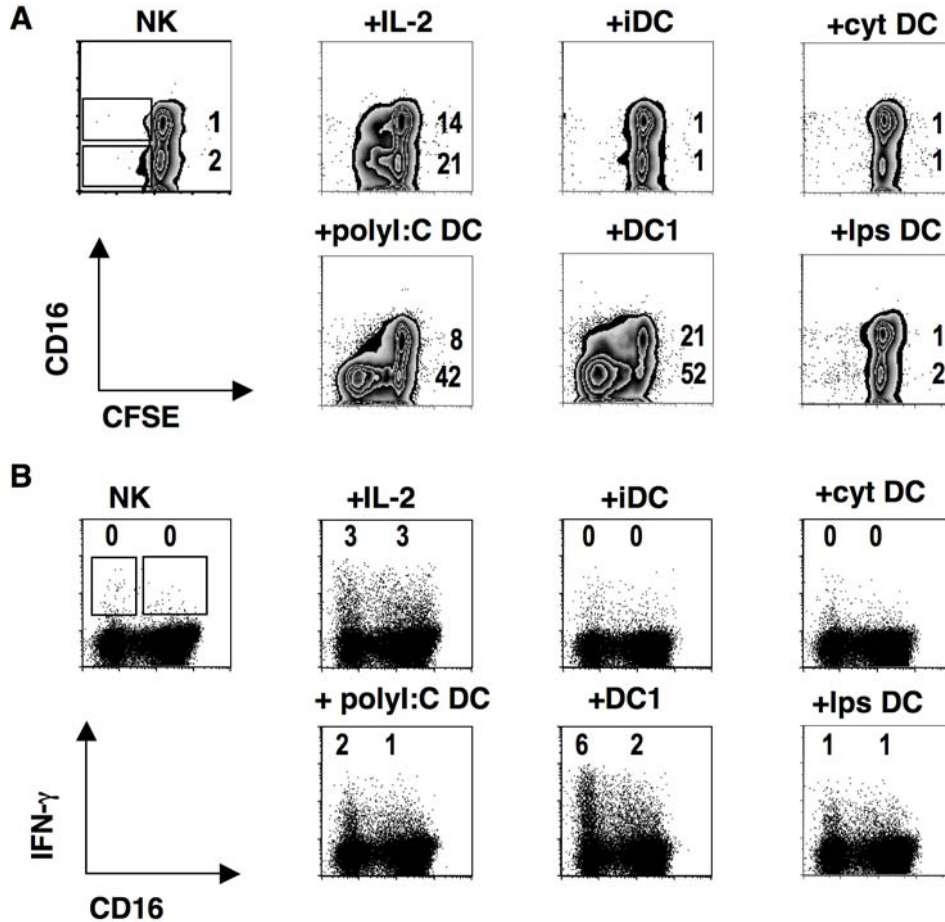
Figure 6: DCs activate NK cells most efficiently after maturation with the dsRNA analog poly(I:C). Differently matured DCs were compared to immature DCs for their ability to induce NK cell proliferation (A) and IFN-γ secretion (B). The tested maturation stimuli included i) the standard proinflammatory cytokine cocktail consisting of IL-1β, IL-6, TNF-α, and prostaglandin E2 (cyt DC), ii) the TLR3 and mda-5 agonist poly(I:C) (poly(I:C) DC), iii) a cocktail of inflammatory cytokines (IL-1β, TNF-α), IFN-α, IFN-γ, and poly(I:C) (DC1), iv) the TLR4 agonist LPS (lps DC). (A) CFSE-labeled NK cells and DCs were cultured for 6d at a ratio of 5:1 and proliferation was analyzed by gating on CD3$^-$CD56$^+$ cells. Percentages of CFSE dilute CD16$^+$ and CD16$^-$ NK cells are indicated. (B) NK cells and DCs were cultured for 20 h at a ratio of 2:1 and BFA was added for the last 8 h. IFN-γ production of CD3$^-$CD56$^+$ cells was analyzed. Percentages of IFN-γ positive NK cells are indicated. Similar results were obtained in three independent experiments.

Table 5: Surface expression of DC maturation markers on monocyte-derived DCs

Maturation stimulus	HLA-DR	CD25	CD80	CD83	CD86	IL-15Rα	IL-15
none	307[a]	87	301	104	412	4	21
cyt	435	265	546	407	690	15	62
poly(I:C)	429	267	602	390	745	37	75
DC1	429	347	620	412	742	68	72
LPS	403	269	558	429	671	20	52
dsRNA	354	205	450	167	621	8	23

[a]: Values indicate mean flourescence intensities (substracted from isotype control staining)

Similar results were obtained in at least four experiments

3.1.2 Production of NK cell stimulatory cytokines upon DC maturation with viral stimuli.

To characterize the mechanism of DC-mediated NK cell activation, we compared the production of NK cell stimulatory cytokines by DCs after maturation. Previous work indicated important roles for IL-12, IL-15, and IL-18 in the activation of NK cell proliferation and IFN-γ secretion. We detected only little (< 50 pg/ml) or no secretion of the bioactive form of IL-12 (IL-12p70) by cyt DCs or iDCs, respectively, whereas poly(I:C) DCs and DC1s produced high amounts of this cytokine (up to 5000 pg/ml) (Table 6). LPS-matured DCs produced intermediate amounts of IL-12p70. Monocyte-derived DCs matured with EBV-derived dsRNA produced slightly higher IL-12 levels than LPS matured DCs (up to 200pg/ml). The addition of recombinant IL-12p70 at comparable levels (≥ 100pg/ml) induced both proliferation and IFN-γ production by NK cells (data not shown). IL-15 secretion and surface expression of IL-15 and IL-15Rα were also primarily induced by incubation of DCs with poly(I:C) (Table 5, Table 6). IL-18 secretion was not detectable for all DC preparations tested (Table 5). In line with these

observations, IFN-γ secretion of NK cells stimulated by DC1s was mainly dependent on

IL-12 (80 %, $p < 0.01$) and to a lesser degree on IL-18 (15%, $p < 0.05$) (Figure 7). Similar

results were obtained with poly(I:C) DCs (data not shown). Consistent with the

hypothesis that poly(I:C)-matured DCs mainly elicited IFN-γ secretion of NK cells via

their high levels of secreted IL-12, this NK cell/DC interaction was not sensitive to

transwell separation (Figure 7B). Furthermore, we found that NK cell proliferation upon

co-culture with DC1s (Figure 7C) and poly(I:C) DCs (data not shown) could be blocked

with an IL-12 specific antibody by 90% or 80%, respectively ($p < 0.01$ for both), and

again transwell experiments showed that direct cell contact was not required (data not

shown). Blocking of IL-15 also significantly decreased numbers of surviving NK cells

when combined with anti-IL-12 antibodies ($p < 0.01$), however blocking of IL-15 or IL-18

alone did not significantly decrease survival (Figure 7D). In addition, antibody blocking of

IL-2 did not influence DC induced proliferation and IFN-γ production by NK cells (Figure

8). These data suggest that poly(I:C), and EBV-derived dsRNA elicit IL-12 production,

which in turn stimulates NK cell proliferation and IFN-γ production by NK cells.

Table 6: Secretion of IL-12, IL-15, and IL-18 by DCs

Maturation stimulus	IL-12	IL-15[a]	IL-18[a]
none	<16.25[b]	12 ± 11	40 ± 32
cyt	29 ± 25	78 ± 40	55 ± 23
poly(I:C)	2445 ± 985	159 ± 68	44 ± 11
DC1	4123 ± 1098	289 ± 84	50 ± 19
LPS	69 ± 40	n.d.	n.d.
dsRNA	85 ± 35	n.d.	n.d.

[a]: Samples were 10-fold concentrated

[b]: Values indicate concentration of cytokine in pg/ml

n.d.: not determined

Results represent data from 6 experiments
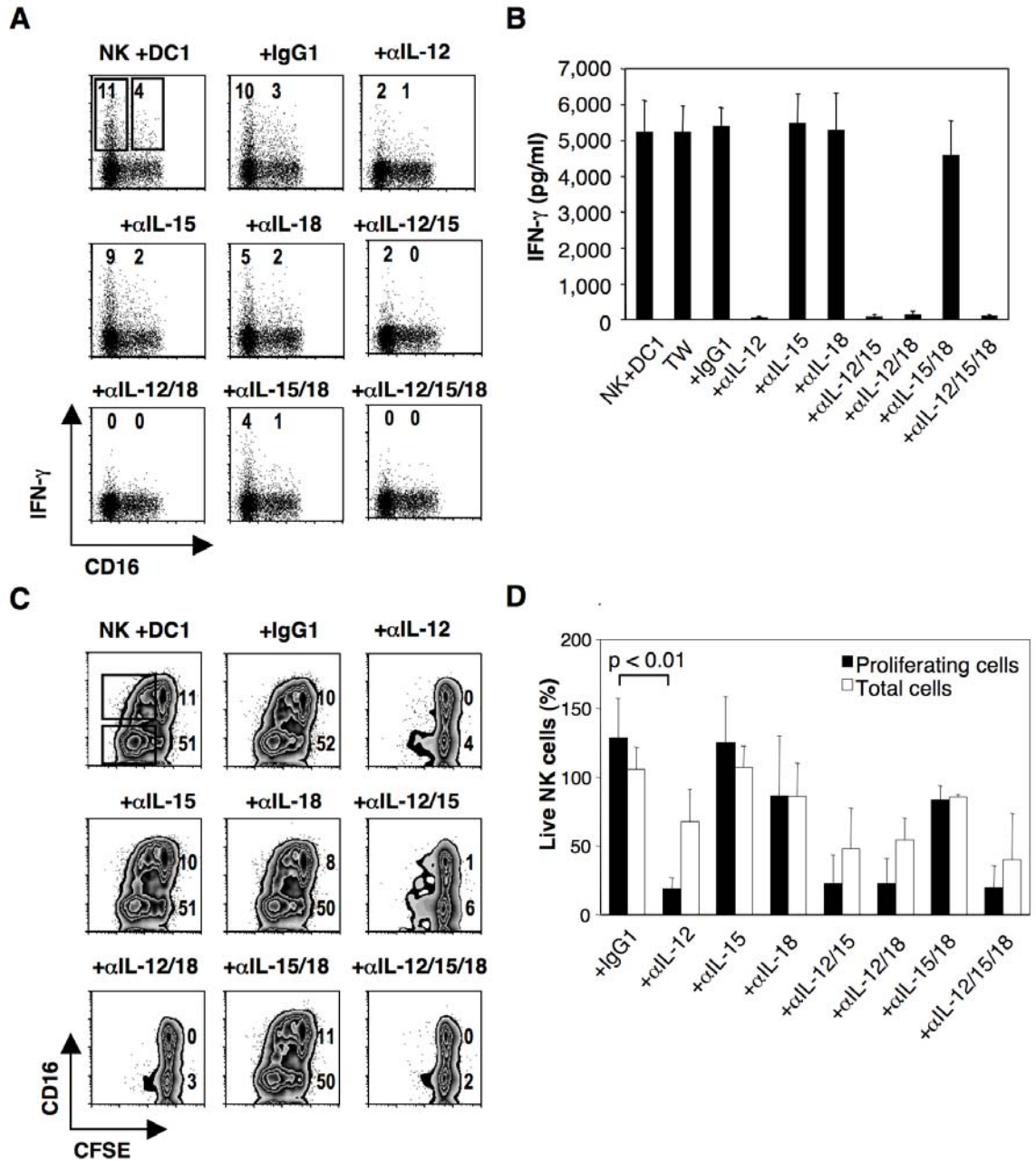
Figure 7: NK activation by poly(I:C)-matured DCs is IL-12 dependent and is mainly restricted to CD56^brightCD16^- NK cells. (A) Blood NK cells were activated by DC1s in the presence or absence of blocking antibodies and IFN-γ production of CD3^-CD56^+ cells was assayed by intracellular cytokine staining. Percentages of IFN-γ positive NK cells are indicated. (B) NK cells were cultured directly or separated by transwell with DC1s. In addition, cytokines were blocked in transwell experiments using blocking antibodies. IFN-γ levels were measured by ELISA (mean ±s.d.) (C) NK cells were cultured with DC1s in the presence or absence of blocking antibodies for 6 d and CFSE dilution of CD3^-CD56^+ cells was analyzed. Percentages of CFSE dilute CD16^+and CD16^- NK cells are indicated. (D) After 6 d of NK cell-DC co-cultures, live cells were counted and subsequently, numbers of total and surviving CD3^-CD56^+ NK cells were determined by measuring ratios of total and proliferating NK cells of total live cells. Data represents numbers of proliferating and total NK cells compared to controls without antibody blocking (mean ±s.d.). Mouse-IgG1 was used in all experiments as isotype control. Data in (A)-(D) represent results of at least three independent experiments.
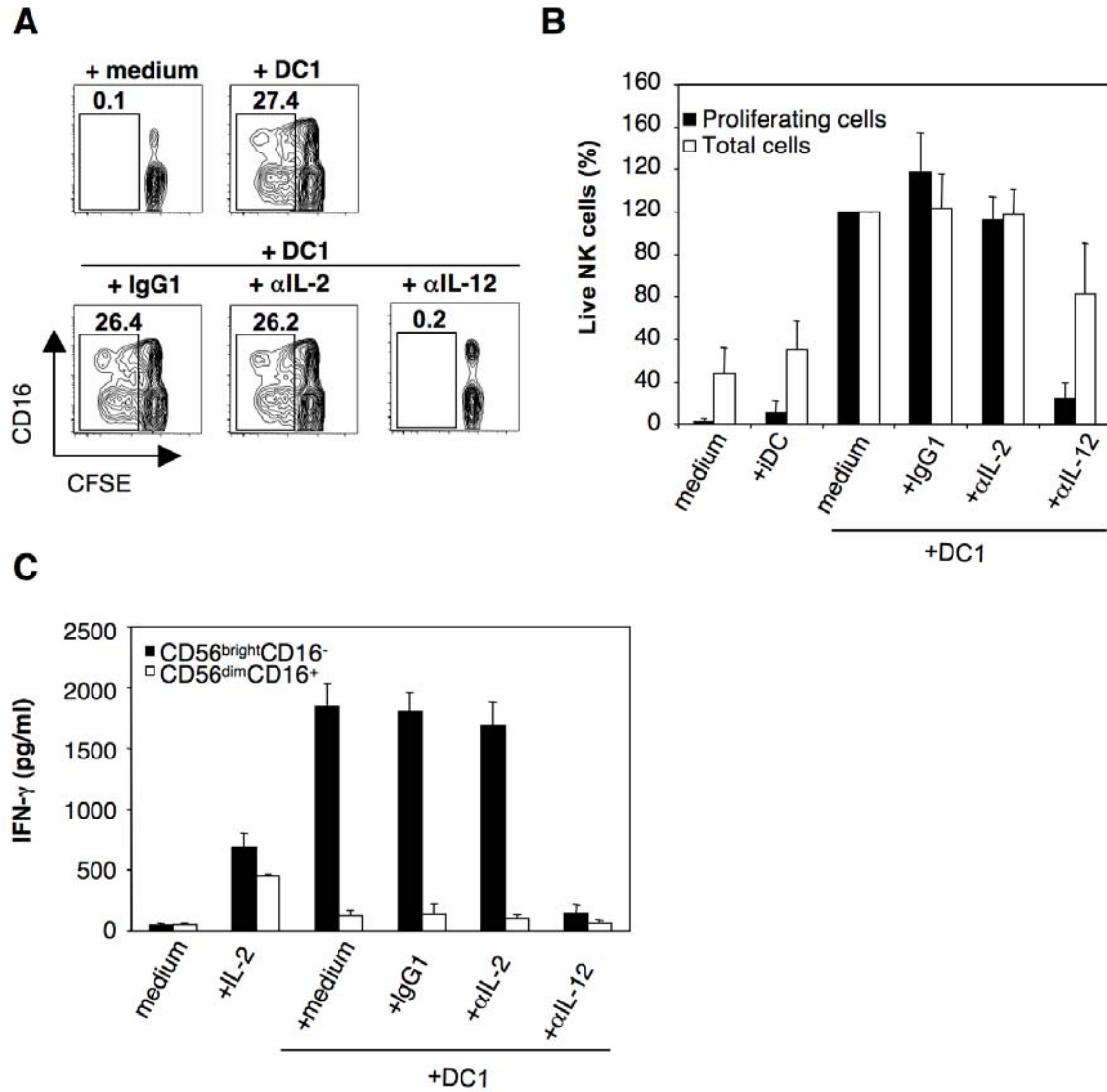
Figure 8: IL-2 is not responsible for DC1 induced NK cell proliferation and IFN-γ production. (A) NK cells were cultured with iDCs or DC1s in the presence or absence of blocking antibodies for 6 d and CFSE dilution of CD3⁻CD56⁺ cells was analyzed. Percentages of CFSE-dilute NK cells are indicated. (B) After 6 d of NK cell-DC co-cultures, live cells were counted and subsequently, numbers of total and surviving CD3⁻ CD56⁺ NK cells were determined by measuring ratios of total and proliferating NK cells of total live cells. Data represents numbers of proliferating and total NK cells compared to DC1 co-culture without antibody blocking (mean ± s.d.). Data in (A) and (B) represent results of three independent experiments. (C) Sorted NK cell subsets were cultured with medium, IL-2 (500 U/ml) or DC1s in the presence or absence of blocking antibodies for 20 h and IFN-g was analyzed by ELISA. Data in (C) represent results of two independent experiments done in duplicates (mean ± s.d.). Mouse-IgG1 was used in all experiments as isotype control.

3.1.3 Restriction of EBV-induced B cell transformation by NK cells upon activation by poly(I:C) matured DCs.

In contrast to previous studies focusing on IL-2 activation of NK cells, we addressed the question whether NK cells restrict EBV-induced B cell transformation after activation by cells of the innate immune system. Because interactions between NK cells and DCs have been shown to be essential for virus control in murine models of herpes virus infections (Andrews et al., 2003; Kassim et al., 2006; Orange et al., 1995), we specifically investigated whether DCs can activate NK cells to limit EBV-mediated B cell transformation. For these experiments we used monocyte-derived DCs, since they can be generated at sufficient numbers to allow functional experiments. When we infected purified B cells with EBV and co-cultured them with resting purified peripheral blood NK cells at ratios of 5:1 (NK to B cells) we could not observe any restriction of B cell transformation. Similarly, addition of iDCs or cyt DCs induced only limited NK cell-mediated inhibition of EBV-transformed B cell outgrowth (6 or 13%, respectively) (Figure 9A, B and C). However, in cultures with NK cells and poly(I:C) DCs or DC1s, we observed a 49 or 55 % (both $p < 0.01$) reduction of the number of transformed B cells, respectively (Figure 9C). The DC preparations used in this study had no significant direct effect on B cell transformation by EBV (data not shown). This demonstrated that DC-activated NK cells can inhibit B cell transformation by EBV.
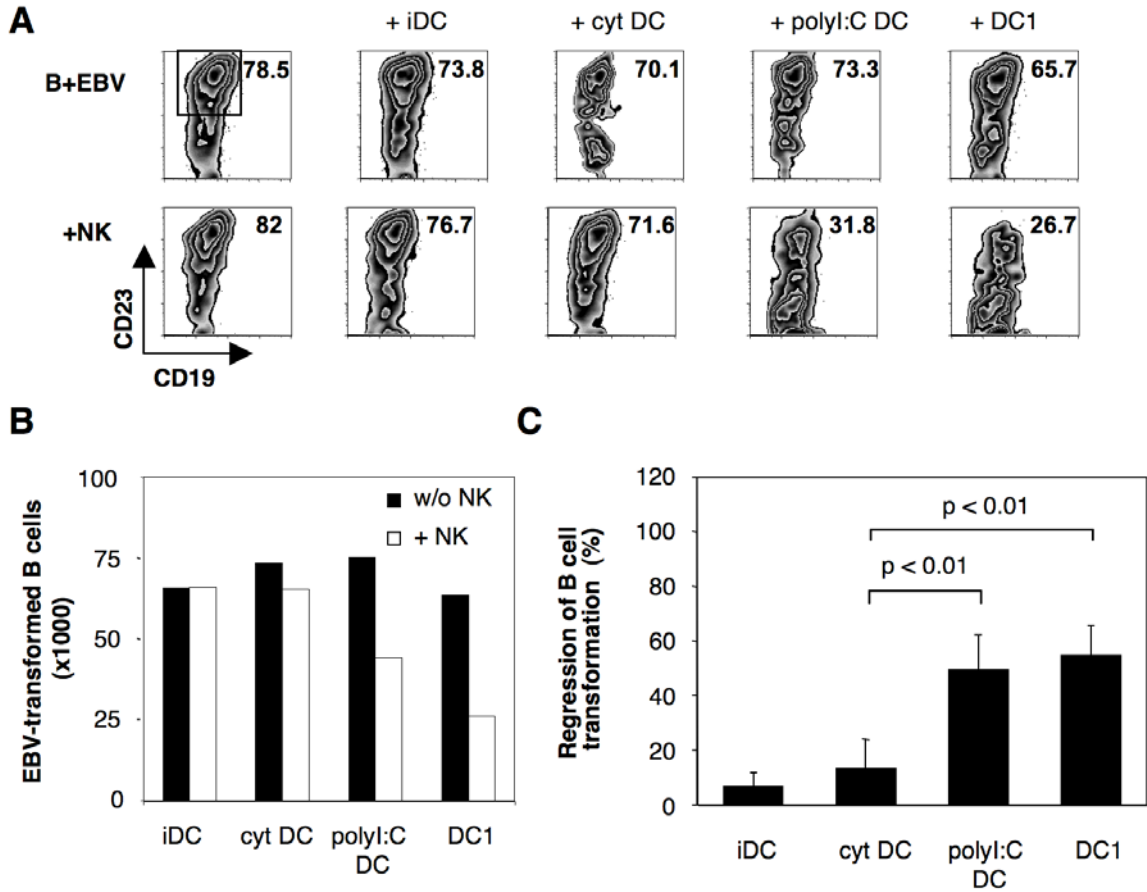
Figure 9: NK cells from blood activated by poly(I:C)-matured DCs limit EBV-mediated B cell transformation. (A) B cells were infected with EBV and cultured for 12 d alone, with DCs or NK cells and with DCs plus NK cells. The indicated percentage of B cell transformation was evaluated by determining the percentage of transformed $CD19^+CD23^+$B cells within gated $CD19^+CD21^+$ B cells. (B and C) Total numbers of transformed B cells were determined from live cell numbers and the percentage of transformed $CD19^+CD23^+$B cells in the different cultures. Total transformed B cell numbers for one representative experiment (B), and differences in total transformed B cell numbers for all experiments as regression (C), with and without NK cells were plotted for the indicated DC maturation conditions. Data represent results from eight independent experiments (mean ± s.e.m.).

