SAPIENZA
UNIVERSITÀ DI ROMA

Facoltà di Farmacia e Medicina

Dottorato di Ricerca in Scienze Immunologiche
XXVI ciclo

Role of chemokine/chemokine receptor axes in the regulation of bone marrow NK cell localization in physiological and pathological conditions

Relatore:
Prof. Giovanni Bernardini

Candidato:
Dott. Andrea Ponzetta

Coordinatore: Prof.ssa Angela Santoni

Anno Accademico 2012/13

Index

1. Introduction

1.1. NK cells

1.1.1. Definition of an NK cell – An historical overview

The first definition of what a Natural Killer (NK) cell is dates back to 1975, when Kiessling and Herberman groups indipendently discovered that "lymphocytes of undefined nature" were capable of "spontaneous cytotoxic activity" against different tumor cell lines[1, 2]. The term "natural" was added to distinguish this mechanism from the one observed until then with T and B lymphocytes, which in turn was specific and MHC-restricted[3]. In the following years several investigators tried to better characterize not only the function, but also the phenotype and the developmental origin of NK cells: their definition then switched to "large granular lymphocytes" belonging to a "separate lymphocyte lineage, with both cytotoxicity and effector functions"[4]. This statement has been, and still is, generally accepted within the scientific community, even if a more recent attempt to update it has been carried on by Vivier group, on the basis of the multitude of information nowadays available on this cell type: their formalization of what a NK cells is would be "an NKp46+ CD3-, IL-15-dependent, IL-12-responsive lymphocyte, present in all mammalian species. Upon maturation, the NK cell is an early source of IFN-γ and a potent cytotoxic lymphocyte in response to various cellular targets, which lack constitutive self molecules and that express stress-induced self molecules or microbial molecules"[5].

Regardless of the complexity of such a definition, it clearly shows how much is currently known on NK cells.

Firstly, what does an NK cell do? It has been classically described as a powerful component of the innate branch of the immune system, involved in the immune surveillance and in the response against tumor cells and virus-infected cells[6].

Since their discovery, NK cells were described as effector lymphocytes that recognized cells that did not express the Major Histocompatibility Complex I (MHC-I) molecule, in an

opposite fashion from what happened for T lymphocytes, whose target recognition was instead "MHC-restricted"[7]. This huge difference was later elucidated by the work of Karre and colleagues, that for the first time hypothesized the "missing self" theory [8]. According to this revolutionary view, NK cells were indeed able to discriminate transformed (and dangerous) cells by self cells through specific receptors for MHC-I molecules: the recognition of those molecules by means of specific receptors caused the inhibition of NK cell cytotoxic activity (against self), while the absence of the surface expression of MHC-I on any cell would trigger the NK cell response, activating the "natural" cytolysis.

Later on, it was described how NK cells were also able to target MHC-I expressing tumors[9, 10, 11], and this raised questions on alternative mechanisms of cell recognition used by the NK population[12]. In fact, today it is known that there are several activatory signals that stimulate NK cell activation and degranulation capacity. Altogether, these findings brought to the concept that "a balance of inhibitory and activatory signals regulates NK cell responses"[13], widely accepted today.

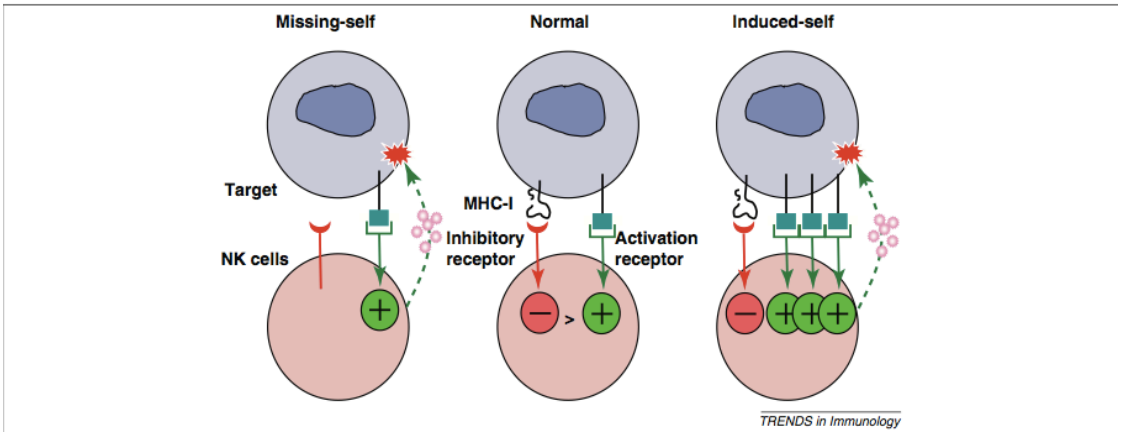


Figure 1 - ***Regulation of NK cell effector responses***. The balance between inhibitory and activating receptors determines the fate of target cell. Under normal circumstances, MHC-I receptors dominate over activating receptors. When MHC-I is downregulated (missing self, left), the inhibition signal is lost and the NK cell releases cytotoxic granules. If the target cell is "stressed" its activating ligands are overexpressed and inhibiting signals are overcome (induced self, right)[14].

1.1.2. NK cell receptors

As shown in Figure 1, the result of the encounter of an NK cell with a potential target cell depends on the integration of a multitude of signals, deriving from the wide array of inhibitory and activatory receptors engaged in that moment.

As this work regards the mouse immune system, the following part will be mainly focused on murine NK cells, with a particular attention on the difference with the human immune system when needed.

The most peculiar set of receptors present on NK cell surface is probably the one belonging to "the NK complex", a C-type lectin superfamily of at least nine highly related genes present on mouse chromosome 6, that are able to bind polymorphic H-2 class-I molecules, and can either be activatory or inhibitory[15].

Activating receptors. Among the activatory receptors, Ly49H is important for the recognition of mouse cytomegalovirus (MCMV)[16, 17, 18], an herpesvirus which is particularly susceptible to NK cell immune surveillance. The epitope recognized by Ly49H is a viral protein expressed by MCMV-infected cells, m157.

Activating NK cell receptors are also able to recognize "stress signals", the most important of which are Rae-1 (RNA export homolog 1), H60 (histocompatibility-60) and MULT1 (murine ULBP-like trancript 1), the ligands of NKG2D[19]: these molecules are frequently overexpressed by transformed cells[20, 21], virus-infected cells[22] or even by cells that suffered from genotoxic-stress, or DNA-damage[23]. NKG2D belongs to the NKG2 (Natural Killer Group 2) family, which contains four genes in total (NKG2A, NKG2C, NKG2E and NKG2D/F)[24], that together with CD94 are part of the C-type lectin superfamily.

NK cells can also sense microbial particles through Toll-like receptors (TLRs), such as double-strand DNA (viral origin - TLR3) and CpG (bacterial origin – TLR9)[25], even though there are doubts regarding NK cell intrinsic capacity to respond to TLR ligands, as the interaction with accessory cells like dendritic cells (DCs) significantly increases NK cell

activation. In fact, this might hide an indirect role for NK cells *in vivo* upon stimulation with microbial molecules[26, 27].

Other activating receptors are those called natural cytotoxicity receptors (NCRs), the NKp46 (NCR1), NKp44 (NCR2) and NKp30 (NCR3)[28], which are not only strong activators of NK cell function, but, in the case of NKp46, a potential marker for the NK cell lineage [5, 29](with the exception of IL-22+ Ror-γT+ innate lymphoid cells residing in the gut[30, 31] and of discrete subsets of T cells[31]). As NKp46 is well-conserved across species (differently from other classic NK lineage markers, such as CD56 in humans and NK1.1 in C57BL/6 mice), it might represent the real NK cell marker. For what concerns NCR ligands, they are not very well defined: an interaction between influenza virus hemagglutinins and Sendai virus hemagglutinin/neuraminidase with NKp46 has been reported some years ago[32], but its biological relevance remains to be elucidated. Furthermore, NKp46 seems to mediate cytotoxicity also against Mycobacterium Tubercolosis infected cells[33], while in human NKp30 has been shown to ligate the HCMV tegument protein pp65, leading to the inhibition of HCMV infected cells[34]. The difficulty in finding a good candidate as NCR ligand are increased by the absence of a murine ortholog for NKp44, and by the fact that NKp30 is a pseudogene in mus musculus, but in general NCRs are believed to be mainly involved against tumor cells[31].

Although exclusive, at least until now, for humans, the killer cell immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LILRs) role shoud be reminded: they are type I glycoproteins and members of the Ig superfamily[35, 36], are able to bind, like Ly49s, human leukocyte antigen-A (HLA-A), HLA-B and HLA-C, and their genetic locus is highly variable, polygenic and polymorphic within human population; in addition, depending on their structure and on the adaptor molecules they are associated with, KIRs can both inhibit and activate NK cell function upon the receptor engagement[19].

Taken together, the receptor families described above are those responsible for the so called "natural cytotoxicity", or the capacity to recognize non-self or stressed cells by means of germinally encoded proteins.

An alternative pathway leading to NK cell activation is the antibody-dependent cell cytotoxicity (ADCC), which is exerted through CD16 (FcγRIIIA): CD16 is a low affinity receptor for IgG that recognizes antibody-coated cells, signalling through its associated molecules FcεRI γ chain and T cell receptor (TCR) ζ chain, triggering granule release and cytokine production[37]. CD16 stimulation is so powerful that can lead to NK cell activation by itself, similarly to what happens with the multipotent TCR and BCR receptors (along with costimulatory molecules), while none of the other activatory NK cell receptors are able to elicit a response by themselves (and are thus defined "coactivatory")[38].

Inhibitory receptors. Many of the members of the inhibitory receptor group belong to the families cited above, like KIRs and LILRs in humans, and Ly49s in mice. They recognize both MHC-I-related and –unrelated molecules, and the inhibitory signal deriving from the engagement of these receptors counterbalances the stimuli that come from activatory receptors. Examples of inhibitory receptors are Ly49A-I (with the exception of Ly49D and Ly49H), which are specific for MHC-I molecules, and KLRG1, a member of C-type lectin-like receptor family, which binds E-, N- and L-cadherins.

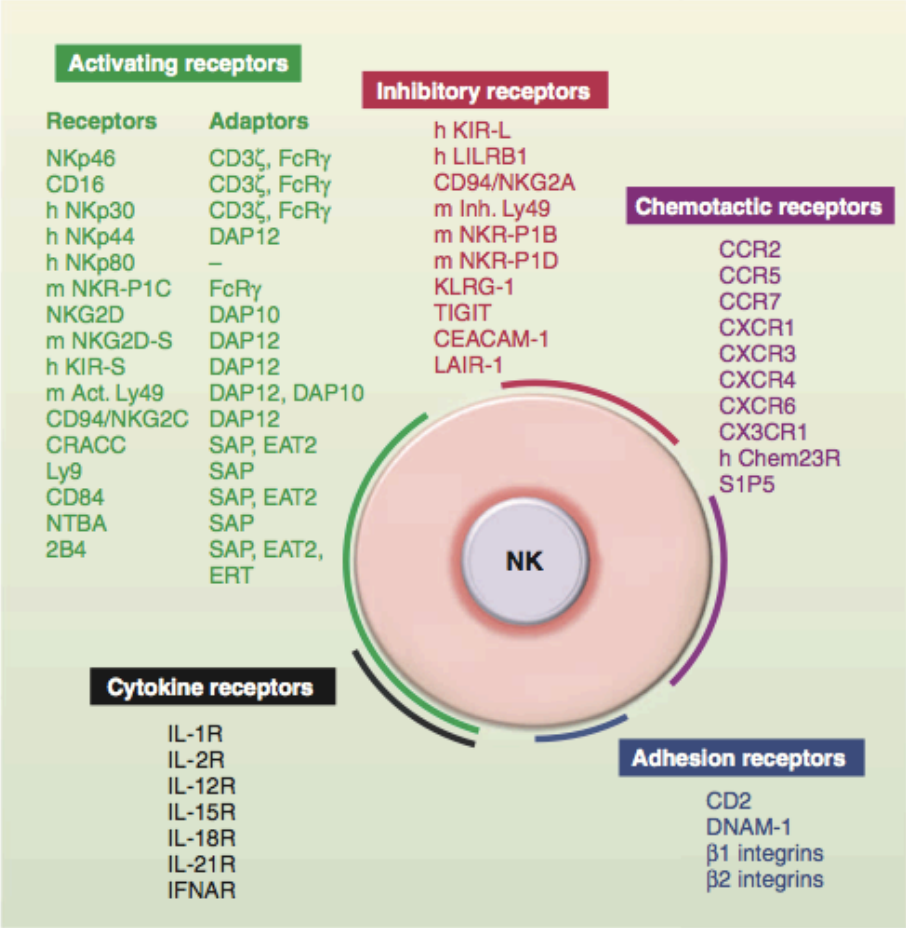


Figure 2 – *NK cell receptors*. A scheme of human and murine NK cell surface molecules, divided in activating (with their coupled adaptors), inhibitory, cytokinic, chemotactic and adhesion receptors. The majority of them are conserved in the two species[39].

Tipically the signaling trasduced by either an inhibitory or an activatory receptor depends on the presence of specific aminoacidic motifs, known as immunoreceptor tyrosine-based inhibitory motif (ITIM) on the receptor cytoplasmic tail or of immunoreceptor tyrosin-based activatory motif (ITAM) present on associated adaptor proteins, respectively. If an inhibitory receptor binds its ligand, the tyrosine on the ITIM domain is phosphorylated by a kinase (likely a Src-family kinase) and then recruits phosphatases through the recognition of SH-2 domains, decreasing the Ca^{2+} cytoplasmic concentration, and thus cell activation.

Conversely, if an activatory receptor is stimulated the tyrosines present on the ITAM motifs of FcεRIϒ, CD3ζ, DAP10 or DAP12 is phosphorylated and recognized by Syk

kinases and ZAP70 kinases, activating a wide range of intracellular events, leading to chemokine and cytokine gene trascription and cell degranulation[19].

The process of NK cell activation can trigger several responses, depending on the receptors engaged, on the environment, on interactions with other immune cells, and on the phenotype of the NK cell. The most characterized of those responses are the release of cytotoxic granules containing perforin (protein able to generate pores on the target cell membrane, disrupting the transmembrane ionic gradient and inducing cell lysis), granzymes, and the secretion of interferon-γ (IFNγ), a cytokine that interferes with viral replication and addresses the inflammation towards a type-1 response[4, 40]. Besides, NK cells can produce proinflammatory cytokines such as interleukin-1 (IL-1), IL-12, IL-15, IL-18 and tumor necrosis factor-α (TNF-α) or immunosuppressive molecules, like IL-5, IL-10 and IL-13. Furthermore growth factors like granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and IL-3 can be secreted. Finally, NK cells are an efficient source of chemokines, including CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), XCL1 (lymphotactin) and CXCL8 (IL-8)[39, 41].

1.1.3. Between innate and adaptive immunity

Interactions with other immune cells. In the last decade, it has been widely acknowledged that NK cells are not mere aspecific effector lymphocytes anymore, but are able to mediate numerous functions classically attributed to the adaptive immunity: for example NK cells can act like regulatory cells, interacting with and, more importantly, impacting on other cellular components of the immune system (Figure 3), be it of the innate (DCs, neutrophils, macrophages)[42] or of the adaptive branch[40, 43, 44]. In fact, NK cells have been shown to qualitatively and quantitavely regulate T and B cell responses activating or killing antigen-presenting cells (APCs) and regulatory T cells (Tregs)[45, 46], modulating the cytotoxicity of effector T cells[47], and inducing the isotype switching in T helper cells[40] as well as B cells[48].

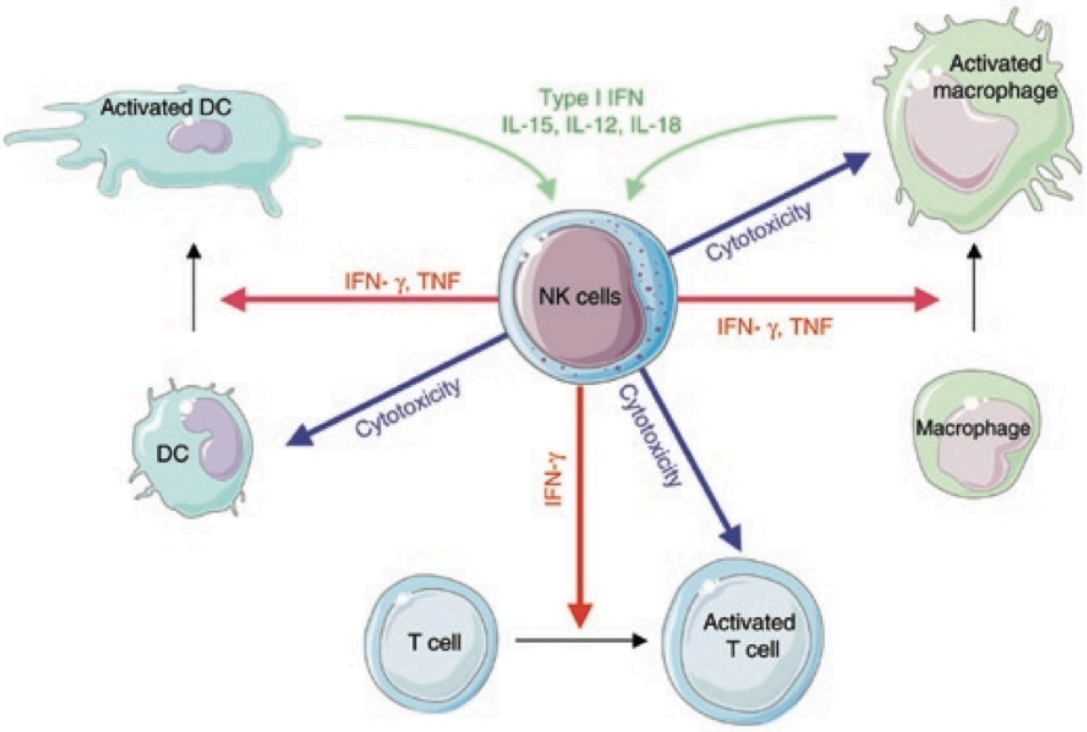


Figure 3 - *NK cell regulation of immune responses.* After stimulation (priming) with different cytokines (i.e. IL-15). NK cells can exert boosting effects (red arrows) on DC maturation or macrophage activation, but can also mediate cytolysis against activated CD4+ T cells, immature DCs or activated macrophages (blue arrows)[49].

Memory NK cells. Among other typical features of the adaptive immunity, the discovery of putative memory NK cell has been of much importance: it has been described in classic models of antigen-specific memory such as hapten-induced contact hypersensitivity (CHS) and viral infections, that NK cells are able to display a "learned" response (that requires an episode of sensitization) and to "remember" sensitizing antigens after a long period of time (in this case 4 months)[50]. This findings clearly demonstrate the existence of an NK cell subset distinct from conventional ones, which half-life has been shown to be of about 17 days[51]. In this study the authors also describe the tissue localization of memory NK cells, which in fact reside within the liver in the CHS model, while are spread in all lymphoid organs, and particularly in the spleen, in the MCMV-infection model. The markers for these (different) types of memory NK cells are CXCR6 (the receptor for CXCL16) and Ly49H, respectively. It is also shown that long-lived, antigen-specific NK cells are present in other inflammation models, such as vesicular stomatitis virus (VSV), influenza and,

curiously, HIV-1[52]. Although some characteristics of this new NK cell subset are already described, much remains, at least partially, unexplained, like the mechanism of antigen recognition, the stimuli needed for their survival, or the dynamics of their activation upon antigen encounter. Furthermore, little is known about their equivalent in human, even if some studies seem to point in that direction[53].

Polarization. An aspect reminiscent of T and B cell biology is certainly the capacity of these cells to have a polarized response in consequence of environmental cues (i.e. Th1, Th2, Th17, Th22 for CD4+ T helper cells). Recently some groups have shown how this polarization can be obtained also with NK cells, both in humans and in mouse models[54, 55], defining NK1 and NK2 subsets according to their ability to produce and secrete in vitro IFN-γ or IL-5 and IL-13 (but not IL-4), respectively. However, the mechanisms leading to the production of type 1 or type 2 cytokines are not well defined, as for example IFN-γ can be produced, together with IL-13, by IL-12+IL-18-stimulated murine NK cells[56]. Moreover, the transcription factors determining NK cell polarization are still on the way to be demonstrated, even if some evidence would suggest that GATA3, ROG and STAT6 are NK2-polarizing factors, while T-bet, and STAT4 are NK1 differentiation inducers[55]. An interesting view on NK cell (but it also applies to T cells) polarization process has been given by Perussia et al.[57]: the authors challenge the classical model of lymphocyte development, called "branching 1-2", according to which naive (and N0, or non-primed NK cells) CD4 T cells still retain the potential to be polarized to any inflammatory type (1, 2, 17, 22), and only by subsequent exposition to environmental factors are irreversibly directed towards their terminal phenotype. Conversely, it is proposed that most immature cells have a type 2 phenotype, which can, or can not, switch to an intermediate stage (here called type 0), and afterwards to type 1 NK cells. There are several evidences supporting this model , such as the ability of NK1 cells to produce IL-10 upon stimulation with different stimuli[54, 58, 59], that so far has not been observed in NK2 cells. Furthermore, while it is possible to convert an NK2 to an NK1 phenotype, the opposite has not been possible, suggesting that the NK1 might be the terminal state of NK cell differentiation, at least for

what concerns physiological conditions. Although these are intriguing and stimulating possibilities, definitive proofs in in vivo studies are needed, since both in homeostatic and pathological conditions, NK cell phenotype and functionality seem to be significantly plastic.

Education and tolerance. In order to avoid self-reactivity, thymocytes are selected in the thymus, where clones that do not recognize, or recognize too efficiently, self ligands bound to MHC-I molecules are negatively selected and consequently undergo apoptosis. This process endows mature T cells with the capacity to discriminate self and non self molecules, escaping autoimmunity. Furthermore, naive T cells are not fully functional, and must be "primed" through the recognition of a specific ligand, as well as by the signalling triggered by cytokines and co-stimulatory molecules (such as CD28): only after this T cells reach their terminal maturation stage.

In parallel with the ability to polarize their response, probably the most important similarity of NK cell biology and adaptive immunity is the existence of an "education" process during NK cell maturation and development. The necessity for such a mechanism has emerged for several reasons. Firstly, when it has been highlighted that 1) NK cells express activatory receptors for self-MHC molecules [19, 60, 61] and 2) there are NK cells that lack of at least one inhibitory receptor for self-MHC molecules [62, 63]: indeed, the classical definition of the NK cell activation state as "result of the integration of the signals deriving from activatory and inhibitory signals" did not explain why, then, NK cells did not show manifestations of autoimmunity.

Secondly, because of the data showing that the NK cell population displayed various degrees of functionality, depending on their developmental stage.

The term education defines the "host effects on NK cell function which could include MHC-dependent and MHC-independent effects" [14], and includes both the acquisition of complete functionality (licensing) and the capacity to recognize and thus spare cells expressing self molecules, also known as tolerance.

The processes leading to licensing and tolerance are still being debated, as there still is no

conclusive evidence that clearly demonstrates how those properties are obtained by NK cells. However, two main models have been proposed by Yokoyama and Raulet group: the first hypothesis postulates that the binding to self MHC-1 ligands with inhibitory receptors promotes NK cell licensing [63], and is referred to as "arming" model.

This is the simplest model of the two, it is supported by data showing that MHC-I deficient mice - derived NK cells have a significant functional impairment [64, 65], and that the ITIM motif contained in the cytoplasmic tail of the inhibitory receptors is required to reach a licensed state[63]. Though this hypothesis has been strenghtened also by human studies [66, 67] doubts remain on the possibility that a inhibitory signal derived from the engagement of a self MHC-I receptor, coupled with phosphatases activity might as well lead to a positive signal, represented by the acquisition of functional maturation [14].

An alternative hypothesis for the licensing mechanism is known as "disarming" model [68, 69], and proposes that all NK cells are initially responsive, but the absence of an inhibitory signal deriving from a self MHC-I receptor might unbalance the chronic stimulation with a putative self-specific activatory ligand, leading the cell to an anergic state, similar to the one observed in T cells. The presence of an unknown activatory ligand is supported by the fact that MHC-I deficient cells are efficiently killed by WT NK cells [65], and by studies with transgenic mice (i.e. overexpressing NKG2D ligands), and on humans [41]. Nevertheless, the disarming model poses some questions, in the first place regarding the unknown activatory self ligand, and then concerning the experimental models used to demonstrate this hypothesis, which might present important limitations [14].

A third model, known as "rheostat" model has been proposed by Karre group, and postulates that the process of NK cell education and licensing is not an "on and off" switch, but conversely is a gradual acquisition of functional competence, proportionally to the number of self MHC-I receptors expressed by a certain cell, and by the affinity of each receptor for self MHC-I ligands[70]. This is supported by evidences showing that NK cells expressing two or more inhibitory receptors for MHC-I respond more strongly and more frequently than those expressing only one receptor [70, 71]. Education is thus likely to be a quantitative process, regulated by the binding of inhibitory receptors to self MHC-I

molecules, either opposing chronic activating receptor stimulation, or by trasmitting yet undefined activating signals themselves[72].

1.1.4. NK cell development

Commitment and maturation – from HSCs to immature NK cells. While there has been extensive and fruitful research regarding the molecular mechanisms underlying NK cell activity and function, those concerning NK cell development are probably largely uncovered[73], though alterations of the process of NK cell maturation can lead to critical pathologies, such as immune deficiencies, autoimmunity and NK cell malignancies, that are extremely difficult to diagnose and to eradicate[74, 75, 76].

Nevertheless, it has been known since more than 25 years that the bone marrow (BM) is the main site of NK cell development, this notion coming from studies in which BM ablation with several approaches, such as treatment with bone-seeking isotopes and following estrogen-induced osteopetrosis[77, 78] caused a severe impairment of NK cell population number and functionality in mice. In addition, other hematopoietic lineages, such as myeloid and, to some extent, lymphoid cells, follow their maturation pathway within the BM, starting from the common progenitor called hematopoietic stem cell (HSC). The reasons for this phenomenon might reside in the peculiar microenvironment that BM displays, made of soluble stimuli, feeding-stromal cells, and low oxygen pressure[79]: in fact, it has been progressively clearer that NK cell development is a process dependent on cell-intrinsic and cell-extrinsic factors (to say, transcription factors and interaction with other cells), and within the latter group both cell-contact with stromal cells and the binding of soluble cytokines have an important role for the complete NK cell maturation.

As it happens for other cell types, the NK cell development is generally thought to occur starting from a multipotent progenitor, the HSC, and then to pass through several stages of progressive differentiation, during which their "lineage restriction" accordingly increases.

The first steps in the classical view of NK cell development are represented by the transition from the HSC to the early lymphoid progenitor (ELP) and then to the common lymphoid progenitor (CLP), which are identified as lineage-negative (Lin-), c-kit+ Flt3+ and Lin- c-kit+ IL-7Rα+, respectively[80, 81]: this passage is referred to as "commitment", and involves the impossibility to differentiate to other hematopoietic lineages, in this case the myeloid family. Indeed, ELPs and CLPs can still become B and T lymphocytes, or NK cells.

An additional phase before the definitive specification to the NK cell lineage might be attributed to a bipotent T/NK cell precursor (TNKP), that has been identified in murine fetal liver (Lin- c-kit+ NK1.1+) and in murine and human fetal thymus (CD34+ CD7+ CD1a-)[82, 83, 84], but it is not clear whether a part of the NK developing population (rather than all NK cells) might acquire this phenotype, or even whether the differentiation potential towards NK and T cells is equivalent at this stage[79].

What most of the scientific community agrees on is the phenotypic signature of the NK cell precursor (NKP) present in the fetal thymus and in the BM of adult mice, which expresses the common β chain of the IL-2 and IL-15 receptors (CD122), and lacks the surface expression of T lymphocyte co-receptors CD4 and CD8[85, 86], so that NKP global phenotype is Lin- NK1.1- DX5- CD122+. The acquisition of this molecule endows NKPs with the capacity to respond to IL-15, which has been demonstrated to be necessary for the complete NK cell maturation, from the NKP stage onwards[87], while it has been demonstrated that the generation of NKPs from HSC does not depend on the effect of IL-15[88]. Most likely the effect mediated by IL-15 occurs in the BM, which is a rich IL-15-producing cells environment. The presentation of IL-15 is believed to be mediated by other hematopoietic (i.e. DCs)[89] and non-hematopoietic (stromal)[79] cells, that present the complex IL-15/IL-15Rα in trans to the IL-15Rβ/γ present on the NK cell surface; the IL-15β/γ-receptor is able to recognize its ligand even in absence of the α-subunit, but with a much lower affinity, and thus biological efficacy.

Interestingly, NKPs have been found also outside the BM, as they can be detected in murine thymus, spleen, and lymph nodes (LN)[85, 90], but it is still unknown whether these

cells are generated in situ or else they originate in the BM (or in the fetal liver) and then traffick to the peripheral organs passing by the circulation.

Other NK cell specific surface markers have been proposed to be expressed by the NKPs population, in particular NKG2D (but also 2B4)[73, 88]: actually, since only 12% of NKPs give rise to NK cells in vitro[85], and because about 10% of NKPs are NKG2D+, it has been hypothesized that real NKPs shoud be defined as Lin- CD122+ NKG2D+.

An example of extra-BM NKPs has been described also in humans, where a $CD34^{dim}$ CD45RA+ integrin $\alpha_4\beta_7^{high}$ precursor that was present in peripheral blood and was highly enriched in LNs, was shown to retain the capacity to generate in vitro $CD56^{bright}$ NK cells, after stimulation with IL-2 and IL-15[91].

As of now, there is no certainty on which are the transcription factors needed for the commitment to become NKPs[73], but a complex interplay seems to exist among some of them. Studying gene-deficient mice, it has been demonstrated that Ets-1, PU.1 and MEF are, though partially, important for the formation of a complete and mature pool of NK cells, especially in the early phase of the development[92, 93, 94]. The demonstration of a determined role for each one of these factors is complicated by a certain degree of redundancy that might occur in case of deletion of just one of them.

An exception to this redundancy is represented by the basic leucine zipper transcription factor E4BP4, whose absence leads to the complete lack of NK cells, in every organ, similarly to what is observed in IL15-KO mice[95]. Gascoyne and colleagues demonstrated that E4BP4 acts downstream of IL15, but its deficiency does not affect the development of other IL-15-dependent lineages, like CD8+ T cells and NKT cells, pointing out its NK-cell specificity.

Moreover, two members of the inhibitors of DNA binding (Id proteins 2 and 3) family have been associated with the regulation of HSCs (or ELP or CLP) transition to NKP stage, but a mechanism for this was not found[96].

The following step in the classical model of NK cell development is the generation of immature NK cells (iNK), which consists of irreversibly committed cells that have not yet gained most of the functional properties and do not express the surface molecules that

characterize mature peripheral NK cells[79]. In the scheme of temporal acquisition of surfece markers, NKR-P1C (recognized by anti-NK1.1 mAb PK136) is the first to appear on iNKs, followed by the CD94/NKG2A/C/E complex and some Ly49 receptors[73]. Consequently, the typical phenotype of an iNK is CD122+ NKG2D+ NK1.1+ DX5- (and also $CD43^{low}$ $CD11b^{low}$), and these cells can be found mainly in the BM but are also enriched in the liver in adult mice. The peculiarity of hepatic iNK cells is that they express TNF-related apoptosis-inducing ligand (TRAIL), through which they can trigger cell death in susceptible targets[97]. Although phenotypically liver NK cells might resemble immature cells, recently there is accumulating evidence that NK1.1+ DX5- (CD49a+) hepatic NK cells represent a completely different subset from the conventional splenic DX5- NK cells[98]: for example, it has been shown that DX5- hepatic NK cells are tissue-resident, while DX5+ are circulating; furthermore, DX5- cells show lower proliferative rates than their DX5+ counterpart, and are most likely those responsible for memory NK cell response. Overall, hepatic iNKs should probably not be the same as iNK found in other organs, like BM.

Generation and mantainance of mature NK cells. The acquisition of the expression of markers like the integrin DX5/CD49b, CD27, CD11b, CD43, other Ly49 receptors, and KLRG1 marks the further transition to a more differentiated NK cell. As for the other phases of the developmental process, also this one is transcriptionally regulated, and the most important transcription factors involved here are GATA-3, T-bet, Eomes and IRF-2, even if their exact contribution is still not well defined[73].

Along with transcriptional factors, stromal-derived and soluble molecules can exert a regulatory role on NK cell development. Lymphotoxin-α is a cytokine of the TNFα family that can form homotrimers (LTα3) binding TNFR-I and TNFR-II, or associate with its β-isoform, form heterotrimers (LTα1β2), binding LTβR on stromal cells: it has been shown that to have a complete NK developmental process, the interaction between stromal cells expressing LTβR and LTα1β2 produced by NK cells is required[99], and its function is probably upstream of IL-15 requirement, since it was shown that it can trigger IL-15 transcription in the BM[100].

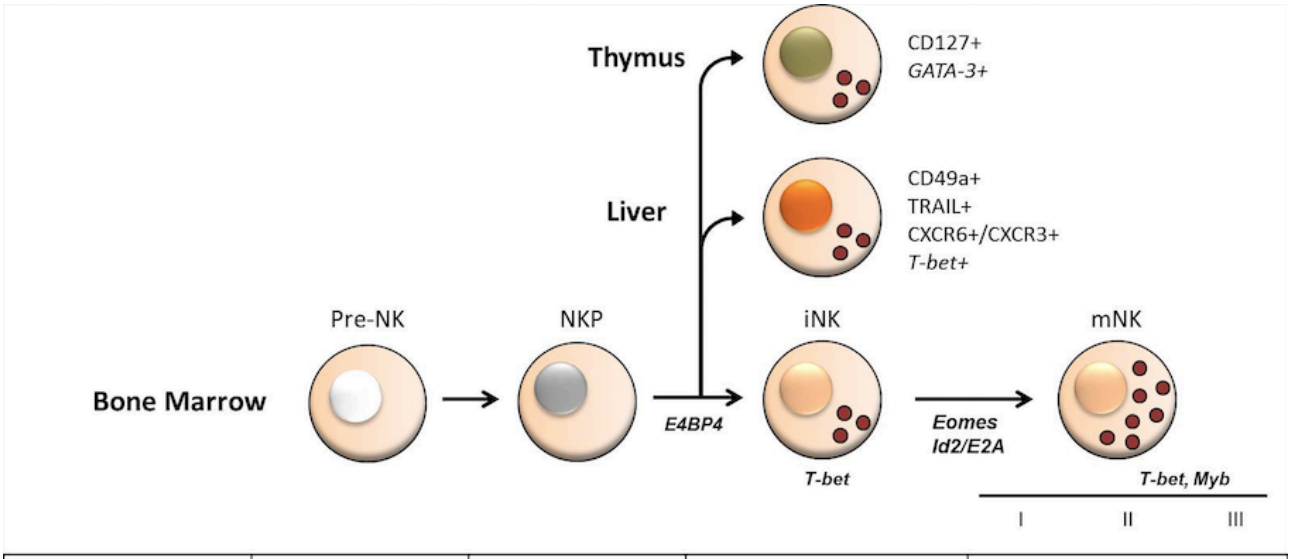
Other relevant factors playing a role in very early phase of NK cell development are c-kitL and Flt3, like they do in other lineages, while IL7 might be particularly important for the maturation of thymic NK cells.

In a simplistic vision of NK cell development and trafficking into lymphoid and non lymphoid organs, once these cells complete their developmental process in the BM, they would reach peripheral organs and exert their patrolling function, and eventually become senescent and undergo apoptosis. What happens in vivo is quite different, since specific signals are required in order to maintain peripheral NK cell pool, and again the most important of these molecules is IL-15[51, 101, 102], which has been shown to induce NK cell survival, proliferation and further differentiation.

An opposite function is attributed to transforming growth factor-β (TGF-β), which modulates negatively NK cell maintanance in the periphery, at least in homeostatic conditions, counterbalancing the effect induced by IL-15[103].

Every maturation step described above refers only (perhaps with the exception of the hepatic NK cells) to the classical paradygm of NK cell differentiation in the BM. Recently an alternative pathway of NK cell development has been shown to take place in the thymus[104], generating a phenotypically and functionally different subset of NK cell, characterized by the expression of the α-chain of IL7 receptor (CD127) and high amounts of the transcription factor GATA-3. In this study, thymic NK cells (tNK) were identified as CD11b^{low} CD16- CD69^{high} Ly49^{low}, and displayed a lower lytic activity versus the NK-sensitive lymphoma cell line YAC-1, thus looking similar to conventional immature NK cells. Nevertheless, it was also shown how their cytokine production potential was not only more powerful, but even wider as compared to classical splenic NK cells, suggesting that thymic NK cells could represent a distinct NK cell subset. The authors demonstrated the necessity for GATA-3 and IL7 presence to achieve a normal tNK reservoire in adult mice, while BM and peripheral NK cells were unaffected by the absence of such molecules. As for their tissue localization, tNK cells have been proposed to reside mainly within the lymph nodes, as CD127+ represented about 30% of total NK cells, while in BM, spleen and

liver this frequency was not superior to 5%. Interestingly, CD127 and GATA-3 expression was preferentially observed on CD56^{bright} CD16- human NK cell subset, implying a possible link between CD127+ NK cells in human and mouse.



CD244	+	+	+	+	+	+	+
CD122	-	+	+	+	+	+	+
NK1.1	-	-	+	+	+	+	+
CD27	-	-	+/-	+	+	+	+/-
CD49b	-	-	-	-	+	+	+
CD11b	-	-	+/-	+/-	+/-	+	+
KLRG1	-	-	-	-	-	-	+

Figure 4 – ***Different pathways of NK cell development.*** The whole process of NK cell maturation, from HSC to mature NK (mNK), takes place in the BM, though alternative developmental routes have been described in the liver and in thymus. Typical markers of each organ-specific NK cell are described, and transcription factors that regulate the transition from a differentiation stage to the next are shown in italics (top panel). The earliest NK cell commitment marker is considered to be CD244 (Pre-NK), while mNKs are characterized by CD49b. Within mNK, CD27, CD11b and KLRG1 define additional mNK cell subsets (adapted from [73, 79])

Mature NK cell subsets – A further dissection. NK cell population heterogeneity is not limited only to the cited developmental stages, but has a further degree of complexity since even within what is currently defined a mature subset several subpopulations differing in functionality, proliferative potential (and consequently maturation degree) and tissue localization can be identified.

The first study in which the existence of an heterogeneous response in the NK cell population to sensitive targets dates back to 1983, and was conducted on human NK cells[105].

Some years later, the same group reported that the surface expression density of the CD56 marker ($CD56^{bright}$ and $CD56^{dim}$) could be used to discriminate between the two subsets[106], and since then there has been an extensive research aimed at the characterization of the different properties of these subpopulations.

Murine NK cells do not express the CD56 gene, thus a comparison between human and NK cell subsets is often difficult.

However, as already discussed, there are many correlative studies that show how the expression of molecules such as Ly49 receptors and others are acquired during maturation, but a sequential "A to B" paradygm was not estabilished until few years ago, when the presence of discrete stages of NK cell maturation in mice, corresponding to the progressive acquisition of full functional competences, was formalized. In the following years it has been shown how the $CD11b^{high}$ NK cell population could be further divided according to the surface expression of the CD27, a member of the TNF receptor superfamily, in $CD11b^{high}$CD27+ and $CD11b^{high}$CD27- subsets[107], and that these subsets were developmentally related[108]. Interestingly, CD27 was also found to be selectively expressed by the $CD56^{bright}$ human NK cell subset, which is thought to be more immature than its $CD56^{dim}$ (CD27-) counterpart, thus resembling the situation observed in mice[109].

Indeed, the proliferative potential, through the assessment of BrdU incorporation during homeostatic immune reconstitution conditions, was proven to be high in $CD11b^{low}$CD27- (double negative, DN) subset, and to decrease gradually in $CD11b^{low}$CD27+, $CD11b^{high}$CD27+(double positive, DP) and $CD11b^{high}$CD27- subsets, indicating a hierarchycal order in the generation of terminally mature NK cells[108].

An additional study evidenced how the expression of the inhibitory receptor KLRG1 was almost exclusively restricted to the most mature $CD11b^{high}$CD27- subset, and was

associated with low cytotoxic capacity against NKG2D ligands expressing targets, and decreased capacity to migrate to several chemokines, such as CXCL10 and CXCL11[110].

These reports also highlighted the differential tissue distribution of NK cell subpopulations: the more immature subsets, namely DN and CD11b^{low}CD27+ cells, were mainly represented in BM and lymph nodes, where their frequency reached 50% of total NK cells. Conversely, CD11b^{high} cells (both CD27+ and CD27-) were more recurrent in liver, spleen, lungs and blood, up to the 85% of thewhole NK cell population[107]. This shift in the subset balance in different organs can be interpreted in several ways: firstly, it reflects the importance of the BM and LN as NK cell-generating sites, in contrast with other peripheral organs, to which more mature cells migrate and exert their immune function, so that immature cells accumulate in BM and LN, while mature ones reside preferentially in the periphery.

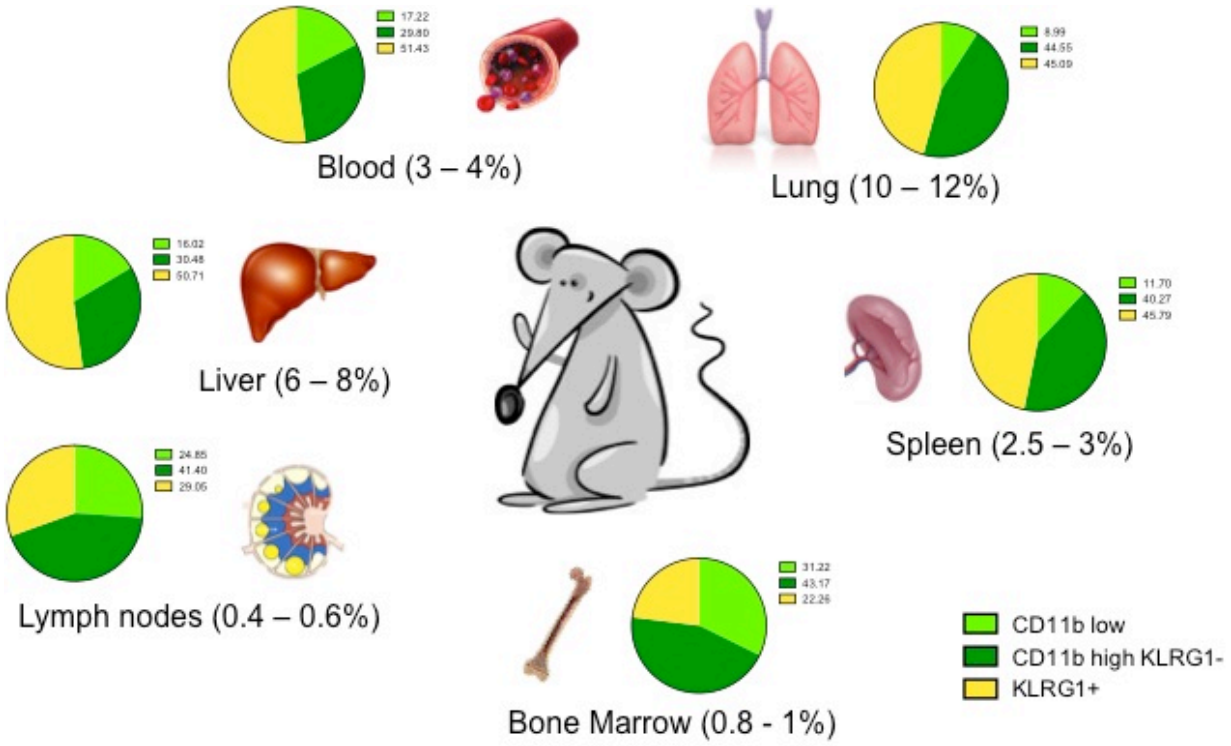


Figure 5 – ***Anatomical localization of mNK cell subsets.*** Outline of NK cell subset distribution in 6-10 weeks old C57BL/6J mice, according to CD11b and KLRG1 expression. The most mature subset (CD11b^{high}/KLRG1+ cells, in yellow) reside preferentially in spleen, blood and non lymphoid organs such as lung and liver. In

contrast, CD11b^{low}/KLRG1- and CD11b^{high}/KLRG1- represent great part of NK cell population in BM and lymph nodes, where NK cell development occurs.

Secondly, it indicates that the functional differences (i.e. the capacity to produce cytokines versus the ability to kill target cells, or even to interact with other hematopoietic cells) are exploited according to the organ characteristics: for example, CD11b^{high}CD27-KLRG1+ NK cells, account for more than 75% of the NK cell population in the lung, express high levels of inhibitory Ly49 receptors and thus are inefficiently activated, but at the same time are continuously exposed to exogenous molecules commonly present in the pulmonary airways, and are probably ideal to avoid a chronic inflammatory state in response to those stimuli. Moreover, DP cells have been shown to be the most efficient in the IFN-γ secretion in response to IL-12 and/or IL-18, and might play a predominant role in the DC activation and promotion of CD4+ T cell Th1 priming in the lymph nodes, where their frequency is about 45% of total NK cells.

Lastly, an important aspect in the regulation of the different NK cell localization in the body is represented by their heterogeneous expression of chemokine receptors. It is known that organs like BM, liver, blood, lymph nodes and lungs express tissue-specific chemotactic factors, that selectively attract cells able to recognize those factors via the corresponding chemokine receptors, both in inflammatory and in steady state conditions. For example, the trafficking to the lymph nodes is modulated, among others, by the chemokines CXCL9, CXCL10 and CXCL11, and their receptor CXCR3 is mainly expressed by the CD11b^{low}CD27+ and DP subset, which together represent the majority of LN NK cells. Likewise, the liver sinusoids have been described to express CXCL16, a peculiar chemokine that exists in membrane-bound and soluble form, and consequently DX5-CD11b^{low} CD43^{low} hepatic NK cell subset is CXCR6^{high}[111].

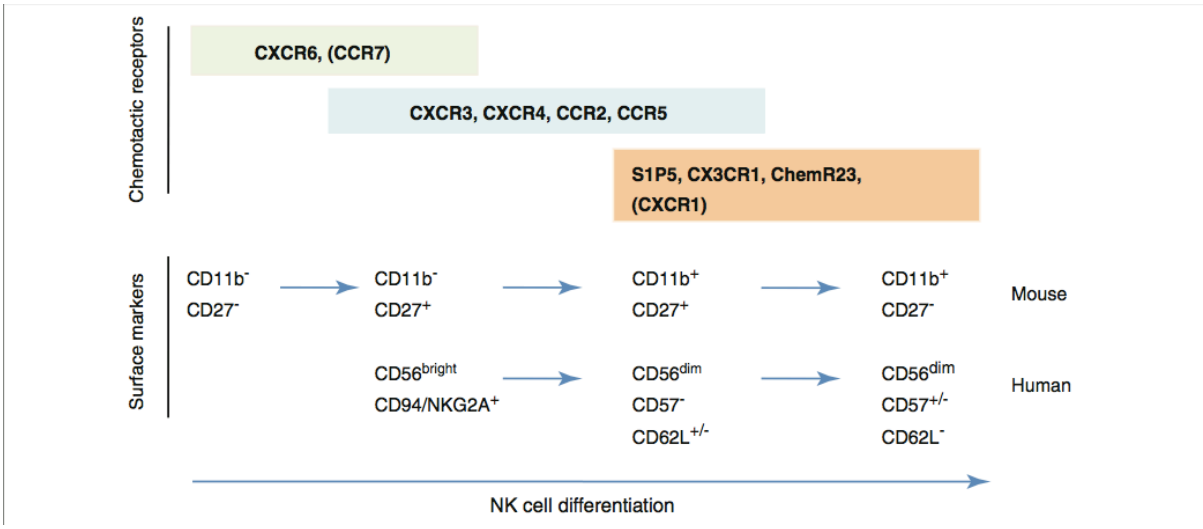


Figure 6 – ***Expression profile of chemotactic receptors of NK cell subsets***. Each stage of NK cell development is characterized by specific chemokine receptors. Those in brackets are expressed in human but not in murine NK cells [112].

BM represents an interesting example of how the modulation of several chemokine receptors complements NK cell development and also explains their positioning within the organ compartments. In fact, the pattern of chemokine receptor expression changes along with NK cell development, likely reflecting the need for different cues to which cells are exposed within the BM, but also in peripheral organs.

NK cell maturation correlates with the downmodulation of CXCR4, a key player in BM NK cell retention[113] and development[114]: its ligand CXCL12 is presented to NK cells by CAR (CXCL12 abundant reticular) cells, which are localized in BM parenchyma[115], and induce CXCR4+ NK cell retention, possibly while providing pro-survival and maturative stimuli (e.g. IL-15)[114]. CXCR4 is robustly expressed by iNKs and by the earliest stages of mNKs, while is progressively lost on CD27- mNKs[113].

S1P is a secreted lysophospholipid bound extensively to albumin and other plasma proteins, which has been recently demonstrated to regulate cell trafficking to BM in steady state conditions[116]. The balance of anabolic (sphingosine kinases) and catabolic (Sphingosine lyase and phosphatases) processes determine an in vivo gradient of S1P, with high concentrations in extracellular fuids and low levels in tissues[117, 118]. Out of the five S1P receptors (S1P1-5), just S1P1 and S1P5 are expressed on NK cells, though the function of the former is unknown yet. Contrarily to CXCR4 expression pattern, S1P5

positively correlates with NK cell development, being acquired later during maturation. s1p5-deficient mice display a significant reduction of peripheral NK cells (spleen, blood, lung), countered by the increase in BM and LN NK cell number, and thus S1P5 is believed to represent a BM (and LN) egress signal for mature CD27- NK cells, following the chemotactic gradient of S1P[116].

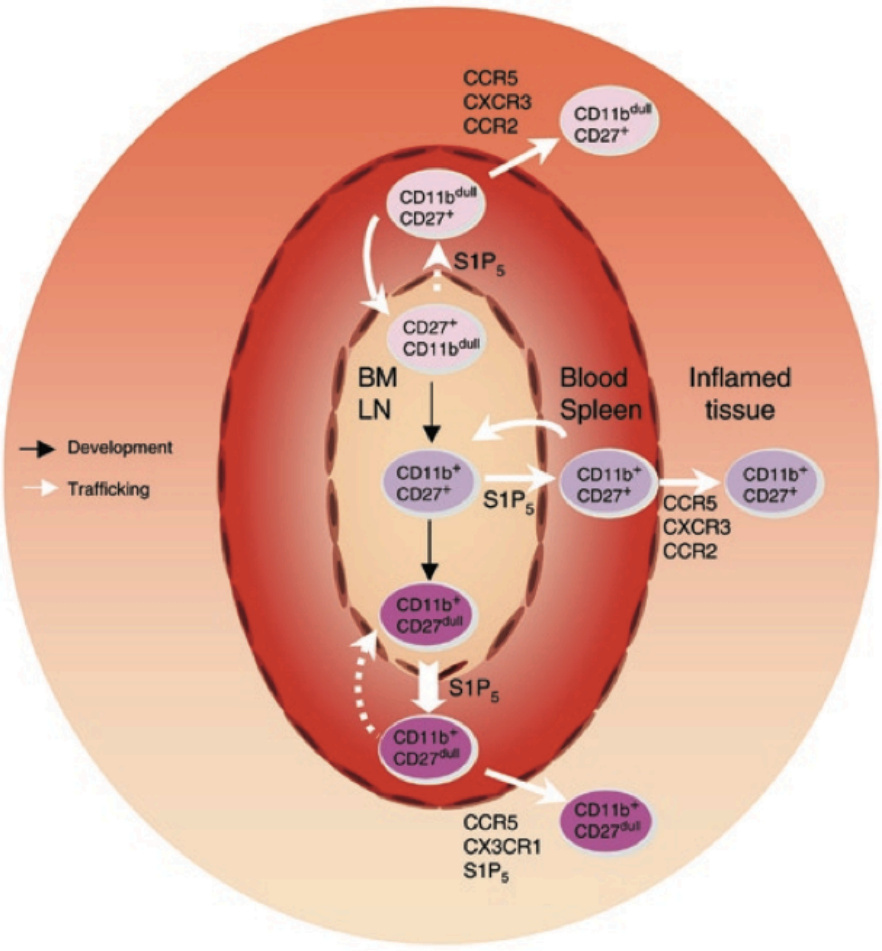


Figure 7 – *NK cell subset trafficking properties*. The exit signal from BM and LNs to the blood can be provided by S1P5, particularly at CD11b^{high}/CD27- NK cell maturation stage. Once in the peripheral circulation, CXCR3 (mainly on CD11b^{low}/CD27+ NK cells), CCR2, CCR5 and CX3CR1 (just for CD27- NK cells) drive cell trafficking into inflamed tissues[49].

Collectively, this deep specialization in differentiation stage, biological functions, and tissue localization of the different mature NK cell subsets seems to be important for innate

and adaptive immune system activity, and this diversity could be exploited in therapies against infectious diseases, tumors and autoimmunity.

1.2. Drivers of leukocyte migration: chemokines and their receptors

1.2.1. General concepts – Chemokine structure, nomenclature and classification

Perhaps the most important factor that directs cell migration and tissue localization is represented by chemotactic cytokines, or in short chemokines. This family of small proteins (their molecular weight ranges from 8 to 10 kDa), includes more than 50 members, which are evolutionarily conserved and structurally related. Through several criteria, chemokines can be divided in subgroups, or subfamilies.

About 25-50% of the aminoacidic sequence is shared by all chemokines, and in particular the N-terminus displays two highly conserved cysteines, important for the formation of structurally relevant disulfide bonds, and according to the position of these specific aminoacids, 4 different classes can be identified: CXC, CC, CX3C and XC (where the X stands for any aminoacid). Currently chemokines are named conformingly to their class (i.e. CXCL1 stands for CXC chemokine ligand 1), simplifying the previous nomenclature system which was based on the molecule function (i.e. CCL2 was previously known as monocyte chemoattrattant protein-1, MCP-1). In addition, this method applies to respective chemokine receptors, that are classified in accordance with the class to which their ligand(s) belong to and a progressive number (i.e. CXCR3 stands for CXC chemokine receptor 3, and binds only CXC chemokines).

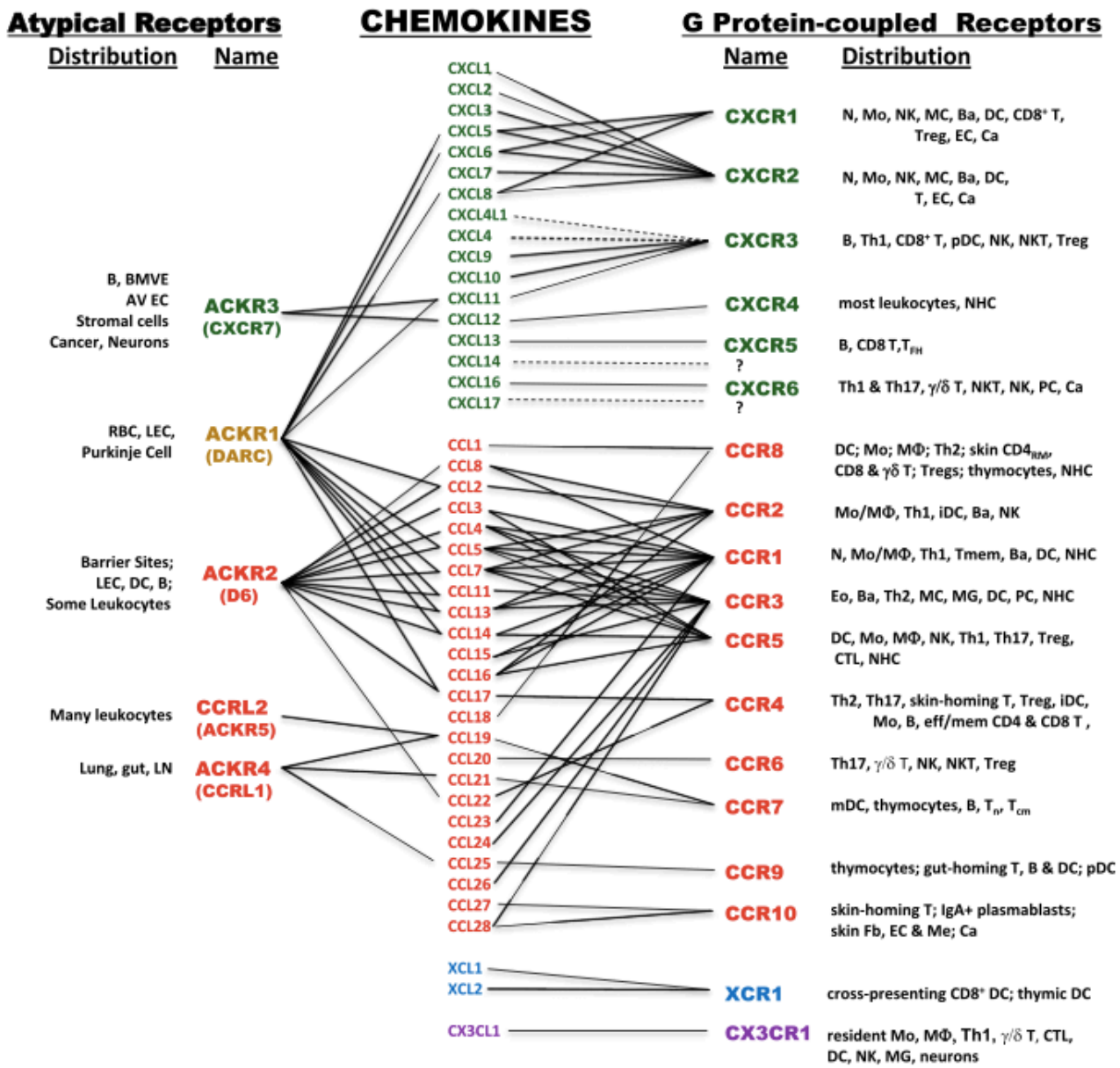


Figure 8 – ***The complexity of chemokine system.*** Black lines describe each chemokine receptor specificity for ligands and leukocytes, highlighting the redundancy and promiscuity of this system. In fact, different chemokines can be specific for the same receptor, and a single chemokine can bind more than one receptor. There are exceptions such CX3CR1, CXCR6 and presumably CXCR5, which are considered "monogamous" ligand-receptor pairs. An updated overview of the currently known atypical receptors is pictured on the left side, while classical signaling receptors are on the right side[119].

Within CXC chemokines, ELR- and ELR+ subclasses can be identified, depending on the presence or the absence of a glutammic acid-leucine-arginine sequence upstream of the first conserved cysteine. CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8 (which was the first chemokine to be discovered[120]) are ELR+, while CXCL4, CXCL9,

CXCL10, CXCL11 and CXCL12 are ELR-. All of them are tyipically responsible for neutrophil, T cells, fibroblasts and hematopoietic precursors recruitment, but are particularly related to angiogenesis[121]: in fact, ELR- CXC chemokines are classically considered antiangiogenic, while ELR+ promote angiogenesis[122].

Another parameter used to characterize chemokines is related to their function: there can be inflammatory, homeostatic or dual-function (mixed) chemokines, even if this classic differentiation is continuously challenged by new studies where the borders of such categories are quite blurred. For example, CX3CL1 is considered homeostatic in the central nervous system (CNS), but inflammatory in the periphery[123, 124]. Nevertheless, it is undeniable that most chemokines are predominantly, if not completely, important either in the steady state or during inflammation.

Inflammatory chemokines are expressed by leukocytes and non hematopoietic cells only upon activation[125, 126], and their expression is triggered by diverse stimuli, such as TNF, IFNγ, microbial products or trauma. Importantly, this class of chemokine is produced mainly in inflamed tissues, during phlogosis, and thus is spatially and temporally related to the inflammation. On the contrary, homeostatic chemokines are constitutively expressed, and regulate the leukocyte basal movement through the organs (CCL19, CCL21 and CXCL13) as well as the complex mechanisms underlying hematopoietic cell development, for example in the thymus and in the bone marrow (CCL25 and CXCL12, respectively). Moreover, some chemokines are abundant in specific tissues, such as skin, lungs and intestinal mucosa, and are likely responsible for the normal patrolling function exerted by many organ-specialyzed leukocytes during the steady state.

1.2.2. Chemokine receptors

Chemokines exert their biological function binding their receptor (or receptors), that is a 7 transmembrane protein, of the ϒ-subfamily of the rhodopsin-like receptors. Chemokine receptors can be divided in the following groups: G-protein coupled receptors (GPCR),

which are usually coupled to α_i (but also α_q and α_{11}) G_α-subunit, and non-signaling atypical chemokine receptors.

1.2.3. Regulatory mechanisms of chemokine signaling

The signal triggered by the binding of a chemokine to its own receptors involves a series of molecules, that vary depending on the identity of the G protein α subunit. Generally, GPCR activation causes the dissociation of the α and the $\beta\gamma$ subunits: $\beta\gamma$ (but also α, in case of a α_i-coupled receptor) can thus activate phosphatidil inositol 3-kinase (PI3K) and phospholipase C (PLC), eliciting calcium mobilization from intracellular compartments and diacylglycerol (DAG) production from membrane phosphatidil inositol trisphosphate (PIP_3). In addition, PLC activates RAP1 guanine-nucleotide exchange factors (GEFs) CALDAG-GEFs (calcium- and diacylglycerol-regulated GEFs). Furthermore, G proteins containing α_i activate DOCK2 (dedicator of cytokinesis 2), while those that contain $\alpha_{12/13}$ can activate the small GTPase RHO-A (RAS homologue A)[127]. These events lead to a wide range of cellular responses, for example the integrin activation on the cell surface, due to the actin cytoskeleton remodeling, a process called inside-out signalling. Chemokine stimulation through its receptor can also trigger gene transcription (pro-inflammatory, pro-survival or anti-apoptotic genes), cell polarization, formation of a leading-edge and of a uropod[128].

Intracellular pathway. The fine tuning of chemokine signalling is ensured by multiple levels of control: in first place, after receptor engagment and consequent cell activation, the stimulation is terminated by the intrinsic GTPase activity of α subunits, that can hydrolyze GTP and reunite with $\beta\gamma$ to return to the initial conformation of inactive heterotrimers. This process can be controlled by proteins known as regulators of G-protein signaling (RGS)[129], and can both enhance and inhibit GTPase activity of the α subunit, depending on the chemokine receptor.

Another mechanism that regulate chemokine signaling is receptor desensitization, which consists in the lack of the activity of the receptor due to the excessive phosphorylation mediated by G-protein-coupled receptor kinases (GRKs) of serine residues on the C-terminal portion of the receptor[130]. Following prolonged exposure to its ligands, a chemokine receptor can also be downregulated, through the internalization in clatrin-coated pits (CCP) or in caveolae by β-arrestin or adaptin1[131, 132, 133]. As with desensitization, the molecules mediating internalization recognize and dock to phosphorylated serines (and threonines) on the carboxy-terminal portion of the GPCR, even though the specific aminoacids recognized are different, allowing a distinct regulation.