3.1.4 Low numbers of tonsillar NK cells restrict EBV induced B cell transformation.

Tonsils are the primary infection sites for EBV and harbor enriched populations of $CD56^{bright}CD16^-$ NK cells, which can be efficiently activated by DCs. Therefore, we investigated whether tonsillar NK cells can restrict EBV-induced B cell transformation. For this purpose, we depleted tonsillar mononuclear cells of $CD3^+$ T cells by cell sorting

and compared the numbers of transformed B cells after EBV infection to cultures that were depleted of both CD3$^+$ T and CD56$^+$ NK cells (Figure 10A and B). Without addition of DCs and with the addition of allogeneic iDCs or cyt DCs, we did not observe any significant difference in the number of transformed B cells after 12 days (Figure 10A and B). However, we observed a 35 % or 42 % (p < 0.03 and p<0.01) reduction of the number of transformed B cells after addition of allogeneic poly(I:C) DCs or DC1s, respectively (Figure 10B). These data suggest that tonsillar NK cells are able to restrict B cell transformation by EBV after stimulation by poly(I:C) DCs and DC1s. NK cells are present in tonsils at lower frequencies than in peripheral blood (0.3 % compared to 10 %), corresponding to only 3000 NK cells in a tonsillar B cell transformation assay with 6 x 10^5 B cells (NK to B cell ratio of 1:200). However, the ratio between CD56^{dim}CD16$^+$ and CD56^{bright}CD16$^-$ NK cells is almost reversed between these organs with 75% of all NK cells being CD56^{bright}CD16$^-$ in tonsils and only 5% being CD56^{bright}CD16$^-$ in peripheral blood (Ferlazzo and Münz, 2004). To compare directly the abilities of NK cells from blood and tonsils to restrict B cell transformation, we tested different ratios of blood NK cells to B cells. In addition to an intermediate ratio (NK:B = 1:1), we mimicked NK cell to B cell ratios found in tonsils (NK:B = 1:200; 60% B and 0.3% NK cells). We also added sufficient peripheral blood NK cells to mimic the CD56^{bright}CD16$^-$ NK cell to B cell ratio found in tonsil (NK:B = 1:13.3; CD56^{bright}CD16$^-$ NK cells are 15fold enriched in bulk tonsillar NK cells compared to blood NK cells: 75% in tonsil and 5% in blood). Already at a ratio of 1:1 we could only detect a limited 6 % reduction of B cell transformation compared to controls (Figure 10C and D). These findings indicate that tonsillar NK cells are 1000-fold more efficient in controlling B cell transformation than peripheral blood NK cells.
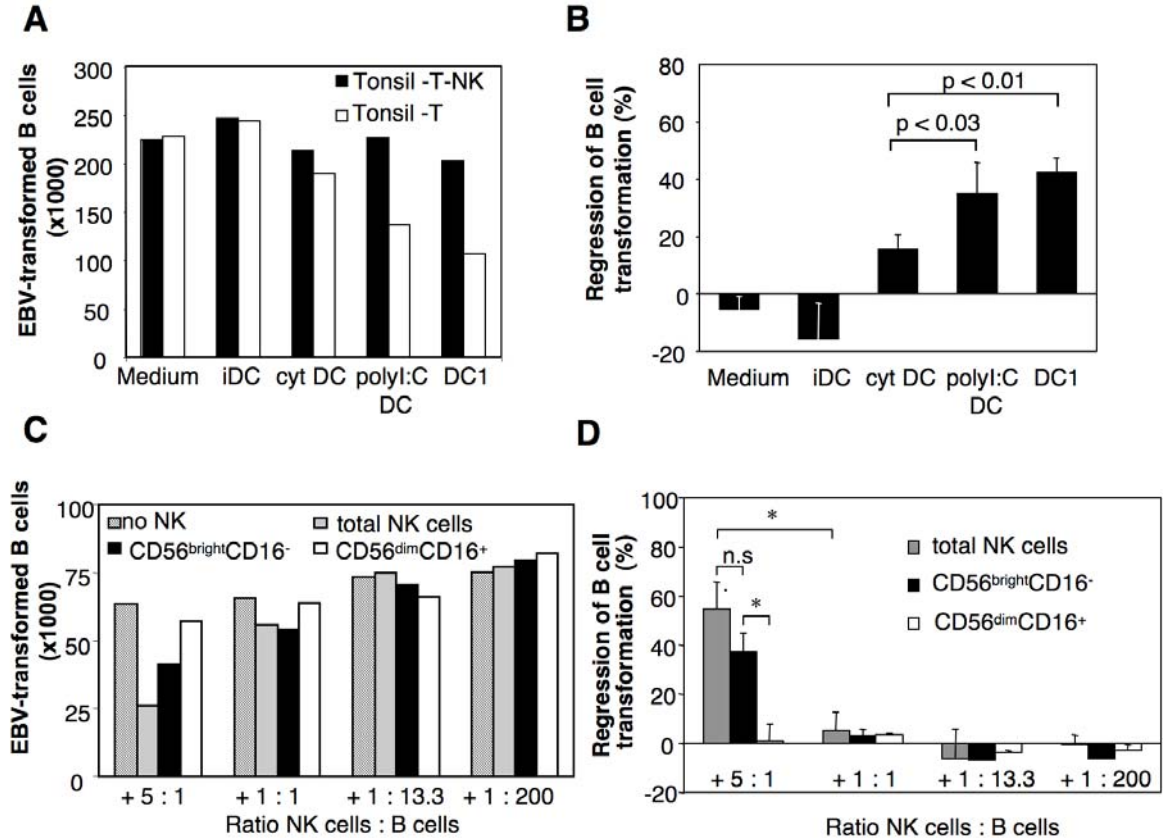
Figure 10: NK cells from tonsil activated by poly(I:C)-matured DCs limit EBV-mediated B cell transformation at lower numbers than blood NK cells. (A and B) Mononuclear cells from tonsils were depleted of CD3^{+} T cells or CD3^{+} T and CD56^{+} NK cells. Total numbers of transformed B cells were determined from live cell numbers and the percentage of transformed CD19^{+}CD23^{+} B cells in the different cultures. Total transformed B cell numbers for one representative experiment (A), and differences in total transformed B cell numbers for all experiments as regression (B), with and without NK cells were plotted for the indicated DC maturation conditions. (C and D). B cells were infected with EBV and cultured for 12 d alone, with DC1s or NK cells and DCs at the indicated NK cell to B cell ratios. Total NK cells were compared to sorted CD56^{bright}CD16^{-} and CD56^{dim}CD16^{+} NK cells in their ability to limit EBV-mediated B cell transformation. To reflect the frequencies of NK subsets in peripheral blood for the different ratios, total transformed B cell numbers for one representative experiment (C) and regression for all experiments (D), mediated by total NK cells was compared to the effect seen with 90 % of CD56^{dim}CD16^{+} NK cells or 10% of CD56^{bright}CD16^{-} NK cells (*: $p < 0.01$).

3.1.5 CD56^{bright}CD16^{-} NK cells restrict EBV induced B cell transformation most efficiently.

To study which subset of NK cells limits B cell transformation, we sorted NK cell subsets from blood and activated them with DC1s. While sorted CD56^{dim}CD16^{+} NK cells did not

mediate restriction of B cell transformation (NK:B = 4.5:1), low numbers of CD56^{bright}CD16$^-$ NK cells (NK:B = 1:2) were found to inhibit B cell transformation (34 %) similar to 10-fold higher numbers of bulk NK cells (Figure 10 C and D). Next, we sorted NK cell subsets from tonsils and observed that again CD56^{bright}CD16$^-$ NK cells but not CD56^{dim}CD16$^+$ NK cells were efficiently limiting B cell transformation after activation by DC1 (Figure 11A and B). At 10-fold lower numbers, tonsillar CD56^{bright}CD16$^-$ cells inhibited B cell transformation more (48.3 % vs. 34 %) than their counterparts in blood. Since CD56^{bright}CD16$^-$ NK cells are 15-fold enriched in tonsils compared to blood, NK cell mediated restriction is achieved with at least 150-fold lower bulk NK cell numbers in this organ. Further increasing the number of tonsillar NK cells 5-fold (NK:B = 1:40) led to restriction of B cell transformation by 69.7 % (Figure 11B). Moreover, high numbers of splenic CD56^{bright}CD16$^-$ NK cells (NK:B = 5:1), a mixture of blood and secondary lymphoid organ NK cells, when activated with matured autologous myeloid CD11c^{high} DCs isolated from spleen, restricted B cell transformation by 67 % (Figure 11C and D). Since we observed that at lower ratios of blood NK cells to B cells, even purified CD56^{bright}CD16$^-$ peripheral blood NK cells were unable to limit B cell transformation after activation by poly(I:C) DCs and DC1s, whereas tonsillar NK cells were still able to restrict EBV-induced B cell transformation, we concluded that tonsillar CD56^{bright}CD16$^-$ NK cells are functionally different from their counterparts in peripheral blood, and inhibit EBV induced B cell transformation more efficiently.
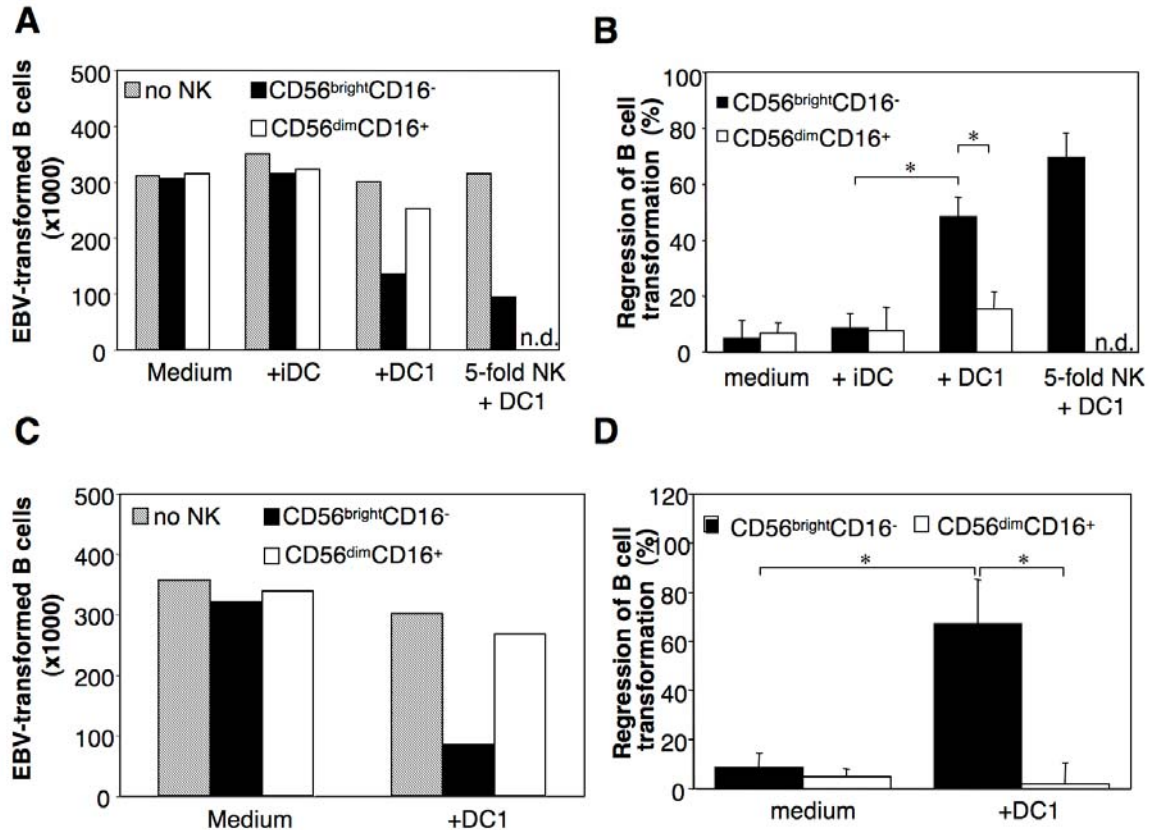
Figure 11: CD56^{bright}CD16$^-$ cells from tonsil and spleen limit EBV-mediated B cell transformation after activation by DCs. (A and B) Tonsillar B cells were infected with EBV and cultured for 12 d alone or with autologous purified NK cell subsets at 2 ratios (NK: 5,000, and 5-fold NK: 25,000) in the absence or presence of allogeneic iDCs or DC1s (*: p < 0.01). Total transformed B cell numbers for one representative experiment (A), and regression for all experiments are shown (B). (C and D) Splenic B cells were infected with EBV and cultured for 12 d alone or with splenic NK cell subsets in the absence or presence of autologous splenic DCs matured with poly(I:C), TNF-α, IL-1β, IFN-α and IFN-γ (*: p < 0.01). Total transformed B cell numbers for one representative experiment (C), and regression for all experiments are shown (D). Data in (A) to (D) represents results from three independent experiments (mean ± s.e.m.).

3.1.6 Tonsillar and lymph node NK cells produce higher levels of the anti-viral cytokine IFN-γ than peripheral blood NK cells.

Human NK cells from secondary lymphoid organs such as tonsils produce IFN-γ rapidly upon activation and this antiviral cytokine contributes directly to control early infection in murine models of herpes virus infection (Orange et al., 1995). When we compared the production of IFN-γ upon NK cell/DC co-culture, we observed that NK cells from tonsil and lymph node produced significantly more IFN-γ than their equivalents from blood or

spleen (Figure 12A). Comparing CD56^{bright}CD16$^-$ NK cells, tonsillar and lymph node cells produced 5-fold more IFN-γ than peripheral blood cells, which amounted to a 50-fold difference when bulk NK cell cultures were analyzed due to the enrichment of CD56^{bright}CD16$^-$ NK cells in these organs. It had recently been reported that IL-18 exposed blood NK cells develop into a CD56^{bright}CD83$^+$CCR7$^+$ NK cell subset with superior IFN-γ production (Mailliard et al., 2005). In order to test if an enrichment of this NK cell subset could account for the superior ability of tonsillar NK cells to produce IFN-γ, we analyzed CD83 and CCR7 expression on tonsillar NK cells (Figure 13). Confirming our previously published data (Ferlazzo et al., 2004b), we found no CCR7 expression on tonsillar NK cells, and only a minor population expressed CD83. Therefore, an enrichment of CD83$^+$CCR7$^+$ NK cells with superior IFN-γ production does not explain why NK cells from secondary lymphoid organs produce more IFN-γ than their peripheral blood counterparts.
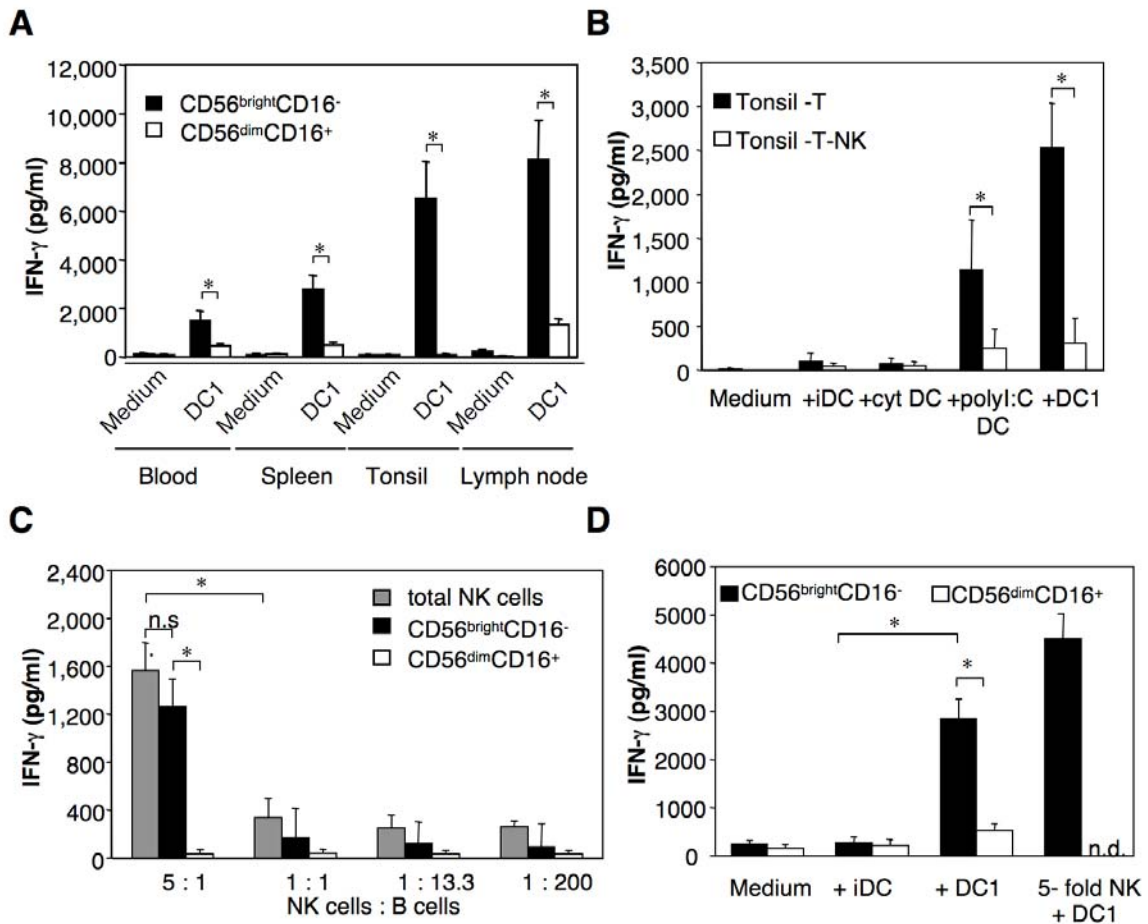
Figure 12: IFN-γ secreted by NK cells restricts EBV-mediated B cell transformation. (A) Sorted NK cell subsets from blood, spleen, tonsil and lymph node were cultured alone or with DC1s, and IFN-γ levels were quantified by ELISA after 20h. (B) IFN-γ secreted by tonsillar NK cells after activation by differently matured DCs during the regression assay was detected in supernatants by ELISA. (C) IFN-γ secreted by peripheral blood NK cells and NK cell subsets during regression assays was detected in supernatants by IFN-γ ELISA (Tonsil–T: tonsillar cultures depleted of T cells, Tonsil–T-NK: tonsillar cultures depleted of T and NK cells). (D) IFN-γ secreted by tonsillar NK cell subsets during regression assays was detected in supernatants by IFN-γ ELISA. Results from at least three independent experiments were summarized (mean ± s.d.) (*: p < 0.03).
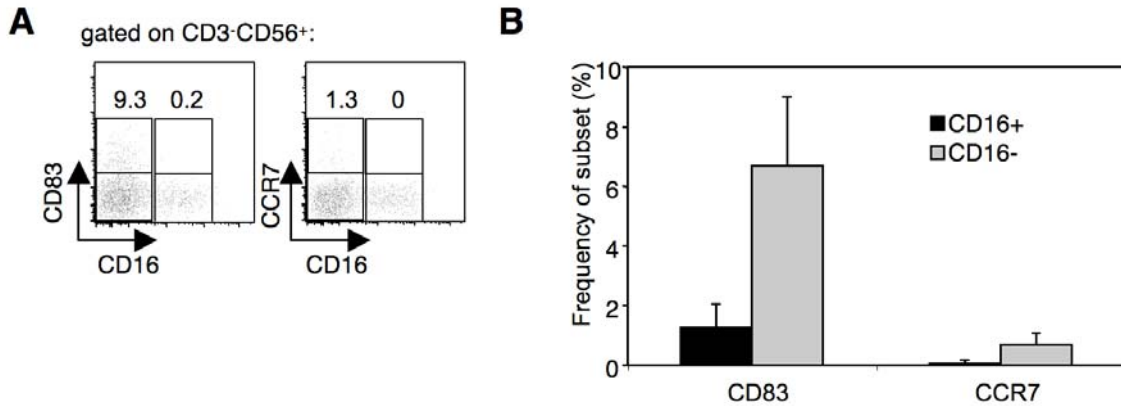
Figure 13: Tonsillar NK cells do not express CCR7 and only a low number expresses CD83.
Mononuclear cells from tonsil were stained with CD3 and CD56 to identify CD3-CD56+ NK cells. In addition cells were stained with antibodies against CD16 and CD83 or CCR7. (A) shows a representative experiment, and (B) summarizes data from four independent experiments (mean ± s.d.).