Association with GAGs. In vivo, chemokine effects are further modulated by the association to glycosamminoglicans (GAGs). In fact, chemokines are seldom present in a "nude" state, but are instead associated to carbohydrate structures such as heparan sulfate, chondroitin sulfate, dermatan sulfate or keratan sulfate, which are believed to be important in processes like the formation of the chemotactic gradient[134], chemokine halflife[135], haptotaxis or chemokine neutralization[129]. Although every chemokine can virtually bind every GAG, specific associations have been shown to be preferred, probably depending on the chemokine tridimensional structure and on the compartment in which the chemokine is produced and secreted. GAG "decoration" has been shown to affect important features like receptor affinity, and can consequently be included in the group of the regulators of chemokine activity.

Tertiary structure. Although the great majority of chemokines exist only as soluble proteins, there are two exceptions, CX3CL1 and CXCL16, that are also present in a membrane-bound form, and are thus tethered to the cell surface. Both proteins have an extracellular chemotactic domain, a transmembrane domain and a cytoplasmic tail, and can be shed by different enzymes[136, 137]. The two forms in which those CX3CL1 and CXCL16 are presented to responsive cells can influence cell migration differently, and the

degree of their shedding by metalloproteases represents an additional level of chemokine modulation.

Quaternary structure. The ability of a given chemokine to bind to its receptor is also determined by their quaternary structure. It is widely documented that both chemokine and chemokine receptors can form homodimers and heterodimers, or even higher order multimers, alone in solution or in association with GAGs[138, 139, 140] although it is still unclear what are the exact consequences for this phenomenon. It has been shown that the formation of chemokine oligomers in vivo can enhance leukocyte recruitment[141, 142], likely because of an association to GAG binding. Other groups have reported the synergistic action of heteromeric chemokine aggregates, increasing chemotactic activity and cell activation[143, 144, 145]. At the same time, chemokine receptors oligomerization is not well understood, but it is known that it can influence their surface expression or their binding and signalling capacity through allosteric modulation[146]. At any rate, it is believed that this superior structural organization and interaction will introduce a further degree of complexity into chemokine-induced signaling[129].

Post-translational modifications. In the delicate balance of the chemokine system, particular attention should be paid to chemokine alternative forms, derived by the proteolitic action of dipeptidylpeptidase IV (DPPIV, or CD26). This enzyme is expressed by many hematopoietic cells, and is highly selective for peptides containing a Proline residue at position 2 of the N-terminus, which is a common feature of one third of the known chemokines[147]. There are also other proteases able to cut chemokines, such as Cathepsins (G and D) and elastases, and they can cleave in the core or even in the C-terminal region of peptidic sequence. The products of the protease action can have antagonistic effect, enhanced agonistic effect, loss of receptor binding capacity, or even no effect[129]. For example, proteolitic cleavage of CXCR3 ligands CXCL10 and CXCL11 by CD26 results not only in a 10-fold reduction of chemotactic potency[148], but also in an antagonistic effect of CXCR3 signaling. Conversely, the truncated forms of CCL6, CCL9,

CCL15 and CCL23 have an increased affinity for CCR1 as compared to their full-lenght form[149], and CCL4 can even acquire specificity for CCR1 (although as an antagonist) following N-terminus cleavage, while its full-lenght form does not bind this receptor at all[150].

1.2.4. Atypical chemokine receptors: from walk-on to main characters

During inflammation, great amounts of pro-inflammatory factors are released in the extracelluar space, and chemokines make no exception. An important role in maintaining acceptable chemokine concentrations in inflamed tissues is played by atypical chemokine receptors (ACKRs), which in the past have been also known as decoys, interceptors, scavengers or chemokine-binding. Recently there has been growing interest concerning their function in many pathological contexts, such as microbial infection and tumors, but also in physiological processes, for example leukocyte recirculation to peripheral lymph nodes. ACKRs differ from classical signalling chemokine receptors under many aspects: structurally, the sequence motif DRYLAIV, highly conserved in the former ones, is either absent or poorly conserved in the latter ones. This parameter is currently used (among others) to identify new putative ACKRs (such as the new entries ACKR6 and C5L2). Another distinctive property of ACKRs is their different signaling pathway, that does not pass through a G protein, but is still able to internalize the bound ligand: this process is called "scavenging", and represents the most important feature of these proteins. For ACKRs, ligand internalization by endocytosis is β-arrestin-dependent, and can lead to the phosphorylation of the actin-binding protein cofilin through the Rac-p21-activated kinase 1-LIM kinase 1 cascade[151]. However, following chemokine recognition no ACKR is able to induce cytoplasmic calcium release, activation of PI3K pathway, or any other G protein-dependent signaling cascade, and this is another paradigm of ACKR function, useful in the identification of new members of this protein family.

The majority of ACKRs degrade the internalized chemokines through the fusion of the endocytic vesicle with a lysosomial vesicle, resulting in the complete clearance of the protein from the environment. ACKR1-mediated sequestration, though, does not lead to chemokine degradation, suggesting a different regulatory mechanisms for chemokine scavenging in specific compartments or for specific chemokines[152].

Being their function opposite to signaling chemokine receptors, it is also conceivable that different cell types express ACKRs. In fact, ACKR1 is not found on leukocytes, but only on endothelial cells of venules and small veins, and costitutively on erithrocytes[153, 154]. Similarly, ACKR2 is widely expressed in "barrier" tissues, such as skin, gut, placenta or lung[119].

ACKRs can bind broad spectra of chemokines, but usually belonging to the same functional class: for instance, ACKR1 is highly promiscuous as it presents affinity for more than 20 chemokines (CC and CXC chemokines), primarily of the inflammatory group[155, 156], while ACKR4 binds only homeostatic chemokines (CCL19, CCL21 and more weakly CXCL13)[157]. The existence of ACKR that are able to specifically bind homeostatic chemokines reflects the importance of this chemokine regulation system also during the steady state, and specifically during development. ACKR3 recognizes CXCL12 and, as for CXCR4, ACKR3-deficiency, particularly in endothelial cells, is lethal in mice[158], indicating an important function in the formation of normal cardiac structures for ACKR3. Evidences for ACKR3 role in other developmental programs have been found in zebrafish, where it regulates CXCL12 gradient, necessary for primordial germ cell migration[159]. Interestingly, it has also been shown that ACKR3 can heterodimerize with CXCR4, downmodulating the G protein-dependent signaling activity after CXCL12 ligation, and shifting the towards the arrestin axis[160]. Differently, ACKR4 targeted disruption generates healthy mice, although they display a more rapid onset of the multiple sclerosis model EAE (experimental autoimmune encephalomyelitis)[161], likely attributable to the impaired capacity of DCs and thymic precursors to home to lymph nodes and to thymus, respectively[162].

Furthermore, the "silent" chemokine receptors are involved in cancerogenesis, being relevant in the control of cancer-related inflammation, especially ACKR3, which has been

related to tumors of both hematopoietic (lymphomas) and mesenchymal (sarcomas, prostate and breast cancer) origin[119].

1.2.5. The tight link between chemokines and cancer immunology

Inflammation has recently been recognized as a hallmark of cancer [163, 164], for several reasons: first, inflammatory diseases increase tumor susceptibility (extrinsic pathway), and, secondly, in epidemiologically unrelated cancers induced by genetic events (intrinsic pathway) there is a common inflammatory signature[165, 166]. Chemokines are part of the network of inlammatory mediators associated to neoplastic events, especially those belonging to the inflammatory group (CC and CXC, mostly), and their involvement can be deduced from a pletora of evidences. For instance, the degree of tumor associated macrophages (TAMs), which often is proportional to the disease severity, is unequivocally linked to CCL2/CCR2 axis. Moreover, while at first the tissue where blood-borne metastatic cells homed to was believed to be regulated uniquely by mechanical factors, it has been proven that many types of cancer preferentially metastasize to specific organs (BM, lymph nodes, lung, liver), while other organs were rarely interested by metastatic events (stomach, kidneys)[167]. In addition, mice deficient for the aforementioned ACKR2, which scavenges most of inflammatory CC chemokines, showed increased susceptibility to skin carcinogenesis and colitis-associated cancer (a paradigmatic example of inflammation-cancer connection)[168, 169].

Generally speaking, chemokines can influence tumor growth, the tissue of metastasis, and anti-tumor immune responses in two ways: directly acting on cancer cells or modulating the behavior (i.e. migratory properties) of tumor-infliltrating leukocytes, but it is likely that in vivo both phenomena might be observed at the same time.

Chemokines and cancer cells. Regarding the former mechanism, the acquisition of chemokine receptor expression is intrinsic in the molecular pathways that characterize the tumorigenesis. In fact, it has been shown that downstream of RET (papillary thyroid

carcinoma), beta-catenin (breast cancer), Ras, mutant p53, Myc and Notch1[170, 171, 172, 173, 174, 175] several chemokines and related receptors are targeted: for example in acute myelogenous leukemia high levels of the transcription factor MEF2C causes upregulation of the expression of CCL2, CCL3 and CCL4[176], directing invasiveness of cancer cells. Additionally, through a GWAS (genome wide association scan) it was evidenced that genetic instability at *cxcr4* locus, with functional consequences at the protein level, correlated with incidence of familial forms of chronic lymphocytic leukaemia (CLL)[177]. Conversely, when a tumor-suppressor gene expression such as TGFβ was lost in breast cancer cells, it resulted in increased secretion of inflammatory chemokines CXCL1, CXCL5 and CCL20[178, 179].

Being neoangiogenesis fundamental for tumor progression, as it provides oxygen and nutrients to the highly demanding cancer cells, many studies have been conducted to assess CXC chemokines' role in this process. The pro-angiogenic signals can directly come from tumor cells or indirectly from leukocytes recruited by the tumor microenvironment[180].

As already mentioned above, ELR+ CXC chemokines display a proangiogenic effect, especially on CXCR1+/CXCR2+ endothelial cells: the formation of new blood vessels is promoted increasing endothelial cells resistance to pro-apoptotic stimuli or enhancing their proliferative potential, after CXCL5 and CXCL8 ligation. CXCR2 activation can induce production and secretion of metalloproteases like MMP-2 and MMP-9, which are responsible for extracellular matrix remodeling, but also of key molecules for angiogenesis such as VEGF and FGF2[181]. A synergistic effect of CXCL12 and VEGF in angiogenesis enhancement has been evidenced by Kryczek and colleagues in human ovarian cancers, and CXCR4 triggering by CXCL12 has been related to augmented endothelial cell survival and consequent increase of vascularization[182]. Besides, CXCL12 can attract endothelial cell precursors from blood or BM, father expanding CXCR4 involvement in vessel formation[183]. An opposite role has been proven for CXCR3 ligands CXCL9, CXCL10 and CXCL11, which block endothelial cell proliferation in several tumors[184].

Proangiogenic chemokines can as well exert their functions indirectly, for example recruiting neutrophils at the early stage of the disease[185], TAMs, myeloid-derived suppressor cells (MDSCs), DCs and Tie-2-expressing macrophages. These cells can synthetize a great variety of pro-tumoral moieties, among which there are VEGF, PDGF (platelet-derived growth factor), TGFβ, metalloproteases and CXCL8 itself[186, 187].

Despite the great number of strategies used by transformed cells to create new routes for oxygen and nutrients delivery, one of the most common features of tumor microenvironment is hypoxia, mainly because of the disruption of tissue architecture and for the high metabolic request of dividing cells. Hypoxia-inducible factor-1 α (HIF-1α) is rapidly expressed when the oxygen pressure decreases under normoxic levels, determining several responses, including promotion of vascularization. CXCR4 is activated downstream the signaling pathway initiated by HIF-1α, and is thus upregulated in hypoxic conditions[188], but is also induced in case of gene mutations of von Hippel-Lindau (VHL), a negative regulator of HIF-1α[189].

In ovarian cancer, it has been shown that HIF-1α stimulates secretion of CCL28, a chemoattractant for T regulatory cells (Tregs) (and type-2 polarized cells, generally), thus estabilishing an immunosuppressive environment and increasing intratumoral vascular density through production of VEGFA[190].

The concept of "cellular highways" emerged recently[191], and takes into account the pivotal role played by homeostatic chemokine receptors in directing the normal leukocytic trafficking, in physiological conditions. As a result, there are tissue-specific chemokinic axes (e.g. CXCR4/CXCL12 in the CNS and BM, CCR7/CCL19-CCL21 in secondary lymphoid tissues, CCR9/CCL25 in the small intestine and CCR10/CCL27 in the skin), which can univocally address responsive cells if needed. Tumor cells evolved mechanisms to exploit this system, hijacking the highways to metastasize in distant tissues[192]. The most common chemokine receptors expressed by the great majority of tumor cell types are by far CXCR4 and CCR7[191].

The first case was reported by Muller and colleagues[193], and since then CXCR4 was extensively investigated, and found upregulated on more than 20 different tumor histotypes[166], where almost invariably represents a poor prognostic sign and correlates with lung, liver and BM metastasis[192]. CXCR4 is also the only chemokine receptor whose expression was found on cancer stem cells (CSCs), in glioblastoma and in pancreatic cancer, and in the latter case CD133+/CXCR4+ cells were identified at the front of tumor invasion, and determined the metastatic phenotype of individual tumors[194, 195]. Besides the ability to follow chemokine gradients displayed by metastatic cancer cells, it was reported that at certain stages of tumor development and spread, malignant cells produce chemokines (CXCL12, primarily) for which they express the appropriate receptor[196, 197], endowing them with a survival advantage over non-transformed cells present in the microenvironment[192], generating an autocrine signaling loop that confers resistance to chemotherapy-induced apoptosis, and promotes proliferation[198].

CCR7 expression in breast, melanoma, gastric and non-small cell lung cancer, head and neck tumor and colorectal carcinomas are associated with lower survival rates in patients and higher risk of lymph nodal metastasis. CCR7 mRNA levels are also linked to tumor stage, while CCR7 was demonstrated to mediate brain colonization of leukemic cells in T-cell acute lymphoblastic leukemia (T-ALL)[175, 193].

Other CXC chemokine receptors have been involved in the promotion and guidance of tumor infiltration and metastatic process, such as CXCR1 and CXCR2 in malignant melanoma[199], CXCR3 in B-cell chronic lymphocytic leukemia (B-CLL)[200], CXCR5 in liver metastasis of colorectal carcinoma[201], and CXCR6 in prostate cancer[202]. Similarly, CC receptors promote metastasis to skin (CCR4 and CCR10), to intestine (CCR9) and liver (CCR6), while CX3CR1 is associated to bone colonization and perineural invasion of pancreatic adenocarcinoma[166].

The chemokine ability to trigger pro-survival signals in cancer cells through the activation of MAP/Erk kinase pathway has already been mentioned for CXCR4, but it has also been reported for other chemokine receptors, in a wide array of oncologic diseases.

Interestingly, even the opposite phenomenon was observed, as CCR5-mediated p53 activation inhibited in vitro proliferation in breast carcinoma samples and cell lines, and furthermore the nonsense CCR5Δ32 mutation frequency in humans is more frequent in patients with larger tumor size and lower disease-free survival[203].

Shaping the immune response in the tumor microenvironment. The other side of the coin in the bond between chemokines and cancer refers to leukocyte recruitment at the tumor site. While the immune system is supposed to appropriately, specifically and quickly respond to neoplastic hazard, tumor cells can contribute to the shaping of the infiltrate, recruiting more convenient and tolerant cell types.

In this regard, an important contribution to tumor promotion and progression is given by TAMs, which are associated to advanced stage of disease, induction of neoangiogenesis and inhibition of T cell response[187, 204]. CC chemokines, in particular CCL2, CCL5 and, in some cases CCL7, are the main actors in TAMs trafficking to tumor site, and strongly correlate with number and frequency of TAMs in situ. Both neoplastic cells and surrounding stromal cells are responsible for CC chemokines production, and high levels of CCL2 and CCL5 are indeed present also in sera of carcinoma patients[205]. Among the roles played by TAMs in tumor growth, there is also the recruitment of other immune-suppressive cell types, such as Tregs, through the release of CCL22 in ovarian cancer[206], CCL20 in colorectal cancer[207], or CCL1, CCL17 and CCL22 in other pathological conditions[208].
CCL2/CCR2 axis has been proven to be important even for the migration of MDSCs to tumor site[209], together with CXCR4/CXCL12 and CXCR2/CXCL5[179], and in some cases (melanoma) CCL21[210].

Less clear is the effect of the neutrophils, as their presence in the leukocytic infiltrate has been associated to poor prognosis, as happens for TAMs, MDSCs and Tregs[211], and thus a polarization towards the so called N2 phenotype has been hypothesized. Sideways,

tumors that artificially over-express CXCL8, the major neutrophil-attractant chemokine, show better clinical outcomes when injected into recipient mice[212].

DCs are found in several types of tumors, and are generally considered beneficial for the anti-tumor response, though they often display an immature state and a refractoriness to activation/maturation signals. CCL20, CCL5, CCL19 and even CXCL12 are the chemokines known to be important for DC recruitment in tumors, like pancreatic carcinoma, papillary thyroid carcinoma, breast carcinoma and ovarian carcinoma[210].

As opposed to ELR+ CXC and many CC chemokines, CXC ELR- chemokines such as CXC3 ligands CXCL9, CXCL10 and CXCL11 are in many cases responsible for the promotion of NK cell and T cell migration to tumor site, generally resulting in antitumor responses. These chemokines are induced by type I and type II IFNs, and are thus symptomatic of a functional type 1 polarization of the cancer-related inflammation[210]. Their levels within the tumor correlates with effector cell number, creating a positive feedback loop, as NK cells, CD4+ and CD8+ T cells are strong producers of these proteins upon activation. CX3CL1 and CXCL16 are other recruiters of NK, T and NKT cells, and are expressed in renal cancer, neuroblastoma and colorectal carcinoma[213, 214]. CCR5-specific chemokines like CCL3, CCL4 and CCL5 play an important role in antitumor imunity, more or less overlapping the function of CXCR3 ligands.

A different strategy employed by the immune system to oppose tumor growth is the formation of tertiary lymphoid structures (TLS), where an efficient adaptive response has been observed in lung cancers, and associated with patient long-term survival[215]. The de novo synthesis of TLS is promoted by CCL19, CCL21, CCL17, CCL22 and CXCL13, that can additionally be involved in the interplay between B cells, T cells and DCs to produce a specific antitumor response.

ACKRs in tumor immunology. Given the multifaceted role of chemokines in tumor immunology, it is not surprising that atypical chemokine receptors could significantly

influence cancer-related inflammation. As of now, data concerning the most studied ACKRs are available in the literature at this regard (ACKR1, ACKR2 and ACKR3), and interestingly show that ACKRs involvement is non redundant and varied. It is currently known that ACKR1, that acts as "chemokine sink"[216], clears from the circulation the chemokines that recruit immune suppressive cells, such as TAMs (CCL2) or MDSC (CXC ELR+), to tumor site; consequently when in a melanoma murine model ACKR1 was overexpressed on endothelial cells, cancer cell apoptotic rate was increased, while angiogenic rate significantly diminished[217]. Besides its inflammatory and proangiogenic chemokine scavenging function, it has ben proposed that ACKR1 might favour anti-tumor response as well by directly inducing apoptosis in neoplastic cells through the interaction with the tetraspanin molecule KAI1/CD82[218]. Collectively, ACKR1 role is believed to be befeficial for tumor immune response.

The function of ACKR2 is on the same lines of ACKR1, as its expression frequently results inversely correlated with TAMs infiltrate, and directly correlated with disease-free survival[219].

In contrast, ACKR3 role seems to be likely pro-tumoral, as its overexpression on cancer cells promotes adhesion molecule production (such as cadherin-11 and CD44), enhancing invasion and angiogenesis[220]. ACKR3, like its functional counterpart CXCR4, is found on several tumor types, and it is a direct target in the pathway of Hypermethylated in Cancer 1 (HIC1), a tumor suppressor gene early inactivated during tumor progression[221].

Chemokine post-translational modifications. An additional control level that might be regulated by cancer cells to manipulate immune response is represented by chemokine post-translational modifications. As mentioned above, chemokines can be cleaved by proteases, citrullinated, deiminated, nitrated and glycosilated[222], and although this issue is still quite unexplored, recent data indicate that tumor progression can also pass through chemokine processing. For example, the soluble form of CXCL16 has been shown to promote CXCR6+ cell proliferation and migration, while reduced cancer cell proliferation and detachment rate if present in its transmembrane form[223].

Furthermore, CCL2 nitration/nitrosylation induced by reactive nitrogen species (RNS) within the tumor has been found to impair cytotoxic T lymphocytes (CTLs) recruitment, while TAMs capacity to migrate to the chemokinic gradient was unaffected[224]. Indeed, this novel tumor immune escape strategy could be of interest for future therapeutic intervention protocols.

Chemokine and tumors – the example of HHV8 and perspectives. A paradigmatic case of the importance of the chemokine system in tumor progression is represented by the mechanism of action of the etiologic agent of Kaposi sarcoma (KS) and other hematologic malignancies, the human herpesvirus 8 (HHV8). Viral proteins divert host immune response in two ways: the first through the costitutive production of the viral receptor vGPCR, which interacts with both ELR+ and ELR- CXC chemokines, enhancing autonomous cell proliferation and promoting the classic KS lesions. The second mechanism of action is mediated by three viral proteins (vMIP-I, vMIP-II and vMIP-III) that mimic the structure of endogenous CC chemokines, and are able to bind CCR3, CCR8 and CCR4, recruiting Tregs and other type 2 polarized immune cells[225].

Collectively, it appears clear that cancer related inflammation might be, and currently is, an optimal target for the oncologic therapy. Efforts are being made to test chemokine receptor antagonists (i.e. AMD3100[226]), or neutralizing mAbs specific for certain chemokines (i.e. αCCL2[227]), but it appears likely that the study of alternative mechanisms of immune evasion orchestrated by cancer cells, such as the proliferative advantage given by the autocrine chemokine/chemokine receptor loop, or the promotion of chemokine post-translational processing, can contribute to the understanding of the link between neoplasia and the chemokine system.

1.3. Multiple myeloma

1.3.1. General information, disease stages and molecular markers

Multiple myeloma (MM) is a neoplastic plasma-cell disease, characterized by the clonal proliferation of malignant plasma cells prevalently in the bone marrow[228], and less frequently in extramedullary tissues. Among the signs of this disorder, the most important are the presence of monoclonal protein in the blood and urine and associated organ disfunction.

MM represents about 1% of total neoplastic diseases, and 13% of hematologic cancers, is usually diagnosed later in life (around 70 years of age), and currently the treatments rely on autologous stem cell transplantation and subministration of several classes of drugs, such as alkylating agents (melphalan), IMiDs (thalidomide, lenalidomide) or proteasome inhibitors (bortezomib). New treatment protocols have improved the managment of MM course, and extended patients survival[229], though this pathology is still classified as incurable.

Commonly, the pathology evolves from an initial age-dependent pre-malignant stage known as monoclonal gammopathy of undetermined clinical significance (MGUS), which affects 2% of the population above the age of 50[230], with a risk of progression to MM of 1%. MGUS displays abnormal monoclonal spike, but not organ disfunction, and usually precedes the phase of smoldering MM (SMM), in which the involvement of bone marrow plasma cells can reach 30% of total cells, and the risk of advancement to symptomatic MM is 10-20% per year[231].

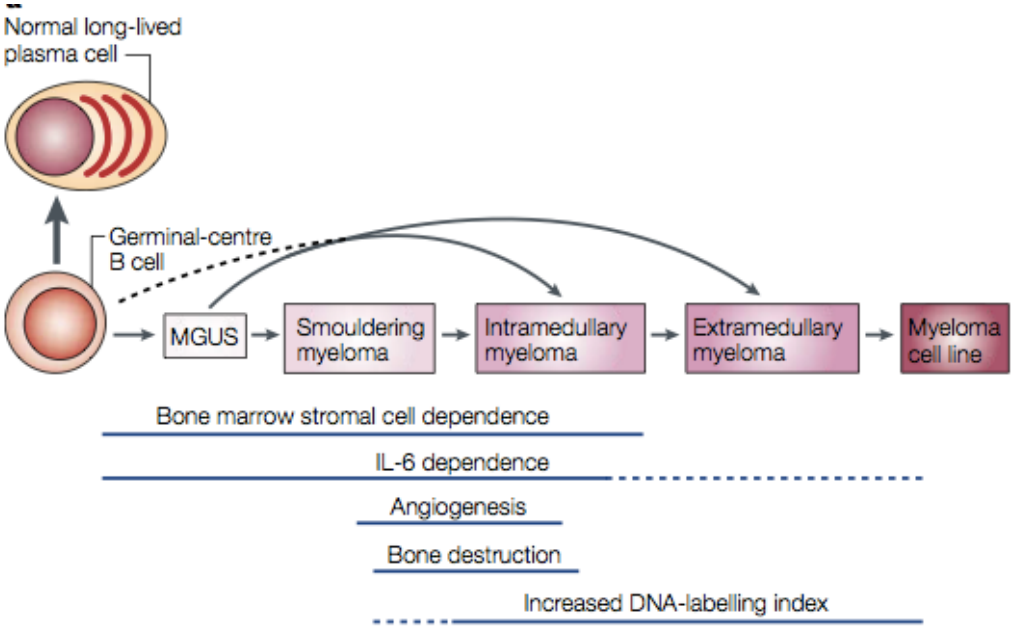


Figure 9 – *Pathogenesis and stages of Multiple Myeloma.* MM arises from a germinal centre-B cell, in 30-50% of cases passing by the benign MGUS phase and often by the Smouldering phase. With MM progression, tumor cells obtain the ability to colonize extramedullary tissues (blood, pleural fluid, skin) and thus to metastasize. Below, the most important features of each MM stage, such as angiogenetic and osteolytic bone resorption potential are shown. Moreover, some of the extramedullary MM can estabilish immortalized cell lines in vitro[232].

The transition to the terminal stage of MM is due to many oncologic events: probably the earliest one is the dysregulation of the cyclin D gene[233], which is already observed at the MGUS step. Indeed, many genetic abnormalities occur in plasma cells, and can be divided in primary and secondary, depending on the phase in which they most frequently occur.

Primary translocations are early observed in MM pathogenesis, and involve the juxtaposition of the immunoglobulin switch region on oncogenes, like c-MAF and MMSET on chromosome 14, in which case resulting in the deregulation of MMSET and, in some cases, FGFR3.

Differently from other B-cell malignancies, this kind of chromosomal modifications simultaneously dysregulates a great variety of genes. Secondary events are characterized by c-Myc dysregulation, activating mutations in NRAS and KRAS2, as well as monosomy of chromorome 13, inactivation of cyclin-dependent kinase inhibitors CDKN2A and CDKN2C[232]. Moreover, epigenetic alterations (i.e. gene methylation) and microRNA

expression have been related to MM pathogenesis. Besides, MM patients can be classified also according to a transcriptional signature, as recently evidenced by Zhan and colleagues[234].

1.3.2. The importance of bone marrow microenvironment

The almost exclusive localization of MM cells in bone marrow is due to the peculiar microenvironment present in this organ, both in terms of cellular and non cellular components.

As an example, bone marrow highly expresses CXCL12, which represents a pivotal chemotactic cue for CXCR4+ MM cells, whose organ retention and capacity to grow significantly relies on this receptor. Within bone marrow cellular component, hematopoietic and non hematopoietic cells can be found, the former consisting in T, B, NK and NKT lymphocytes, DCs, and, during MM progression, MDSCs. In turn, the latter includes adypocytes, bone marrow stromal cells (BMSCs), endothelial cells (ECs), osteoclasts (OCs) and osteoblasts.

MM cells are able to generate significant modifications in their surroundings, actively contributing to the formation of bone lesions and neovascularization through the interaction with BMSCs and ECs, while they inhibit osteoblasts thus impairing bone neosynthesis. In parallel, the normal immune response is shifted towards tolerance or a type 2, or as recently shown, type 17 cancer-related inflammation. These condition leads to the formation of the so called "MM niche", or in other words an environment that allows tumor growth, cell survival and drug resistance[235].

Non-hematopoietic components of the MM niche. A great variety of adhesion molecules are expressed by myeloma cells and enable them to interact with BMSCs and the components of the extracellular matrix (ECM): very late antigen-4 (VLA-4) on MM cells binds to vascular cell adhesion molecule 1 (VCAM1) on BMSCs, while the VLA-4 together with CD138, also known as syndecan-1 mediate the interaction of MM cells to ECM

components, such as collagen I and fibronectin. Other important proteins involved in MM adhesion are CD44, LFA-1, CD56 and MPC-1, besides the fundamental role played by CXCR4[236]. This complex set of connections triggers anti-apoptotic signals in MM cells and upregulates cell-cycle regulating proteins[237], favoring tumor growth.

Upon interaction with MM cells, BMSCs activate the NFkB pathway and produce IL-6: this cytokine upregulates the expression and secretion of pro-angiogenic factors, such as VEGF, which acts on MM cells, creating a paracrine loop that optimizes MM growth conditions[238]. IL-6 release, combined with VEGF and insulin-like growth factor-1 (IGF-1) production, is also induced in MM cells and BMSCs following Notch pathway activation, which is correlated to MM proliferation and survival[239]. Besides, IL-6 plays a role in the formation of bone lesions[240], it is induced by pro-inflammatory cytokines such as TNFα in BMSCs, and determines the polarization of CD4+ T cells towards a Th17 phenotype, further contributing to disease progression. Collectively, IL-6 stands out as a key molecule in the MM milieu, as it is produced by paracrine (BMSCs and osteoblasts) and autocrine mechanisms, and accordingly its serum levels inversely correlate with disease-free survival[241].
Interestingly, it has been shown that IL-6 and TNFα, together with bFGF and TGFβ, are potent inducers of VEGF in BMSCs and MM cells, demonstrating once more the close relation among the several factors involved in tumor pathogenesis[242].

As previously mentioned, bone lesions are a hallmark of multiple myeloma, and are caused by a large number of factors. The balance between bone production and mineralization (osteoblasts activity) and bone resorption (OCs activity) is frequently lost, because of osteoclast formation and/or hyperactivation. Perhaps the most important molecule involved in this process is Receptor activator of NK-kB ligand (RANKL), a member of TNF family, produced by MM cells. RANKL is an essential growth factor for OCs, since through the binding to its receptor RANK on OCs, it mediates OC differentiation and inhibits apoptosis[243]. The action of RANKL is attenuated by

osteoprotegerin (OPG), a soluble decoy for RANKL produced by BMSCs, whose concentration in BM plasma is found decreased in MM patients, resulting in OC activation and bone destruction[244].

Besides RANKL, the chemokine CCL3 was identified as an important player in OC activation for the first time by Choi and colleagues[245], as it stimulates osteoclastogenesis, in concert with RANKL and IL-6[246]. Another CC chemokine, CCL5, has been demostrated to mediate osteoclastogenesis, thus suggesting a broader role for CCR1 and/or CCR5 ligands in the process of bone destruction[247]. Moreover, CCL3 and CCL4 might influence contact-dependent MM cells-mediated osteoclastogenesis, since CCR1 and CCR5 binding on BMSCs induces the expression of adhesion molecules such as VCAM-1, that, in turn, upregulates RANKL and downmodulates OPG[248].

Among the factors that trigger the formation of bone lesions IL-3 might be important, as it exerts its function in a dual mode. From one hand it activates OCs, while on the other it impairs osteoblastogenesis through an indirect mechanism, which involves the monocytic lineage[249]. Additionally, TNFα, CXCL12, hepatocyte growth factor (HGF) and thrombospondin-1 have been reported to be upregulated in bone lesions, and are likely implicated in this process[250, 251, 252].