3.1.7 IFN-γ levels correlate with NK cell induced restriction of B cell transformation by EBV.

Higher IFN-γ secretion by tonsillar NK cells was also apparent when we then quantified the levels of IFN-γ in the culture supernatants of the B cell transformation assay after 12 days. We detected high levels in co-cultures of NK cells from blood with poly(I:C) DCs (960 pg/ml; data not shown) or DC1s (1560 pg/ml; Figure 12C), but only with the highest numbers of NK cells (NK:B = 5:1). However, IFN-γ levels were even higher in B cell transformation cultures with bulk tonsillar NK cells and poly(I:C) DCs (1140 pg/ml) or DC1s (2500 pg/ml), and this IFN-γ secretion was NK cell dependent (Figure 12B). Furthermore, we detected similar levels of IFN-γ also in cultures with sorted CD56^{bright}CD16$^-$ NK cells from blood, tonsil and spleen (Figure 12C, D and data now shown), reaching up to 4000pg/ml IFN-γ concentrations with purified tonsillar CD56^{bright}CD16$^-$ NK cells. Therefore, only DC/NK cell co-cultures with poly(I:C) matured DCs and either high peripheral blood or low tonsillar NK cell numbers produce IFN-γ

concentrations above 1000 pg/ml, and only these high IFN-γ levels correlate with control of EBV transformed B cells.

3.1.8 Restriction of EBV induced B cell transformation by NK cells relies on IFN-γ.

In order to estimate the contribution of NK cell-produced IFN-γ on control of EBV-infected B cells, we added recombinant IFN-γ to purified and EBV infected B cells from blood, spleen and tonsil. We detected fewer transformed B cells in cultures with high levels of IFN-γ (from 1000 to 10000 pg/ml) compared to controls with low levels (from 10 pg/ml to 200 pg/ml) of IFN-γ or without IFN-γ (Figure 14A). Indeed, 42% restriction of B cell transformation ($p < 0.02$) was observed in tonsillar B cell cultures with 5000 pg/ml IFN-γ, an IFN-γ concentration that was produced by tonsillar NK cells upon culture with poly(I:C) DCs and DC1s, and similar to measured IFN-γ levels in tonsil cell cultures (Figures 12A and D). However, IFN-γ mediated restriction of B cell transformation by EBV only limits, but does not eradicate EBV infection, since even with IFN-γ concentrations exceeding 10,000 pg/ml, we never observed more than 80% inhibition of B cell transformation by EBV (data not shown). Finally, DC activated NK cell were still able to mediate restriction when separated by transwell from EBV infected B cells, and we could block inhibition of B cell transformation by over 60 % using blocking antibodies against IFN-γ (Figure 14B). Therefore, IFN-γ contributes to NK cell-mediated restriction of EBV-induced B cell transformation.
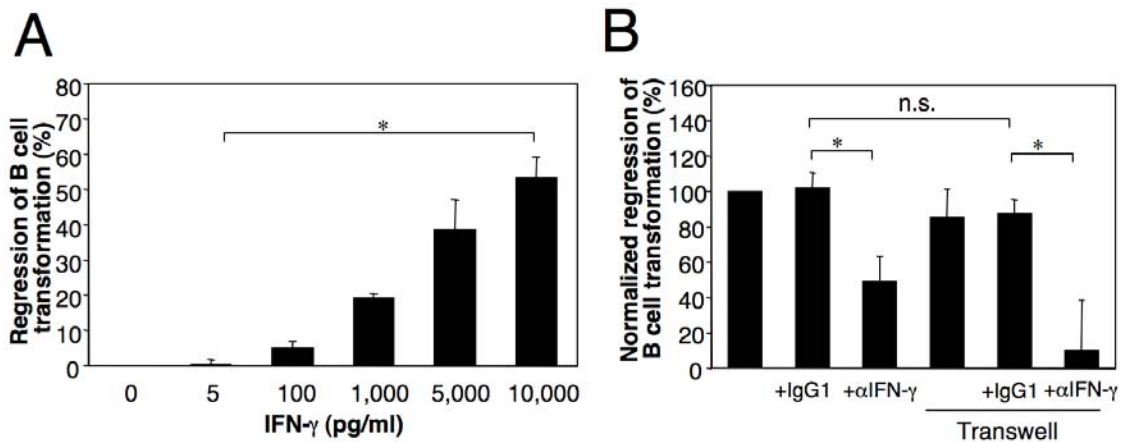
Figure 14: IFN-γ secreted by NK cells is sufficient and necessary to restrict EBV-mediated B cell transformation. (A) Peripheral blood B cells were infected with EBV and increasing concentrations of IFN-γ were added. Regression of B cell transformation was analyzed after 12 d by comparing numbers of transformed B cells with and without IFN-γ. (B) Blocking antibodies against IFN-γ were added to regression assay with B cells, NK cells, and DC1s from peripheral blood. In addition, NK cells were separated from B cells and DCs by transwell membranes. Results from at least three independent experiments were summarized (mean ± s.d.) (*: $p < 0.03$).

3.1.9 Myeloid DCs sense EBV directly and elicit IFN-γ secretion by NK cells to levels protective against EBV induced B cell transformation.

In order to extend our findings from monocyte-derived DCs to human blood DCs and from poly(I:C) to maturation by EBV, we exposed sorted human CD11c⁺ myeloid DCs to EBV particles directly (DC:EBV MOI = 1:1). We observed 189 ± 20 pg/ml IL-12 secretion and upregulation of the maturation marker CD83 upon co-culture of myeloid DCs with EBV (Figure 15A and B). Both infectious and heat-inactivated EBV elicited this DC maturation (Figure 15A). DC maturation by EBV was not due to endotoxin contamination of the EBV virus preparations, since we detected less than in 0.1 ng endotoxin in 1×10^5 EBV RIU, a concentration insufficient for human DC maturation (Figure 15A). Furthermore polymyxin B, which inhibits TLR4 stimulation by LPS (Duff and Atkins, 1982), had no effect on EBV mediated DC maturation, but significantly inhibited DC maturation by LPS (Figure 15A). While EBV induced IL-12 levels were lower than IL-12

concentrations in response to poly(I:C) and to high levels of LPS (1286 ± 188 pg/ml and 763 ± 87 pg/ml, respectively; Figure 15A), EBV matured DCs stimulated purified autologous NK cells to secrete IFN-γ in excess of 4000 pg/ml via IL-12 (Figure 15C). These IFN-γ concentrations are high enough to inhibit B cell transformation by EBV *in vitro* (Figure 14A). These data suggest that human myeloid DCs can be matured by EBV and then activate NK cells to produce protective amounts of IFN-γ.
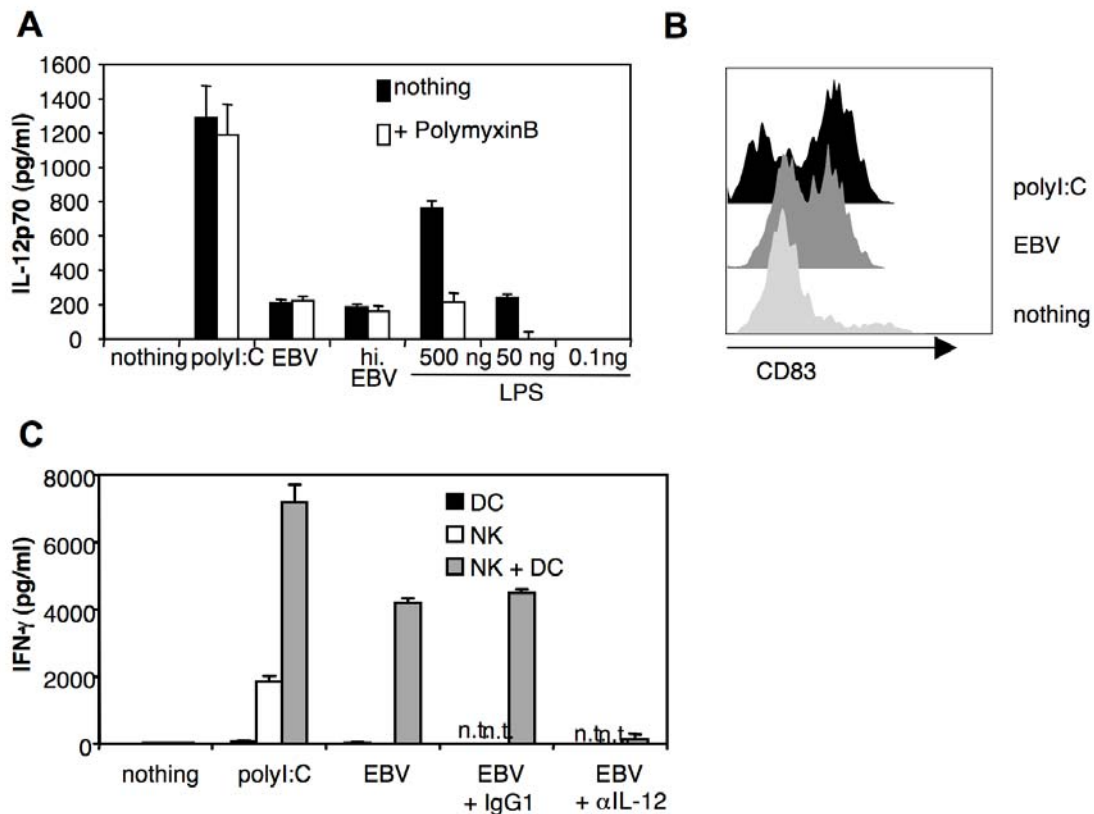
Figure 15: Myeloid DCs can sense EBV and activate subsequently NK cells via IL-12. (A) CD11c+ myeloid DCs (1x10^5) were purified to 99.8 % purity by flow cytometric sorting and exposed to poly(I:C) (25 µg/ml), EBV (non-treated or heat-inactivated) (5x10^5 RIU/ml) and LPS in the absence and presence of polymyxin B (25 µg/ml), an inhibitor of LPS-mediated TLR4 activation. IL-12p70 was detected by ELISA 24 h later. (B) Purified DCs were exposed to poly(I:C) (25 µg/ml) or EBV (5x10^5 RIU/m) and upregulation of the DC maturation marker CD83 was detected by flow cytometry 24 h later. (C) Flow-sorted CD11c+ DCs and NK cells were cultured together or separate in the presence of poly(I:C) (25 µg/ml), or EBV (5x10^5 RIU/ml). IFN-γ was detected by ELISA 24 h later. Additionally, IL-12 was blocked in selected experiments using blocking antibody (n.t.: not tested). Data represents results from six (A, except heat-inactivated EBV) or three independent experiments performed in duplicates.

3.1.10 IFN-γ regulates B cell transforming EBV latency.

In order to investigate how IFN-γ restricts B cell transformation, we infected B cells with EBV and compared total cell numbers, proliferation and expression of EBV-encoded genes between untreated and IFN-γ-treated cells. We started observing significant differences in cell numbers from day 4 (Figure 16A). This coincided with beginning EBV-infected B cell proliferation, which was delayed when cells were treated with IFN-γ (Figure 16B). Comparing expression of different EBV-encoded genes showed that EBER1, EBNA2, and EBNA1 were similarly up-regulated in untreated and IFN-γ-treated EBV infected B cells. In contrast, we observed a delayed up-regulation of LMP1 in IFN-γ-treated cultures (Figure 16C and data not shown). Quantitative RT-PCR demonstrated that expression of LMP1 was reduced by 28% and 49% at days 3 and 5 post infection, while at later stages similar LMP1 levels were observed with and without IFN-γ addition, when normalized to GAPDH (Figure 16D). Notably, recombinant IFN-γ did not mediate restriction of B cell transformation when added later than 96h after EBV infection (Figure 16E), and also did not inhibit growth of established EBV-transformed B cell lines (data not shown). We did not observe any effect of IFN-γ on B cell viability as measured by counting live cells up to day 6 post-infection, and levels of the EBV receptor CD21 were not affected by IFN-γ treatment (data not shown). Therefore, we suggest that DC activated NK cells limit B cell transformation by EBV via regulation of EBV latent infection, at least in part via delaying LMP1 expression via IFN-γ.
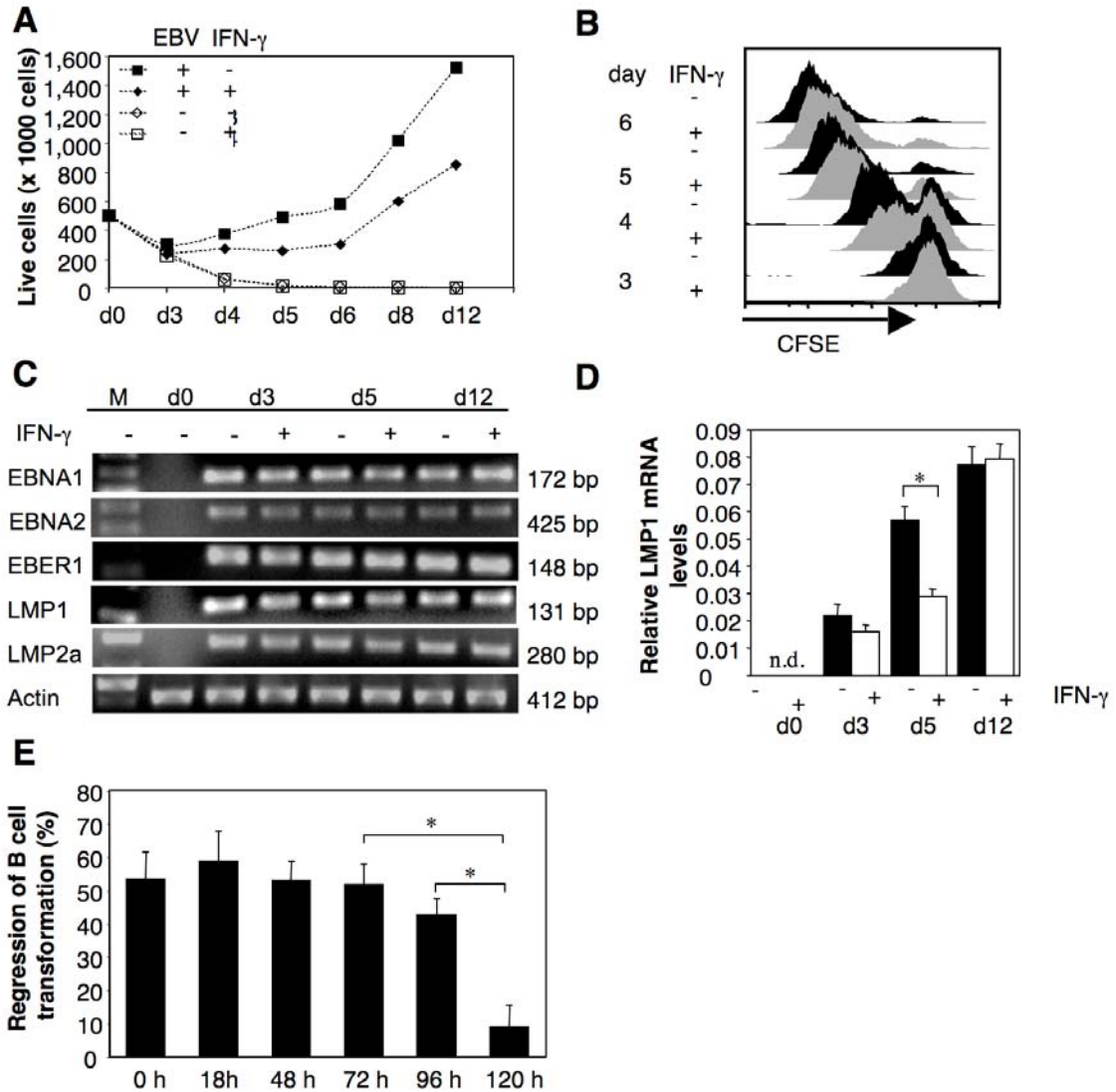
Figure 16: IFN-γ impairs transformation of B cells by EBV. (A) Cell numbers were compared at different time points between control and IFN-γ-treated B cells with and without EBV infection. (B) CFSE-labeled B cells were infected with EBV and proliferation was compared between controls and IFN-γ-treated samples at the indicated time points. (C) Expression of EBV-encoded genes was quantified by RT-PCR at different time points and compared between controls and IFN-γ-treated cells. M: 100 bp ladder (D) Expression of the oncogene LMP1 was quantified by RealTime-PCR and normalized to GAPDH expression. Results represent data from at least three independent experiments. (E) Peripheral blood B cells were infected with EBV and 10,000 pg/ml IFN-γ was added at the indicated time points. Regression of B cell transformation was analyzed after 12 d by comparing numbers of transformed B cells with and without IFN-γ.

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3.2 Studying immune responses against EBV *in vivo*

3.2.1 Immune reconstitution of NOD-*scid* $\gamma_c^{-/-}$ mice injected with human CD34$^+$ hematopoietic stem cells

In order to generate mice susceptible to EBV infection and hence potentially capable of generating EBV specific human immune responses *in vivo*, we compared previously described mouse models for human immune system reconstitution (NOD-*scid* $\gamma_c^{-/-}$ and Rag2$^{-/-}\gamma_c^{-/-}$ mice) (Gimeno et al., 2004; Ishikawa et al., 2005; Shultz et al., 2005; Traggiai et al., 2004). For this purpose, we engrafted irradiated newborn Rag2$^{-/-}\gamma_c^{-/-}$ and NOD-*scid* $\gamma_c^{-/-}$ mice with human fetal liver-derived CD34$^+$ HSCs. We observed higher levels of primarily human T and NK cell reconstitution in NOD-*scid* $\gamma_c^{-/-}$ mice (Figure 17A) and therefore pursued this model, to which we will refer as hu-NSG mice in the remainder of the text. In hu-NSG mice, we consistently achieved reconstitution of 20 to 50 mice from the same graft with similar reconstitution efficiency of B cell and T cell frequencies in peripheral blood after three months (Figure 17B). In reconstituted mice, frequencies of human CD45$^+$ were routinely above 60 % of total splenocytes after four months of reconstitution (Figure 17C) with the majority (80-90%) being B and T cells (ratio 2:1 for B:T cells, and 3:2 for CD4$^+$ to CD8$^+$ T cells). Additionally, CD123$^+$ plasmacytoid (1-2%) and CD11c$^+$ myeloid dendritic cells (1-2%), monocytes (1-2%) and NK cells (2-5%) were also detected (Figure 17C and see section 3.3.1). Furthermore, human CD45$^+$ cells and all major subsets of human immune cells were detected in thymus, mesenteric lymph node, bone marrow, liver, and lung of hu-NSG mice (data not shown).
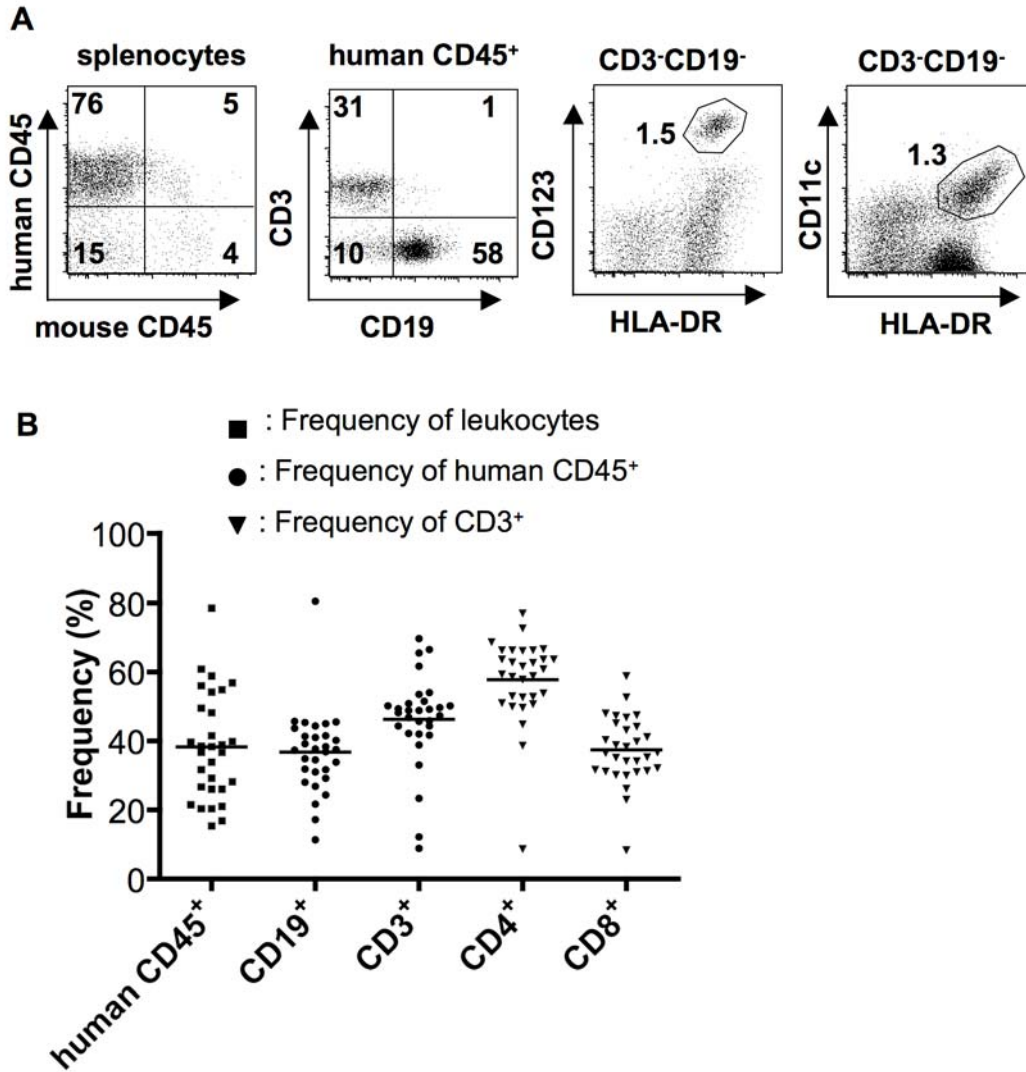
Figure 17: Multilineage reconstitution of NSG mice with human immune cells. Newborn NSG mice were reconstituted with CD34$^+$ stem cells and were analyzed 12 weeks later for the presence of human cells. (A) Total splenocytes were analyzed fro the presence of human leukocytes, B cells , T cells and DC subsets. One representative example is displayed. (B) Summary of reconstitution in the peripheral blood of several litters of NSG mice that were reconstituted with cells from the same donor.

Compared to the structured architecture in human secondary lymphoid organs such as lymph nodes or tonsils, immune cells in the spleen of hu-NSG mice showed a primitive organization into white and red pulp (Figure 18). In summary, in line with previous findings, we successfully achieved human multi-lineage reconstitution in NOD-*scid* $\gamma_c^{-/-}$

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mice after neonatal HSC transfer, thus generating a small animal model with the potential to generate human immune responses *in vivo*.
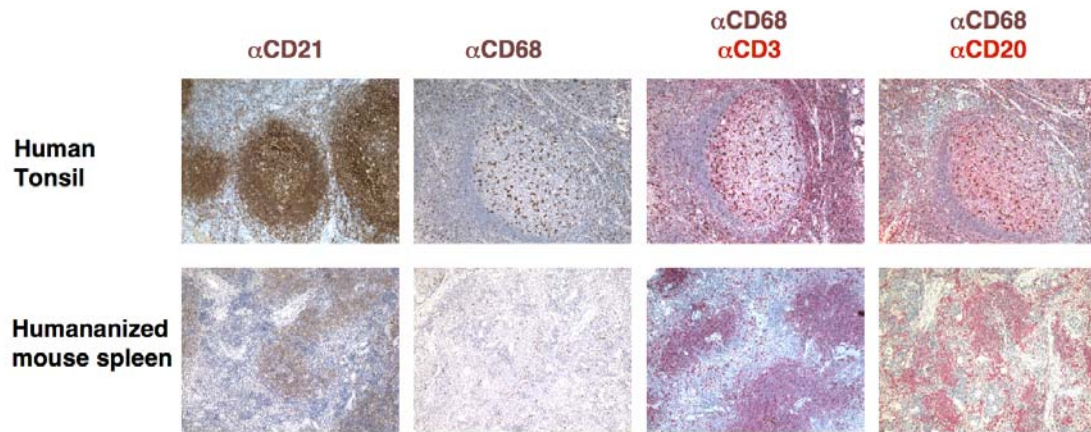
Figure 18: Primitive structural organization of the secondary lymphoid organs in hu-NSG mice. Immunohistological characterization of the spleen of humanized mice four month after reconstitution in comparison to a human tonsil (10x magnification). The indicated stainings characterize B (CD21, CD20), T (CD3), and myeloid cells (CD68).

3.2.2   EBV infection of humanized mice

In order to test the ability of the reconstituted human immune system to generate pathogen specific immune control, we infected hu-NSG mice with EBV, a human tumorvirus that is efficiently immune controlled in the majority of infected individuals. We chose an infectious dose that would roughly reflect the number of virus particles in 100 μl of saliva from a symptomatic EBV converter (Fafi-Kremer et al., 2005). After injection of hu-NSG mice with 10^5 RIU of purified viral particles intraperitoneally, EBV infected cells were readily detectable in the spleen, 4 weeks post-infection by in situ hybridization for EBERs, while we did not detect any EBER$^+$ cells in mock-infected animals (Figure 19A). At later time points, we also detected EBER$^+$ cells in the lymph nodes and livers of most infected animals (Figure 19A). In the spleen of infected animals, EBER$^+$ cells were consistently surrounded by CD3$^+$ T cells (Figure 19B). Furthermore, when EBER$^+$ cells

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were detected in non-lymphoid organs such as liver and kidney, these infected cells were again in close proximity to CD3$^+$ T cells, suggesting that T cells can home to similar peripheral sites as EBV infected cells in hu-NSG mice (Figure 19B). In order to characterize the latency type of EBV infected cells, we stained spleen sections for the two EBV-encoded proteins EBNA2 and LMP1. We did not detect any LMP1 single positive cells, but we found similar frequencies of EBNA2$^+$ compared to EBER$^+$ cells, some of which also expressed LMP1, indicating a latency III of EBV infection (Figure 19C). These results suggest that hu-NSG mice establish latent EBV infection, which constitutes the basis of B cell transformation by EBV.
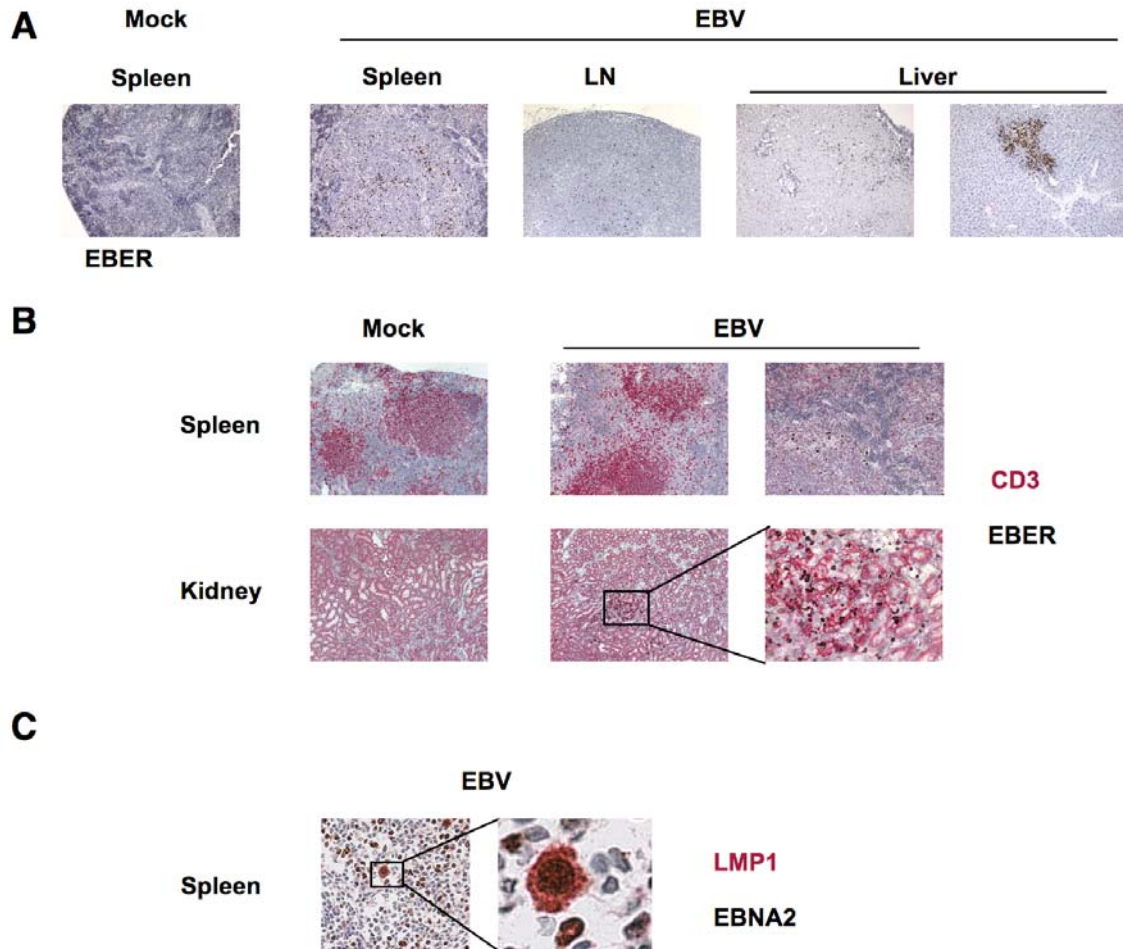
Figure 19: EBV infected cells are detected in hu-NSG mice in multiple organs and express EBNA2 and LMP1. (A) EBV infected cells could be detected in the indicated organs by EBER hybridization (10x magnification). (B) EBER$^+$ cells were surrounded by T cells (CD3$^+$) both in spleen and after migration to the kidney. (Right panels 40x magnification, others are 10x magnification) (C) EBNA2$^+$ cells co-expressed LMP1 in the spleen (Left panel is 40x magnification, right panel is a magnification of the left panel).

3.2.3 Development of HLA restricted EBV specific human T cell responses in infected hu-NSG mice

Since we rarely observed tumors after six weeks of infection in hu-NSG mice, we determined whether they developed EBV specific T cell responses, which are believed to protect healthy human carriers from EBV associated malignancies (Hislop et al., 2007; Khanna and Burrows, 2000). For this purpose, we first analyzed the splenic lymphocyte composition of the infected animals by flow cytometry. We found dramatic expansions of

CD3+ T cells among the human CD45+ leukocytes after EBV infection, which was accompanied by a corresponding increase in the percentage of CD8+ cells among the human T cells (Figure 20A). On average, we observed a statistically significant two-fold expansion of splenic CD8+ CD3+ T cells in 10 independent experiments with a total of 40 mice (Figure 20B). In addition, both in the CD8+ and the CD4+ compartments, there was a marked upregulation of HLA-DR and CD45RO surface expression, indicating an activated memory phenotype of the expanded T cells, which is also seen in humans during symptomatic primary EBV infection (Hislop et al., 2007; Khanna and Burrows, 2000).
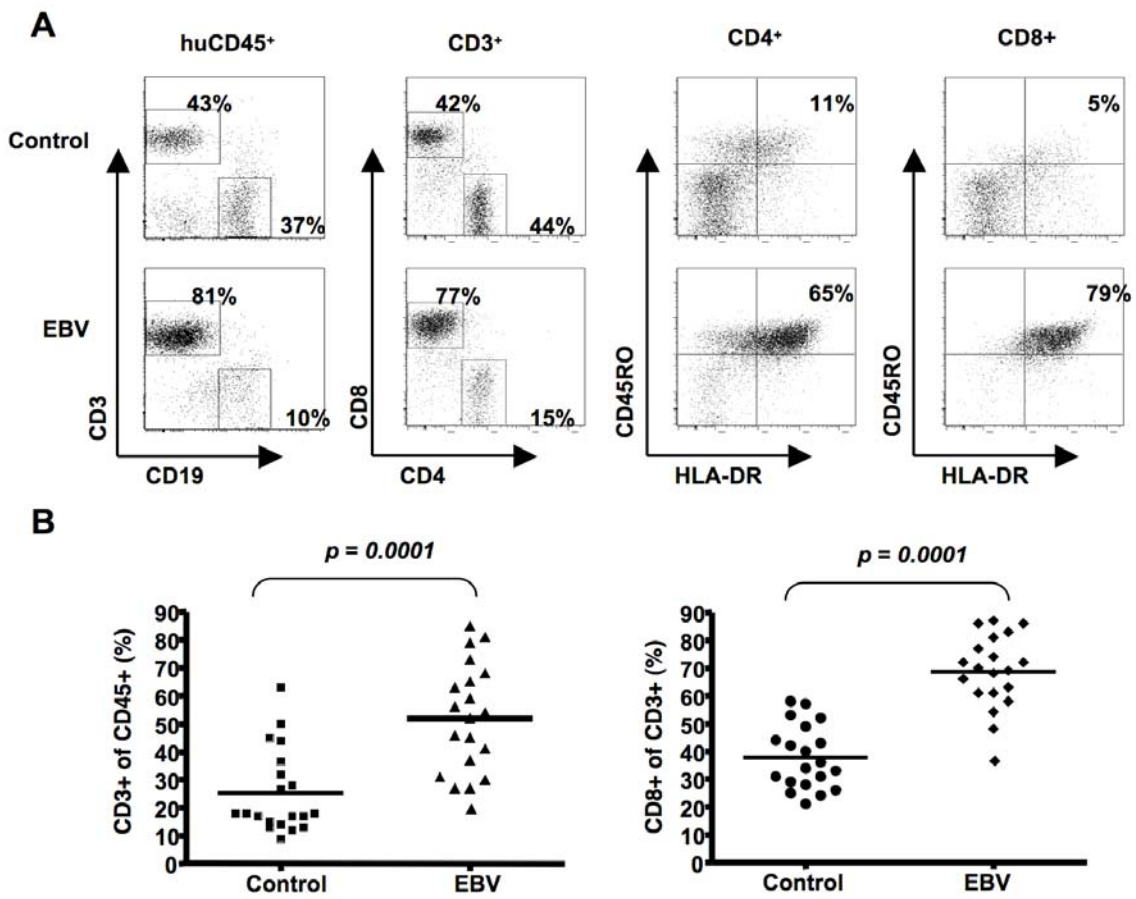
Figure 20: Expansion of human CD3+ T cells after EBV infection (A) Splenocytes from control or EBV infected animals were harvested six weeks after infection. Frequencies of lymphocyte subsets were

determined by flow cytometry. Activation and memory phenotype of both the CD4^{+} and CD8^{+} T cells was monitored by measuring the upregulation of the HLA-DR and CD45RO surface markers, respectively. Representative data of 10 experiments are shown. (B) Summary of CD3^{+}CD8^{+} T cell expansion for 40 mice in 10 different experiments.
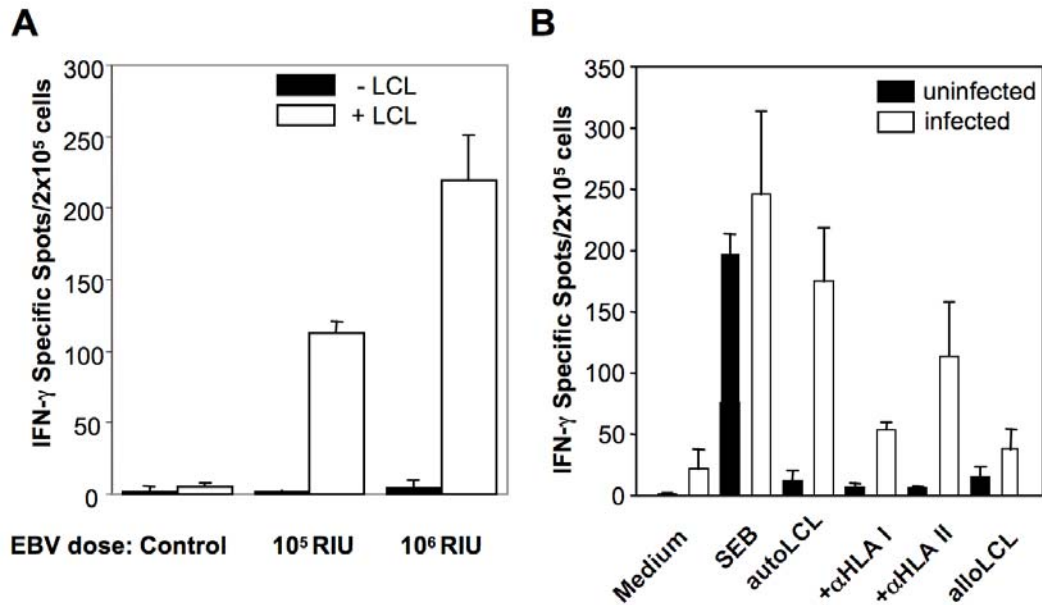
Figure 21: Dose dependent induction of HLA restricted T cell responses against autologous EBV transformed B cells in infected hu-NSG mice (A) Reconstituted NSG mice were infected with 10^5 or 10^6 Raji infectious units of EBV. Six weeks after infection, human B cell depleted splenocytes were incubated with autologous EBV transformed B cells (LCL) to measure EBV specific IFN-γ secretion using ELISPOT assays. IFN-γ specific spots per 10^5 cells are shown for a representative experiment with 3 mice in each group. One representative of 6 experiments is shown. (B) Humanized NSG mice were infected with 10^6 Raji units of EBV. Six weeks after infection, splenocytes were harvested from control and infected animals and T cell reactivity was evaluated by IFN-γ ELISPOT assays under similar conditions as described in (A). Staphylococcal enterotoxin B (SEB) superantigen and allogenic LCLs were used as positive and negative controls, respectively. Human HLA restriction was determined using inhibitory antibodies against HLA I and II as indicated. One representative of 2 experiments is shown.

We next examined whether T cells from infected animals would respond to autologous

EBV transformed B cells presenting viral antigens by human MHC molecules. For this

purpose, we first established lymphoblastoid cell lines (LCL) by *in vitro* infection of B

cells derived from a littermate mouse reconstituted with cells from the same HSC donor

used for reconstitution of the EBV infected animals. B cell depleted splenocytes isolated

from control and infected animals were incubated with these autologous LCL and IFN-γ

secretion was monitored by ELISPOT assays. A significant amount of IFN-γ production

was detected in response to autologous LCL whereas responses to allogeneic LCL were comparable to background IFN-γ production (Figure 21A and B). In addition, EBV specific T cells responses were much more vigorous from animals infected with higher doses of EBV (Figure 21A) suggesting that T cells can be primed in a dose dependent manner. LCL recognition could be blocked by pretreatment with antibodies against either HLA-A/B/C, HLA-DR/DP/DQ, or combinations thereof (Figure 21B and data not shown), clearly indicating that HLA restricted, EBV specific CD8$^+$ and CD4$^+$ human T cells were primed in hu-NSG mice upon infection with EBV.

3.2.4 Isolation of EBV-specific T cell clones

In order to analyze the peptide epitope specificity and effector functions of the *in vivo* primed EBV specific T cell responses, we isolated splenocytes from infected hu-NSG mice reconstituted with HLA-A2$^+$ HSCs 10 weeks post EBV infection. After labeling with CFSE, these splenocytes were stimulated with either autologous LCLs or a pool of 33 peptides derived from lytic and latent EBV antigens (Figure 21A). To enrich for LCL-specific or peptide-specific cells, CFSE^{low} T cells were sorted after 6 days by flow cytometry, cloned by limiting dilution, and finally retested with either autologous LCLs or the pool of 33 EBV derived peptides in IFN-γ ELISPOT assays, respectively. We were able to identify three CD8$^+$ T cell clones that recognized the library of 33 peptides, and subsequently tested them against a matrix of smaller peptide libraries allowing us to identify the individual peptides recognized by the clones (Figure 22A). All three clones recognized a peptide derived from LMP1, LMP1$_{167-176}$ (Figure 22A), which has been described to be recognized by CD8$^+$ T cells in EBV$^+$ infected HLA-A2$^+$ individuals (Khanna and Burrows, 2000). Titration of the cognate peptide on one of the clones (CD8-LMP1) showed recognition down to 10 nM indicating high affinity towards the

peptide (Figure 22B). We furthermore identified CD4$^+$ and CD8$^+$ T cell clones that recognized the autologous LCL in IFN-γ ELISPOT assays. Subsequently, we compared the cytotoxicity of three of these LCL specific clones (#1-3) and one of the LMP1 specific CD8$^+$ T cell clones (CD8-LMP1) against autologous LCL, and found that one of the two CD4$^+$ T cell clones, the LCL specific CD8$^+$ T cell clone, and the LMP1 specific CD8$^+$ T cell clone were able to lyse autologous LCL at similar effector to target ratios (Figure 22C). In addition, these T cells also degranulated and secreted IFN-γ in response to LCL recognition (Figure 22D). These data indicate that multifunctional cytotoxic EBV specific T cells are primed in hu-NSG that can kill EBV transformed B cells.
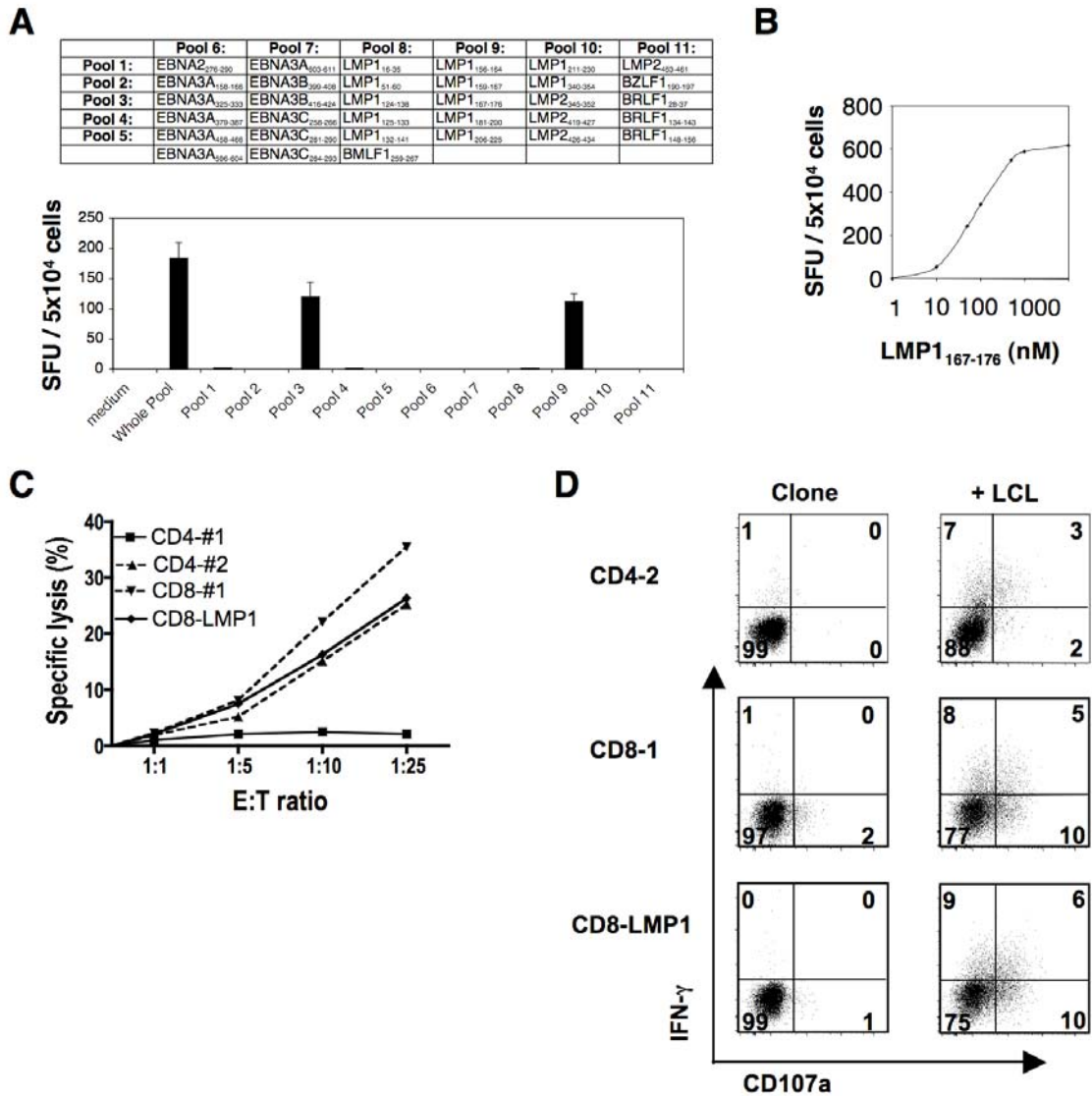
Figure 22: **Isolation of EBV specific T cell clones from infected hu-NSG mice.** (A) T cell clones were established by limiting dilution cloning from sorted CFSE^{low} T cells, that had proliferated in response to EBV transformed B cells (LCL) or EBV derived peptides, The library of 33 EBV peptides that was used for the initial T cell proliferation was divided into the indicated matrix of peptide pools and used to assess the fine specificity of obtained T cell clones. Reactivity of one out of three CD8^{+} T cell clones specific for the HLA-A2-restricted peptide LMP1$_{167-176}$ in IFN-γ ELISPOT is shown. (B) Epitope affinity was determined by cognate peptide titration on LMP1$_{167-176}$ specific CD8^{+} T cells in IFN-γ ELISPOT assays. One representative of 2 experiments is shown. (C) The cytotoxicity of LMP1$_{167-176}$ specific (CD8-LMP1) and LCL specific (#1-3) CD4^{+} and CD8^{+} T cell clones against autologous EBV transformed B cells (LCL) was assessed by flow cytometric To-Pro-3-iodide exclusion assays at the indicated effector to target ratios (E:T). One representative of 3 experiments is shown. (D) Degranulation and IFN-γ production was evaluated after co-culture with autologous LCLs by flow cytometric surface staining for CD107a and intracellular IFN-γ staining. One representative of 3 experiments is shown.