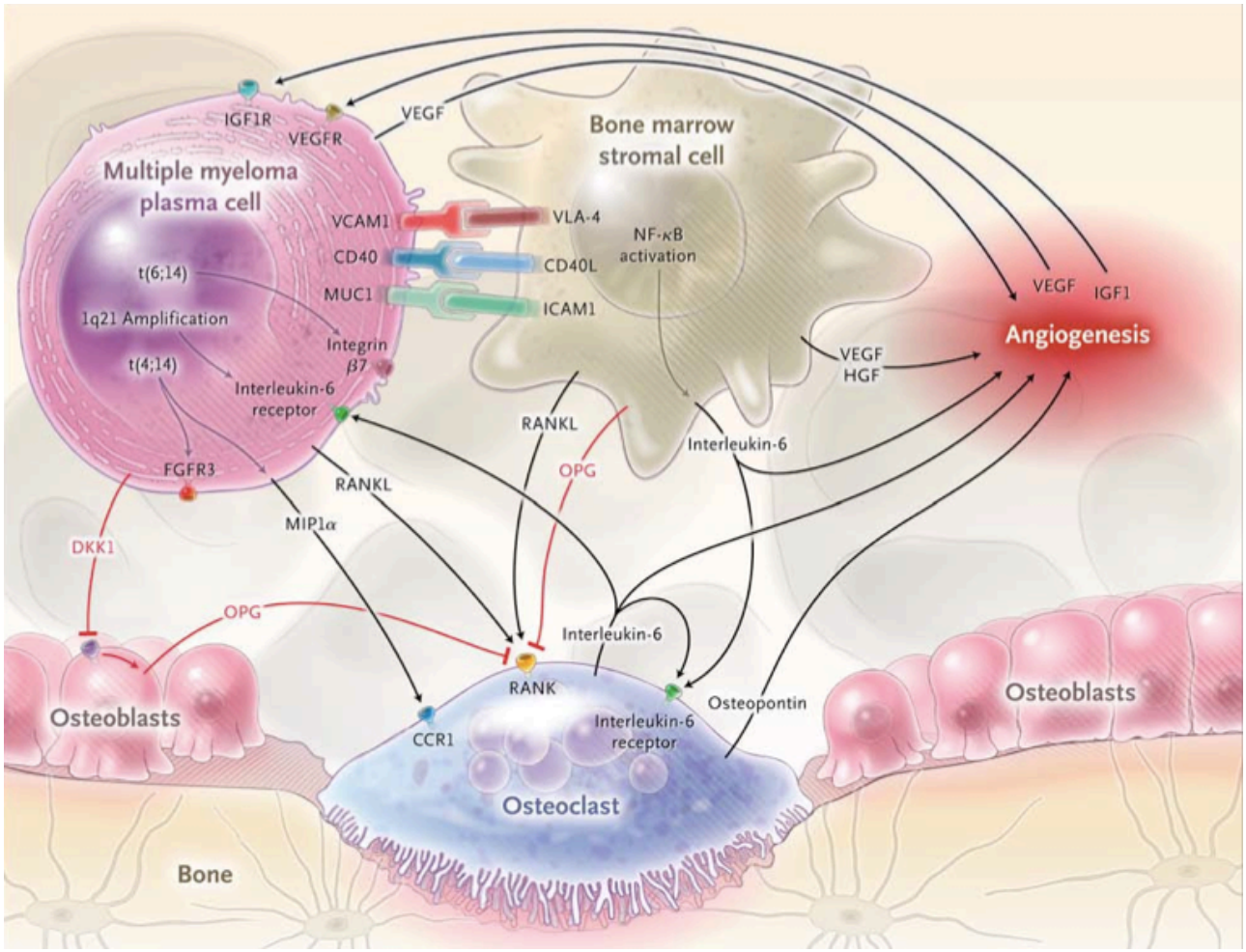


Figure 10 – ***MM cells deeply influence and shape BM microenvironment.*** Tumor cells promote a wide array of affects within the BM, for example modulating osteoclast activity and differentiation through IL-6, RANKL or CCL3. Their effects on osteoblast activity (DKK1) and on stromal cells (VCAM1) are also displayed, resulting in bone resorption, neoangiogenesis and MM cell survival[228].

The contribution of osteoblasts to MM pathogenesis is probably less clear; in fact, they can support myeloma growth through the production of IL-6 and OPG, which can inhibit TRAIL-mediated MM cell apoptosis by binding to RANKL and to TRAIL[253]. At the same time, osteoblastic differentiation is blocked by several factors, including the Wnt-signaling antagonist DKK1 (produced by MM cells), secreted frizzled protein-2 (sFRP2), sFRP3, TNFα and sclerostin[254, 255, 256, 257], but also osteoblastic proapoptotic molecules, such as IGF-BP4, IL-7 and, as recently suggested, TGFβ and activin A[258, 259, 260, 261].

It is likey for those mechanisms that the total osteoblast number within bone lesions in MM patients is most frequently reduced as compared to healthy subjects, and correlates negatively with tumor progression and disease stage[262].

How the leukocytes (try to) respond: role of the immune system. The other half of the story is related to the influence that MM cells have on what should be an efficient and productive anti-tumor response. Indeed, the concept of MM niche is defined also by the presence of tolerant leukocytes and the exclusion of most dangerous ones (or even by their functional impairment), a process that is referred to as tumor immuno-editing.

A great deal of the immune response against cancer is carried out by T lymphocytes: the bone marrow is an important reservoir for effector/memory T lymphocytes (T_{EM}), and a DC-based cell vaccine has shown that bone lesions contain tumor-specific, potentially active, T cells[263]. Moreover, other groups have focused on the role of marrow-infiltrating lymphocytes (MILs), that are able to produce high levels of IFNγ, thus blunting OC outgrowth, when cocoltured in vitro[264], and to display a higher tumor specificity than PBLs stimulated with anti-CD3/anti-CD28 beads[265].

Nevertheless, many T cell dysfunctions have been described in relation to MM, such as CD4+ T cell lymphopenia, which represents a marker of advanced disease, lower survival rate and increased risk of relapse[266 267]. In addition, the normal proportion of CD4 and CD8 T cells has been found inverted, while, within CD4 T cell population, an abnormlly high Th1:Th2 cell ratio has been reported[268].

Furthermore, soluble molecules like TGFβ can suppress CD8 T cell effector function, including the production of perforin, granzyme A and B, FasL, and IFNγ, through a IL-2 autocrine pathway[269], while the impaired expression of co-stimulatory molecules (CD28 and CD40L) and signal trasduction proteins (CD3ξ and ZAP-70) is associated to more advanced stages of the disease[270].

An important role in MM progression is thought to be played also by other CD4+ T cell subpopulations, specifically Th17 and Tregs. IL-6 is among the cytokines that induce both

of those phenotypes; for instance, IL-6 in combination with IL-23 and TGFβ determines Th17 differentiation, via a MM cell STAT3-dependent mechanism[271]. As for Tregs, their actual function in MM has not been clearly determined yet. Contrasting data evidenced either the paucity[272] or the increase[273] of this population in MM patients, and at least some of those differences could be possibly due to the examined compartment or to the criteria used to define Tregs [241]. At any rate, MM cells are able to induce Tregs formation both directly, through the expression of ICOS-L[274], and indirectly, in an APC-dependent manner, often mediated by DCs[275], suggesting that Treg contribution might be significant.

Another key component of BM infiltrate in MM are the dendritic cells, whose primary task shoud be to cross-present tumor related antigens (TRA), activate effector cells and recruit leukocytes to the tumor site. As described for other types of malignancies, the dysregulation of DC function mainly consists of low maturation capacity, with consequential impairment of T cell activation, and in reduced number of circulating precursors, both of myeloid (CD11c+) and plasmacytoid (CD11c- CD123+) lineage. This results in an immature phenotype displayed by in situ DCs, with lower expression levels of HLA-DR, CD40 and CD80, and lower capacity to induce allogeneic T cell proliferation and to present tumor antigens to specific lymphocytes[276]. Besides, DCs from MM patients with stable disease fail to upregulate CD80/CD86 expression in response to IL-2 and CD40L[277].

The factors that promote DC maturative and functional impairment include IL6, since IL6Rα-deficient DCs have been shown to be functionlly competent and to mediate protective immunity[278], but also IL-10 and TGFβ, through a p38-dependent mechanism[279]. Moreover, HGF, VEGF and β2-microglobulin have been reported to be involved in DC functional inhibition[280].

A deeper understanding of the processes that lead to DCs functional defect is fundamental for the development of novel therapeutic vaccination approaches, which so far have proven to produce interesting results in vivo[241].

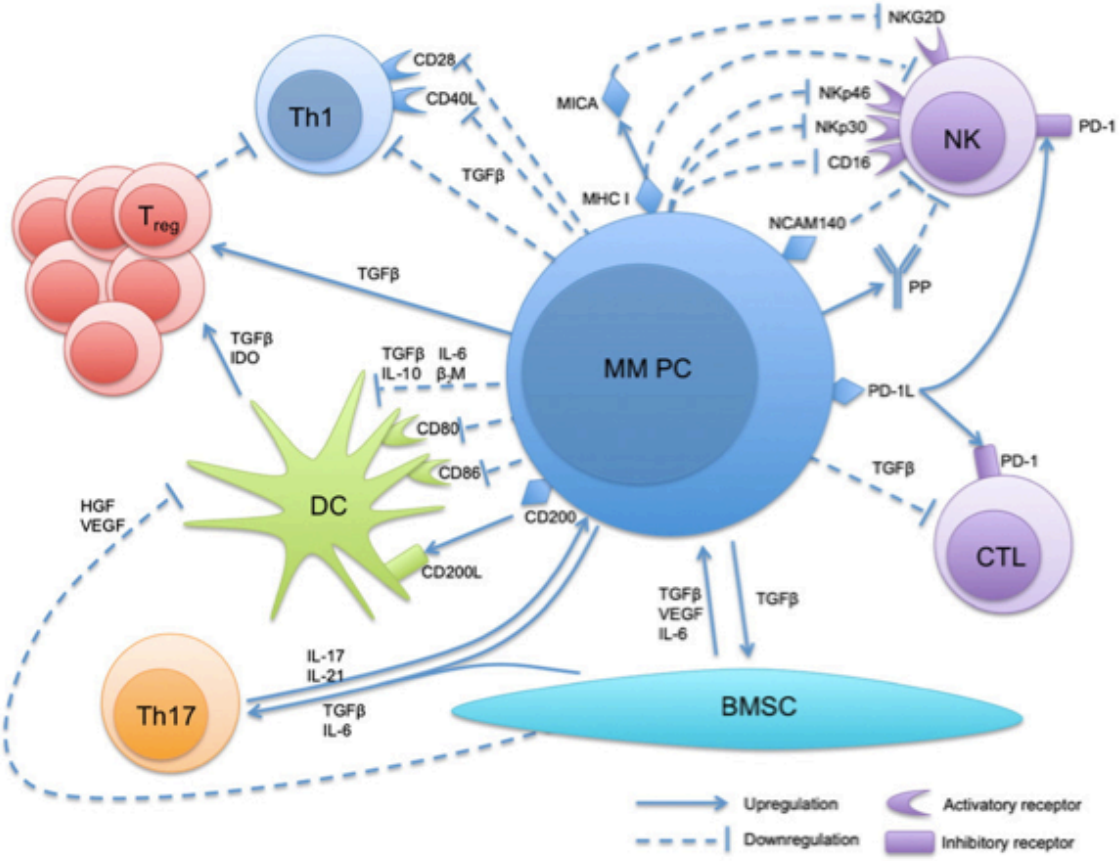


Figure 11 – ***Immune response is modulated by tumor cells.*** Schematic representation of the interplay between MM cells and leukocytes within BM. TGFβ and PD-1L can suppress T and NK cell effector response, together with high expression of MHC-I (which blocks NK cells) and the impairment of DCs maturation (thus interfering with T cell activation). In parallel, T regs and Th17 are actively induced from MM cells, favouring a tolerant environment[280].

Accumulating evidences link NK cells to MM, portraying those innate lymphocytes as important sentinels in myeloma-related immune response. The ability of NK cells to kill MM cells in vitro has been documented years ago by Frohn and colleagues[281], and has been further investigated by other groups, in search for an alternative strategy to treat this disease.

On a molecular level, NK cells recognize and are able to mediate cytotoxicity against MM cells mainly through activating receptors, such as NKG2D and DNAM-1, since it has been described that myeloma cells express high levels of MIC-A, MIC-B (NKG2D ligands)[282], PVR and Nectin-2[283]. Intriguingly, it has been described that different clones of MM express either NKG2D or DNAM-1 ligands, but not both at the same time, suggesting an

immune selection of the NK cells during tumor progression[282]. In fact, while NKG2D ligands are generally present solely on the surface of transformed cells, DNAM-1 ligands can be found also on the membrane of healthy, normal cells, adding further complexity to the mechanisms underlying NK cell-MM interaction. Moreover, NCRs (NKp46 in particular) are involved in MM recognition and clearance by NK cells, although the specific ligands that mediate the binding have not been defined yet[283].

Besides, tumor cells can escape the immune control exerted by NK cells even by increasing the membrane shedding of NKG2D ligand MIC-A[284]. This process is mediated by ERp5, a disulphydic isomerase whose expression is induced in MM patients. MIC-A cleavage generates a soluble form of the ligand, that is able to impair NKG2D-dependent NK cell activation and degranulation, representing an important form of defense for MM cells. Nevertheless, the immune system has accordingly adapted to this strategy, through the formation of antibodies specific for MIC-A soluble form (sMIC-A), and indeed the titer of these antibodies has been found increased in patients with active MM as compared to subjects with MGUS[284].

An important regulator of MIC-A expression on MM cells is glycogen synthase kinase-3 (GSK-3), that downmodulates MIC-A through the activation of the signaling transducer STAT3[285]. In this work, the authors proposed that the inhibition of GSK-3, a serine/threonine kinase on which many signaling pathways converge, might be exploited in vivo to enhance NK cell-mediated cytotoxicity.

In the last years, a line of research focused on the role of chemotherapeutic drugs administered at low doses on the upregulation of NK cell activating ligands. Indeed, it has been demonstrated that sublethal doses of melphalan and doxorubicin, genotoxic agents currently used in MM therapy, induce NKG2D and DNAM-1 ligand overexpression on tumor cells in vitro, through an ATM (Ataxia-Telangectasia mutated)/ ATR (ATM- and RAD3- related)-dependent mechanism, associated to cellular senescence[286].

As emerging data support the importance of NK cell control on MM progression, it appears clear that, for some reason, endogenous BM and peripheral NK cells are unable to efficiently counter tumor growth. At this regard, NK cell adoptive transfer therapeutic

protocols are being tested, in order to have an appropriate number of functional (primed) NK cells. This strategy could be used after the classic chemotherapy, thus exploiting the MIC-A, MIC-B and DNAM-1 overexpression on MM cells, or could completely bypass it in the future. Alici and colleagues tried successfully to expand and activate NK cells in vitro with IL-2 stimulation, before transplanting them in a murine model of MM, reporting a significant achievement in terms of tumor growth and survival[287]. The same group attempted to expand in vitro human NK cells derived from peripheral blood of newly diagnosed MM patients, and tested their functionality ex vivo against primary MM cells, in an autologous setting, obtaining encouraging results[288]. The same strategy has proven to be successful in a humanized murine model of multiple myeloma, where transplanted NK cells derived from human healthy donors were able to reduce tumor growth and bone lesion[289].

A different approach bases on the capacity of the NK cells to detect the "missing self": in other words, NK cells from haplo-identical donors can be transplanted in patients after autologous stem cell transplantation (ASCT) exploiting the HLA (recipient)/KIR (donor) mismatch. In this condition, NK cells display a strong graft versus leukemia (GvL) effect, and a lower Graft versus Host disease (GvHD). That aside, the quantity of haploidentical NK cells was not sufficient to obtain a complete remission in that conditions; moreover, the difficulty in finding an appropriate HLA/KIR mismatched donor make this therapeutic approach quite demanding[290].

1.3.3. 5TMM – A reliable murine model of Multiple Myeloma

SCID-hu models. Several murine model for MM are described in literature, and a group of them involves SCID (severe combined immunodeficiency) mice, which present the advantage of having human myelomatous cells transplanted[291, 292, 293, 294, 295]: SCID-hu (humanized) mice can be useful to test in vivo the cellular response and/or systemic toxicity of novel pharmacological compounds, but present evident limits in relation to the interaction between human MM cells and the environment, or with the endogenous

immune system. Moreover, commonly these models are heterotopic, as tumor cells are often injected sottocutaneously, or even in artificial scaffolds that mimic human bone structure.

Transgenic models. An alternative approach of development for murine models of MM is represented by mice with genetic mutations that cause the overexpression of oncogenes, in particular the ones related to B cell lineage. MAF, XBP-1s and c-Myc are some of these genes, and have been targeted for the generation of such models[296, 297, 298]. Although these genetically modified mice reproduce some features of human multiple myeloma, the causal relationship between gene mutation and disease estabilishment has not yet been demonstrated, thus the genetic alteration may not fully represent the MM progression.

The 5TMM model. The 5TMM model in mice was first described by Radl and colleagues several years ago (in fact it is also called Radl model)[299], and consists in the i.v. transplantation in young C57BL/KaLwRij mice (6-10 weeks old) of a cell line derived from the BM of syngeneic elderly mice, that spontaneously develop a pathology very similar to human MM.

About 33% of MM patients develop the pathology passing first by the MGUS phase, while the remaining cases present de novo myelomas, or, in other words, MM caused by the direct transformation of plasma cells into over myeloma[300], and 5TMM model belongs to the latter category[301].

Many of the characteristics of human MM heva been detected in 5TMM models, for example osteolytic bone lesions, increased angiogenesis and the presence of paraprotein in serum[302, 303, 304, 305]. Several cell lines have been derived from C57BL/KaLwRij MM-bearing mice, and have been used for 5TMM models, the most studied of which are 5T2 and 5T33. The first induces a slower, less aggressive and more BM-tropic MM (clinical symptoms detectable

12 weeks after injection), while the second grows more rapidly, and displays the typical hallmarks of MM already at the 4th week after transplantation[303].

In this work the 5TMM model was used, with the i.v. transplantation of 5TGM1 line, which derives from 5T33, and presents the advantage of proliferating easily in vitro without IL-6 (like 5T33) or feeder cells (like 5T2)[306]. Being even more aggressive than 5T33 cell line, 5TGM1 are capable of colonize spleen and liver, probably for their hematopoietic nature in mice, besides of course BM. Several studies reported that the homing preferences of transplanted MM cells can be distinguished after the first 18 hours, when they accumulate in BM, spleen and liver, while decreasing in blood and lung[307]. Many molecules have been involved with the initial homing process of 5TGM1 to BM, such as CCL2, CCL3, IGF-1, CD44v, CXCL12 and CXCR3 ligands[308, 309, 310, 311, 312], and have provided important insights in MM pathogenesis.

Overall the 5TGM1 5TMM model represents a useful tool to characterize the multi-organ progression and estabilishment of Multiple Myeloma, and also to evaluate its interactions with the numerous components of the immune system.

2. Aim of the thesis

BM is the organ where NK cell development mainly occurs, and thus where virtually all NK cell differentiation stages can be found. There are several factors influencing NK cell development, including transcription factors, cytokines and interaction with other cells, belonging both to the hematopoietic and to the stromal compartment.
The role of chemokines and chemokine receptors has recently emerged as part of the components that drive NK cell maturation process, likely driving the positioning of responsive subsets in the correct BM niche. In particular, homeostatic chemokine axes seem to be significantly involved in this phenomenon, as for example CXCR4 expression is progressively downregulated in various NK cell subsets during development[313], while S1P5, one of the five receptors for the chemotactic lipidic molecule sphingosine-1 phosphate (S1P), is strongly expressed by NK cells prevalently during the terminal phase of their maturation in the BM[116].

The role of CX3CR1 in NK cells was previously investigated in inflammatory contexts, such as NK cell recruitment to the brain in experimental autoimmune encephalomyelitis (EAE)[314] and CMV infection[315]. Besides, CX3CL1 was found to induce NK cell cytotoxicity[316], to mediate melanoma lung metastasis clearance by NK cells in vivo[317], and to enhance cardiac allograft rejection[318]. Lastly, CX3CR1 has been demonstrated to have a role in NK-DC interaction during inflammatory conditions[319].

In the steady state CX3CR1 has been studied mainly in relation to the myeloid lineage, as it has been reported to play an important role in DCs development and monocyte biology[320,321,322]. So far, there are no data regarding CX3CR1 role in NK cell development, trafficking or functional properties in homeostatic conditions.

Through the use of a transgenic mouse model, our group focused its attention on CX3CR1 on NK cells, where it has a similar expression pattern to that of S1P5, as it is preferentially expressed by the CD11b+/KLRG1+ subset[323], which is abundantly located in the sinusoidal compartment of the BM. As this receptor expression is developmentally and spatially regulated, in the first part of this thesis the phenotypical and functional consequences of CX3CR1 deficiency on murine NK cells will be described, with a particular emphasis on the BM trafficking capacities of different NK cell subsets. The effect of CX3CR1 absence on NK cells was investigated both during steady state conditions and in a model of acute hepatic inflammation, namely Poly(I:C) subministration. The results obtained in this framework shed light on the involvement of CX3CR1 in NK cell retention within the BM.

While the trafficking capacities to BM and, eventually, to metastatic sites of multiple myeloma cells have been extensively studied[241, 308, 309], so far little is known about the migratory properties of immune cells during tumor growth, both in situ and in the peripheral organs. The level of several chemokines has been found altered in MM[324, 325], but the direct consequences of these environmental modifications on leukocytic, and more specifically, on NK cell recruitment to tumor site have not been assessed yet.

Thus, given the notable importance of the BM microenvironment for NK cell development and functional maturation, and considering the modifications induced by multiple myeloma growth in the same organ, the second part of this work will deal with the reciprocal influence between NK cells and MM cells during tumor progression in BM microenvironment. To this aim, a murine model of multiple myeloma was first characterized and validated, and then used to evaluate several features of NK cell behavior, including their killing efficacy against tumor cells, their expression of maturation markers and their capacity to reach and reside within the BM.

The balance between chemokine receptor expression, both homeostatic (i.e. CXCR4) and inflammatory (i.e. CXCR3) ones, and the environmental concentration of the respective

ligands has a critical relevance in this latter aspect. Moreover, the knowledge of the mechanisms that guide lymphocyte retention within the tumor site at different stages of MM progression will be extremely valuable in light of a future NK cell-based adoptive transfer therapy for this incurable disease.

3. Methods

Mouse Models. Male and female CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ mice were purchased from JAX Mice and Services (Bar Harbor, Maine, USA), and were bred and housed in the animal facility of the Istituto Nazionale Tumori Regina Elena, in Rome. For all CX3CR1 experiments, female mice between 6 and 12 weeks of age were used. Female C57BL/KaLwRij mice were housed in the animal facility of the Istituto Superiore di Sanità (ISS), in Rome. At the age of 5 weeks mice were injected with 2x10^6 5TGM1-LUC cells, and then sacrificed after 2, 3 or 4 weeks of tumor growth. Multiple myeloma progression was assessed at the moment of the animal sacrifice by staining BM and splenic cells with IgG2b-specific antibodies. Wild type C57BL/6 mice Ly5.1 (CD45.1) and Ly5.2 (CD45.2) were purchased from Charles River (Calco, Italy) and used for adoptive transfer experiments. Animals were kept under specific pathogen-free conditions, in accordance with institutional guidelines for animal care and use. Mice were killed by cervical dislocation and BM, spleen, liver, blood and inguinal LNs were collected.

Cell preparation and NK cell enrichment. For FACS staining experiments, BM, spleen, liver, LNs and blood were processed as described in [323]: briefly, BM cells were isolated by extensive flushing of femurs and tibias with PBS, while cells from spleen, liver and LNs were obtained by mechanical disruption on a 70-μm cell strainer (Falcon, Becton Dickinson) with a rubber syringe plunger in RPMI 10% FBS. Hepatic white cells were further separated using Lympholyte (CL5031, Cedarlane Labs) and then washed and resupended in RPMI 10% FBS. Peripheral blood samples were obtained by tail bleeding and collected in heparin containing tubes.

The NK cell enrichment from BM and spleen was performed in two steps: first, CD3+ and CD19+ (and Gr-1+ only for BM) cells were depleted with Dynabeads Biotin Binder Kit, Invitrogen (Paisley, UK) according to manufacturer's instructions. Secondly, DX5+ cells

were positively selected using CD49b (DX5) MicroBeads Kit, Miltenyi (Bergisch Gladbach, Germany). NK cell frequency was always > 80% of total cells.

Antibodies, cytokines and reagents. Several antibodies directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) 5.5, allophycocyanin (APC), PE-cyanine (cy)7, APC-eFluor 780, or biotin, and specific for the following antigens (clone name in parentheses) were used in this study: NK1.1 (PK136), CD3ε (145-2C11), CD49b (DX5), CD11b (M1/70), KLRG1 (2F1), CD19 (6D5), c-Kit(2B8), DNAM-1 (TX42.1), CD127 (A7R34), IFN-γ (XMG1.2), CD45.1 (A20), CD45.2 (104), CXCR4 (2B11), CXCR3 (220803), CCR1 (643854), CCR5 (C34-3448), CD107a (1D4B), BrdU (3D4), CD138 (281-2), IgG2b (RMG2b-1) and respective isotype controls. Abs and Pe-cy7, and PE-conjugated streptavidin were purchased from Pharmingen (Becton Dickinson, San Diego, CA), eBioscience (San Diego, CA), Biolegend (San Diego, CA) and R&D Systems (Minneapolis, MN). Alexa Fluor 647- and FITC-conjugated Annexin V was purchased from Biolegend. PE-conjugated mouse anti-human IgG was purchased from Jackson Immunoresearch Laboratories. Purified rat anti-mouse Ly49G2 (4D11), mouse anti-mouse Ly49C/I (5E6) mAb were from Pharmingen.
Recombinant mouse IL-12 and human CXCL12, IL-15 and IL-2 were from Peprotech EC (London, UK). Bovine Serum Albumin (BSA), Polyinosinic-Polycytidylic acid (Poly-I:C), bromo-deoxyuridine (BrdU), saponin, AMD-3100 and PKH26 were from Sigma-Aldrich (St. Louis, MO).

In vitro functional assays. In order to perform degranulation assays, BM and/or splenocytes from CX3CR1$^{+/GFP}$, CX3CR1$^{GFP/GFP}$ or healthy C57BL/KaLwRij female mice were collected, washed in PBS and incubated overnight in complete medium supplemented with IL-15 (100 ng/ml) at 10^6 cells/ml. Stimulated cells were washed in RPMI 10% FBS, Hepes 10 mM, and $5x10^5$ cells were resuspended alone, with the mouse lymphoma cell line YAC-1 or with 5TGM1 MM cell line in the same medium supplemented with monensin (0.1 M) and IL-2 (500 U/ml) at 1:2 effector:target (E:T) ratio. Cells were then

incubated at 37°C, 5% CO_2 4 h in 96 well-plate. During the last 3h, PE- or FITC-conjugated anti-mouse CD107a or IgG was added directly into each well. Next, cells were harvested and stained with anti-CD3ε, anti-NK1.1 and anti-KLRG1 mAbs, and then analyzed by FACS.

Intracellular staining for IFN-γ was performed as described in [323] with cells isolated from BM and spleen and cultured for 18 h in complete medium, in presence of IL-2 (500 U/ml) and IL-12 (100 ng/ml) or of IL-2 alone (500 U/ml). Brefeldin A (10 mg/ml) was added during the last 6 hours of incubation.

In competitive in vitro experiments, splenocytes from Ly5.1+/Ly5.2+ CX3CR1$^{+/GFP}$ mice and Ly5.1-/Ly5.2+ CX3CR1$^{GFP/GFP}$ mice were mixed at 1:1 ratio before cytokine stimulation. Cells derived from the two mouse strains were identified by anti-CD45.1 mAb staining.

Chemotaxis assays of BM (5×10^5) and spleen (1×10^6) cells were performed in 5 μm-pore Transwell insert using the indicated concentrations of CXCL12 as described in [323].

For the cytotoxicity assay, YAC cells were treated for 1h at 37°C with 1mCi/mL of ^{51}Cr per 10^6 cells; target cells were then washed and co-incubated with enriched (or sorted) NK cells from BM or spleen for 4 hours at different effector-target ratio in MW96. Radioactivity was analysed with a β-counter, and spontaneous ^{51}Cr release was also assessed. Killing frequency was evaluated by dividing the number of killed targets by the number of effector cells for each E:T ratio.

Immunostaining and FACS analysis. Cells from the indicated organs were washed and resuspended in staining buffer (PBS without Ca^{2+} Mg^{2+}, BSA 0,2%, EDTA 2mM and NaN_3 0,025%). The anti-CD16/32 (clone 24G2) mAb was added for 10 minutes on ice to prevent non-specific and Fc-mediated binding. Then, cells were stained with the indicated mAbs diluted in staining buffer for 20 minutes on ice.

Staining for CXCR4 was performed after cell incubation for 2 h at 37°C in 5% CO_2 to optimize membrane expression of the potentially internalized receptor. For all chemokine receptor staining, cells were incubated 40 minutes on ice.

Intracellular staining for IgG2b was performed by fixing cells with 1% paraformaldehyde (PFA) in PBS, and by permeabilizing them with 0,2% TWEEN in PBS. Cells were incubated with biotinilated anti-IgG2b specific mAb, washed, and stained with PE-conjugated streptavidin.
Samples were analyzed using a FACSCanto II (BD Biosciences) and data were elaborated using Diva Version 6.1.3 (BD Biosciences) or FlowJo Version 7.6 software (TreeStar).

ELISA assay. Several chemokine concentration levels were assessed in BM supernatants and sera. BM supernatants were obtained flushing BM cells in ice-cold PBS, and centrifuging cells twice, at 200 and 8000 rcf, and were stored at -80°C. To assess the concentration of CXCL12, CXCL9 and CXCL10 we made use of specific DuoSet Kits (R&D, Minneapolis, US) according to manufacturer's instruction. For BM supernatants, chemokine concentrations were normalized to total protein concentration present in the extracellular fluid, assessed with Bio-Rad Protein Assay Kit, Biorad (Hercules, CA).
BM supernatants data are expressed as pg of protein/mg of total extracellular proteins, while as pg of protein/mL for serum chemokine level.

Adoptive transfer experiments

CX3CR1-related experiments. Sorted KLRG1+/CX3CR1+ and KLRG1+/CX3CR1- (purity > 95%) splenic NK cells from $CX3CR1^{+/GFP}$ mice were adoptively transferred by i.v. tail injection into CD45.1/.1 C57BL/6 WT female mice, and BM, spleen and liver were collected after 12 days. Similarly, total splenocytes from $CX3CR1^{+/GFP}$ mice were i.v. injected into WT recipients, and organs were collected after 18 hours to detect the localization of GFP-expressing NK cells.
Splenocytes from CD45.1/.2 $CX3CR1^{+/GFP}$ and CD45.2/.2 $CX3CR1^{GFP/GFP}$ mice were collected, mixed at 1:1 ratio and stained with the cell fluorescent dye PKH26. $15x10^6$ splenocytes were i.v. injected in C57/BL6 recipient mice. An aliquot was saved and stained to assess the input ratio (calculated as $[CD45.1-]_{cells}/[CD45.1+]_{cells}$).

After 150 minutes, 24 and 36 hours, BM, spleen, liver, LN and blood cells were collected, and transferred NK cells were identified by immunofluorescence and FACS as PKH26+ NK1.1+ CD3- cells. To discriminate between $CX3CR1^{+/GFP}$ and $CX3CR1^{GFP/GFP}$, PKH26+ donor cells were identified as CD45.1+ ($CX3CR1^{+/GFP}$) or CD45.1- ($CX3CR1^{GFP/GFP}$). The homing index was calculated dividing the $[CD45.1-]_{organ}$/ $[CD45.1+]_{organ}$ cell ratio by $[CD45.1-]_{input}$/ $[CD45.1+]_{input}$ ratio among the PKH26 labeled cells.