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3.2.5 Disseminated EBV associated malignancies in T cell depleted hu-NSG mice

To study the role of these *in vivo* primed EBV specific T cells in controlling EBV infection and EBV associated malignances in hu-NSG mice, we depleted T cells prior to EBV infection using antibodies against the CD4 and CD8 cell surface antigens (data not shown). Four to five weeks post infection, mice were analyzed for the development of tumors as well as for EBV viral loads. While we observed small splenic tumors in only 3 of the 17 EBV infected animals with or without isotype control antibody injection, all 11 T cell depleted animals developed disseminated EBV positive tumors in spleen, mesenteric lymph node, kidney, or liver (Figure 23A). Histological analysis showed expansion of white pulp regions in the enlarged spleens of T cell depleted and infected mice, which contained almost exclusively EBER$^+$ CD20$^+$ B cells (Figure 23B and data not shown). The majority of these cells expressed EBNA2, once again indicating a latency III expression pattern (Figure 23B). In addition, we observed a similar phenotype of the EBER$^+$ cells located in the LN, liver, and kidney. Furthermore, viral DNA loads increased significantly in T cell depleted mice compared to infected mice ($3.2x10^6$ vs. $1.9x10^5$, p = 0.003) indicating uncontrolled EBV infection after T cell depletion. In summary, these results indicate that hu-NSG mice are able to prime human EBV specific T cell responses that protect mice against uncontrolled EBV infection and EBV associated malignancies.
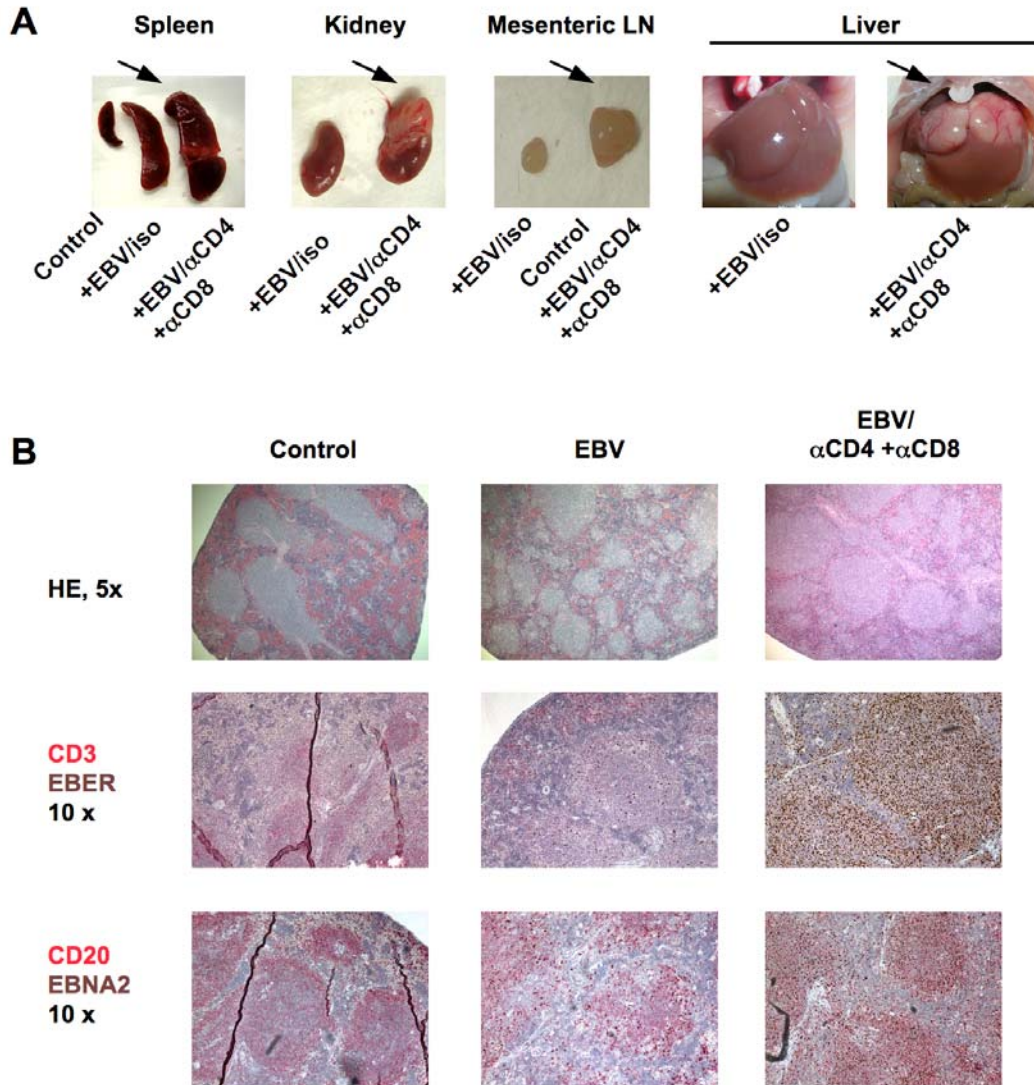
Figure 23: Development of EBV associated tumors after T cell depletion in EBV infected hu-NSG mice. (A) Disseminated tumors in EBV infected animals following T cell depletion. T cell depletion in EBV infected hu-NSG mice resulted in splenomegaly and EBV positive tumors either in kidney, mesenteric lymph node, or liver (arrows). T cell depleted and EBV infected hu-NSG mice (EBV/αCD4+αCD8, n=11) were compared to EBV infected hu-NSG mice (EBV, n=13), EBV infected hu-NSG mice treated with isotype control antibodies (EBV/iso, n=4) and uninfected hu-NSG mice (control, n=11). Representative pictures are shown. (B) Immunohistological characterization of representative spleen sections of T cell depleted and EBV infected, EBV infected and mock treated or uninfected hu-NSG mice. Splenic architecture was assessed by hematoxylin and eosin staining (HE), EBV infected cells were identified by either EBER in situ hybridization (EBER) or staining with EBNA2 specific antibodies (EBNA2), and T and B cell content was characterized by CD3 and CD20 specific antibody staining, respectively (HE 5x magnification, CD3/EBER and CD20/EBNA2 10x magnification).
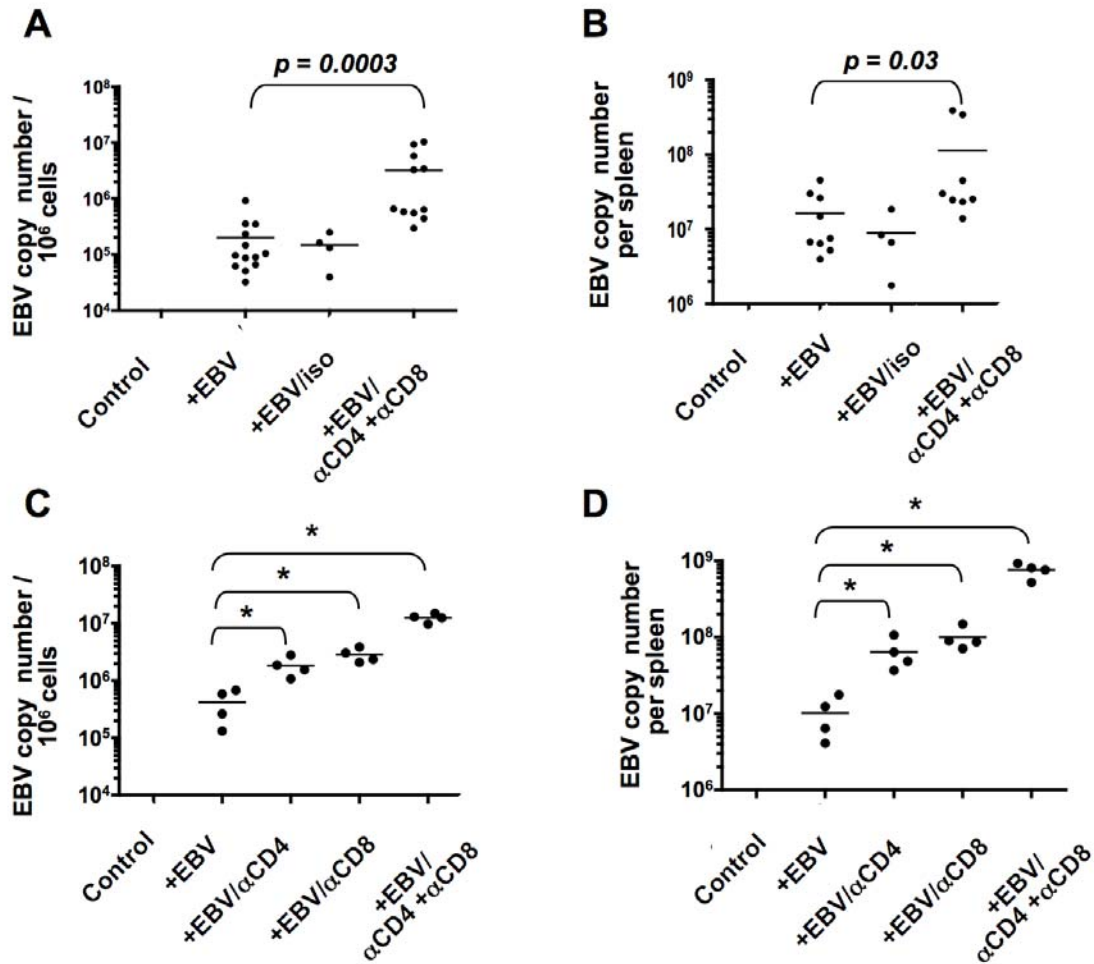
Figure 24: Elevated viral loads in T cell depleted and EBV infected hu-NSG mice (A) Splenic EBV loads were determined by quantitative real-time PCR four weeks after EBV infection. Viral titers were calculated from three independent experiments with a total of 39 animals (see above). No EBV titers were detected in uninfected hu-NSG mice (control). (B) Total splenic EBV loads were determined by multiplying splenic viral loads determined as in (C) with total splenocyte numbers determined by counting. Results are shown for seven control mice, nine EBV infected mice, four EBV infected and isotype control antibody treated mice, and eight T cell depleted and EBV infected mice. (C) EBV episome copy numbers in 10^6 splenocytes were determined in EBV infected hu-NSG mice after CD4$^+$ (αCD4) and CD8$^+$ T cell (αCD8) single depletions, as well as double depletion (αCD4+αCD8). Composite data of two independent experiments are shown. (D) Total viral loads per spleen were calculated from the viral copy numbers per 10^6 cells multiplied by the total counted splenocyte numbers. Statistical significance for all data was assessed with the Mann-Whitney U test, *: $p < 0.03$.

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3.2.6 EBV-specific CD4+ and CD8+ T cells contribute both to the immune control of EBV *in vivo*

In humans, *in vitro* studies have demonstrated the presence of both CD4+ and CD8+ EBV-specific T cells in healthy individuals, however the contributions of each of them to successful immune control of EBV *in vivo* has not been analyzed. In order to compare the contributions of CD4+ and CD8+ T cells to the T cell-mediated immune control of EBV infection, we then depleted T cell subsets separately. Viral titers increased significantly both after the depletion of CD4+ and CD8+ T cells compare to EBV-infected animals (EBV: $6.4x10^5$, αCD4: $1.9x10^6$, αCD8: $3.2x10e^6$, αCD4+αCD8: $1.3x10^7$ per 10^6 splenocytes, p < 0.03, and EBV: $1.5x10^7$, αCD4: $7.2x10^7$, αCD8: $1.2x10e^8$, αCD4+CD8: $8.6x10^8$ per spleen, p < 0.03) (Figure 24) increased significantly in all groups. However, neither the separate depletion of CD4+ and CD8+ T cells increased the viral titers as much as the depletion of both CD4+ and CD8+ T cells in these experiments. Notably, we observed in all (n=4) pan-T cell-depleted and in 3 of 4 CD8-depleted animals the occurrence of tumors in the spleens and mesenteric lymph nodes, but only in 1 of 4 CD4 depleted animals. This indicates that both CD4+ and CD8+ T cells contribute to the immune control of EBV *in vivo*, but additional experiments will be necessary to analyze how CD4+ T cells participate in the immune response.

3.2.7 Improved detection of EBV-peptide specific CD8 T cell responses in HLA-A2 transgenic hu-NSG mice

Since we were able to identify EBV derived peptide epitopes for our isolated T cell clones only occasionally from EBV infected hu-NSG mice, we aimed to bias EBV specific T cell recognition to peptides epitopes that dominate EBV specific immune control in humans by introducing a HLA-A2 transgene into NSG mice (NSG-A2). We reconstituted a group of regular NSG mice and NSG mice transgenic for HLA-A2 with CD34+ cells

from the same HLA-A2$^+$ donor (hu-NSG and hu-NSG-A2, respectively). Human immune cells and in particular human CD4$^+$ and CD8$^+$ T cells developed in both groups of mice with similar frequencies and distributions (Figure 25).
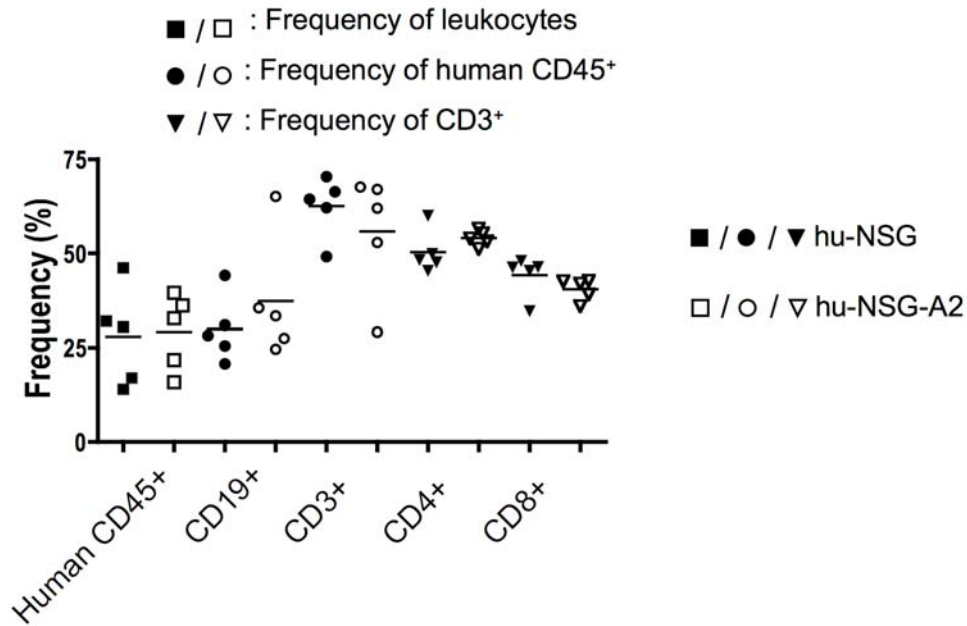
Figure 25: Similar reconstitution of hu-NSG and hu-NSG-A2 mice. Littermate wildtype and HLA-A2 transgenic newborn NSG mice (n=5 each) were reconstituted with HLA-A2$^+$ stem cells and were analyzed 12 weeks later for the presence of human cells. Expression of the HLA-A2 transgene on mouse cells was verified by flow cytometry.

We infected hu-NSG and hu-NSG-A2 mice with EBV and 5 weeks p.i. we stimulated splenocytes from these mice with two pools of EBV-derived HLA-A2-restricted peptides, one containing 8 lytic epitopes and one containing 12 latent epitopes. We detected in all (n = 5) EBV-infected hu-NSG-A2 mice IFN-γ secretion by ELISPOT after stimulation with the lytic pool, but only in 4 of 5 mice after stimulation with the latent pool (Figure 26A). Notably, these latent responses were significant lower than the lytic responses (11 vs. 70 SFU/2x10^5 cells, p = 0.04). Moreover, no IFN-γ secreting cells were detected in any of the control mice or in EBV-infected hu-NSG mice reconstituted with HLA-A2$^+$ matching

(n=2) or non-matching (n=8) CD34$^+$ cells. Interestingly, after stimulation with autologous LCLs, splenocytes from both EBV-infected hu-NSG and hu-NSG-A2 responded similarly (107 vs. 124 SFU/2x10^5 cells). In order to detect EBV-specific CD8$^+$ T cells directly, splenocytes were stained with a control tetramers (HIV GAG$_{SLY}$), and two well-characterized EBV tetramers derived from a lytic epitope (BRLF1$_{YVL}$) and a latent epitope (LMP2$_{CLG}$), respectively. While we did not detect any tetramer-positive cells in control hu-NSG and hu-NSG-A2 mice as well as EBV-infected hu-NSG mice, 3 of 5 EBV-infected hu-NSG-A2 mice developed up to 2.7% BRLF1$_{YVL}$ tetramer positive CD8$^+$ T cells (Figure 26B and C). These tetramer-positive cells had an activated phenotype being exclusively CD45RO$^+$HLA-DR$^+$. But, also in these mice we did not observe any cells positive for the LMP$_{CLG}$ tetramer, possibly because their frequency was too low. These experiments suggest that HLA transgenic hu-NSG mice bias primary T cell responses of reconstituted human immune system components towards recognition of EBV derived peptide epitopes that are dominant during EBV infection in humans
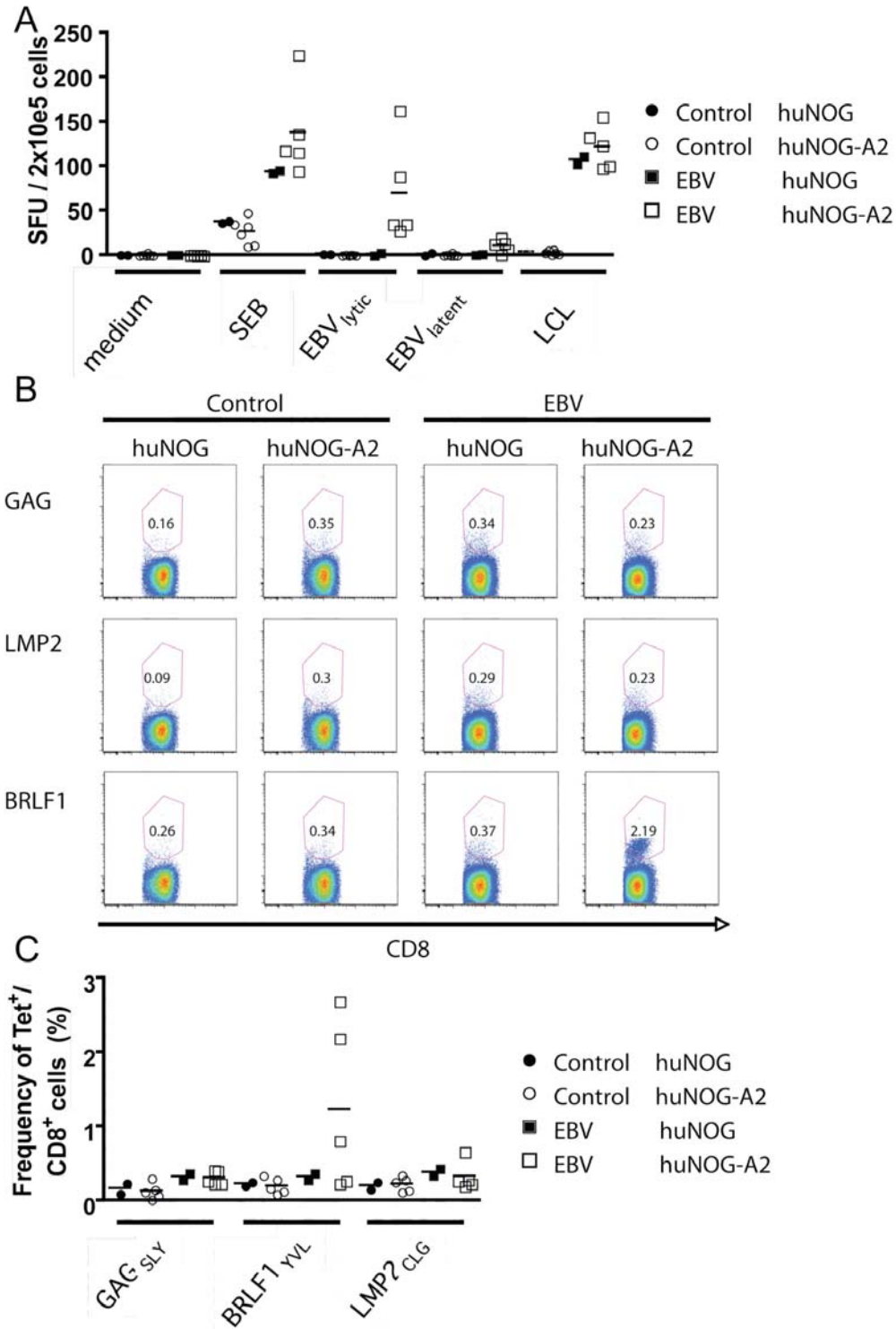
Figure 26: Enhanced priming of CD8+ T cell responses against dominant EBV peptides in HLA-A2 transgenic hu-NSG mice. (A) HLA-A2 transgenic and non-transgenic NSG mice were reconstituted with HLA-A2+ CD34+ HSCs from the same donor. 4 weeks after EBV infection or mock treatment, splenocytes were restimulated for IFN-γ ELISPOT assays with medium alone, SEB as a positive control, the autologous EBV transformed B cell line (LCL), 8 lytic EBV antigen derived dominant CD8+ T cell epitopes and 12 latent

EBV antigen derived CD8$^+$ T cell epitopes, which had been defined as dominant CD8$^+$ T cell epitopes in human EBV carriers. The data summarize two independent experiments. (B and C) In parallel, tetramer staining on splenocytes of EBV infected or control mice was performed *ex vivo*. Tetramers of HLA-A*0201 with the HIV gag77-85 (GAG$_{SLY}$), EBV LMP2 aa426-434 (LMP2$_{CLG}$) or EBV BRLF1 aa109-117 (BRLF1$_{YVL}$) peptides were used in co-staining with anti-CD8 and analyzed by flow cytometry. (B) shows a representative experiment and (C) the summary of two independent experiments.

3.3   Studying NK cell function *in vivo* in hu-NSG mice

3.3.1   Distribution and phenotype of NK cells in hu-NSG mice

NK cells are important effector cells contributing to the host response against pathogens and tumors. To assess their functions *in vivo*, we first decided to analyze their distribution and phenotype. In human PBMCs, NK cells are routinely identified as CD3$^-$CD56$^+$ cells, however, in the peripheral blood of hu-NSG mice we detected only few CD3$^-$CD56$^+$ cells (routinely <0.5%, data not shown). Recent research characterizing mouse NK cells has established the surface marker NKp46 as an universal NK cells marker. Hence, we decided to analyze peripheral blood and spleen for the presence of CD3$^-$NKp46$^+$ cells (Figure 27 and data nor shown). Interestingly, 12 weeks after reconstitution around 2-5 % of CD45$^+$ cells in the blood were CD3$^-$NKp46$^+$ NK cells. While around 75 % to 95 % of CD3$^-$CD56$^+$ cells expressed also NKp46, only 50 % of CD3$^-$NKp46$^+$ cells expressed CD56 (Figure 27) indicating that NKp46 is a better marker for NK cells in hu-NSG mice. We further analyzed these cells and found that they expressed activating receptors such as NKp30, and NKG2D, and also inhibitory receptors such as NKG2A/CD94, or KIRs. Notably, the frequency of CD3$^-$NKp46$^+$ expressing C-type specific KIRs was relatively low after 12 weeks, but based on a limited numbers of mice, it seems that this frequency increases with time. Another interesting observation is that around 50 % of CD3$^-$NKp46$^+$ cells do not express CD16 and this subset is therefore enriched in reconstituted mice. Finally, in addition to blood and spleen, NK cells were also detected at similar frequencies in organs such as lymph

nodes, lung, and liver, only in the bone marrow NK cells were present a lower frequencies (Figure 28 A and B). The observed phenotype of NK cells in these organs resembles that of NK cells in blood or spleen. In summary, CD3⁻NKp46⁺ cells expressing a number of additional NK cell markers develop in hu-NSG mice, but they seem to be enriched in early development stages like CD56⁺CD16⁻, possibly due to the lack of NK cell differentiation signals.
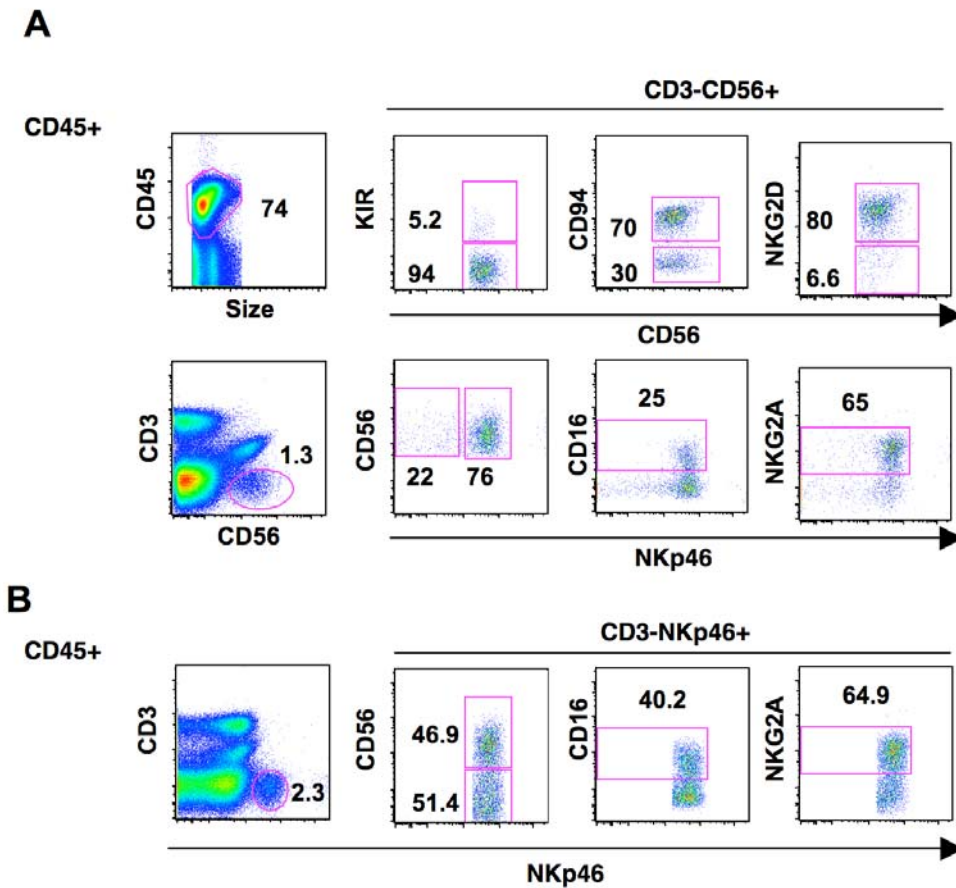
Figure 27: NKp46⁺ is superior compared to CD56 to identify human NK cells in hu-NSG mice. 12 weeks after reconstitution, splenocytes were used to identify NK cells by gating on (A) CD3⁻CD56⁺ cells and (B) CD3⁻NKp46⁺ cells. Then, CD3⁻CD56⁺ cells and CD3⁻NKp46⁺ were analyzed for additional NK cell markers. Data shown are from one representative mouse out of six mice reconstituted with two different fetal livers (n = 3 each).
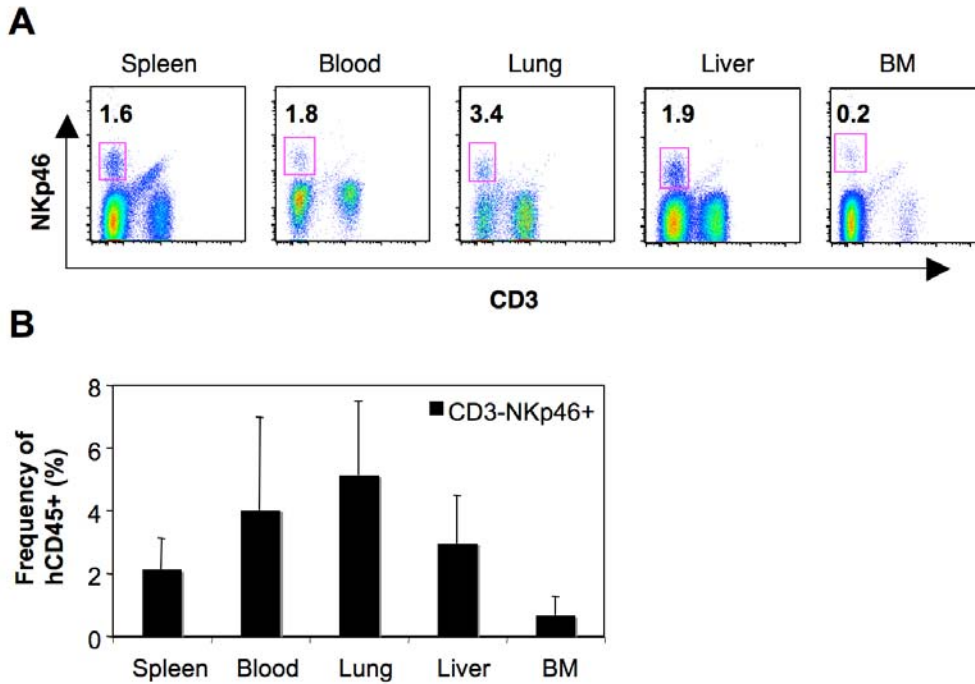
Figure 28: CD3⁻NKp46⁺ NK cells are present in multiple organs in hu-NSG mice. (A) CD45⁺ cells from the spleen, heparinized blood, perfused lung and liver as well as bone-marrow from one representative mouse were analyzed 12 weeks after reconstitution, numbers indicate the frequency of CD3⁻NKp46⁺ cells of CD45⁺ cells. (B) Summary of six mice reconstituted with two different fetal livers (n = 3 each).

3.3.2 Functional analysis of NK cells from hu-NSG mice *in vitro*

NK cells in mouse and man have a number of important functions during immune responses, the two most important being the killing of target cells such as virus-infected cells and secondly, the secretion of cytokines such as IFN-γ that modulate functions of immune cells such as DCs and have direct effects on pathogen-infected cells. To characterize the functional capabilities of NK cells developing in hu-NSG mice, bulk splenocytes were co-cultured with K562 and CEM cells, two classical NK target cell lines. PBMCs from healthy donors were used as control and when co-cultured with K562 and CEM cells, around 30 % of NK cells degranulated. After co-culture with K562 cells, NK cells also produced IFN-γ demonstrating full functional maturation as previously described (Figure 29B). However, significant fewer NK cells from hu-NSG degranulated

and no IFN-γ production was observed indicating a lower frequency of functional NK cells (Figure 29A and B). Furthermore, compared to CD56^{dim}CD16^{+} NK cells from PBMCs, NK cells from hu-NSG contained very little perforin and granzyme B and no difference was observed between CD16^{+} and CD16- cells (data not shown).
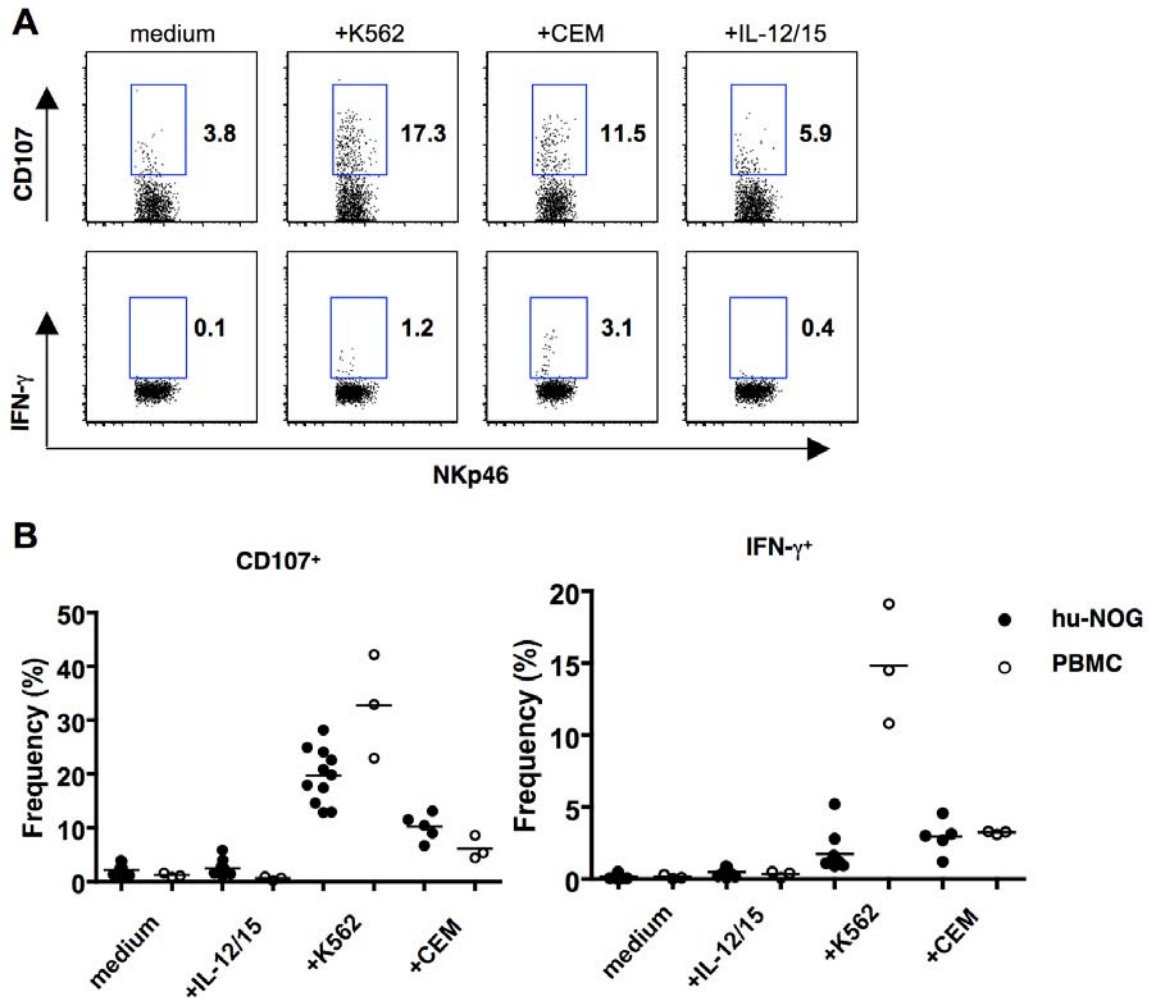
Figure 29: NK cells from hu-NSG mice have an impaired ability to produce IFN-γ after stimulation with K562 cells. (A) Splenocytes from hu-NSG mice were cultured for 6 hrs with medium, with K562 cells or CEM cells at an E:T ratio of 10:1 and with IL-12 and IL-15 (1 ng/ml each) in the presence of monensin (5 ug/ml). Numbers indicate the frequency of IFN-γ^{+} cells of CD45^{+}CD3^{-}NKp46^{+} NK cells. (B) After stimulation as in (A), CD107a surface expression and IFN-g production of NK cells from hu-NSG mice was compared to NK cells fo peripheral blood from human donors. Data represent results from three independent experiments including eleven mice and three human donors.

This lack of proteins of the cytolytic machinery was also directly observed in assays against K562 cells. Freshly isolated splenocytes were not able to efficiently lyse K562 cells, in contrast, NK cell lines established from these splenocytes were able to lyse a number of classical NK target cell lines such as K562, CEM and LCL721.221 (Figure 30 and data not shown). These results indicate that NK cells in hu-NSG mice are resting or immature as they lack natural cytotoxicity, which is a property of regular circulating NK cells in humans.
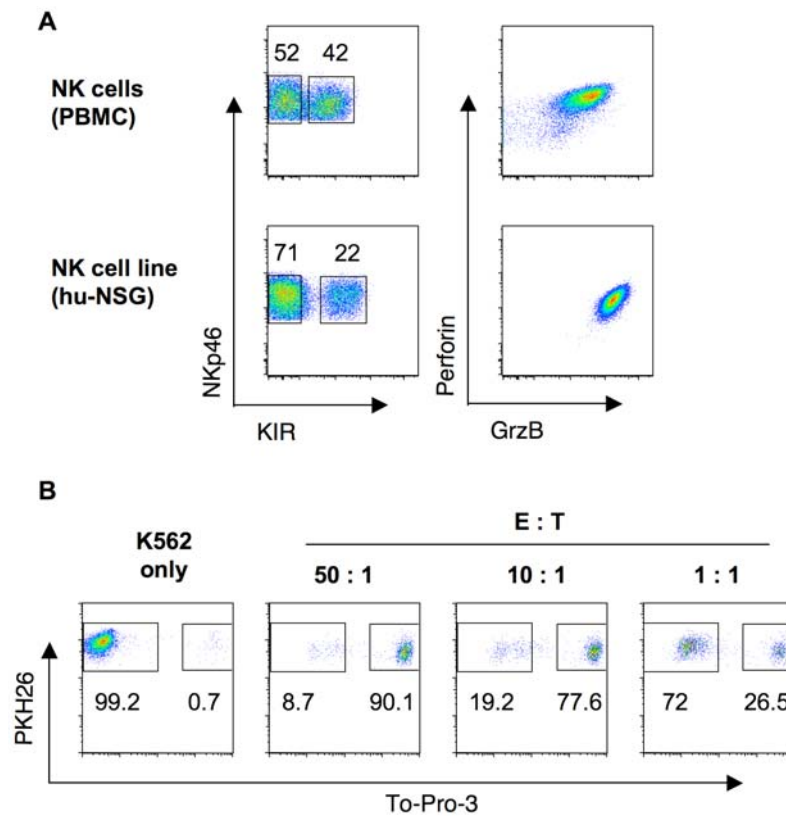
Figure 30: IL-2-activated NK cell lines are highly cytotoxic. Polyclonal NK cell lines were generated from splenocytes of hu-NSG in low-dose IL-2. After 7 days, expression of perforin and granzyme B was compared to NK cells in control PBMCs (A) and cytolytic ability gainst K562 cells was determined (B). Representative data from two independent experiments.

3.3.3 Activation of NK cells by poly(I:C) and IL-15

Recently, it was shown that resting murine NK cells have only very limited reactivity towards target cells in the steady state, but NK cells upregulated both cytotoxicity and IFN-γ production towards target cells upon short-term (<24 hrs) *in vivo* and *in vitro* activation (Fehniger et al., 2007). Hence, splenocytes were cultured for 24hrs with poly(I:C) and their reactivity towards K562 and CEM cells was compared to cells cultured in medium alone. Indeed, poly(I:C)-activated NK cells showed a significantly higher reactivity towards K562 and CEM cells than control cells (Figure 31A and B). Since IL-15 was recently described to be involved in the activation of NK cell effector functions in murine NK cells, we then tested if human NK cells from hu-NSG mice could be similarly activated by IL-12 and IL-15. Culture for 24 hrs in IL-12 and IL-15 together but also in IL-15 alone, increased both degranulation and IFN-γ towards K562 cells to levels similar of poly(I:C) (Figure 31A and B), indicating that in the steady state NK cells in hu-NSG mice are less functional mature compared to healthy individuals, but that after activation they are efficient effector cells. Notably, culture of splenocytes with IL-15 and poly(I:C) also increased intracellular levels of perforin and granzyme B. (Figure 32) This is of particular interest as murine NK cells also do not express perforin and granzyme B protein in the steady state, but only after activation with IL-15.
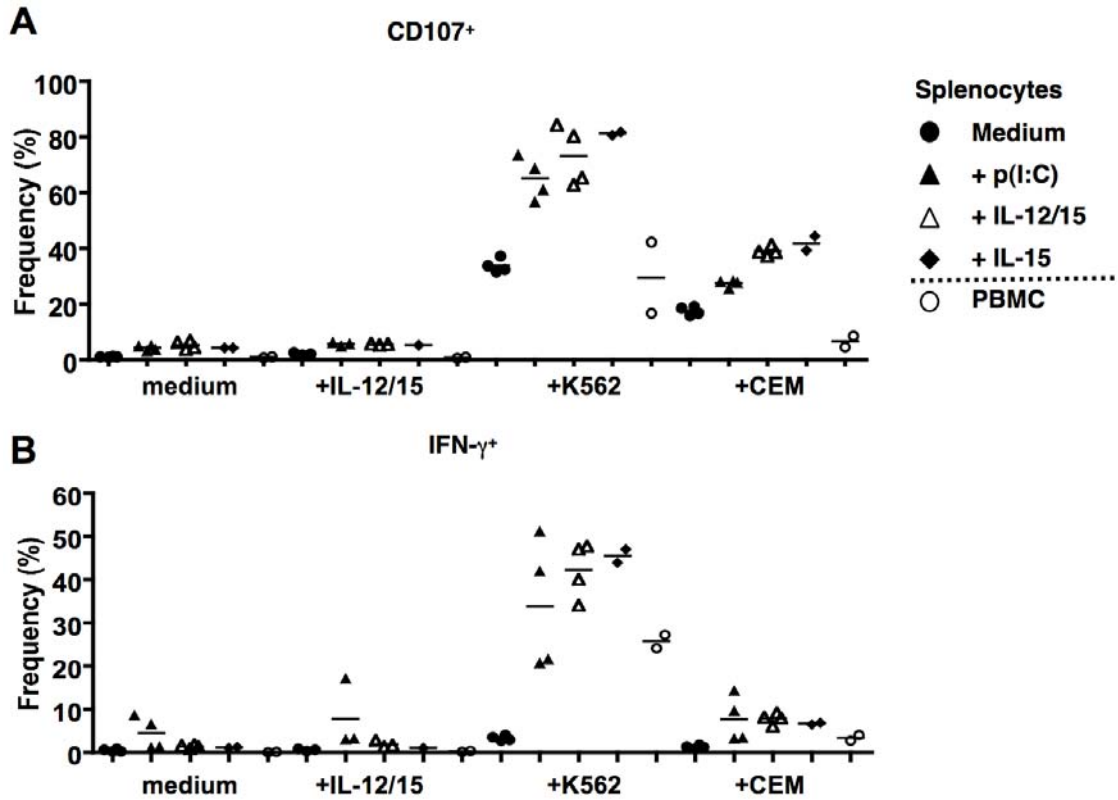
Figure 31: After *in vitro* pre-activation human NK cells from hu-NSG develop the ability to produce IFN-γ after co-culture with K562 cells. Splenocytes were pre-cultured for 24 hrs in medium alone or with p(I:C)(25 µg/ml), IL12 and IL-15, or IL-15 (1 ng/ml each). Then, splenocytes were cultured for 6 hrs with medium, with K562 cells or CEM cells at an E:T ratio of 10:1 and with IL-12 and IL-15 (1 ng/ml each) in the presence of monensin (5 ug/ml). (A) CD107a surface expression and (B) IFN-γ production of NK cells from hu-NSG mice was compared to NK cells fo peripheral blood from human donors. Data represent results from two independent experiments including four mice and two human donors.
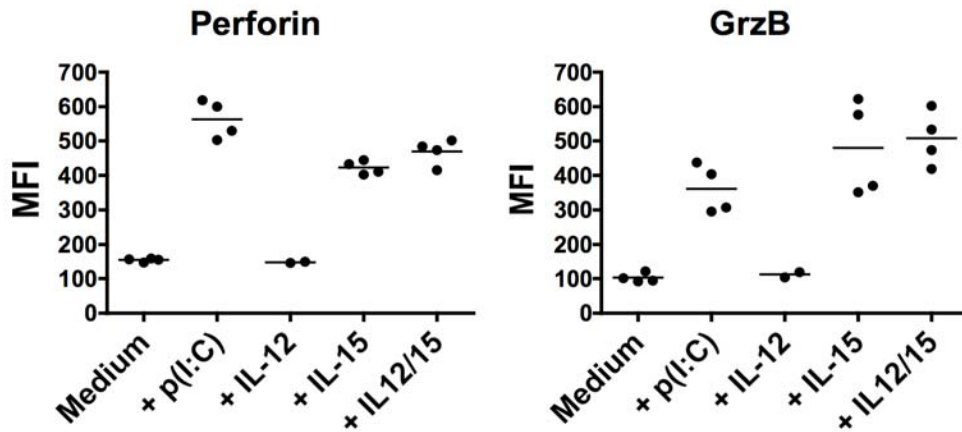
Figure 32: Increased expression of perforin and granzyme B protein in NK cells after preactivation *in vitro*. Splenocytes were pre-cultured for 24 hrs in medium alone or with p(I:C)(25 μg/ml), IL12 and IL-15, or IL-15 (1 ng/ml each). Intracellular expression of perforin and granzyme B in CD3⁻NKp46⁺ NK cells was analyzed by flow cytometry. Data represent results from one experiment including four mice.