In another set of experiments, 10^6 sorted PKH26–labeled KLRG1+GFP- (purity > 85%, no GFP+ cell) or KLRG1+GFP+ splenic NK cells (purity > 95%) from $CX3CR1^{+/GFP}$ (CD45.1/.2) and $CX3CR1^{GFP/GFP}$ (CD45.2/.2) donor mice were i.v. injected into C57/BL6 recipient mice. After 12h and 36h, BM, spleen, liver and blood were collected, and transferred cells were identified as PKH26+ CD45.1+ ($CX3CR1^{+/GFP}$) or PKH26+ CD45.1- ($CX3CR1^{GFP/GFP}$).

In a third set of experiments, C57/BL6 recipient mice were i.v. injected with $5x10^6$ enriched splenic NK cells from $CX3CR1^{+/GFP}$ CD45.1/.2 and $CX3CR1^{GFP/GFP}$ CD45.2/.2 donor mice at 1:1 ratio. Enrichment of NK cells was performed by depleting CD3+ and CD19+ cells with Dynabeads Biotin Binder (Life Technologies), and NK cell percentage ranged around 40-45%.

MM-related experiments. Total splenocytes from 8 weeks old female C57BL/KaLwRij were stained intracellular fluorescent dye PKH26, and $12x10^6$ PKH+ cells were injected in control and tumor-bearing mice, after 3 weeks of tumor growth. BM, spleen, lymph nodes, liver and blood cells were collected after 18 hours, and were stained with CD3-, NK1.1- and KLRG1-specific conjugated antibodies.

Alternatively, C57BL/6 splenic NK cells were enriched with Dynabeads Biotin Binder Kit, were stained with CFSE 2 μM, and transferred in control and tumor-bearing mice. BM, spleen, lymph nodes, liver and blood cells were collected after 150 minutes or 18 hours, and stained with CD3-, NK1.1-, KLRG1-, CXCR4- or CXCR3-specific antibodies.

In a third set of experiments, $15x10^6$ splenocytes from CD45.1/.1 C57BL/6J WT and from CD45.2/.2 $CXCR3^{-/-}$ mice in a 1:1 ratio were stained with CFSE 2 μM and i.v. injected in control or tumor-bearing mice. After 150 minutes or 18 hours BM, spleen, liver and blood

cells were collected and stained as before. Donor-derived cells were identified according to their CFSE- expression and CD45 allelic variant.

Data were analyzed as percentage of NK cells or NK cell subsets in the organ compared to total injected counterpart, and as KLRG1-/KLRG1+ ratio within the NK cell transferred population, corrected for the KLRG1-/KLRG1+ ratio detected in the input.

To assess whether KLRG1- NK cells acquired KLRG1 expression during the 18 hours of adoptive transfer in vivo, splenic NK cells were first enriched depleting CD3+ and CD19+ cells, and then KLRG1- cells were sorted, stained with CFSE and injected into control and tumor-bearing mice. After 18 hours, BM, spleen and liver were collected, and transferred cells were analyzed for KLRG1+ expression.

Labeling of sinusoidal BM NK cell populations and in vivo mice treatments. BM sinusoidal NK cells were identified using the procedure described by Pereira and colleagues[326]. Briefly, 2 minutes before sacrifice, recipient mice were i.v. injected with 1 μg of PE-conjugated anti-CD45 or anti-α2 integrin chain (DX5) mAb diluted in PBS as described in [323]. After cell collection and staining, parenchymal and sinusoidal NK cells can be identified as PE- and PE+ cells, respectively.

AMD-3100 (100 μg diluted in 50 μL of PBS) was subcutaneously injected, as previously described[113]. At the end of the treatment, mice were sacrificed and their organs collected for immunostaining and FACS analysis.

For MM experiments, NK cell depletion was obtained by intraperitoneally injecting anti-NK1.1 mAb (clone PK136) (100 μg/mouse/injection) at day -2 before 5TGM1 transplantation, and the depletion was maintained with subsequent injections 2, 7 and 14 days after 5TGM1 infusion.

In vivo proliferation and survival assays. One milligram of BrdU diluted in 200 μl PBS was in vivo administered to mice by intraperitoneal (i.p.) injection every 8 h for three times. Mice were sacrificed, and BM and spleen cells were collected and stained with anti-NK1.1, -CD3ε, and -KLRG1 specific mAbs. Following fixation and permeabilization, samples were

washed and treated with 0.45 μg/ml DNase. Samples were then incubated for 1 h at room temperature (RT) with anti-BrdU specific mAb, washed and analyzed by flow cytometry. Annexin V staining was performed on BM and splenic cells following the manufacturer's instructions.

Bone marrow chimeras. Eight week-old female C57BL/6 (CD45.1+/CD45.2-) mice were irradiated with 900 rads (in two 450 rad doses with an interval of 3 h). For single BM chimeras, irradiated mice were reconstituted by i.v. injection of $2x10^7$ BM cells derived from either CD45.1/.2 $CX3CR1^{+/GFP}$ or CD45.2/.2 $CX3CR1^{GFP/GFP}$ mice. For mixed BM chimeras, 2x107 BM cells from CD45.1/.2 $CX3CR1^{+/GFP}$ and CD45.2/.2 $CX3CR1^{GFP/GFP}$ or CD45.2/.2 $CX3CR1^{+/GFP}$ mice were i.v. injected in irradiated hosts in a 1:1 ratio. In some experiments, irradiated mice were reconstituted with BM cells from NK cell-depleted mice. Depletion of NK cells was here obtained in vivo by i.p. injection of anti-NK1.1 mAb (clone PK136) (100 μg /mouse/injection) at day -2 and -1 in donor mice before sacrifice. NK cell depletion was always controlled by FACS staining for CD3/NK1.1 the PBMCs of donor mice before reconstitution. Organs of recipient mice were collected after 5 weeks, and donor-derived cells were identified with anti-CD45.2 and anti-CD45.1 mAbs.

Poly-(I:C) treatment. $CX3CR1^{+/GFP}$ and $CX3CR1^{GFP/GFP}$ mice were i.p. injected with 150 μg of Poly-(I:C), diluted in PBS. BM, spleen and liver cells were collected at 6, 18 and 72 hours after the injection. The total number of BM cells corresponded to the cell count from two tibias and femurs. Cells were stained with anti-NK1.1-, anti-KLRG1-, and anti-CD3ε-specific mAbs to enumerate total NK cells (CD3- NK1.1+) and subsets of NK cells. NK cell tissue redistribution during inflammation was then quantified as percentage of NK cell number in Poly-(I:C)-treated versus untreated control mice.

Statistics. Unpaired student's t test was used to compare experimental groups, and a p value below 0.05 was considered statistically significant. To analyze competitive in vitro

assays or adoptive transfers, paired student's t test was used to compare experimental groups, and likewise a p value below 0.05 was considered statistically significant.

4. Results

4.1 First part – CX3CR1 in NK cell development and function

4.1.1. Terminally differentiated NK cells express CX3CR1 in BM and in peripheral organs

We initially evaluated CX3CR1 expression on NK cells using a genetically modified mouse model, in which GFP cDNA replaced one copy of *cx3cr1* gene, but was still under its original transcriptional promoter[327]. This model allowed us to track CX3CR1-expressing cells by analysing GFP positivity with a flow cytometric approach, distinguishing NK cells as CD3-/NK1.1+ lymphocytes, and within this population cell subsets were defined according to CD11b and KLRG1 expression.

Figure 12b shows the CX3CR1+ NK cell frequency is high in peripheral organs (liver and spleen), while their proportion is much lower within BM. Likewise, adoptively transferred CX3CR1-expressing NK cells preferentially localized in extramedullary organs 18 hours after injection (Figure 12c). When BM NK cell subsets were analysed, CX3CR1 expression was found to be developmentally regulated, as it progressively increased from CD11b^{low}/KLRG1- (6.3±1.5 %) to CD11b^{high}/KLRG1- (10.8±2.3 %), reaching its maximum levels in the CD11b^{high}/KLRG1+ subset (27.8±4.1 %), although the latter subset expressed much higher levels of CX3CR1 in spleen (43±3.2 %) and liver (50±3 %). Conversely, in spleen and liver the frequency of CX3CR1-expressing NK cells was equal in CD11b^{low}/KLRG1- and CD11b^{high}/KLRG1- subsets, but was markedly increased in CD11b^{high}/KLRG1+ population (Figure 12e).

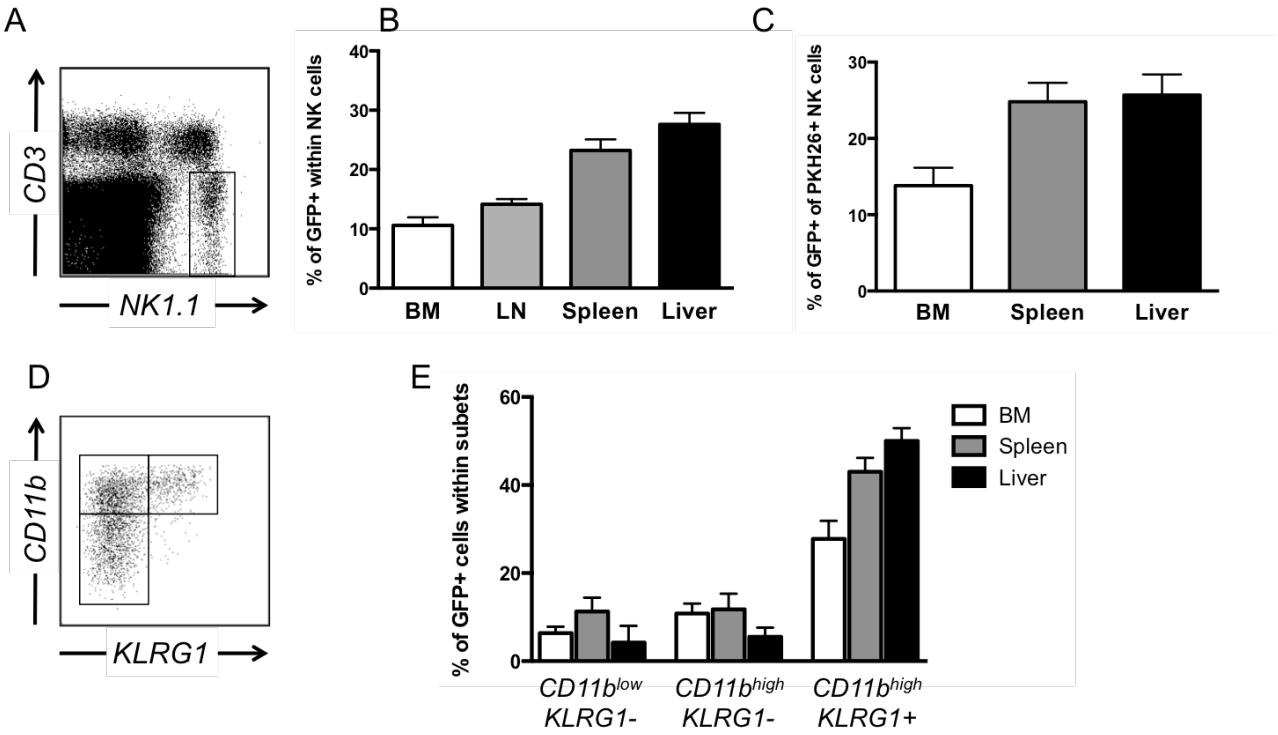


Figure 12 – *CX3CR1 is preferentially expressed by peripheral NK cells and more mature NK cell subsets.* CD3-/NK1.1+ lymphocytes (a) from BM, inguinal lymph nodes, spleen and liver of CX3CR1$^{+/GFP}$ mice were analyzed for GFP expression (b). CX3CR1+/GFP-derived splenocytes were stained with PKH-26 and adoptively transferred into WT hosts. The frequency of GFP+ cells among PKH-26+ cells was assessed 18 hours after transfer, in the represented organs (c). Representative dot plot of a staining for CD11b and KLRG1 (d) to analyze GFP expression within NK cell subsets (e). Data are expressed as mean values ± SEM. At least 6 CX3CR1$^{+/GFP}$ and WT donor mice were used in three independent experiments.

4.1.2. CXCR4 and CX3CR1 expression levels inversely correlate on NK cells

Given the heterogeneous levels of CX3CR1 within NK cell subpopulations and their different trafficking capacities, the following experiments were aimed at determining whether other chemokine receptors could be differentially regulated in the subsets of interest. In accordance with the previous literature, KLRG1- BM NK cells (that from here on will include CD11b^{low}/KLRG1- and CD11b^{high}/KLRG1- NK cells) had a higher CXCR4 expression levels in comparison to KLRG1+ cells, but an additional difference was recorded between KLRG1+/CX3CR1- and KLRG1+/CX3CR1+ NK cells (Figure 13a,b),

suggesting a further level of CXCR4 regulation. This phenotypic variation was also functionally significant, as in in vitro chemotaxis assay against CXCL12 the NK cell subpopulations behaved accordingly to their CXCR4 expression, displaying a higher migration index in the KLRG1- subset and a lower responsiveness to CXCL12 in KLRG1+/CX3CR1+ subset (Figure 13c).

This functional feature identified two additional NK cell subsets that differed for their distinct trafficking capacities, within the KLRG1+ NK cell population.

The differential CXCR4 expression might indeed suggest a dissimilarity in BM compartmentalization of the two subsets, as CXCL12 is highly produced on CXCL12 abundant reticular (CAR) cells, which are present in the marrow parenchymal compartment, while are absent in the vascular part, also known as BM sinusoidal compartment. By means of a novel method that allows the identification of sinusoidal vs parenchymal BM cells[326], we show that NK cell subsets are differentially distributed in the two BM compartments, as within KLRG1-, KLRG1+/CX3CR1- and KLRG1+/CX3CR1+ NK cells 11%, 44% and 87%, respectively, were localized in the sinusoidal space (Figure 13d). Notably, the KLRG1- subset represents the vast majority of total BM NK cells, meaning that about 86% of BM NK cells are parenchymal, while only a little proportion, substantially consisting of KLRG1+ fraction, is sinusoidal.

Considering the data obtained so far, we hypothesized that the differential BM positioning of the three NK cell subsets in analysis might be regulated, at least partially, by their diverse expression levels of CXCR4. To test this hypothesis, we s.c. administered AMD3100, a pharmacologic inhibitor of CXCR4, which is known to mobilize, among other cell types, BM NK cells to the peripheral blood[113]. To analyse changes in NK cell BM compartimentalization, PE-conjugated anti-DX5 antibody was i.v. injected one hour after AMD3100 administration, and mice were then sacrificed. Figure 13g,h shows how the CXCR4 inhibitor administration induced a significant shift in KLRG1- NK cell subset localization from parenchyma to the sinusoid, while both KLRG1+/CX3CR1- and KLRG1+/CX3CR1+ NK cells were unaffected by the treatment. Taken together, these data demonstrate that CXCR4 function is fundamental for NK cell retention in BM

parenchyma, particularly for KLRG1- subset, while it seems to be irrelevant for their positioning in the sinusoids. This conclusion suggests the existence a CXCR4-dependent directional transfer from parenchymal to sinusoidal space to take place during NK cell maturation, which preludes to the definitive exit of terminally differentiated NK cells (i.e. KLRG1+ cells) from BM to the peripheral circulation.

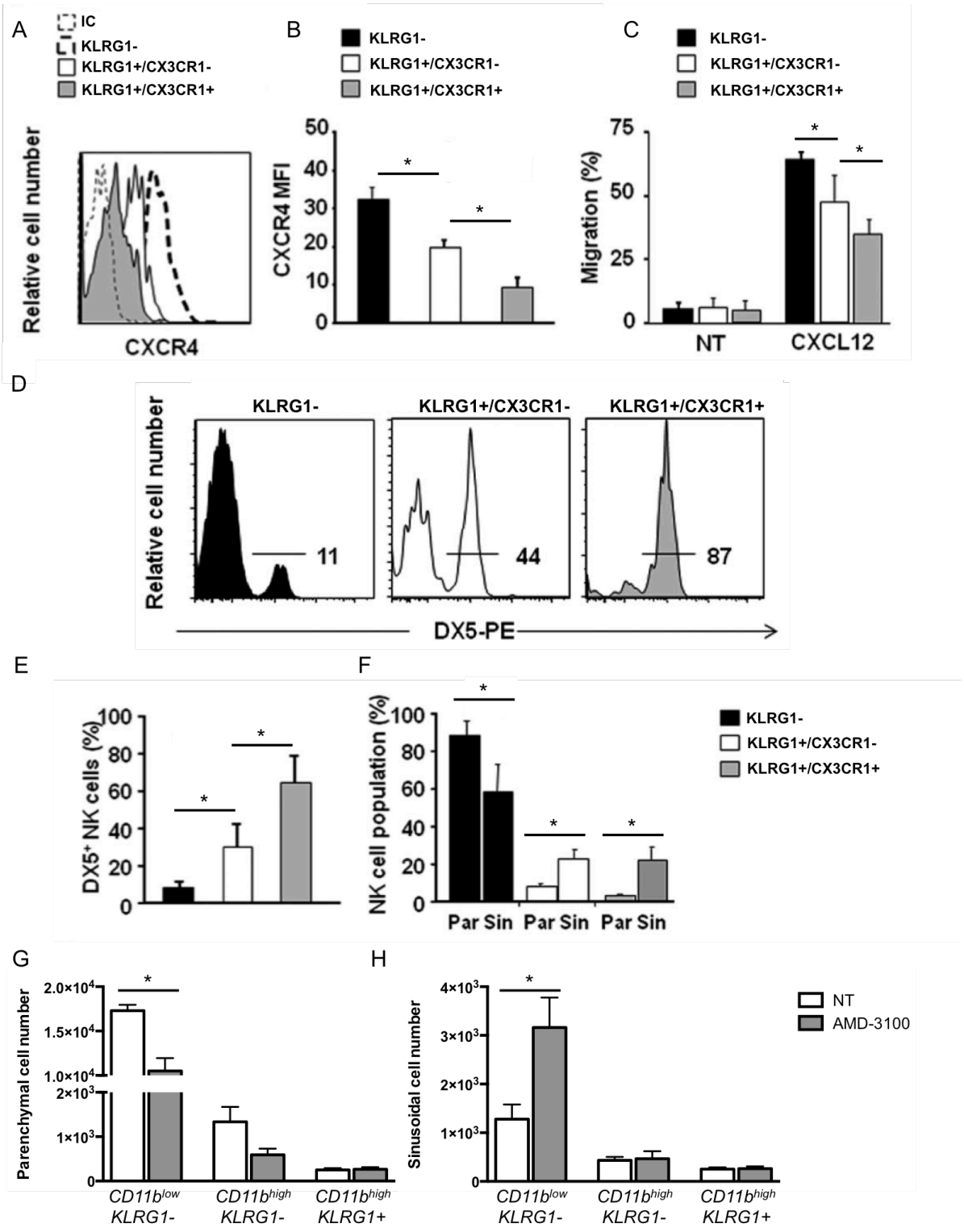


Figure 13 – ***KLRG1+/CX3CR1+ NK cells express low levels of CXCR4 and mainly localize in BM sinusoids.*** Overlay of CXCR4 expression (a) and representation of mean values of median fluorescence intensity (MFI) on NK cell subsets (b). In vitro chemotaxis assays were performed with CX3CR1$^{+/GFP}$ splenocytes, against medium or CXCL12 (200 ng/mL), for 2 hours. Data are displayed as frequency of migrated cells over input cells (c). d and e show representative histograms and the quantification of the sinusoidal fraction of NK cell subsets. The relative contribution of the NK cell subsets was then considered within total parenchymal or sinusoidal BM NK cells (f).

AMD-3100 was subcutaneously administered to CX3CR1+/GFP mice, and 1 hour later PE-conjugated anti-DX5 mAb was i.v. injected, before mice sacrifice. Parenchymal (g) and sinusoidal (h) localization of NK cell subsets was then assessed by FACS. Data are expressed in number of cells for each subpopulation. Each bar graph show data from at least 6 mice from 3 independent experiments. * = $p < 0.05$.

4.1.3. CX3CR1 identifies a terminally mature KLRG1+ NK cell subset with reduced effector capacities

The distinct migration properties of the two KLRG1+ NK cell subsets identified by the CX3CR1 expression could also imply the presence of functional differences, in terms of IFNγ production upon cytokine stimulation or cell cytotoxicity against NK cell-sensitive targets, such as YAC-1 thymoma line. Thus, BM- and spleen-derived cells were cultured overnight with IL-2 + IL-12 or IL-2 alone as a negative control, to assess NK cell IFNγ production. Figure 14 shows that KLRG1+/CX3CR1- NK cells from both organs displayed a higher cytokine production rate than their KLRG1+/CX3CR1+ counterpart. Nevertheless, this experimental approach takes into account also the effect of other cell types, both hematopoietic and non-hematopoietic, upon the stimulation with IL-2 ± IL-12. Thus, to assess unequivocally the intrinsic NK cell functional properties, splenic KLRG1+/CX3CR1- and KLRG1+/CX3CR1+ NK cells were sorted and treated overnightwith IL-2 ± IL-12 or with IL-15, to study IFNγ production and their ability lo kill target cells, respectively. Both assays showed the reduced functionality of KLRG1+/CX3CR1+ NK cells, as compared to the KLRG1+/CX3CR1- counterpart.

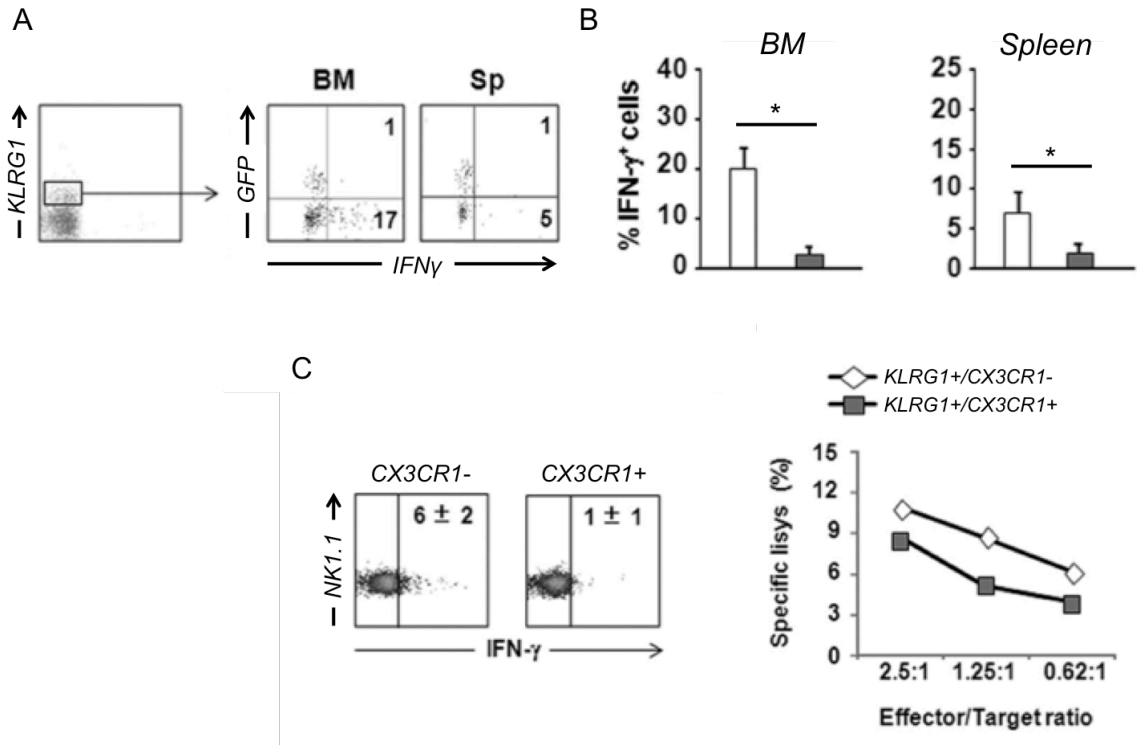


Figure 14 – ***Distinct functional properties of CX3CR1+ and CX3CR1- KLRG1+ NK cells.*** a) Previously activated with IL-2 with or without IL-12 BM cells or splenocytes from CX3CR1$^{+/GFP}$ mice were incubated with Brefeldin A for 6 hours and then stained for intracellular IFNγ. KLRG1+ NK cells were analysed for their expression of CX3CR1/GFP and IFNγ (a). Histograms in b represent the quantification of three separate experiments shown in a for BM and spleen. Then, the two KLRG1+ NK cell subsets were sorted from CX3CR1$^{+/GFP}$ splenocytes, and tested for IFNγ production (c, left) and for cytotoxicity against the lymphoma line YAC-1 (c, right). Data are representative of 1 of at least 3 performed experiments. * = $p < 0.05$.

The more pronounced sinusoidal localization, the reduced CXCR4 expression and the upregulation of CX3CR1 on NK cells during maturation suggested that KLRG1+/CX3CR1- and KLRG1+/CX3CR1+ subsets might be developmentally related. Thus, we set up an adoptive transfer experiment where we injected purified KLRG1+/CX3CR1- or KLRG1+/CX3CR1+ NK cells, derived from the spleen of CD45.2+ CX3CR1$^{+/GFP}$ donors, into WT CD45.1+ recipients. After 12 days, organs were collected from recipient mice, and the phenotype of donor-derived NK cells was analyzed. As represented in Figure 15, KLRG1+/CX3CR1- NK cells gave rise to KLRG1+/CX3CR1+ cells, but not the opposite. This result clearly indicates that high levels of CX3CR1 are acquired at later stages of NK cell

differentiation, being KLRG1+/CX3CR1+ population a more mature stage than KLRG1+/CX3CR1- cells. In addition, once CX3CR1 expression is acquired by KLRG1+ cells, is stably maintained on their cell surface.

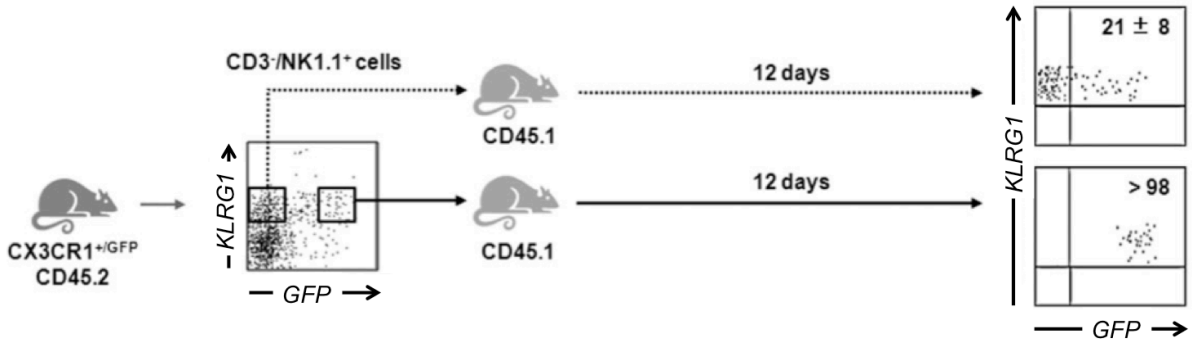


Figure 15 – *KLRG1+/CX3CR1- cells give rise to KLRG1+/CX3CR1+ cells, but not vice versa.* Highly purified KLRG1+/CX3CR1- or KLRG1+/CX3CR1+ from CD45.2+ CX3CR1$^{+/GFP}$ spleen were injected into CD45.1 WT mice, and their frequency was assessed by FACS in the recipient BM, spleen and liver after 12 days, staining for CD3/NK1.1/CD45.1/CD45.2. Number in plots indicate the mean frequency of GFP+ cells among transferred KLRG1+ NK cells. Data are representative of 1 of at least 3 experiments performed.

To further characterize the two subsets, we also determined the surface expression level of several markers, many of which are produced at different times during NK cell development, like c-kit and CD127 (on more immature NK cells) or Ly49 MHC class I receptors (on functional NK cells). In agreement with the data obtained so far, KLRG1+/CX3CR1+ NK cells expressed lower levels of c-kit and higher levels of Ly49 C/I as compared to KLRG1+/CX3CR1- and more so when compared to KLRG1- NK cells, although no differences between the two KLRG1+ subsets were observed concerning CD94, DNAM-1, Ly49H and Ly49G2 (Figure 16).

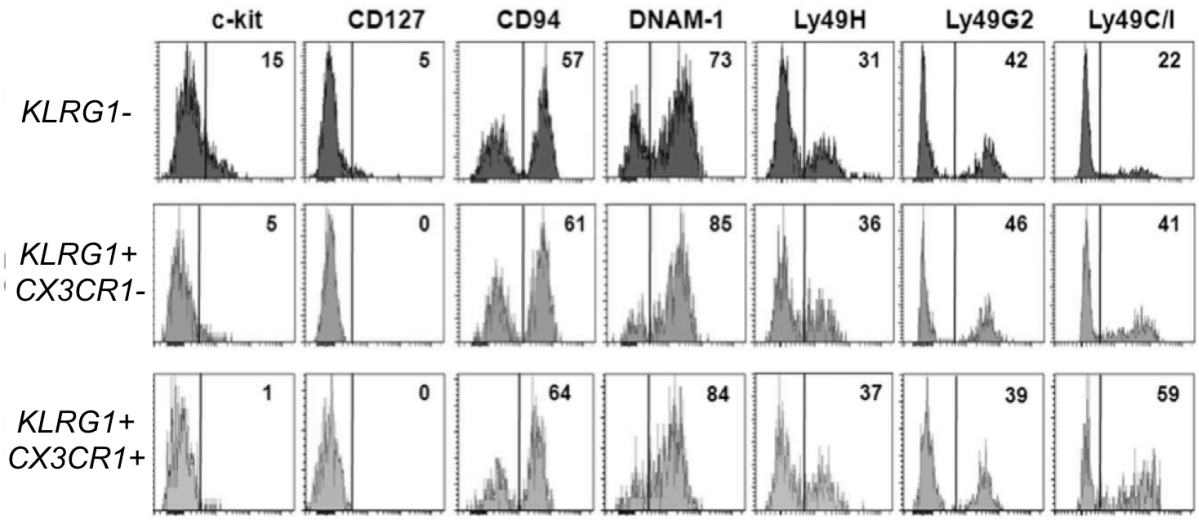


Figure 16 – *Surface receptor profile of NK cell subsets*. Splenocytes from CX3CR1$^{+/GFP}$ mice were stained CD3, NK1.1 and KLRG1, and for the extracellular receptors indicated above. Control for m157-Fc (Ly49H) staining was NKG2D-Fc followed by PE-conjugated anti-human IgG, while anti-Ly49C/I and anti-Ly49G2 staining controls were PE-conjugated anti-mouse IgGa/b and anti-rat IgG, respectively (not shown). Numbers in the histograms represent the positive cells for each subset, of a representative analysis out of 3 separate experiments performed.

Taken together, these data evidenced the existence of two functionally and developmentally distinct NK cell subsets within the KLRG1+ subpopulation, that differently localize in BM compartments. Albeit, the actual function of the receptor CX3CR1, and how and whether it contributes to these differences remained to be determined.

4.1.4. KLRG1+ NK cells accumulated in BM and LNs of mice lacking CX3CR1

The next issue to tackle was the biologic role of the CX3CL1 receptor on NK cells, and to this aim we adopted a so called "loss of function" approach, using CX3CR1$^{GFP/GFP}$ mice, in which both alleles of *cx3cr1* gene were replaced by two copies of GFP cDNA as compared to the heterozygous CX3CR1$^{+/GFP}$ mice, considered as controls. The interpretation of the data deriving from the study of these knock-in mice is complex, since the expression of

GFP does not identify cells with a functional CX3CR1 anymore, but instead cells that mantain the CX3CR1 trascriptional activity (GFP cDNA is placed under *cx3cr1* promoter). Moreover, being most of experimental techniques described in this thesis related to FACS, the issue of GFP fluorescence intensity must be taken in consideration when analyzing changes of GFP+ cells frequency: in fact, having one *gfp* allele instead of two, $CX3CR1^{+/GFP}$-derived GFP+ cells display a lower GFP fluorescence intensity than $CX3CR1^{GFP/GFP}$-derived GFP+ cells. Nevertheless, our experiments were adequately controlled not to be biased by this difference.