Next, we tested if NK cells could be also activated *in vivo* with either IL-15 or poly(I:C). hu-NSG mice were injected with poly(I:C) and IL-15, and effector functions of splenocytes from these mice were compared to those of control mice. Similar to the previous *in vitro* cultures, NK cells from mice injected with both poly(I:C) and IL-15 showed increased reactivity towards K562 cells compared to control NK cells demonstrating that human NK cells reactivitiy increased rapidly *in vivo* (Figure 33). Again, we also observed a rapid increase of the intracellular perforin and granzyme B protein levels after *in vivo* activation (data not shown). In summary, these results indicate that NK cells developing in hu-NSG mice display an unusual phenotype and show in functional assays a resting behavior.
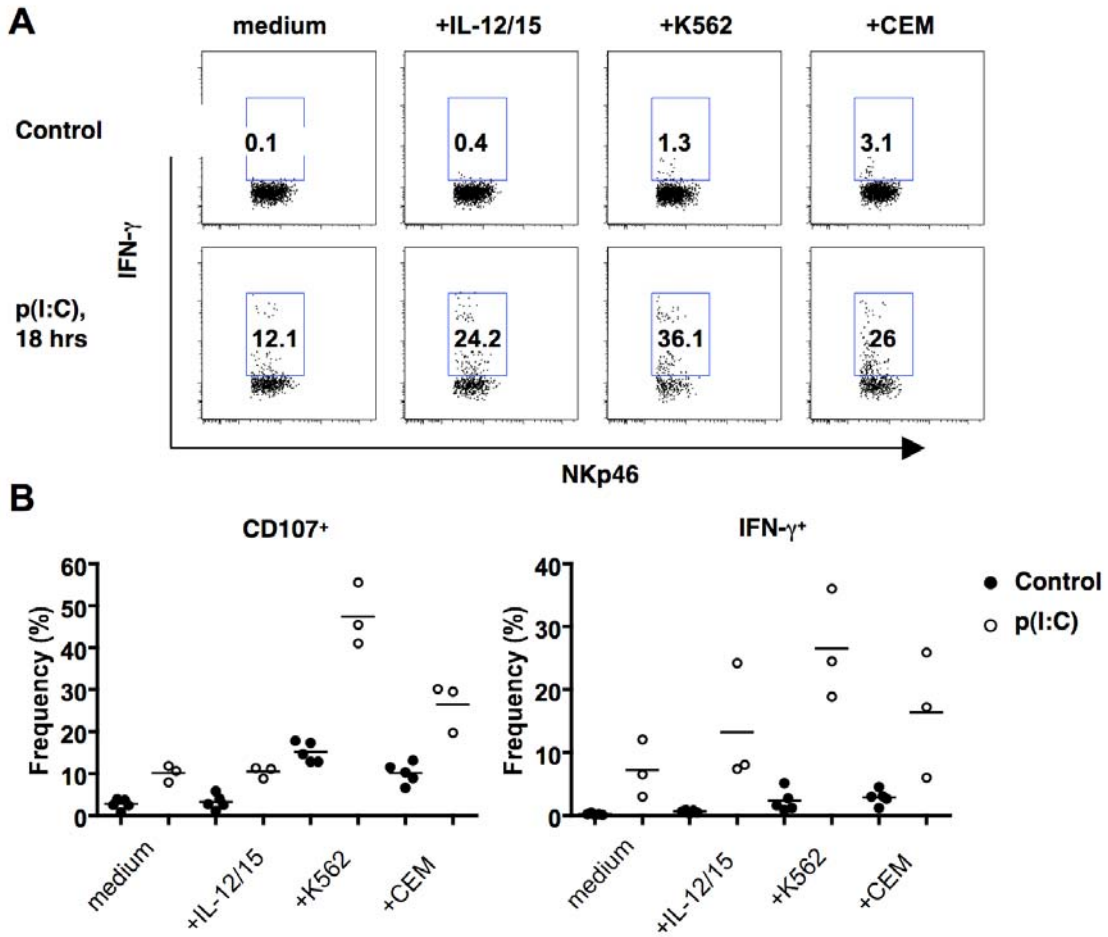
Figure 33: Human NK cells from hu-NSG acquire after *in vivo* activation the ability to produce IFN-γ after co-culture with K562 cells. Hu-NSG mice were injected i.p. with PBS or with p(I:C)(50 µg/mouse). Then, splenocytes were cultured for 6 hrs with medium, with K562 cells or CEM cells at an E:T ratio of 10:1 and with IL-12 and IL-15 (1 ng/ml each) in the presence of monensin (5 ug/ml). CD107a surface expression and IFN-γ production of NK cells from hu-NSG mice was analyzed. (A) shows the representative results from two mice, (B) is the summary of two experiments with a total of eight mice.

4 Discussion

4.1 Innate Immune control of EBV infection

NK cells and DCs are central figures in the innate immune response, and have been shown to interact in early phases of murine herpes virus infections (Andoniou et al., 2005; Andrews et al., 2003; Kassim et al., 2006). In contrast to the mouse, humans possess the CD56^{bright}CD16$^-$ subset of NK cells, which rapidly secretes high IFN-γ levels and strongly proliferates upon activation by DCs (Vitale et al., 2004). These NK cells are enriched in secondary lymphoid organs like tonsils and lymph nodes, and are therefore strategically positioned to rapidly respond to pathogens at these sites (Fehniger et al., 2002; Ferlazzo et al., 2004a; Ferlazzo et al., 2004b). Such a pathogen is the human tumor virus EBV, which enters the human body through the tonsils after transmission via saliva exchange. Within tonsils of healthy virus carriers, the proliferation program, which is also observed in *in vitro* EBV infected B cells, was found in naïve B cells, which travel through the perifollicular T cell zone and follicular mantle zone to encounter antigens (Babcock et al., 2000; McHeyzer-Williams and McHeyzer-Williams, 2005). These areas were also described to harbor DC/NK cell interactions or are close vicinity to those areas (Bajenoff et al., 2006; Ferlazzo et al., 2004a; Garrod et al., 2007). Our data suggest that at these sites, human DCs can activate preferentially CD56^{bright}CD16$^-$ NK cells, which then become able to limit EBV-mediated B cell transformation, mainly by secretion of IFN-γ, and regulate the proliferation program of EBV latency via this cytokine. Restriction of EBV induced B cell transformation by NK cells probably curtails viremia until it can be efficiently immune controlled by the adaptive immune system. These results suggest for the first time an important effector function for tonsillar NK cells early in the primary immune response against human persistent and oncogenic EBV.

4.1.1 Sensing EBV infection by the immune system

iDCs patrol the periphery and act as sentinels for the immune system (Banchereau and Steinman, 1998). Upon direct infection by a pathogen or uptake of pathogen-containing material in conjunction with a maturation stimulus they migrate to secondary lymphoid organs carrying information both in the form of a particular maturation pattern and pathogen constituents. DC maturation changes drastically the properties of DCs converting them into potent activators of both the innate and adaptive immune system. One group of DC receptors that detect pathogenic determinants and trigger the activating functions of DCs are TLRs (Iwasaki and Medzhitov, 2004). In murine herpes virus infections, it has been shown that different pathways synergize for the activation of immune responses against these pathogens. TLR9-deficient mice as well as TLR3- and TLR2-deficient mice have increased MCMV titers, suggesting that the immune system uses complementing recognition systems in herpes virus infection (Andoniou et al., 2005; Krug et al., 2004; Tabeta et al., 2004). Similarly, EBV might activate human DCs by means of several pathways. In addition to TLR9-activating CpG-motifs, by which EBV activates human plasmacytoid DCs (Lim et al., 2006), the EBV genome supports convergent transcription, which occurs also in other DNA viruses such as herpes simplex virus-1 (HSV-1)(Lagunoff and Roizman, 1994; Sample et al., 1989). The resulting virally encoded dsRNAs have been isolated from HSV-1-infected cells (Jacquemont and Roizman, 1975). Indeed, we were able to demonstrate that dsRNA from the convergently transcribed LMP1 and LMP2A antigens of EBV was able to stimulate IL-12 secretion. The measured IL-12 amounts were similar to levels secreted by CD11c$^+$ DCs after exposure to live virus and heat-inactivated EBV particles. Consequently, we suggest that myeloid DCs can detect EBV either directly, or indirectly through EBV-derived dsRNA as TLR3 and mda-5 agonists during primary infection and subsequently

initiate the immune response by activating NK cells and priming of T cells (Gitlin et al., 2006; Schulz et al., 2005). In line with previous studies, we find that phenotypical markers such as MHC class II and costimulatory molecules are equally upregulated with various DC maturation stimuli, while cytokine secretion profiles varied dramatically between different DC maturation conditions (Mailliard et al., 2004). In particular, IL-12p70, a potent stimulator of NK cells and Th1 responses, was produced at higher levels by DCs exposed to EBV, or matured with EBV-derived dsRNA or with maturation cocktails containing the dsRNA analog poly(I:C), compared to immature DCs or DCs matured with proinflammatory cytokines or LPS. Nonetheless, even low levels of IL-12 secreted by LPS-matured DCs through directed secretion into the synapse between NK cells and DCs have been shown to activate NK cells (Borg et al., 2004). Hence, our data suggests that myeloid DCs are able to sense EBV infection and to stimulate NK cell responses during primary EBV infection at least in part via IL-12.

4.1.2 NK cell mediated immune control of EBV infection

Two main functional subsets of NK cells have been described in humans, while counterparts for these NK cell populations have not been identified in the mouse so far (Cooper et al., 2001). The CD56^{dim}CD16$^+$ subset is mainly responsible for natural cytotoxicity and ADCC, while the CD56^{bright}CD16$^-$ subset has been characterized by its unique capacity to produce high amounts of immunoregulatory cytokines, such as TNF, IFN-γ and GM-CSF, upon activation (Cooper et al., 2001). IFN-γ production by CD56^{bright}CD16$^-$ NK cells, as well as NK cell proliferation are rapidly induced by DCs (Vitale et al., 2004). In addition, DCs also augment cytotoxicity of this subset after prolonged activation (Ferlazzo et al., 2004b). Several studies indicate a role for NK cells in the control of EBV infection and in particular in early primary immune responses

(Kaplan and Shope, 1985; Masucci et al., 1983; Moretta et al., 1997; Wilson and Morgan, 2002). However, all *in vitro* studies up to now have used blood NK cells activated by IL-2, which is, at least in humans, mostly secreted by activated T cells and therefore presumably not present during innate immune responses. Hence, we focused on NK activation by DCs as a physiological NK cell stimulus present during the early immune response to primary virus infections. Furthermore, previous studies on the role of NK cells during EBV infection mainly emphasized cytotoxicity, but not IFN-γ secretion of NK cells, although lymph node- and tonsil-resident NK cells primarily release cytokines after activation (Fehniger et al., 2002; Ferlazzo and Munz, 2004; Ferlazzo et al., 2004a). Suggesting a prominent role for NK cell-derived cytokines early during EBV infection, earlier studies have found that recombinant IFN-γ is protective for several days during initial B cell transformation by EBV, whereas in contrast, type I interferons inhibit transformation only during the first hours after infection (Lotz et al., 1985). Moreover, EBV-specific CD4$^+$ and CD8$^+$ T cells have been reported to mediate regression of EBV-transformed B cells despite low to undetectable cytolytic activity (Lee et al., 2004; Shi and Lutz, 2002). Our data support the hypothesis that tonsillar NK cells restrict efficiently EBV-induced B cell transformation via their superior ability to produce IFN-γ upon DC activation. Although IL-12 and IFN-α secreted by poly(I:C) DCs and DC1s upregulate cytotoxicity of NK cells, autologous EBV transformed B cells are not efficiently killed by activated NK cells due to their high MHC class I expression (Pappworth et al., 2007). Instead, the results of our studies support a prominent role for IFN-γ in the innate immune response to EBV by NK cells. First, addition of recombinant IFN-γ to the regression assay decreased the number of transformed B cells. Second, IFN-γ levels, sufficient to restrict EBV-induced B cell transformation, were found in our regression

cultures. Finally, blocking of IFN-γ in regression assays with NK cells significantly decreased the protective effect of NK cells. Hence, we conclude that this cytokine significantly contributes to innate resistance against primary EBV infection. In addition to its direct antiviral activity, IFN-γ secreted by DC-activated NK cells might also shape the EBV-specific adaptive immune response favoring a Th1-polarization which is observed in EBV-positive individuals (Bickham et al., 2001; Laouar et al., 2005; Martin-Fontecha et al., 2004; Morandi et al., 2006). Therefore, contrary to the notion that NK cells respond primarily with cytotoxicity without prior activation, we demonstrate that their main function against a relevant human pathogen consists of cytokine secretion after activation by DCs.

4.1.3 Interference of IFN-γ with the establishment of EBV latency

The effects of IFN-γ in innate immunity are manifold, ranging from strengthening intrinsic immunity via the induction of antiviral factors or degradative pathways in exposed cells, to the activation of other innate lymphocytes such as macrophages (Schroder et al., 2004). EBV transforms B cells by the coordinate expression of EBV latency genes that provide signals for B cell survival and proliferation. Of the eight latent EBV antigens, LMP1 has been suggested to be the main oncogene of the virus, causing epithelial cell transformation *in vitro* and B cell transformation *in vivo* (Dawson et al., 1990; Fahraeus et al., 1990; Kulwichit et al., 1998). When we analyzed viral gene expression upon exposure of cells to IFN-γ, we only detected differences in the levels of LMP1 mRNA, but not in any of the other viral mRNAs characterized. These genes included also the recently described anti-apoptotic proteins BALF1 and BHRF1 essential for the transformation of B cells by EBV (Altmann and Hammerschmidt, 2005). Therefore, IFN-γ induced down-regulation of LMP1 transcription could be one mechanism by which DC-

activated NK cells limit EBV induced B cell expansion. The fairly late transcription of LMP1, compared to other EBV latent antigens, during the establishment of EBV latency could also explain why IFN-γ can restrict B cell transformation by EBV during the first days of primary EBV infection, while others and we found that IFN-γ was not able to inhibit proliferation of fully EBV transformed LCLs (Lotz et al., 1985; and data not shown). Similarly, IFN-γ secretion by NK cells was shown to limit MCMV infection during the first week of infection, and reduced immediate early or late MCMV gene transcription, depending on the infected cell type (Orange et al., 1995; Presti et al., 2001). Therefore, tonsillar NK cells might limit latent EBV infection by IFN-γ mediated down-regulation of LMP1 until adaptive T cell immune responses can eliminate fully EBV-transformed B cells.

4.1.4   NK cell mediated innate immune control of EBV infection

Originally, NK cells were thought to be primarily cytotoxic effector cells. However, recent studies have highlighted cytokine-mediated immunoregulatory and anti-viral functions of NK cells. Interaction between NK cells and DCs were found to be essential for the proper activation of NK cell function during infection with bacteria and viruses. Based on the results of this study, we suggest that human myeloid DCs stimulate NK cells during EBV infection primarily via their ability to secrete IL-12. Activated NK cells are then able to mediate regression of EBV-mediated B cell transformation. Tonsillar NK cells, which, like lymph node NK cells, produce higher levels of IFN-γ than their peripheral blood counterparts after activation by DCs, are superior in inhibiting EBV-induced B cell transformation *in vitro* by down-regulating important components in the proliferation program of EBV latency. These results suggest a novel and important effector function for tonsillar CD56^{bright}CD16$^-$ NK cells upon DC activation in the primary immune response

against EBV. In addition to this non-cytolytic mechanism, a recent study demonstrated that upon exposure to IFN-α NK cells were able to lyse EBV-infected cells during the lytic phase (Pappworth et al., 2007). Notably, another study demonstrated that pDCs are able to sense EBV and activate NK cell by secretion of IFN-α suggesting that NK cells might provide innate protection against EBV at different point during the viral life cycle (Lim et al., 2006). Beyond EBV infection, our data suggest that humans have a strategically well-positioned population of NK cells that directly combat pathogen entry at mucosal sites and might restrict pathogens until they can be cleared or controlled by adaptive immunity.
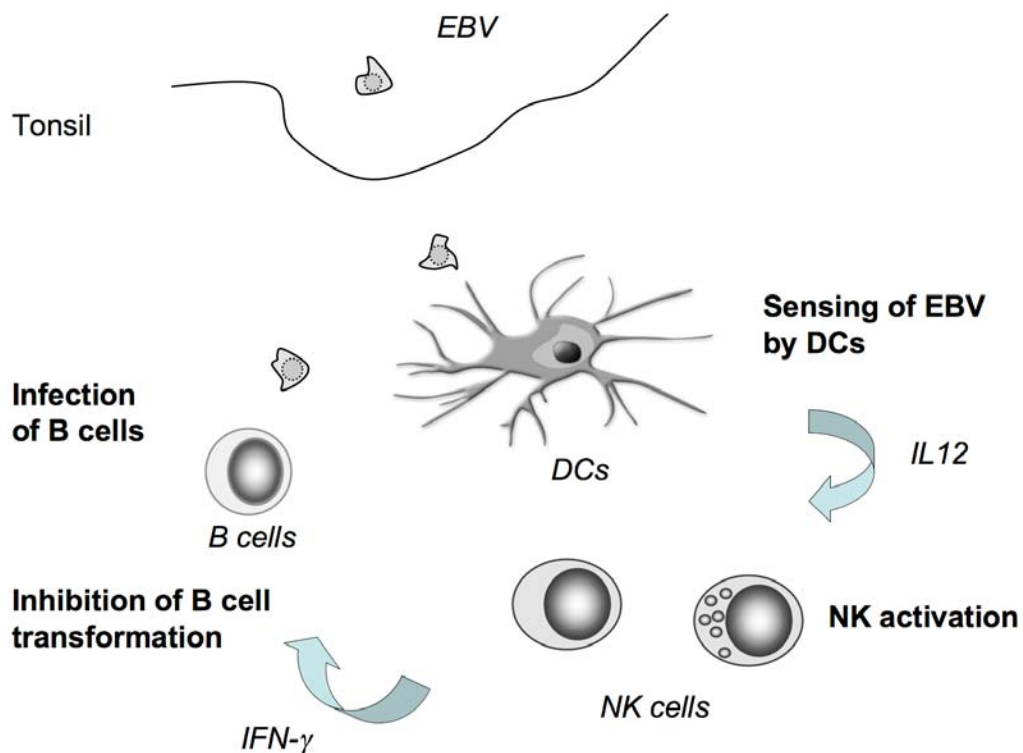
Figure 34: NK cell mediated non-cytolytic immune control of EBV infection after activation by DCs. EBV enters the tonsil and is sensed by cDCs using PRR. DCs mature, upregulate CD83 and start to produce IL12p70. This activates CD56bright CD16- tonsillar NK cells to produce IFN-γ that limits transformation of B cells by EBV.

4.2 Establishment of a humanized mouse models for EBV infection

4.2.1 Multilineage reconstitution of immune cells in hu-NSG mice

Recently, several groups developed independently a number of different severely immunodeficient mouse models that support the multi-lineage reconstitution of human immune cells *in vivo* (Gimeno et al., 2004; Ishikawa et al., 2005; Melkus et al., 2006; Shultz et al., 2005; Traggiai et al., 2004). In our laboratory we obtained higher reconstitution of human cells in NSG mice described originally by Shultz et al than in Rag2$^{-/-}$γ$_c$$^{-/-}$ mice on a mixed 129/Balb/c background described by Gimeno et al. However, it is difficult to generalize that NSG are better hosts as the ability of Rag2$^{-/-}$γ$_c$$^{-/-}$ mice to support reconstitution of human cells strongly depends on the mouse background. Strikingly, C57BL/6 Rag2$^{-/-}$γ$_c$$^{-/-}$ do not support reconstitution and we were not able to test pure-bred Balb/c Rag2$^{-/-}$γ$_c$$^{-/-}$ which support higher reconstitution than mixed 129/Balb/c Rag2$^{-/-}$γ$_c$$^{-/-}$ (R. Flavell, personal communication). However, a polymorphism in the Sirpa gene was recently described to be responsible for improved human hematopoiesis in NOD mice compared to other mouse strains (Takenaka et al., 2007). Therefore, the NOD background might be currently the most suitable for human hematopoietic reconstitution in mice.

We obtained regularly high levels of engraftment in NSG mice as detected in the blood, spleen, and other non-lypmhoid organs such as liver, kidney and the lung. We detected comparable frequencies of cells from all lymphoid lineages (B cells, T cells, NK cells), as well as conventional and plasmacytoid dendritic cells and also CD14$^+$ monocytes in large groups of animals engrafted with cells from the same donor. Notably, CD14$^+$ monocytes were almost absent in spleens of hu-NSG, while they were regularly detected in the blood and bone marrow (data not shown and M. Pack, personal communication). Since

hu-NSG presumably have all the necessary components to raise functional immune responses, we decided to test hu-NSG mice as a model for *in vivo* EBV infection and antiviral T cell-mediated immune control.

4.2.2 EBV infection of hu-NSG mice

Since no similarly oncogenic γ-herpesviruses of the EBV containing genus lymphocryptoviridae has been identified in rodents to date and murine cells do not support the EBV life cycle, an appropriate small animal models for EBV is lacking (Ehlers et al., 2008; Zychlinska et al., 2008). Immunodeficient mice have served as host for development of lymphoproliferative diseases after transfer of PBMCs from sero-positive individuals, and also for de novo infection of B cells developing from transplanted CD34$^+$ stem cells (Fuzzati-Armentero and Duchosal, 1998; Islas-Ohlmayer et al., 2004). However, these early models did not allow performing longitudinal studies of primary EBV infection in the interplay with a functional human immune response. In order to test if hu-NSG can be infected with EBV, we injected increasing doses of EBV intra-peritoneal. We detect CD20$^+$EBER$^+$ B cells in the white pulp region of the spleens in low numbers after 4-6 weeks after infection, but we did not find other EBV$^+$ cell type, which was confirmed in other studies (Cocco et al., 2008; Traggiai et al., 2004; Yajima et al., 2008). EBV$^+$ cells were always surrounded by CD3$^+$ T cells, even in the rare cases when we detected CD20$^+$EBER$^+$ cells in non-lymphoid organs such as liver and kidney. Depending on the dose of EBV used for infection, we detected higher frequencies of EBV$^+$ cells and even stronger expansion CD8$^+$ T cells, resembling IM. Notably, a similar association between EBV dose and CD8 T cell expansion was confirmed in another recent study (Yajima et al., 2008). Hu-NSG infected with lower doses of EBV survived over long periods of time (>12 weeks), while NOD-*scid* mice transplanted with CD34$^+$

cells that do not develop T cells died within 4-5 weeks upon EBV infection indicating the presence of successful immune control in hu-NSG mice (Islas-Ohlmayer et al., 2004). EBV infected B cells were almost always also strongly positive for EBNA2, and to lower levels for LMP1 indicating a latency III expression pattern, again, similar results were observed in two similar studies (Traggiai et al., 2004; Yajima et al., 2008). These results demonstrate that hu-NSG mice can serve as an *in vivo* model for EBV infection and that immune control of EBV seems to curtail disease.

4.2.3   EBV-specific immune responses in humanized mouse models

Previous studies employing different models of humanized mice have demonstrated that humanized mice can be infected with EBV, but failed to demonstrate clearly the induction of protective EBV-specific immune responses (Melkus et al., 2006; Traggiai et al., 2004). In BLT mice, EBV infection elicited low levels of IFN-γ secreting, HLA restricted T cell responses against autologous EBV transformed B cells (Melkus et al., 2006), and in reconstituted BALB/c Rag2$^{-/-}\gamma_c^{-/-}$ mice T cell proliferation against autologous EBV transformed B cells was detected after EBV infection (Traggiai et al., 2004). In none of these animal models, however, the protective value of EBV specific primary T cell responses has been investigated *in vivo*. Depending on the EBV dose used for infection, we detected increasing amounts of IFN-γ producing T cells that were restricted by MHC class I and II demonstrating the priming of human HLA-restricted T cell responses *in vivo* in hu-NSG mice. Notably, the frequency of IFN-γ producing T cells number was about five to tenfold higher than in a previous study (Melkus et al., 2006). We indirectly demonstrated that IFN-γ was mainly secreted CD8$^+$ T cells as it was strongly reduced after using MHC class I blocking antibodies in most experiments. Again, similar results were obtained in the other studies (Melkus et al., 2006; Yajima et al., 2008). In order to

identify the specificities of EBV-specific T cells, we cloned T cells from mice infected with EBV after enrichment by classical limiting dilution. We were able to obtain a number of LCL-specific CD4$^+$ and CD8$^+$ T cell clones that exhibited both cytotoxicity and IFN-γ secretion against autologous EBV transformed B cells. However, we were only able to identify the peptide specificity of one CD8$^+$ T cell clone that recognized a subdominant LMP1 epitope (LMP1$_{167-176}$). These results clearly demonstrate that hu-NSG mice can prime EBV-specific immune responses upon primary EBV infection *in vivo*, but it remained unclear if they were protective.

4.2.4   Priming of protective CD4$^+$ and CD8$^+$ T cell responses in hu-NSG mice

Since we reported that EBV infection in hu-NSG mice elicited cytotoxic and epitope specific primary T cell responses *in vivo*, we decided to test if these T cell responses are truly protective. In our initial experiments we decided to deplete both CD4$^+$ and CD8$^+$ T cells simultaneously by injection of anti-CD4 and anti-CD8 antibodies. Mice that received T cell depleting antibodies had highly increased frequencies of EBV$^+$ tumors in multiple organs and also had increased viral titers in the spleen demonstrating that hu-NSG mice are able to prime protective tumor-specific T cell responses *in vivo*. In order to dissect, if only CD8 T cells are necessary for the immune control of EBV *in vivo*, we also performed experiments in which we compared the depletion of either CD4$^+$ T cells or CD8$^+$ T cells to the depletion of both subsets. Interestingly we found that both CD4$^+$ and CD8 T cells contributed to the control of EBV infection in hu-NSG mice. Based on the results of our *in vivo* experiments, we conclude that CD4$^+$ T cells contribute to the control of early EBV infection, but are not able to restrict viral replication efficiently in the absence of CD8$^+$ T cells. However, upon additional depletion of CD8$^+$ in addition to CD4$^+$ T cells viral titers are further elevated, suggesting that CD4$^+$ T cells can mediate some

immune control of EBV (Heller et al., 2006). The inability of CD8$^+$ T cells to control EBV infection on their own, might reflect the requirement for CD4$^+$ T cell help during primary EBV infection (Bevan, 2004). This analysis demonstrates the usefulness of our *in vivo* model which allows for the first time to dissect protective mechanisms of human lymphocyte compartments *in vivo*.

4.2.5 Limitations of hu-NSG mice as model for EBV infection

Although we demonstrate protective primary immune responses against EBV in hu-NSG mice, there are a number of limitations to this model. Primary immune responses control EBV infection at high levels of viral load, with massive expansion and activation of the CD8$^+$ T cell compartment. These features are reminiscent of symptomatic primary EBV infection. Such a phenotype seems to be even more pronounced in hu-NSG mice, because they carry a tenfold elevated viral load in their splenocytes, compared with approximately 10^4 viral DNA copies per 10^6 peripheral blood mononuclear cells in IM patients (Fafi-Kremer et al., 2005; Hochberg et al., 2004). This of course assumes that the frequency of EBV infected cells is similar in peripheral blood and spleen during IM, as has been shown for healthy virus carriers (Laichalk et al., 2002). The reconstituted human immune system in hu-NSG mice has therefore difficulty controlling EBV infection, similar to chronic active EBV infection (CAEBV) in bone marrow transplantation recipients. Indeed, around 10^5 viral DNA copies per 10^6 peripheral blood mononuclear cells have been observed in more than 50% of CAEBV patients, and these also develop occasionally tumors (Kimura et al., 2001; Miyamura et al., 2008). Nevertheless, the reconstituted human immune system protects most infected mice from EBV associated malignancies. Furthermore, EBV specific T cell response in hu-NSG mice seems to favor subdominant EBV derived peptide epitopes (Hislop et al., 2007), and we were unable to

detect T cells of in humans dominant specificities *ex vivo*. A possible explanation for this might be the sub-optimal selection of human T cells and their TCRs on mouse thymic epithelial cells and on human BM-derived cells. Even so others and we have reported that human T cells recognize EBV-infected B cells after EBV infection of hu-NSG mice, and this recognition can be blocked with antibodies against human MHC molecules, the selection of EBV-specific T cells on human BM-derived cells and due to cross-reactivity with H2 molecules on mouse stromal cells seems to favor different affinities and specificities than those observed in humans with matching MHC type.

Another important process that is impaired in hu-NSG mice is the generation of high-affinity antibodies probably due to the lack of germinal center formation and class-switched humoral immune responses. Although we found low concentrations of human IgG accumulating in the plasma of reconstituted mice over time, we were unable to detect by ELISA EBV specific IgG or IgM responses against the viral capsid antigen (VCA) in EBV infected mice (data now shown). Interestingly, another study found by western blot that 4 of 30 EBV-infected hu-NSG mice developd antibodies against recombinant BFRF3, a component of the VCA (Yajima et al., 2008). We cannot exclude that humoral responses might develop against other EBV antigens in our model and perhaps at later timepoints of infection, as we have observed for EBNA1 in an experimental vaccine study using the same mouse model (Gurer et al., 2008). Accordingly, because more restricted expression patterns of EBV latent antigens have only been found in germinal center B cells of healthy virus carriers and since latency I/II tumors are thought to originate from EBV infected centrocytes or centroblasts, the study of EBV associated Burkitt and Hodgkin lymphomas and T cell responses against these malignancies might be difficult in hu-NSG (Babcock et al., 2000; Kuppers, 2003). Latency II tumors, however, have been observed in EBV infected and human B cell

reconstituted NOD-*scid* mice, suggesting that these hosts allow signaling for germinal center formation. Because these mice are unable to reconstitute human T cells after HSC transfer alone, immunological studies are not possible in this model (Islas-Ohlmayer et al., 2004).

In addition to these limitations regarding the selection and proper maintenance of human B cells and T cells in hu-NSG mice, there are a number of other properties of hu-NSG that need to be further improved to generate humanized mouse models that will be of true value for scientist studying human immunology. As briefly discussed human myeloid cells are rare in humanized mice, most probably because human myeloid progenitors can not compete with their mouse equivalents to repopulate lymphoid and non-lymphoid organs (Manz, 2007). It has been suggested that the genetic replacement of murine cytokines with their human equivalents would favor human myeloid cells and could lead to improved engraftment. Along similar lines, it has been suggested that the *in vivo* maintenance of human CD34$^+$ cells could be also improved leading to increased long-term output of human cells. This could potentially also lead to decreased number of human CD34$^+$ stem cells necessary for reconstitution which could be become a major issue because of limited supply and potential ethical considerations regarding the use of fetal tissues.

Table 7: Limitations of current humanized mouse models

Problem:	Proposed solutions:
Scarce human stem cells are necessary for every mouse	Expansion of HSC or differentiation of HSC from ESC
Impaired maintenance of human hematopoietic stem cells.	Expression of human cytokines or transplantation of mesenchymal stem cells
Human myeloid cell differentiation	Expression of human cytokines with preferentially weak or non-mouse cross-reactivity (GM-CSF, IL-3)
Human T and B cell selection and maintenance	Expression of human MHC class I and II instead of mouse MHC molecules.

4.2.6 Improved detection of EBV-specific immune responses in HLA-A2 transgenic hu-NSG mice

In order to address one of the previously discussed issues, the selection and maintenance of human T cells, we characterized the overall reconstitution and generation of HLA-restricted immune responses in HLA-transgenic NSG mice. We reconstituted HLA-A2 transgenic NSG mice with HLA-A2$^+$ CD34 stem cells and observed similar levels of reconstitution between transgenic and non-transgenic animals. Upon infection with EBV, we detected for the first time the recognition of HLA-A2 restricted in humans dominant T cell epitopes by CD8$^+$ T cells from HLA-A2 transgenic mice. This demonstrates that the short-coming of dominant EBV epitope recognition during infection in NSG mice can be overcome by introducing HLA transgenes, like in our case HLA-A2, into this mouse background. We were able to detect both latent and lytic EBV antigen specific T cell responses against dominant peptide epitopes from EBV infected hu-NSG-A2 mice *ex vivo*. Interestingly and similar to human EBV carriers, lytic EBV specific T cells were detected with nearly one log higher frequencies than latent EBV antigen specificities (Münz, 2005). Therefore, HLA transgenes seem to overcome one of the limitations of human immune responses in hu-NSG mice, and allows this immunocompetent small animal model with human immune system components to develop protective T cell responses against EBV infection with similar specificities to human virus carriers. Because EBV specific T cells are considered to be the cornerstone of immune control against this oncogenic and persistent γ-herpesvirus (Hislop et al., 2007; Khanna and Burrows, 2000), we propose to characterize the innate and adaptive immune responses that lead to this T cell based immune control further. We also plan to evaluate vaccine candidates for eliciting these protective T cell responses against EBV and other pathogens with exclusive tropism for the human hematopoetic lineage. This

includes the human immunodeficiency virus (HIV), which has been shown to establish infection in mice with reconstituted human immune system components (An et al., 2007; Baenziger et al., 2006; Berges et al., 2008; Gorantla et al., 2007; Sun et al., 2007; Watanabe et al., 2007; Zhang et al., 2007)

4.3 NK cell development and function in hu-NSG mice

Extensive studies of murine NK cells demonstrated that NK cells participate *in vivo* in the innate immune responses against pathogens and the immunosurveillance of tumor cells (Biron et al., 1999; Trinchieri, 1989). The developmental pathways leading to generation of NK cells have been mainly analyzed in genetically manipulated mice and provided valuable insight into the generation of subsets of NK cells in the mouse (Huntington et al., 2007). However, the study of human NK cells have been hampered by lack of suitable model systems as *in vitro* models offer some insight into development and functions of human NK cells, but naturally lack the value of *in vivo* experimentation. Importantly, significant differences between NK cells in mice and humans have been noted (Table 1) that increase the difficulty to translate results obtained in mouse models to human immunology and finally to clinical practice (Mestas and Hughes, 2004). A recently developed model attempts to close this gap by generating a small animal model to study function and development of human immune cells (Shultz et al., 2005; Traggiai et al., 2004). We used this model to study adaptive immune responses against EBV, but also reasoned to use this model to study specific aspects of human NK cell function.

4.3.1 Phenotypical analysis of human NK cells in hu-NSG mice

In line with previous reports we detected human $CD3^-CD56^+$ cells in immunodeficient mice reconstituted with human $CD34^+$ stem cells (Figure 27)(Ishikawa et al., 2005;

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Traggiai et al., 2004). However, we realized that by co-staining with anti-NKp46 we could identify cells that were CD3$^-$CD56$^{-/low}$NKp46$^+$ NK cells that expressed uniformly other activating NK receptors such as NKp30 and NKG2D and inhibitory receptors such as CD94/NKG2A.   Hence, we decided in the following to detect human NK cells using NKp46, one of the three human NCRs, that is expressed already early during NK cell development and was also recently proposed as a unique NK cell marker in the mouse (Huntington et al., 2007; Walzer et al., 2007). NK cells were detected in all organs analyzed, demonstrating that human NK cells can migrate to multiple sites in humanized mice. But in contrast to NK cells in human PBMCs, we noted that only around 50 % of NK cells expressed CD16 showing that the CD16$^-$ NK cell subset is enriched in humanized NSG mice. Interestingly in humanized Balb/c Rag2$^{-/-}$γ$_c^{-/-}$ mice, a recent study did not find such an enrichment of CD16$^-$ NK cells (Huntington et al., 2009), while the expression of the CD16 was not analyzed in the other studies (Ishikawa et al., 2005; Traggiai et al., 2004). In comparison, we observed additionally in some organs a significant difference in the frequency in of human NK cells in NSG mice compared to published frequencies in Balb/c Rag2$^{-/-}$γ$_c^{-/-}$ (Huntington et al., 2009). While frequencies in the latter were around 0.2 % of human CD45$^+$ cells in the spleen, we detected frequencies of around 2% of human CD45$^+$ cells. However, in the bone marrow this difference was not detectable, which could potentially indicate that early NK cell development in the bone marrow is similar but that NK cell survival in the periphery is increased in humanized NSG mice. It was hypothesized that the low frequency of NK cells in Balb/c Rag2$^{-/-}$γ$_c^{-/-}$ is potentially due to limiting amounts of IL-15 being trans-presented by IL-15Rα on human myeloid or stroma cells as murine IL-15 does not cross-react on human cells (Huntington et al., 2009). One might speculate that IL-15 levels are

higher in hu-NSG mice compared to Balb/c mice, but this issue remains elusive and needs to be addressed in the future. Nevertheless, based on these results and our own observations, we conclude that hu-NSG may serve as a valuable model to study specific questions related to human NK cell function and development.

4.3.2   Functional analysis of NK cells in hu-NSG mice

NK cells were originally described as naturally-active cells displaying readily effector functions towards tumor and virus-infected cells (Herberman et al., 1975a; Herberman et al., 1975b). However, closer characterization of murine and human NK cells *in vitro*, demonstrated that resting NK cells display minimal effector functions towards tumor cells and after triggering their activating receptors (Bryceson et al., 2006; Fehniger et al., 2007). Indeed, numerous studies have found that NK cell effector functions are increased upon activation by ligands for PRR such as poly(I:C) or synthetic cytokines such as type I interferons, IL-2, IL-12, and IL-15 (Djeu et al., 1979; Gidlund et al., 1978; Orange and Biron, 1996b). Particularly, IL-15 was recently described to be essential for the priming of murine NK cells after exposure to ligands of PRR (Lucas et al., 2007). In order to characterize the functional properties of human NK cell in hu-NSG mice, we cultured splenocytes with two classical NK cell target cell lines, K562 and CEM. NK cells from hu-NSG mice degranulated less than NK cells from human PBMCs after co-culture with K562 cells, and more strikingly, NK cells from hu-NSG mice did not produce any IFN-γ compared to NK cells from PBMCs. In order to test if human NK cells from hu-NSG acquire more effector function after *in vitro* activation, splenocytes were preactivated with poly(I:C) or IL-12 and IL-15. Strikingly, increased numbers of pre-activated NK cell degranulated and produced IFN-γ after exposure to K562 and CEM cells compared to unstimulated cells. These results suggest that human NK cell in hu-NSG mice require

similarly to murine NK cells to receive additional signals to become effective effector cells, in particular since IL-15 alone had a similar effect. To further test this hypothesis, we injected hu-NSG mice with poly(I:C) to activate NK cells *in vivo*. Again, preactivated NK cells displayed increased effector functions with higher numbers of cells degranulating and producing IFN-γ after exposure to K562 and CEM cells. In addition to directly detecting the effector functions of NK cells, we also measured the expression of proteins important for the cytotoxic functions of NK cells, perforin and granzyme B. This is of particular interest since it was recently described that resting murine NK cells do contain perforin and granzyme B mRNA but lack perforin and granzyme B proteins and that activation with IL-15 induces the translation of their mRNAs (Fehniger et al., 2007). Upon culture of splenocytes *in vitro* with poly(I:C) or IL-15, NK cells strongly upregulated the expression of perforin and granzyme B proteins. From these results we conclude that human NK cells in hu-NSG mice require similarly to murine NK cells activating signals to become fully equipped effector cells. One might speculate that the different environment in which hu-NSG mice and healthy human individuals live in and the pathogens that they are exposed to can explain the differences between human NK cells from these hosts. In contrast to hu-NSG mice and other laboratory mice that do live under specific-pathogen-free conditions and receive autoclaved food and water, humans are continuously exposed to various pathogens. These sub-clinical infections might induce a continuous arming or priming of NK cells leading to increased effector functions in the steady-state which can be further enhanced during infection and inflammation. However, the difference between human NK cells from hu-NSG mice and healthy individuals might also be explained by differences in development of human NK cells in hu-NSG mice potentially due to a sub-physiological levels of soluble or trans-presented IL-15. In order to support the first hypothesis, it would be very interesting to analyze the

expression of perforin and granzyme B protein in NK cells from mice living in the wild in comparison to animals kept in the laboratory. In conclusion, hu-NSG mice represent a novel *in vivo* model to study the development of NK cells from CD34$^+$ HSC in a dynamic environment. It will also be of high interest to study the interactions of NK cells with other human immune cells in hu-NSG mice in the steady-state and in the context of viral infections such as EBV.

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