The first step taken was to analyse total NK cell frequency and number in BM, spleen, liver and peripheral lymph nodes (LNs) (Figure 17a). No differences were reported in the first three organs, while a significant increase of NK cell number in LNs was found (10^5 vs $5x10^5$ cells). Interestingly, this increase was mainly attributable to the total LN cell count, which was higher in CX3CR1-deficient mice, while NK cell frequency within lymphocytic population was not significantly altered. Furthermore, the phenotype evidenced an increased proportion of GFP-expressing NK cells in $CX3CR1^{GFP/GFP}$ mice in all the considered organs but the liver (not shown).

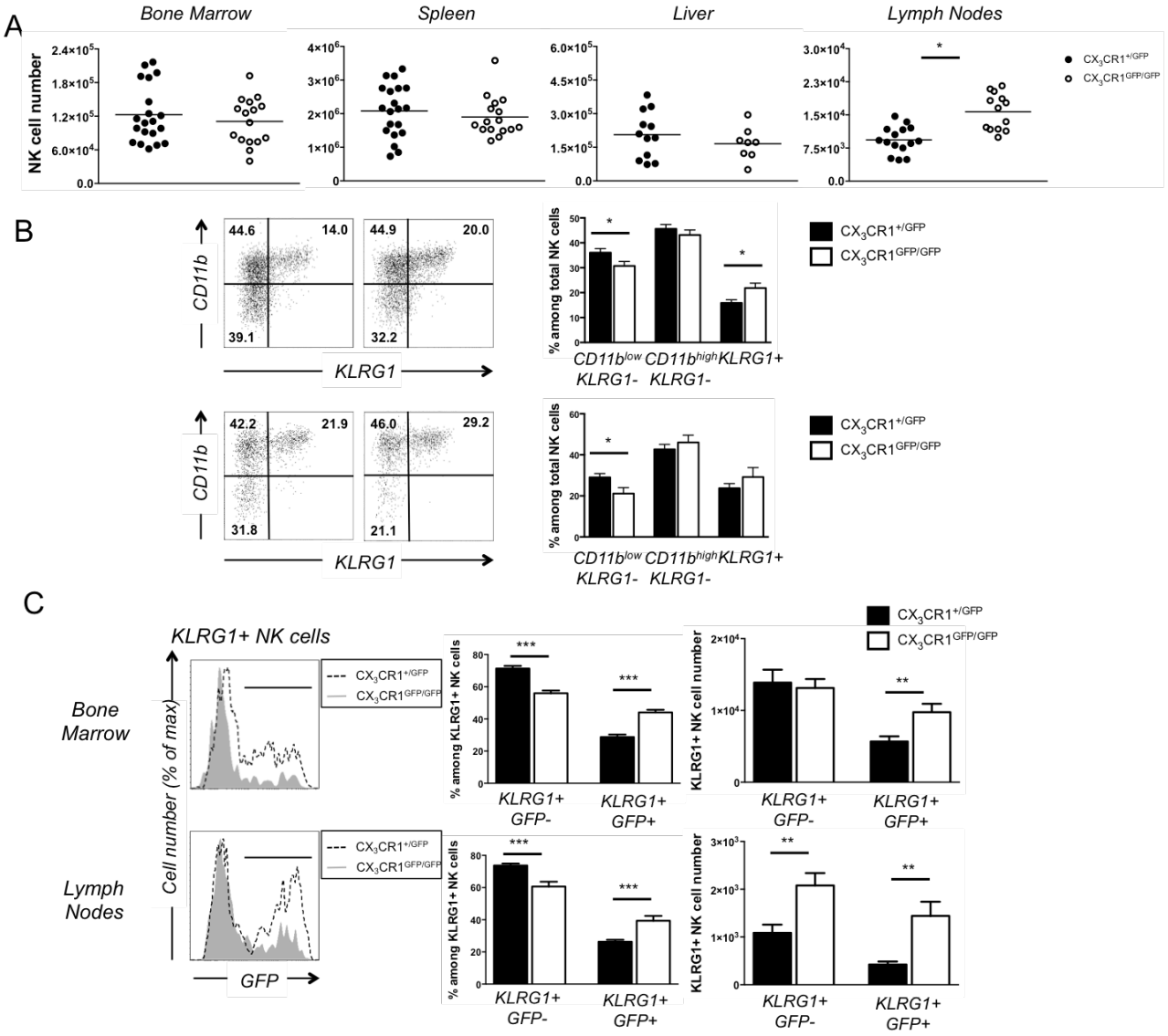


Figure 17 – ***Tissue distribution of NK cell and NK cell subsets in CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ mice.*** Single-cell suspensions were collected from several organs of CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ 6 to 10-week-old mice. BM was collected from one tibia and femur. After staining with selected mAbs, NK cells were gated as CD3-/NK1.1+ among lymphocytes. (a) Circles represent NK cell number in the indicated organ of each mouse. Among NK cells, subsets were identified as CD11b^{low}/KLRG1-, CD11b^{high}/KLRG1-, and CD11b^{high}/KLRG1+ (B), and among KLRG1+ as GFP+ and GFP- cells (c). (b) Dot plots showing NK cell subset composition in BM (upper panel) and LNs (lower panel) of CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ mice are representative of at least eight animals for each genotype in five independent stainings. Numbers in dot plots indicate the frequency of each subset within the NK cell population. Each column of the graphs represents the average frequency ± SEM of the indicated NK cell population among NK cells in BM (upper panel) and LNs (lower panel). (c) Histogram overlays compare the frequency of GFP+ cells within KLRG1+ population into BM and LNs of CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ mice. Graphs represent the average frequency (left panels) and the number (right panels) ± SEM of GFP- and GFP+ cells among KLRG1+ cells in CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ mice. * = $p < 0.05$.

When NK cell population was dissected into cell subsets, according to their expression of CD11b and KLRG1, we registered an increased number of CD11b^{high}/KLRG1+ fraction in the BM (about 1.5 fold), and of CD11b^{high} population (both KLRG1- and KLRG1+) in the LNs of CX3CR1-deficient mice, when compared to heterozygous controls (Figure 17b). Notably, KLRG1+ increase in BM was balanced only by the reduction in CD11b^{low}/KLRG1- cell frequency, the most immature subset, as the functionally competent CD11b^{high}/KLRG1- population did not change.

As CX3CR1 is mainly expressed by mature NK cell subsets, the increase of GFP+ NK cells observed in BM and LNs was likely related to the augmented proportion of KLRG1+ and CD11b^{high} cells in these organs. In turn, KLRG1+ cell number increase in BM of CX3CR1-deficient mice was entirely due to the expansion of the GFP+ fraction, while the KLRG1+/GFP- cell number was unchanged (Figure 17c).

4.1.5. Neither CXCR4 expression nor its function are altered by CX3CR1 deficiency

Because our previous data showed that KLRG1+/CX3CR1- and KLRG1+/CX3CR1+ NK cells expressed different levels of CXCR4 and migrated differently towards a CXCL12 gradient in vitro, we stained BM cells from CX3CR1-deficient and –sufficient mice with anti-CXCR4 mAbs and tested them in in vitro chemotaxis assay. The graphs in Figure 18 make clear that CXCR4 expression levels were unchanged in the two strains, and also that no difference could be detected in terms of CXCL12-directed migration. Thus, the alteration of NK cell subset proportion in the BM of CX3CR1-deficient mice could not be linked to modifications in CXCR4-CXCL12 axis.

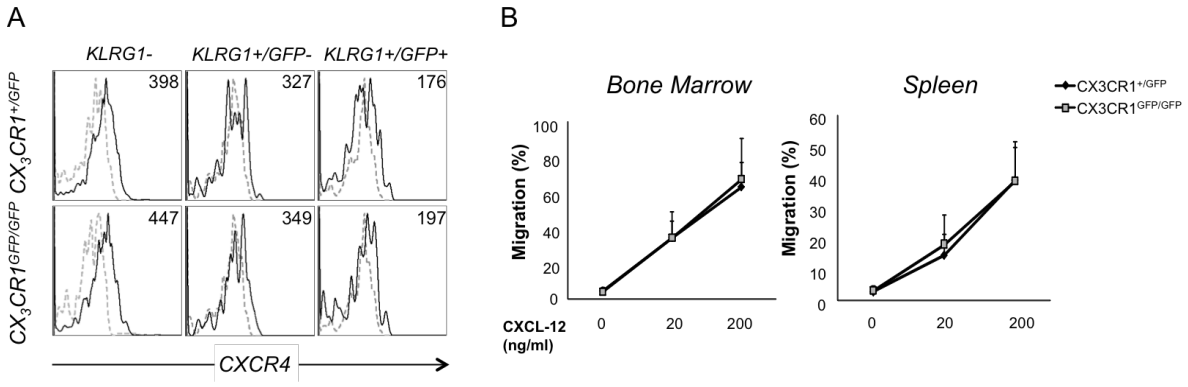


Figure 18 – *CX3CR1 deficiency does not impair CXCR4 expression and function on NK cells.* Immunofluorescence staining of BM cells with anti-NK1.1-, anti-CD3-, anti-KLRG1- and anti-CXCR4-specific (black line) or isotype control (dotted gray line) mAbs was performed, followed by FACS analysis (a). Histogram overlays show CXCR4 expression as compared to isotype control staining and number represents mean fluorescence intensity (MFI) of CXCR4 expression subtracted of isotype control MFI in KLRG1- KLRG1/GFP- and KLRG1+/GFP+ NK cells. One representative experiment of 3 performed is shown. b) Chemotaxis assays to different concentrations of CXCL12 were performed in 5μm pore Transwell insert. After 90 min, migrated cells were collected and stained with anti-NK1.1-, and anti-CD3-specific mAbs and counted by FACS analysis. Shown is the chemotactic response of BM (left) and splenic (right) total NK cells in response to migration medium with no chemokine or containing CXCL12. Data are expressed as percentage of input cells and represent the mean values ± SD from a total of 6 animals analyzed in 3 independent experiments.

4.1.6. NK Cell-intrinsic mechanism of action of CX3CR1

The unbalanced proportion of NK cell subsets, in particular within the BM, might indeed reflect a modulatory role for CX3CR1 in the process of NK cell development. At the same time, since other cell types have been described to express CX3CR1, it was possible that the altered phenotype found in CX3CR1-deficient animals could be due to an indirect effect exerted by other CX3CR1-expressing cells on NK cells in the BM.

To test this possibility, bone marrow chimera experiments were performed, in which WT CD45.1+ mice were sublethally irradiated and then reconstituted with CD45.1+/CD45.2+ CX3CR1$^{+/GFP}$ or CD45.2+ CX3CR1$^{GFP/GFP}$ BM-derived cells. Five weeks after reconstitution, organs were collected and NK cell phenotype was analysed by FACS. Donor-derived cells

were identified as CD45.2+ cells, in contrast to the residual amount of recipient-derived cells, which anyway were CD45.1+.

Figure 19 shows the slight but significant increase in frequency of CX3CR1$^{GFP/GFP}$-derived KLRG1+ NK cells, as compared to CX3CR1$^{+/GFP}$-derived counterpart in BM, but not in the spleen. As observed in adult mice, only the GFP+ fraction was accountable for this alteration, being the BM KLRG1+/GFP- cells almost unchanged in the two chimeras.

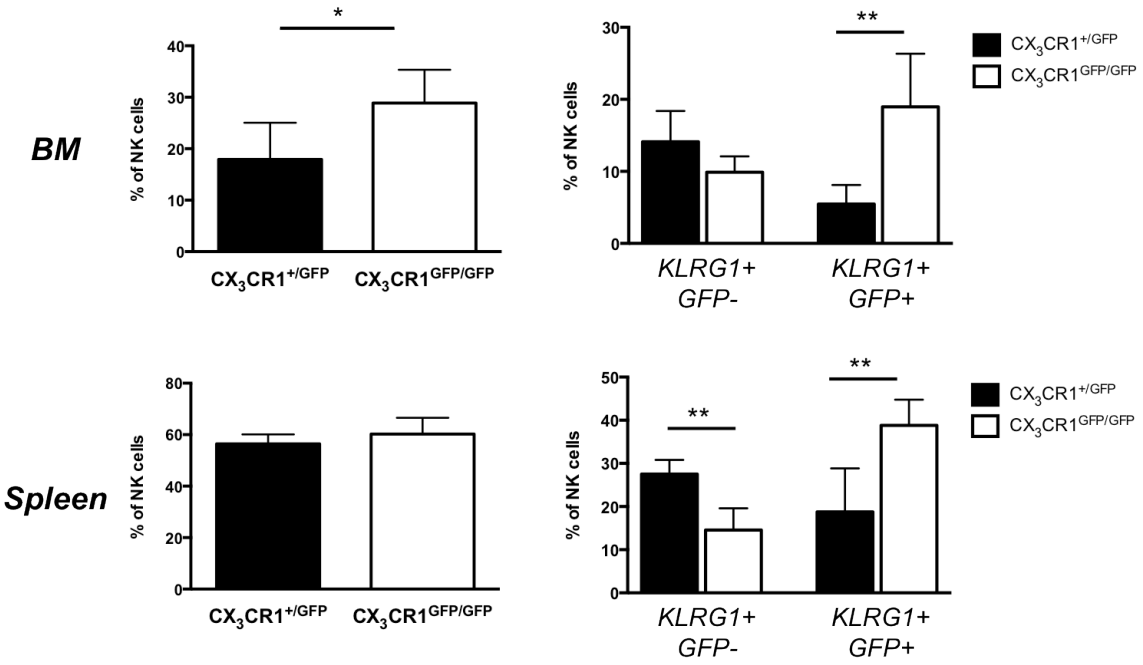


Figure 19 – *KLRG1+ NK cell accumulation is due to CX3CR1 effect on hematopoietic compartment.* CD45.1+ WT mice were irradiated and reconstituted with either CX3CR1$^{+/GFP}$ or CX3CR1$^{GFP/GFP}$ BM cells. After 5 weeks, donor-derived NK cell and NK cell subset frequency in BM and spleen was evaluated by staining with anti-CD3, NK1.1-, KLRG1-, CD45.1- and CD45.2-specific mAbs. In the left panels, the percentage of KLRG1+ cells within total NK cells is shown for BM and spleen, while on the right the frequency of KLRG1+/GFP- and KLRG1+/GFP+ is represented. Bar graphs are representative of at least 5 reconstituted mice for each genotype. * = $p < 0.05$; ** = $p < 0.01$.

The bone marrow chimeric reconstitution allows to estabilish whether the effect observed is, or is not, determined by the hematopoietic compartment, since the stroma in this case belongs to a WT, CX3CR1-sufficient mouse. These data demonstrate that the alteration of BM NK cell subset frequencies reported in CX3CR1-deficient mice is due to the action of

the same receptor on an hematopoietic cell, either resident or derived from BM environment.

However, as other hematopoietic components within the BM, such as the monocyte-macrophage lineage[327], DCs[328], and other lymphocytes[329], have been reported to express CX3CR1, an indirect effect on NK cells mediated by one or more of these cells could not be ruled out yet.

Thus, we generated mixed BM chimeric mice, by the infusion of equal amounts of BM cells from both CD45.1+/CD45.2+ $CX3CR1^{+/GFP}$ and CD45.2+ $CX3CR1^{GFP/GFP}$ genotypes into previously irradiated CD45.1+ WT hosts. In this condition, any phenotypical alteration observed would indeed be due to the direct role of CX3CR1 on NK cells.

The graphs presented in Figure 20b confirm that KLRG1+ NK cell subset expansion in the absence of CX3CR1 was cell intrinsic, as the same KLRG1+ increase was found in mixed BM chimeric mice, after 5 weeks. In addition, once again the KLRG1+/GFP+ was totally responsible for the expansion, as happened in normal mice (Figure 20c).

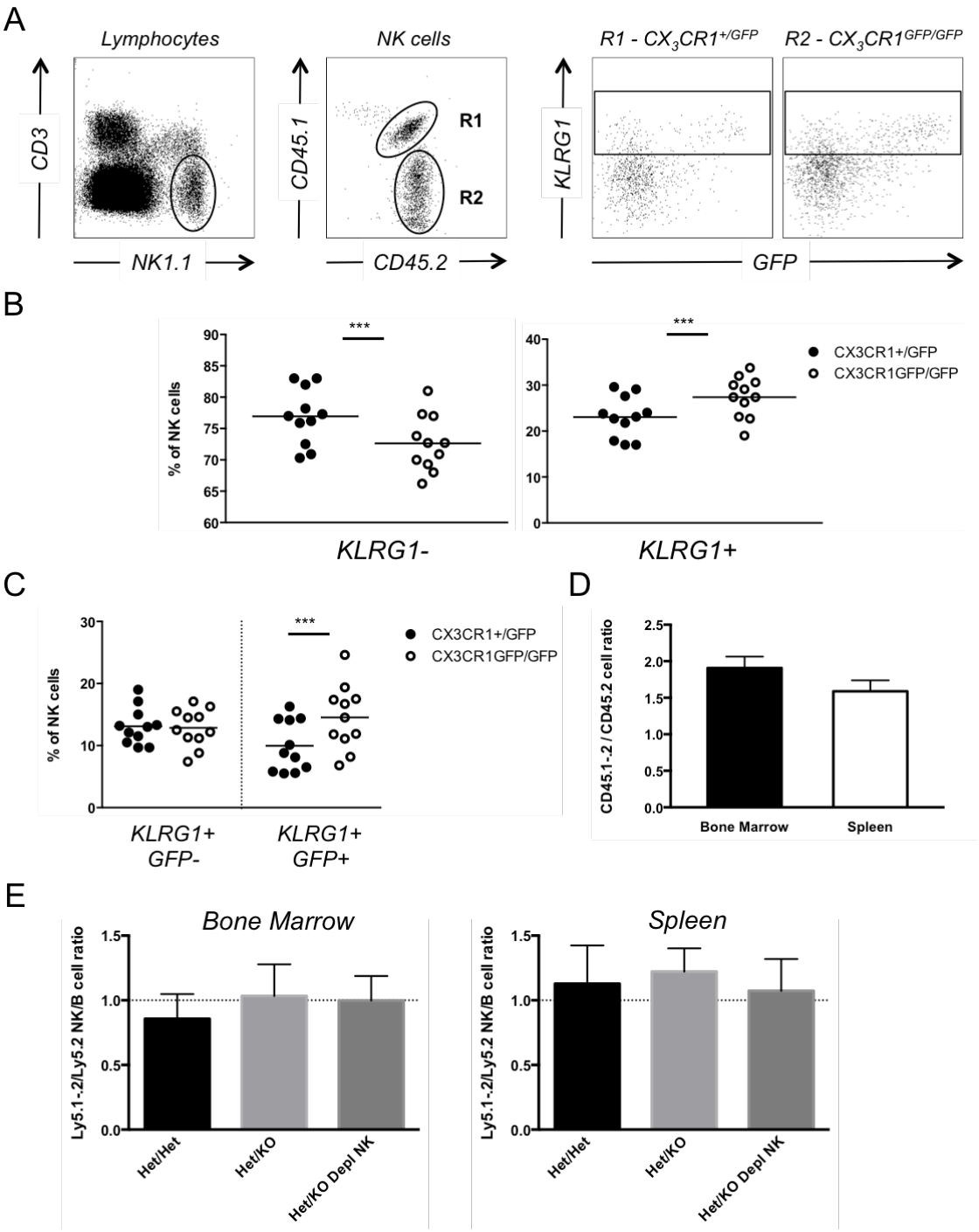


Figure 20 – ***Tissue accumulation of KLRG1+ NK cells is associated to a cell-intrinsic defect.*** BM cells fromCD45.1+/CD45.2+ CX3CR1$^{+/GFP}$ mice and from CD45.1-/CD45.2+ CX3CR1$^{GFP/GFP}$ mice were collected, mixed at 1:1 ratio, and transplanted into lethally irradiated CD45.1+/CD45.2- WT recipients. Organs were collected after 5 weeks, and donor-derived cells were identified with anti-CD45.1 and anti-CD45.2 Abs, with the gating strategy illustrated in (a). Donor-derived KLRG1- and KLRG1+ NK cell distribution in the BM of the recipient mice is shown in (b). c) GFP- and GFP+ NK cell distribution within KLRG1+ population. 11 animals per group were used in 5 independent experiments. The bar graph in (d) represents the total CD45.1+/CD45.2+ versus CD45.1-/CD45.2+ -derived cell ratio for BM and spleen after 5 weeks, for the

experiments illustrated in (b) and (c). e) Circles represent the percentage of the specified subset among NK cells in each mouse. The number of total NK cells derived from donor CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ was divided by the number of the total B (CD19+) cells of the corresponding donor mice. CX3CR1(D)$^{GFP/GFP}$ represents donor mice that were deprived of NK cells using anti-NK1.1–depleting mAb. Normalized Ly5.1.2/Ly5.2 BM and spleen NK cell ratios (R) of Ly5.1 recipient mice transplanted with the Ly5.1+/Ly5.2+ and Ly5.12/Ly5.2+ BM indicated in the horizontal axes are shown for 3 experiments performed with a total of 6 – 11 animals per group. * = $p < 0.05$.

Still, it was possible that putative long-lived mature KLRG1+ NK cells present in the BM of CX3CR1$^{GFP/GFP}$ donors could account for the difference observed after 5 weeks of reconstitution. To rule out this potential misinterpretation, CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ donor mice were first depleted of NK cells, through the administration of the anti-NK1.1 mAb PK136, then BM cells were collected and used as before to reconstitute irradiated WT hosts. Similarly to what was found with non-NK cell depleted donors, CX3CR1-deficient KLRG1+GFP+ NK cell frequency in the BM was increased.

As for the absolute number of donor-derived cells detected in BM chimeras, we evidenced some differences in the reconstitution rate of different genotypes being CD45+ cells derived from CX3CR1$^{GFP/GFP}$ 1.5-1.8 fold than CX3CR1$^{+/GFP}$ cells (Figure 20d). To solve this discrepancy, which could be due to an actual difference in engraftment potential and/or in BM cell maturation, the total NK cell number was adjusted to total B cell number (identified as CD19+ lymphocytes) of each donor genotype. B cells were considered as a control population because they do not express CX3CR1, and thus their engraftment efficiency should not be affected by CX3CR1 absence. The NK/B ratio of the different donor strains were then confronted, and the obtained results are shown in Figure 20e. Being the total ratio equal to 1, it can be concluded that there were no differences in NK cell engraftment efficiency for the different donor strains, neither in the spleen nor in the BM.

4.1.7. CX3CR1 promotes KLRG1+ NK cell egress from the BM

We then tested the possibility that the expansion of a selected NK cell subset could be attributed to an altered homeostatic rate (proliferation/survival), staining NK cells with Annexin V, which recognizes the phosphatidilserine exposed on the outer side of cell membrane in apoptotic cells, or administering Bromo-deoxy-Uridine (BrdU) to mice, in order to detect BrdU incorporation in NK cell DNA (Figure 21). No differences were detected in the frequencies of proliferating and/or apoptotic NK cells, not even when GFP- and GFP+ cell subsets were considered.

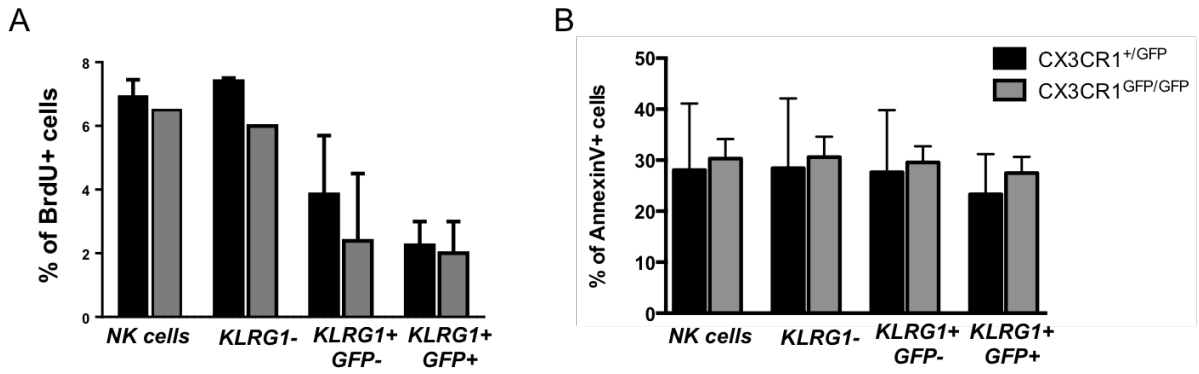


Figure 21 - ***NK cell turnover rate in homeostatic conditions is not altered in absence of CX3CR1.*** a) BrdU (1mg) was administrated to 7-10 weeks old $CX3CR1^{+/GFP}$ and $CX3CR1^{GFP/GFP}$ mice by intraperitoneal injection every 8 hours for 24 hours. Mice were sacrificed and BM cells were isolated and stained with anti-NK1.1-, and anti CD3-specific antibody. Histograms show the fraction of BrdU+ cells within GFP+ and GFP- among total NK cells. A total of five animals per group were analyzed in 3 independent experiments. b) Apoptosis of NK cells in the BM was measured using Annexin V assay. BM cells were isolated and stained with anti-NK1.1-, and anti-CD3-specific antibody and APC-conjugated Annexin V, and the percentage of Annexin V+ cells among GFP- and GFP+ NK cells was calculated by gating on NK1.1+CD3- cells. A total of four animals per group were analyzed in 2 independent experiments

Altogether, these data rule out a modification of NK cell turnover rate, thus we speculated that the NK cell population enhanced maturation degree in the BM of CX3CR1-deficient mice might be due to different trafficking capacities to this organ under homeostatic conditions. To test this possibility, competitive adoptive transfer experiments were

performed, in which equal amounts of CD45.1+/CD45.2+ CX3CR1$^{+/GFP}$ and CD45.2+ CX3CR1$^{+/GFP}$ -derived splenocytes were i.v. injected into WT hosts. To allow their recognition through FACS analysis, transferred cells were previously marked with the fluorescent dye PKH-26, and their genotype was distinguished according to the CD45 allelic variant expressed.

The localization of transferred cells was assessed in BM, spleen and liver at different time points, which enabled us to determine each organ's NK cell homing attitude (2.5 hours after injection) and retention capacity (24 and 36 hours after transfer injection).

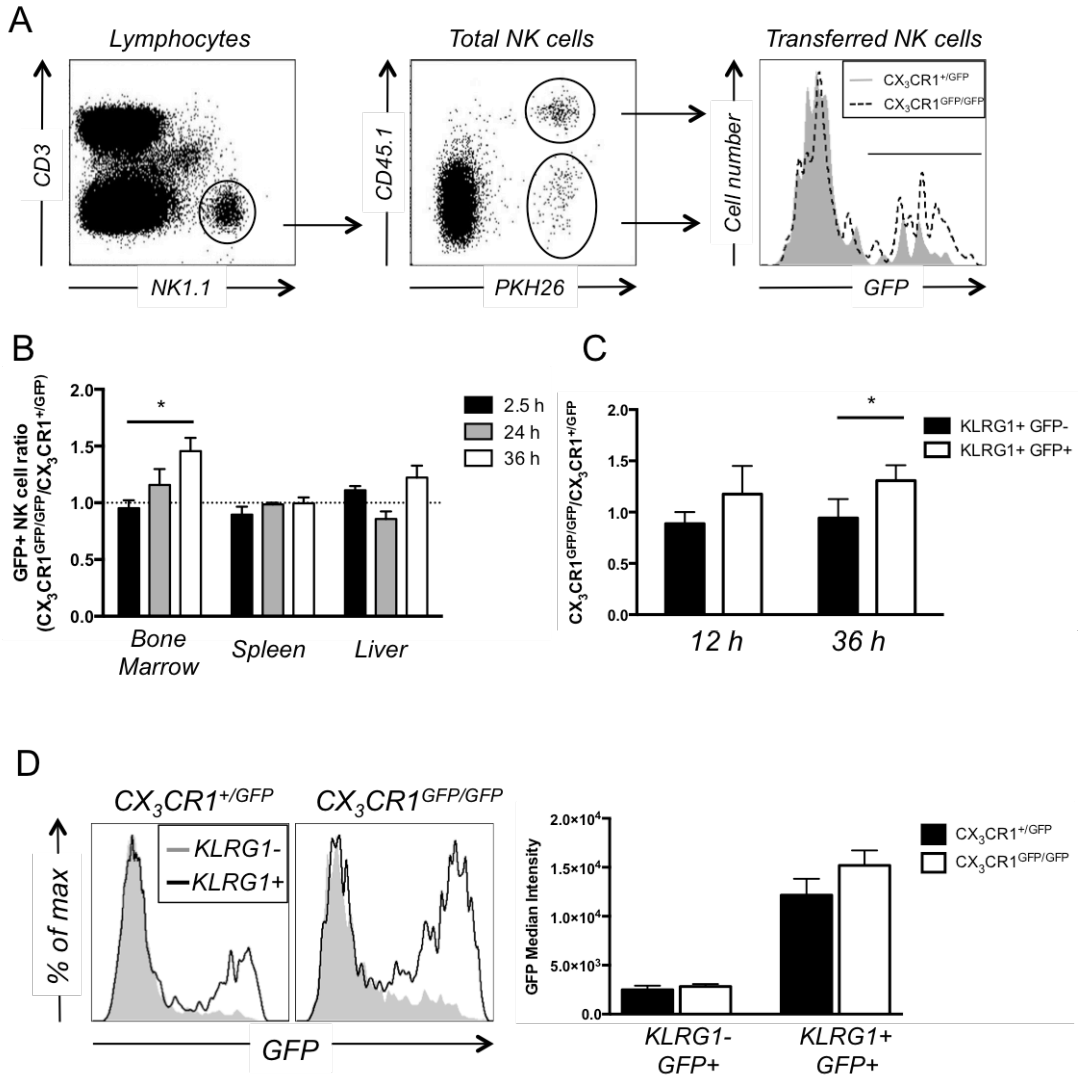


Figure 22 – *CX3CR1 deficiency prolongs NK cell maintenance in the BM.* Competitive homing experiments: (a) PKH26-labeled CD45.1+/CD45.2+ CX3CR1$^{+/GFP}$ and CD45.1-/CD45.2+ CX3CR1$^{GFP/GFP}$ splenocytes were i.v. injected at 1:1 ratio in wild-type Ly5.1-/Ly5.2+ WT mice. After 2.5, 24, and 36 hours, BM,

spleen, liver, and blood were collected, and transferred NK cells were identified as NK1.1+/CD3-/PKH-26+ cells by FACS. CD45.1-specific Abs, which selectively mark cells from CX3CR1+/GFP donors, were used to discriminate between $CX3CR1^{+/GFP}$ and $CX3CR1^{GFP/GFP}$ in recipient mice. Subsequently, we identified the GFP+ fraction within each transferred population. The gating strategy is illustrated above, whereas the graphs in (b) represent the homing index expressed as $CX3CR1^{GFP/GFP}/CX3CR1^{+/GFP}$ ratios of GFP+ NK cells in the different organs at different time points. Data are mean values ± SD of 14 recipient mice analyzed in 4 independent experiments. * = $p < 0.01$. (c) The histogram represents the homing index expressed as $CX3CR1^{GFP/GFP}/CX3CR1^{+/GFP}$ ratios of sorted KLRG1+GFP+ donor NK cells in the different organs of recipient mice at different time points. Data are mean values ± SEM of six recipient mice per group analyzed in two independent experiments. The horizontal line in the graphs defines the point at which the $CX3CR1^{GFP/GFP}$ and $CX3CR1^{+/GFP}$ populations are balanced in the organs. * = $p < 0.05$. The representation of GFP expression levels (left) and their quantification (right) on KLRG1- and KLRG1+ splenic NK cells from $CX3CR1^{+/GFP}$ and $CX3CR1^{GFP/GFP}$ mice are shown in (d).

No significant alteration of recruitment was observed regarding $CX3CR1^{GFP/GFP}$-derived total NK cells in any of the tested organs, and the same result was registered for GFP- NK cells.

However, GFP-expressing NK cells displayed a different behavior, as for what concerned the BM retention capacity. In fact, as shown in Figure 22b, while at earlier time points CX3CR1-sufficient and CX3CR1-deficient GFP+ NK cells equally migrated in BM, a faster reduction of the former's cell number was observed at later times, resulting in the significant increase of the $CX3CR1^{GFP/GFP}/CX3CR1^{+/GFP}$ cell ratio. This finding might indeed point towards an impaired capacity of CX3CR1-deficient cells to exit from the BM in homeostatic conditions.

Nonetheless, in this experiment only GFP+, and not KLRG1+/GFP+, NK cells were analyzed, so there was the chance that, though present in limited proportion, KLRG1-/GFP+ NK cells could influence the result observed. This option was formally excluded for two reasons: first, as presented in Figure 22c, the competitive adoptive transfer experiment was repeated injecting purified KLRG1+/GFP+ NK cells derived from the two mouse strains, with the same outcome 36 hours after injection. Secondly, the histogram in Figure

22d shows that KLRG1-/GFP+ cells cells are much less represented than KLRG1+/GFP+ cells, and display a low GFP expression intensity, making KLRG1-/GFP+ contribution unlikely.

Moreover, the study of the trafficking properties of purified KLRG1+/GFP- and KLRG1+/GFP+ NK cells ruled out even the possibility of a conversion of the former subset into the latter, during the time course analysed, given no newly generated GFP+ cells were found in KLRG1+/GFP- transferred recipients (not shown). Accordingly with these data, KLRG1+/GFP- cells derived from the two strains behaved similarly in all organs and at all time points analysed (Figure 22c).

Altogether, the competitive adoptive transfer experiments indicate a more pronounced capacity of CX3CR1-deficient GFP-expressing KLRG1+ NK cells to be retained in the BM, or, taken backwards, they show an active role for CX3CR1 in driving mature NK cells outside the BM, towards peripheral circulation.

4.1.8. CX3CR1/CX3CL1 axis regulates NK cell distribution within BM

Based on the evidence that KLRG1+/CX3CR1+ NK cells were preferentially localized in BM at the sinusoidal level, possible differences in KLRG1+/GFP+ NK cell compartimentalization in CX3CR1-sufficient and CX3CR1–deficient mice were then tested. First, adult mice were analysed, with the same approach described in Figure 13, for BM NK cell positioning into BM compartments, evidencing a significant increase in KLRG1+/GFP+ vs KLRG1+/GFP- NK cell ratio in the parenchyma of $CX3CR1^{GFP/GFP}$ mice, but not in their sinusoids. As a consequence, when parenchymal compartment was studied, there were more GFP+, but not GFP-, cells within the KLRG1+ NK cell subpopulation of CX3CR1-deficient mice (Fig. 23a right panel). Hence, the sinusoidal vs parenchymal ratio for KLRG1+/GFP+ NK cells was lower for homozygous mice, when compared to their heterozygous controls (not shown). This result demonstrated that the accumulation of KLRG1+/GFP+ in $CX3CR1^{GFP/GFP}$ mice is more pronounced in parenchyma

than in sinusoids. Thus, considering the data obtained so far, it is likely that KLRG1+/GFP+ CX3CR1-deficient NK cell increased BM retention could be associated to this subset alterated positioning in the BM. To prove this assumption, splenocyte competitive adoptive transfer experiments were repeated, this time assessing the donor-derived cell localization at the time points analysed (Figure 23b). When GFP^{high} NK cells (which for the 85% are KLRG1+) were quantified in the parenchyma, a differential kinetics of egress was observed for heterozygous and homozygous-derived cells. In fact, CX3CR1-sufficient cells decreased over time (from 2 to 36 hours after injection) in this compartment, whereas their KO counterpart remained more or less unchanged in the time course considered. This finding mirrors the behaviour reported in Figure 22b, strongly suggesting that not only CX3CR1 is involved in mature NK cell egress from the BM, but it specifically regulates their exit from parenchymal to the sinusoidal microenvironment.

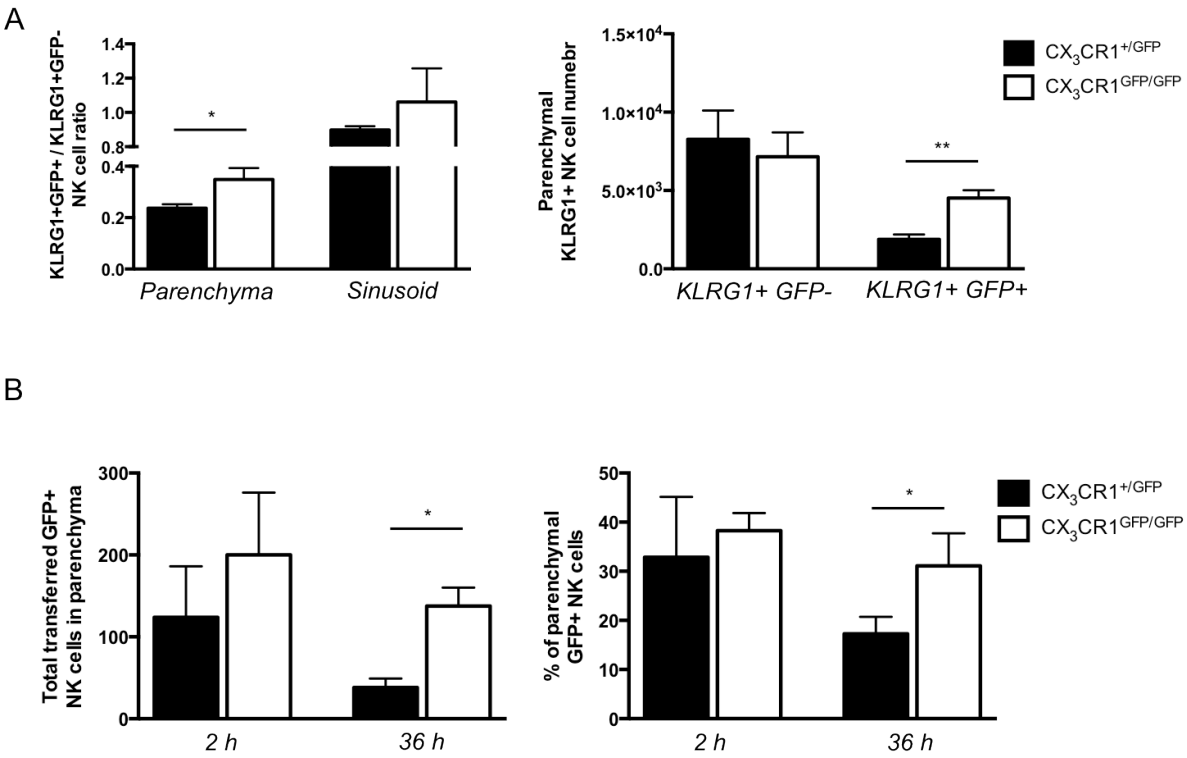


Figure 23 – ***Endogenous and adoptively transferred CX3CR1-deficient GFP+ NK cells are preferentially retained in BM parenchymal compartment***. (a) To test whether CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ NK cells were differentially localized in BM compartments, sinusoidal NK cells were labeled by i.v. injecting 1 mg CD45-PE mAb in 300 ml PBS. Cell suspensions from BM were then quickly collected and stained with CD3-, NK1.1-, and KLRG1–specific mAbs. The percentage of CD45-PE+ and CD45-PE-

cells within KLRG1+/GFP- and KLRG1+/GFP+ NK cells was evaluated. The number of parenchymal (CD45-PE-) NK cells (right panel) and sinusoidal (CD45-PE+) NK cells of the indicated phenotype was calculated. Histograms (left panel) represent the KLRG1+GFP+/KLRG1+GFP- ratio of endogenous NK cell number in $CX3CR1^{+/GFP}$ and in $CX3CR1^{GFP/GFP}$ mice inside the parenchyma and sinusoids. The average values ± SEM of two experiments performed with a total of four animals per group are shown. (b) Enriched splenic NK cells from $CX3CR1^{+/GFP}$ and $CX3CR1^{GFP/GFP}$ were transferred into WT recipient mice and sinusoidal NK cells were labeled by i.v. injection of DX5-PE mAb 2 and 36 h after transfer. Histogram plot shows the number (left) and percentage (right) of parenchymal GFP^{high} NK cells (representing bona-fide KLRG1+GFP+ NK cells) from $CX3CR1^{+/GFP}$ and $CX3CR1^{GFP/GFP}$ donor mice among total BM GFP^{high} cells. Data are mean values ± SEM of five recipient mice per group obtained in two independent experiments. * = $p < 0.05$.

4.1.9. Altered functions of CX3CR1-deficient KLRG1+ NK cells in response to *in vitro* and *in vivo* stimulation

BM parenchyma is a complex environment, where the hematopoiesis takes place, and residing cells are exposed to a great variety of stimuli, that can be cellular (i.e. stromal cells, osteoblasts, etc.) or non-cellular (cytokines, growth factors, or proteins of the extracellular matrix). Consequently, there was the chance that the prolonged exposition of NK cells to these powerful environmental cues might somehow influence their functional capacities, for example in an inflammatory context.

Two different approaches were adopted to verify this speculation, the first in an *in vitro* setting and the second in an *in vivo* model of acute inflammation.

Classical features of NK cell function were then tested, in particular their abiity to degranulate in presence of tumor cells and their capacity to produce IFNγ upon cytokine stimulation.

The results obtained are represented in Figure 24a/b, where it can be seen that while CX3CR1-deficiency does not affect NK cell IFNγ production after IL2+IL12 overnight incubation, it conferes increased potential to react against sensitive targets, YAC-1 in this case.

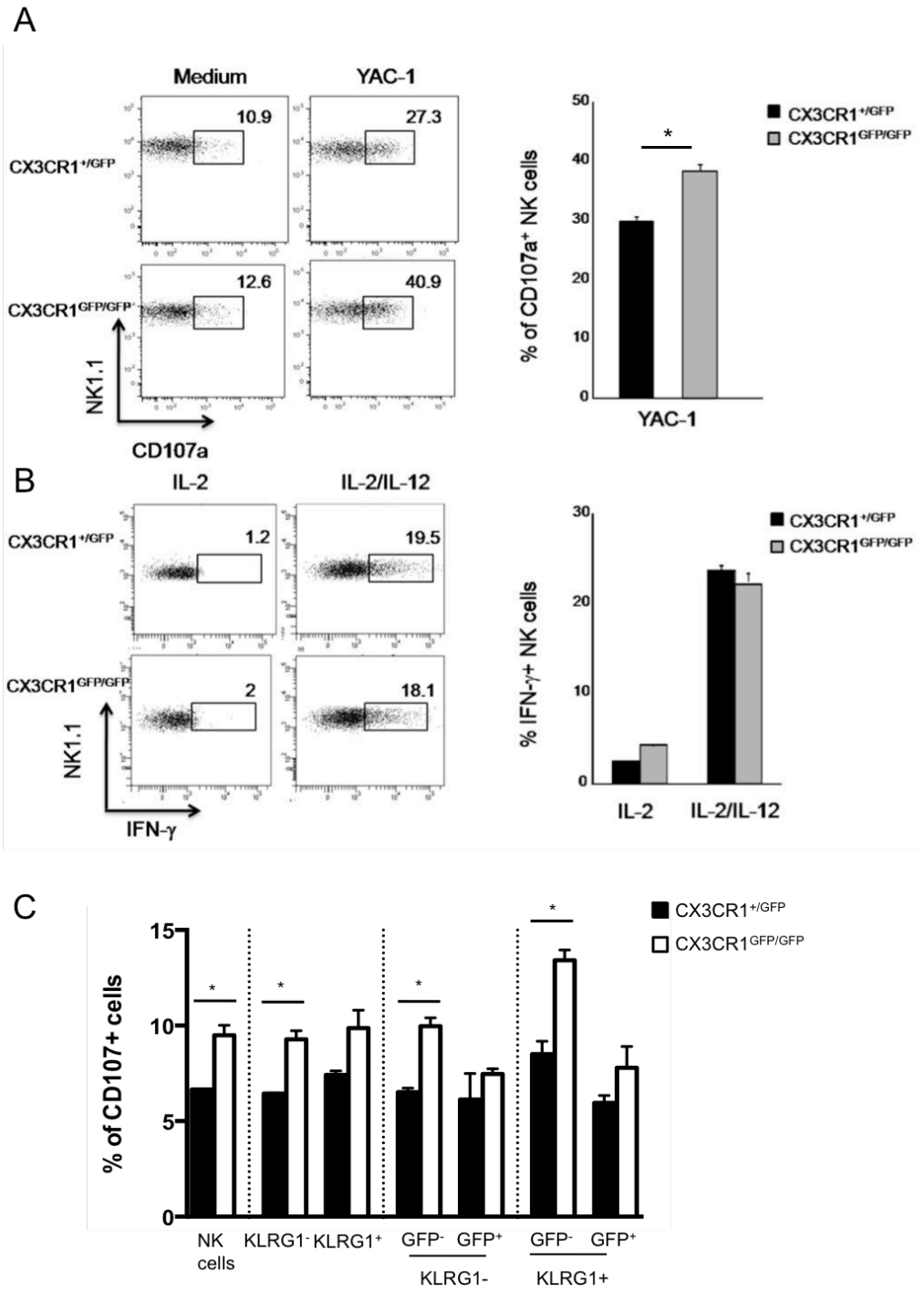


Figure 24 - ***Functional features of NK cells in CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ mice.*** (a) Splenic NK cells from CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ mice were cultured 18 hours in presence of IL-15 (100 ng/ml) and used as effector cells in a 4 hours degranulation assay against YAC-1 target cells in the presence of monensin and PE-conjugated anti-CD107 mAb. Cells were then collected and stained with NK1.1- and CD3ε–specific mAbs. Representative FACS dot plots (left) show NK1.1 and CD107a expression gated on NK1.1+/CD3- lymphocytes. The histogram on the right shows the average percentage ± SD of CD107+ NK cells in a representative experiment out of 5 performed in duplicate with a total of seven animals

per group. Frequency of CD107+ NK cells in the absence of target was subtracted to each treatment. (b) Freshly isolated cells from spleen of CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ mice were incubated for 18 hours, at 37 ℃, 5% CO_2 in presence of IL-2 (500 U) and IL-12 (100 ng/ml). Representative FACS dot plots (left) show NK1.1 and IFNγ expression gated on NK1.1+ CD3- cells. Histograms show the mean values ± SD of the percentage of IFNγ+ total NK cells for a total of five animals per group analyzed in three independent experiments. * = $p < 0.05$. (c) Equal amount of freshly isolated cells from spleen of CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ mice were mixed and stimulated overnight with IL-15 (100 ng/mL) and then used as effector cells as in (a). The histogram shows the mean percentage ± SD of CD107+ NK cells from the two strains, in two performed experiments with a total of four animals per group. Percentage of CD107a+ NK cells in the absence of target was subtracted to each treatment. * = $p < 0.05$.

These experiments were conducted keeping heterozygous and homozygous splenocytes separate, thus it was impossible to state if there was a contribution deriving from other GFP-expressing "helper" cells in CX3CR1$^{GFP/GFP}$ mice. Similarly to the in vivo approach pursued with mixed BM chimeras (Figure 20), the *in cys* effect of CX3CR1 absence on NK cells was investigated by mixing equal amounts of the cells from the two strains, stimulating them overnight with IL-15 and then incubating them with the target cell line in a 1:2 effector:target ratio for 4 hours. The degree of degranulating activity was higher in CX3CR1-deficient NK cells, indicating an intrinsic effect of CX3CR1 loss on NK cells as for what concerns their enhancement in degranulation potential (Figure 24c). Some intriguing findings were observed when NK cell population was dissected into cell subsets: in fact, the increase of CD107a positivity was reported not only on KLRG1+, but also on KLRG1- cells, and furthermore, GFP- cells were mainly responsible of this increase within both KLRG1- and KLRG1+ subpopulations. Although it is difficult to explain such a phenomenon, since GFP- cells do not express CX3CR1 in control mice and thus should not be affected by its deficiency, it is still possible that the alteration in NK cell behavior might arise earlier than GFP acquisition, and more so before KLRG1 expression, during NK cell development in absence of some direct effect mediated by CX3CR1.

As anticipated, the following step was to evaluate potential differences in NK cell behavior during an in vivo model of acute inflammation. Specifically, we used Poly(I:C)-induced hepatic inflammation model, in which a TLR3 agonist that mimics viral dsRNA is described to activate NK cells and to provoke their redistribution in several tissues, such as BM, spleen, and blood, finally driving them to the liver[330]. In our experimental conditions, NK cell frequency and number in those organs were monitored during a 72 hours long time course, with intermediate time points at 6 and 18 hours post-Poly(I:C) subministration.

CX3CR1$^{+/GFP}$mice displayed a substantial mobilization of total NK cell pool in BM and spleen (up to 80% reduction of the initial population after 18 hours) as early as 6 hours post injection, which was paralleled by a corresponding accumulation in the liver, where NK cell number increased even by 3.5 fold. The initial NK cell number was recovered in the BM and in the spleen after 72 hours, whether at that time point the liver still presented a doubled NK cell population, compared to the time 0 condition (Figure 25a).

Significant differences were observed between the two strains, inasmuch CX3CR1-deficient BM NK cells exited more promptly from the BM (6 hours) and were less reduced in the spleen at intermediate time points (6 and 18 hours). It is difficult to adfirm whether the lower mobilization from the spleen meant a slower exit rate from this organ or either a transitional splenic recruitment after BM egress, which could not be observed in the CX3CR1$^{+/GFP}$ mice (or was too quick to be detected). Interestingly, a slight but not significant increase in the splenic NK cell number was also reported after 72 hours, indicating that the first hypothesis might be more likely.

When specific NK cell subset contribution to Poly(I:C)-induced NK cell redistribution was considered, a predominant effect on KLRG1+ NK cell subset was observed, in accordance to the fact that CX3CR1 is mainly expressed by more mature KLRG1+ NK cells (Figure 25b), although significant but less important changes could be detected also for the CD11b^{low}/KLRG1- and CD11b^{high}/KLGR1- subsets (not shown). Indeeed, KLRG1+ NK cell trafficking reflected what was remarked for total NK cells, with the notable difference of a decreased number of CX3CR1-deficient KLRG1+ NK cells in liver after 18 hours, as

compared to the heterozygous cells, which was probably masked by other cell subsets' behavior when total NK cells were considered.

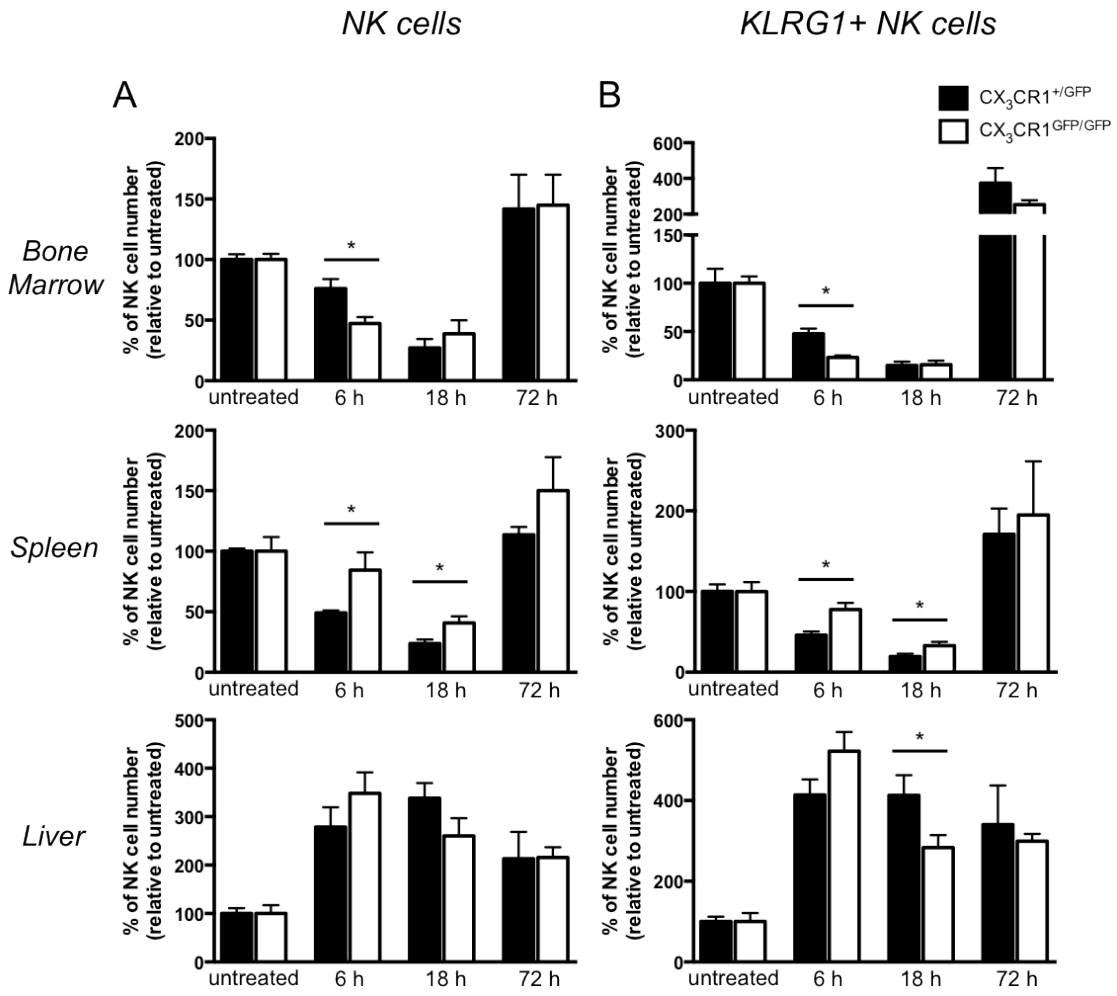


Figure 25 - ***Altered poly(I:C)-in- duced tissue redistribution of NK cells in CX3CR1-deficient mice***. CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ mice were i.p. injected with 150 μg Poly(I:C), and NK cells were collected from BM, spleen, and liver after 6, 18, and 72 hours. To test whether the differences in total NK cell number could be ascribed to a specific cell subset, we also stained our samples for CD11b- and KLRG1- specific mAbs. The histograms represent the results obtained from at least six animals per group in four independent experiments. Data are expressed as percent (mean values ± SEM) of total (a) or KLRG1+ (b) NK cell counts at the different time points vs the NK cell count (total and KLRG1+, respectively) in untreated mice. * = $p < 0.05$.

The degree of hepatic damage resulting from NK cell recruitment to liver can be assessed through the measurement of the enzymatic activity of hepatic enzymes present in the serum, in particular the Alanine Transaminase (ALT), which is released in the extracellular

medium following hepatocyte apoptosis, by means of a biochemical assay we tested ALT activity in blood samples of CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ animals at different time points after Poly(I:C) subministration, in order to determine whether the alterated NK cell trafficking could result in differences in the hepatic damage (not shown). In preliminary experiments, the results of this procedure showed a similar trend for the two strains, as in both sera no ALT activity was detected until 48 hours after injection, while a significant increase was observed at the latest time point (72 hours).

4.2. Second part – NK cell trafficking and function in Multiple Myeloma

Besides CX3CR1, NK cell capacity to reach several anatomical compartments is conferred by a wide array of chemokine receptors expressed on their cell surface[113, 331, 332, 333], such as CXCR3, CXCR4, CXCR6, CCR1, CCR3, CCR4 and CCR5. These receptors are specific for chemokines secreted in diverse physio-pathological conditions, and are also expressed heterogeneously within NK cell subpopulations.

Being the most important district of their development, several developmentally distinct NK cell subsets reside within the BM, also displaying a differential chemokine receptor expression pattern. In Figure 26 the expression profile of some of these receptors is presented, on total NK cells and on KLRG1- and KLRG1+ cell subsets localized in the BM of 8 weeks-old healthy mice. Although the KLRG1- subset is not an homogeneous population, which includes immature CD11b^{low} NK cells as well as fully functional CD11b^{high} NK cells, it can be seen in the presented histograms that most of the chemokines receptors analyzed are preponderantly represented on this fraction of NK cells, while CX3CR1 is the only one found prevalently on mature KLRG1+ cells.

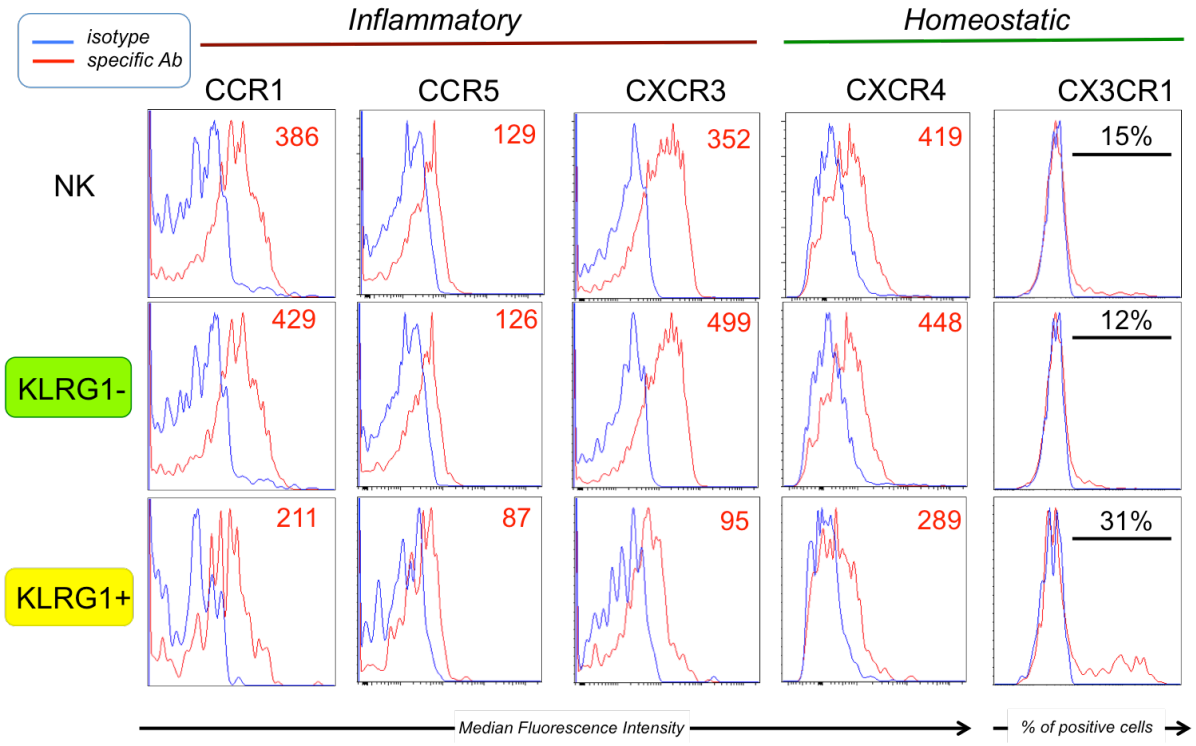


Figure 26 – *Chemokine receptor expression on BM NK cells.* BM cells from healthy C57BL/KaLwRij mice were collected and NK cells and NK cell subsets were stained for with CCR1-, CCR5-, CXCR3- and CXCR4-specific mAbs (red lines) and respective isotype controls (blue lines). CX3CR1 expression level was assessed through the GFP analysis in BM cells from CX3CR1$^{+/GFP}$ mice, in a C57BL/6J background. The same expression levels were detected on C57BL/KaLwRij BM NK cells using human CX3CL1 fused with human Fc, and then staining with anti-human-Fc mAb (not shown). Numbers in histogram plots represent the MFI in one out of at least five independent experiments.

In paragraph 1.2.5., the several strategies exploited by cancer cells to divert immune response from the tumor site through the disregulation of the chemokine balance in the surrounding microenvironment were described. Because of the major role played by NK cells in Multiple Myeloma (MM), and since this pathology mainly interests the BM, the second part of this thesis will deal with the interaction of MM growth and NK cell function and migration, with the perspective of uncovering new aspects of NK cell biology in MM-related inflammation to be employed in the future for NK cell-based immune therapies.

4.2.1. MM cells are sensitive to NK cell action in vitro and in vivo

The first step towards this objective was to estabilish and characterize an appropriate murine model of MM, which as such should mimic the most typical features of the human pathology. The murine model induced by transplantation of 5TMM cells into a syngeneic mouse strain displays the classical hallmarks of MM, including osteolitic lesions, hypergammaglobulinemia, and angiogenesis, and, importantly, grows preferentially in the BM, being thus classified as an orthotopic tumor model.

In this work, 5TGM1 cells, a 5TMM-derived tumor line, were used to estabilish the murine model: $2x10^6$ cells were i.v. transplanted in 6-10 weeks old syngeneic C57BL/KaLwRij female mice, and MM growth was monitored up to 4 weeks after injection. Tumor progression was evaluated through the detection of intracellular production of the monoclonal paraprotein, IgG2b, which is expressed exclusively by 5TGM1 cells. IgG2b levels were monitored 2, 3 and 4 weeks after 5TGM1 injection in BM and in spleen (Figure 28a).

At first, in vitro NK cell capacity to react to 5TGM1 cells was tested: to this aim, splenic and BM-derived NK cells from healthy mice were previously activated by cullturing them overnight with IL-15, and then tested through ^{51}Cr-release cytotoxicity assay and degranulation assay against 5TGM1 cells. Figure 27 shows that NK cells from both BM and spleen are able to express the degranulation marker CD107a, and when NK cell subsets were considered, KLRG1- fraction was observed to be more active than KLRG1+ subpopulation. Accordingly, enriched splenic NK cells were able to exert up to 20% of cytotoxicity towards their target. These results demonstrate the sensitivity of MM cells to NK cells, through a mechanism that, al least in part, is mediated by the release of cytotoxic granules.

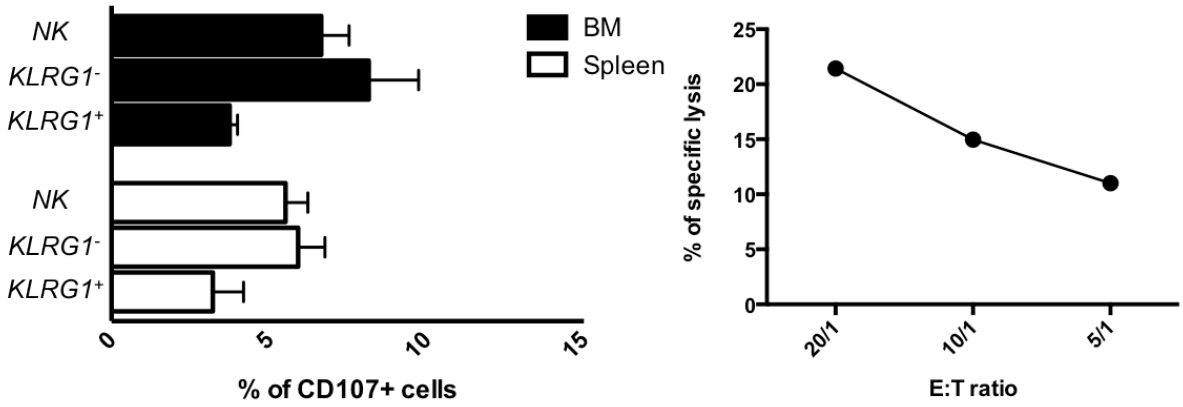


Figure 27 – *5TGM1 activate and are sensitive to NK cells in vitro.* BM and splenic cells from healthy C57BL/KaLwRij mice were activated overnight with IL-15, then washed and incubated with or without 5TGM1 cells for 4 hours, in presence of anti-CD107a mAb, or its isotype control. NK cell degranulation was then assessed by FACS, staining for NK1.1, CD3 and KLRG1. Data are expressed as percentage of CD107a+ cells, and CD107a values obtained in absence 5TGM1 cells were subtracted from those obtained with tumor cells, and are representative of 1 out of 3 independent experiments performed (left). Enriched splenic NK cells were activated overnight with IL-15 and used as effector, while YAC-1 cells were incubated in complete medium for 1 hour in presence of ^{51}Cr. The cytotoxicity lasted 4 hours, and the radioactivity present within supernatants was assessed with a TopCount-NXT counter (right).

Considered the in vitro data, we focused on the analysis of NK cell anti-tumor activity in vivo. MM growth was followed in a 4 week-long time course, during which we could observe a slow proliferation/engraftment rate until the 3rd week, in BM and spleen, and after that a marked increase in the speed of tumor growth. Nevertheless, though in terms of absolute numbers of 5TGM1 BM and spleen may appear similar, the frequency of tumor cells was significantly different already after 3 weeks (2 ± 0.2% in BM and 0.47 ± 0.03% in spleen), and quite distant after 4 weeks (8 ± 1% in BM and 2.4 ± 0.3% in spleen). At four weeks, the alteration in the equilibrium in the BM cellular composition was far deeper than that observed in the spleen, likely having a strong impact on several aspects related to BM, such as the hematopoiesis or the immune response exerted by the BM-residing leukocytes, as it will be shown later on.

Having determined the kinetics of MM progression in our model, the next question was related to the role of NK cells in the immune response against it. To address this issue, NK cells of MM-bearing mice were depleted through i.p. administration of the anti-NK1.1

mAb PK136 both before and after the injection of 5TGM1 cells. This depletion protocol was meant to measure NK cell contribution during the whole course of MM progression, since the initial migration to and colonization of BM to the last phase of estabilished disease. As it is shown in Figure 30b, NK cell absence determined an increase of MM growth both in the spleen and in BM, although the difference was not statistically significant after 4 weeks in the BM, whereas it was evident in the spleen. This effect could likely be linked to the different role played by NK cells in the two different anatomical compartments during MM progression.

At any rate, the induction of a quicker MM growth demonstrates the in vivo action of NK cells in the immune response to tumor.

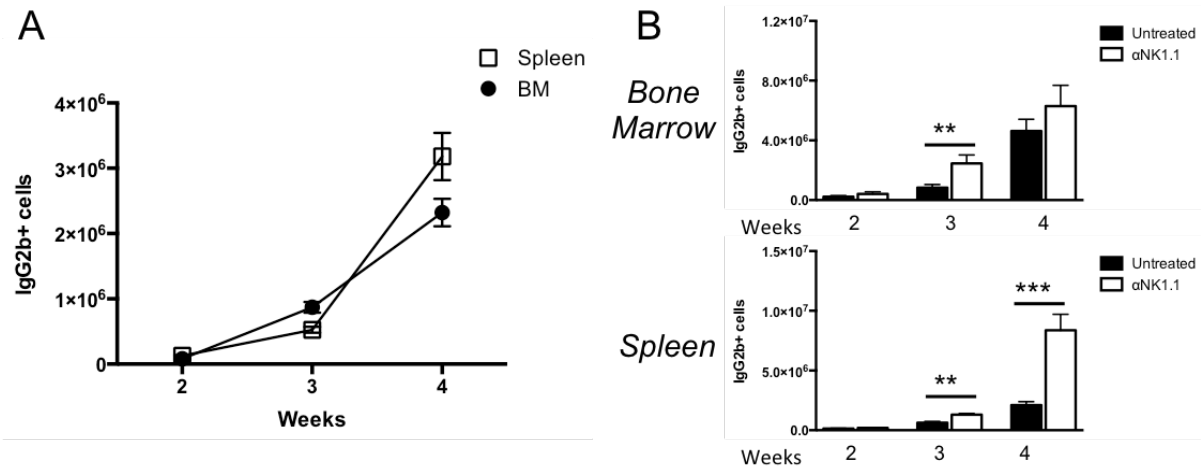


Figure 28 – ***MM growth in vivo is affected by NK cells in BM and spleen.*** BM and spleen cells were collected from healthy and tumor-bearing mice 2, 3 and 4 weeks after 5TGM1 injection, and were intracellularly stained with anti-IgG2b mAbs, specific for tumor cells. Kinetics of MM growth in BM and spleen, shown as mean value ± SEM (left). Effect of NK cell depletion on MM progression at the time points indicated (right). Data are expressed as mean number of IgG2b+ cells in organs ± SEM, and are representative of at least 8 mice for each group, in 3 separate experiments. ** = $p < 0.01$; *** = $p < 0.001$.

4.2.2. NK cell response in vivo is impaired early during tumor growth

As already done in vitro, NK cell degranulation capacity was then assessed ex vivo, by the evaluation of CD107a expression of freshly collected cells from BM and spleen of healthy and MM-bearing mice (Figure 29). Interestingly, splenic NK cells derived from tumor-bearing mice showed an increase of their degranulation rate, at all time points analyzed, while BM NK cells were activated only in the early phases of tumor growth. Indeed, in light of the in vitro experiments, NK cells naturally react against 5TGM1 cells, thus suggesting that the increase of CD107a+ cells observed in the spleen could reflectstheir normal activity. Conversely, the lack of degranulation enhancement reported at later times in the BM is probably due to the dysregulation or suppression of NK cell function, which is related, directly or indirectly, to MM cell extensive proliferation.

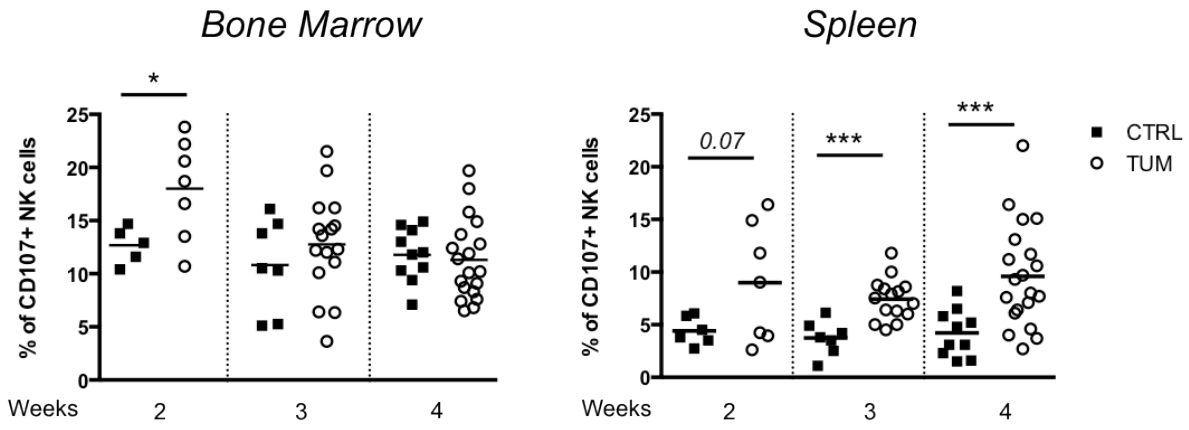


Figure 29 – *NK cell functionality is impaired after 3 weeks in the BM.* Expression of degranulation marker CD107a was assessed by FACS on freshly collected NK cells from BM (left) and spleen (right) of healthy and MM-bearing mice after 2, 3 and 4 weeks of tumor growth. Each symbol represents a single mouse, analysed in at least 2 independent experiments. The horizontal bar represents the mean value. * = $p < 0.05$; *** = $p < 0.001$.

To insert these data in a more detailed framework, the NK cell number and frequency among lymphocytes were assessed in BM and spleen. Mirroring the trend of MM cell growth, a significant NK cell number decrease was reported in BM as early as the 3rd

week, while it was halved by the following week. Of note, not only the NK cell number, but also total lymphocyte count and even total BM cell count was reduced after 4 weeks, but were normal after 3 weeks (not shown). The situation was different in the spleen, where NK cell number remained unchanged until the 3rd week, but suffered a strong reduction at the 4th (decreasing by a third).

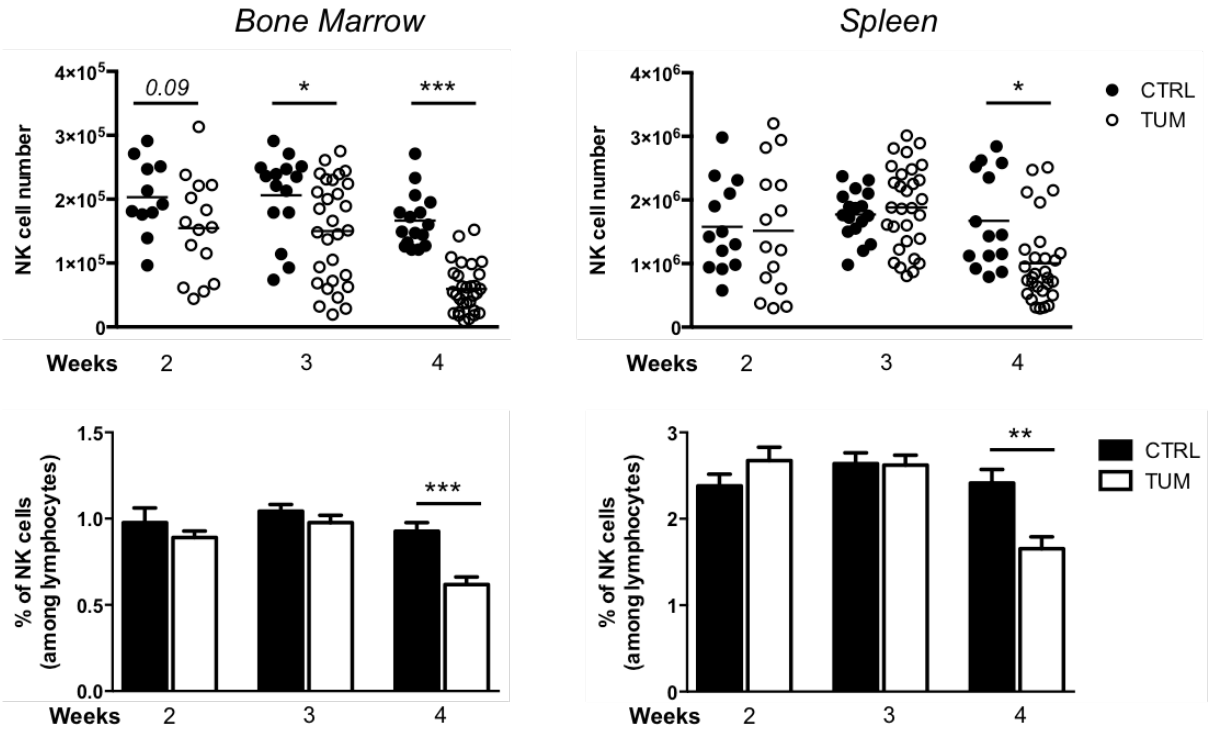


Figure 30 – ***MM growth causes the reduction of BM NK cell number after 3 weeks.*** CD3-NK1.1+ lymphocytes were quantified in BM (left) and spleen (right) of healthy and tumor-bearing mice during disease progression. In the upper panels, graphs show the NK cell number in each group (the horizontal bar represents the mean value), while below the histograms display the mean NK cell frequency among lymphocytes of the same animals ± SEM. Each circle represent a single mouse, analysed in at least 3 independent experiments. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Comparing the result obtained from NK cell phenotyping and MM quantification, a fine correlation between those two parameters was found, which might explain the impaired NK cell degranulation capacity observed after 3 and 4 weeks in the BM (Figure 31).

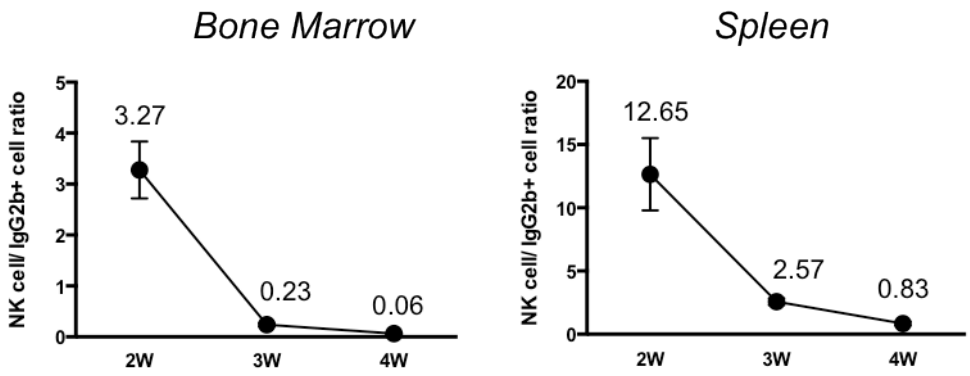


Figure 31 – *NK cells are overwhelmed by 5TGM1 cells in BM after 3 weeks.* Total NK cell number in BM and in the spleen of each tumor-bearing mice after 2, 3 and 4 weeks of tumor growth was divided by the IgG2b+ cell number found in the same mouse. The curve shows the mean values of the resulting NK:IgG2b+ (5TGM1) cell ratios. Data represent at least 10 mice, in 3 independent experiments.

In fact, dividing each animal's NK cell number by tumor cell number detected in BM (or in the spleen) at different time point analyzed, a decreasing curve can be drawn, indicating the presence of 4 MM cells per NK cells in BM already after 3 weeks, and of about 20 MM cells per NK cell after 4 weeks. Obviously, NK cells are not the only players involved in MM-directed immune response, but it is likely that the unfavourable effector:target ratio might put them at a strong disadvantage. In comparison, the spleen displays a far better situation, since the NK cell/IgG2b+ cell ratio slighlty goes below 1 only after 4 weeks.

4.2.3. Myeloma cells alter the proportion of NK cell subsets within BM

When BM NK cell subset composition was analyzed, a preponderant involvement of the less mature KLRG1- subset was found after 3 weeks, while the KLRG1+ subset was more or less maintained intact. Consequently, considering the NK cell subset frequencies $CD11b^{high}$/KLRG1+ subset was doubled, mainly at the expense of $CD11b^{low}$/KLRG1- fraction. If added to NK cell number decrease, this shift in NK cell phenotype might further impair immune system "firepower" against MM growth (Figure 32).

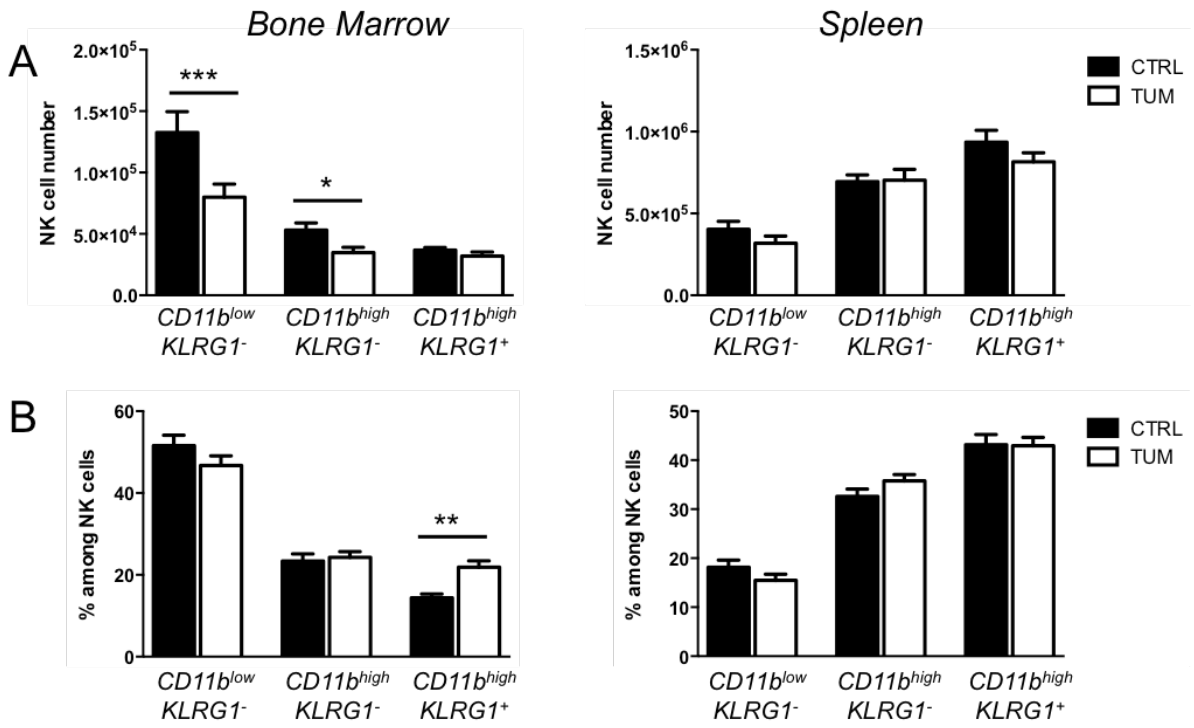


Figure 32 – ***NK cell decrease in BM is selectively caused by KLRG1- subset.*** CD3-/NK1.1+ lymphocytes were dissected in 3 subsets according to their CD11b and KLRG1 expression. a) Histograms show the reduction of both KLRG1- cell subsets in the BM of MM-bearing mice after 3 weeks (left), while KLRG1+ cell number is unchanged. Conversely, KLRG1+ frequency within the BM NK cells in presence of MM is augmented 1.5 fold (b, left), as compared to healthy mice. Graphs represent mean values ± SEM of at least 15 mice, from at least 4 independent experiments. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Differently, after 4 weeks no important difference in NK cell subset frequency was evidenced neither in the BM nor in the spleen, although every subpopulation suffered an important reduction in both organs, probably related to the hematopoiesis block caused by the expansion of MM cells. Indeed, the slightly reduced transition of immature CD11b^{low}/KLRG1- cells into fully functional CD11b^{high}/KLRG1- cells observed in BM might be in agreement with this speculation, and could also cause the similar phenotype in the spleen (Figure 33).

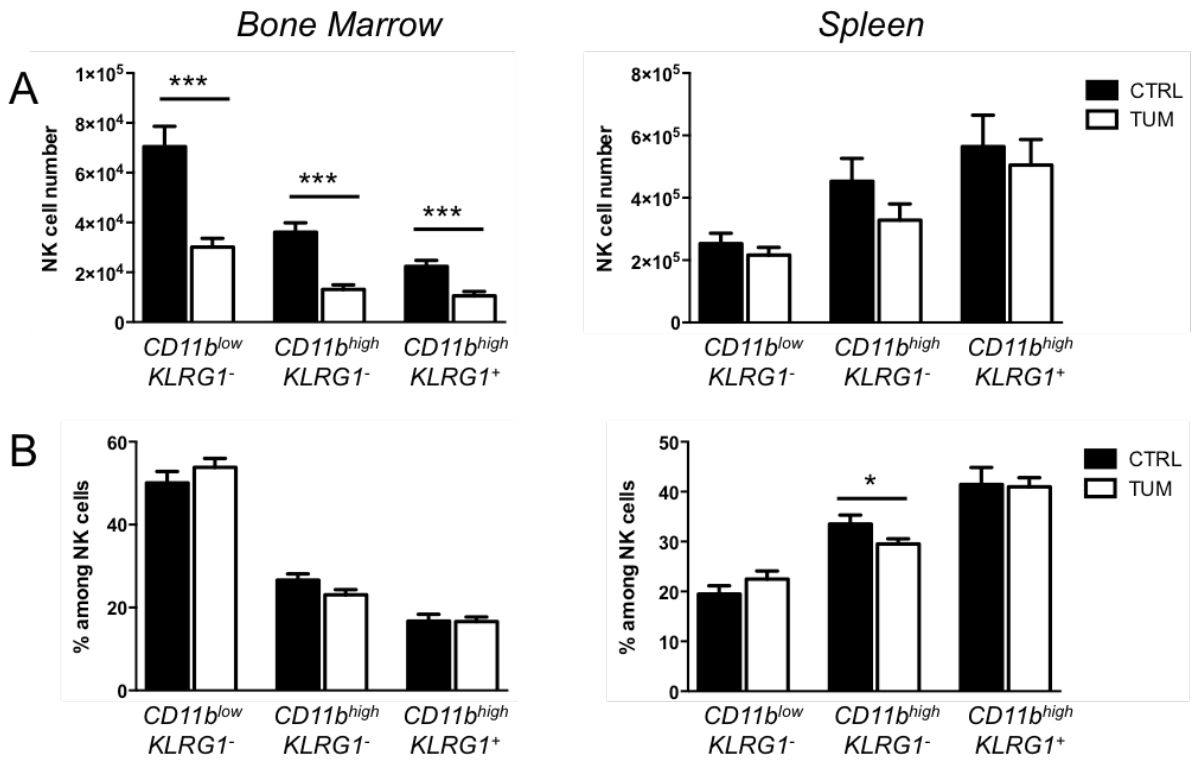


Figure 33 – ***At later stages of MM growth BM NK cell number is greatly reduced.*** All NK cell subsets suffer an important numeric reduction in the BM after 4 weeks of tumor growth (a, left), while in the spleen only a slight tendency can be observed (a, right). NK cell subset balance is unaltered in the BM (b, left), while a decreased frequency of the functional CD11b^{high}/KLRG1- subset in MM-bearing mice is shown in the spleen (b, right). Graphs represent mean values ± SEM of at least 15 mice, from at least 4 independent experiments. * = $p < 0.05$; *** = $p < 0.001$.

The individual contribution of each NK cell subpopulation to total NK cell degranulation degree ex vivo at 2, 3 and 4 weeks was in line with the results shown in Figure 29, and did not evidence any particular alteration between control and tumor-bearing mice (not shown).

4.2.4. KLRG1- NK cell trafficking capacity to BM is impaired in tumor-bearing mice

The results obtained so far described a developing situation regarding NK cells in the BM: in fact, while at the beginning NK cells seem to be functional and able to efficiently hamper tumor growth, at the 3rd week they are reduced in numbers and function, thus paving the way to what will take place at later stages, when they are completely overpowered by MM cells in the BM. Our interest then focused mainly on the 3rd week, which represents an asymptomatic, intermediate phase of tumor progression, that may allow to dissect early mechanisms of subversion of NK cell-mediated function.

In particular, KLRG1- BM NK cell decrease in numbers evidenced at this stage could be possibly due to several, non mutually exclusive, phenomenons: first, a reduction of cell survival, which should, at any rate, not affect KLRG1+ subset. Another option would be the altered NK cell development, which would explain KLRG1+ frequency increase. Lastly, there might be a disregulation of NK cell subsets trafficking capacities, selectively impairing the entry rate of specific NK cell subsets to the tumor site.

The first possibility was tested by staining NK cells derived from BM of healthy and MM-bearing mice with AnnexinV (Figure 34a). No significant differences in the survival rate of total NK cells and of KLRG1- or KLRG1+ NK cell subsets in the two conditions were observed, suggesting that, at least at this stage of MM progression, the apoptotic rate of NK cells did not affect KLRG1- subset numeric reduction.

Next, the hypothesis of the altered trafficking was examined, by adoptively transferring PKH26-marked splenocytes into healthy or MM-bearing mice to evaluate their *in vivo* migration capacity (Figure 34b). The graphs display the reduction in total NK cell BM recruitment in the BM of tumor-bearing mice, that could be exclusively attributed to the KLRG1- fraction of cells, whereas KLRG1+ capacity to traffick to BM was not affected by MM-induced BM conditioning. Conversely, donor-derived NK cells were normally able to

migrate to the spleen, even in presence of tumor cells, and so were KLRG1- and KLRG1+ NK cell subsets.

These data are similar to those related to endogenous KLRG1- NK cells, displayed in Figure 32 (there separated for the expression of CD11b), and indicate that the equilibrium between NK cell entrance and retention in BM is disrupted after 3 weeks of tumor growth. Furthermore, this evidence weakens the hypothesis that the reduced representation of the KLRG1- subset in BM might be due to altered NK cell development, as 18 hours would definitely be insufficient to justify such a phenomenon.

Nevertheless, it could be still conceivable that during the time between the engraftment and the organ collection a fraction of KLRG1- NK cells would acquire KLRG1 expression, thus altering the interpretation of the data deriving from the transfer experiments. To rule out this hypothesis, purified KLRG1- cells were stained with PKH-26 and i.v. transferred into control or tumor-bearing mice. After 18 hours, BM and splenic cells were collected, and the presence of newly generated KLRG1+ NK cells within the engrafted population was determined by FACS (Figure 34c).

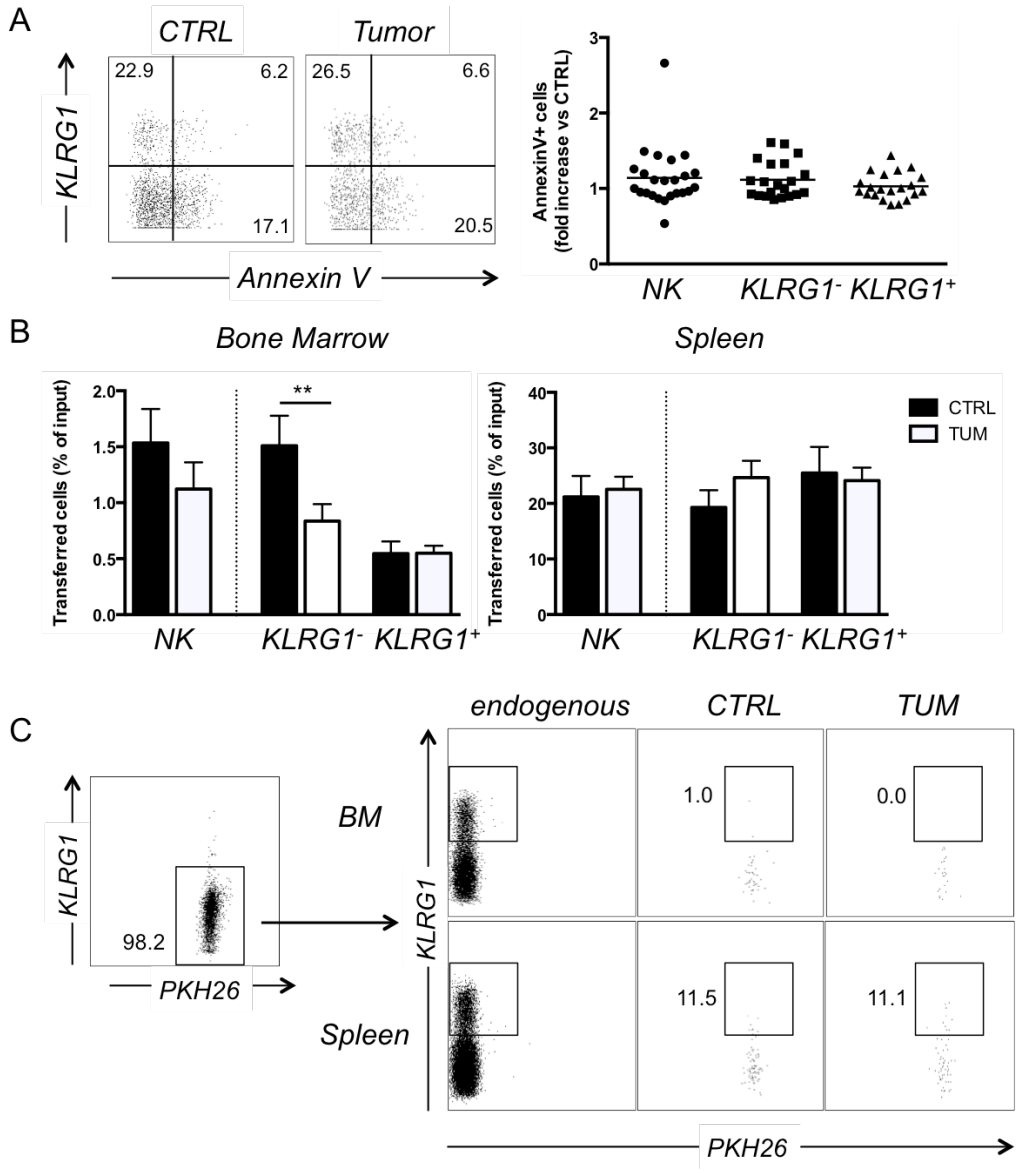


Figure 34 – *Altered capacity of KLRG1- NK cell to migrate to BM in MM-bearing mice.* The apoptotic rate of BM NK cells and NK cell subsets was measured by staining for AnnexinV, as shown in a representative experiment (a, left). Data are expressed as fold increase of AnnexinV+ cells in MM-bearing mice vs those in healthy mice (a, right). Being the ratio close to 1, there was no alteration in the measured apoptotic rate. b) $12x10^6$ splenocytes were stained with PKH26 and adoptively transferred in healthy or tumor-bearing mice after 3 weeks. Transferred cells were detected in BM (left) and spleen (right) of recipient mice 18 hours after transplantation, through PKH-26 expression analysis. Histograms represent the mean values ± SEM of the frequency of transferred cells detected in each organ among total transferred cells. Highly purified (>95%) KLRG1- splenocytes were stained with PKH-26 and adoptively transferred into healthy and MM-bearing mice after 3 weeks. 18 hours later, KLRG1 expression was assessed by FACS on recipient's NK cells (left panels), and on transferred NK cells in healthy and tumor-bearing mice (central and right panels), within BM (above) and spleen (below). Numbers in

dot plots represent the frequency of newly generated KLRG1+ NK cells among the total transferred NK cells in the organ analysed, from one over two independent experiments, with at least 4 mice per each group. ** = $p < 0.01$.

This was not the case, at least for the BM-residing transferred NK cells, and, at any rate, the process of acquisition of KLRG1 that occurred in the spleen did not present differences between control and MM-bearing mice. Taken together, these experiments demonstrate the selective reduction of KLRG1- NK cell homing to BM in presence of MM at the 3rd week of tumor growth, indicating that these cells are probably driven outside the organ at an intermediate phase of disease progression.

4.2.5. The chemokine environment is unbalanced in BM of tumor-bearing mice

To further characterize BM NK cell trafficking properties, the following step was to analyze the expression of some of the chemokine receptors known to affect NK cell function, in particular those involved with cancer-related inflammation (CXCR3, CCR1, CCR5) and with BM retention (CXCR4). Figure 35a,b show that CXCR4, CCR1 and CCR5 present a normal expression profile on BM NK cells derived from MM-bearing mice, while CXCR3 expression was found to be significantly reduced. As pictured in Figure 26, KLRG1- fraction accounts for the majority of the chemokine receptors expressed by NK cells (including CXCR3), and thus it is not surprising that CXCR3 downregulation within the total NK cell pool is prevalently caused by a marked effect on KLRG1- subpopulation (Figure 35c). To portray BM microenvironment in relation to the chemokine system more in detail, the concentration of several chemokines in BM extracellular fluids of healthy and MM-bearing mice was assessed. In particular, we determined the concentration of the specific ligands of the same chemokine receptors analysed on NK cells (Figure 35d,e). CXCR3 ligands CXCL9 and CXCL10 were upregulated in BM extracellular fluids of MM-bearing mice, while CXCL12 concentration was reduced in presence of MM. Interestingly, an increase in CXCL9 concentration was also found in sera of MM-bearing mice, whereas

neither CXCL10 nor CXCL12 were altered. Soluble CX3CL1 was not detected in blood of either control or tumor-bearing mice (not shown). Moreover, preliminary data obtained through Luminex technology indicate a tendency to increase in CCR1/5 ligands CCL2 and CCL3 expression in MM-bearing mice, while CCL5 was not altered (not shown).

While CXCL12 decrease in BM is in agreement with KLRG1- NK cell reduction as their BM retention is supposed to be more dependent on CXCR4 function, the increase of CXCR3 ligands is harder to explain, especially if CXCR3 expression on NK cells is downregulated.

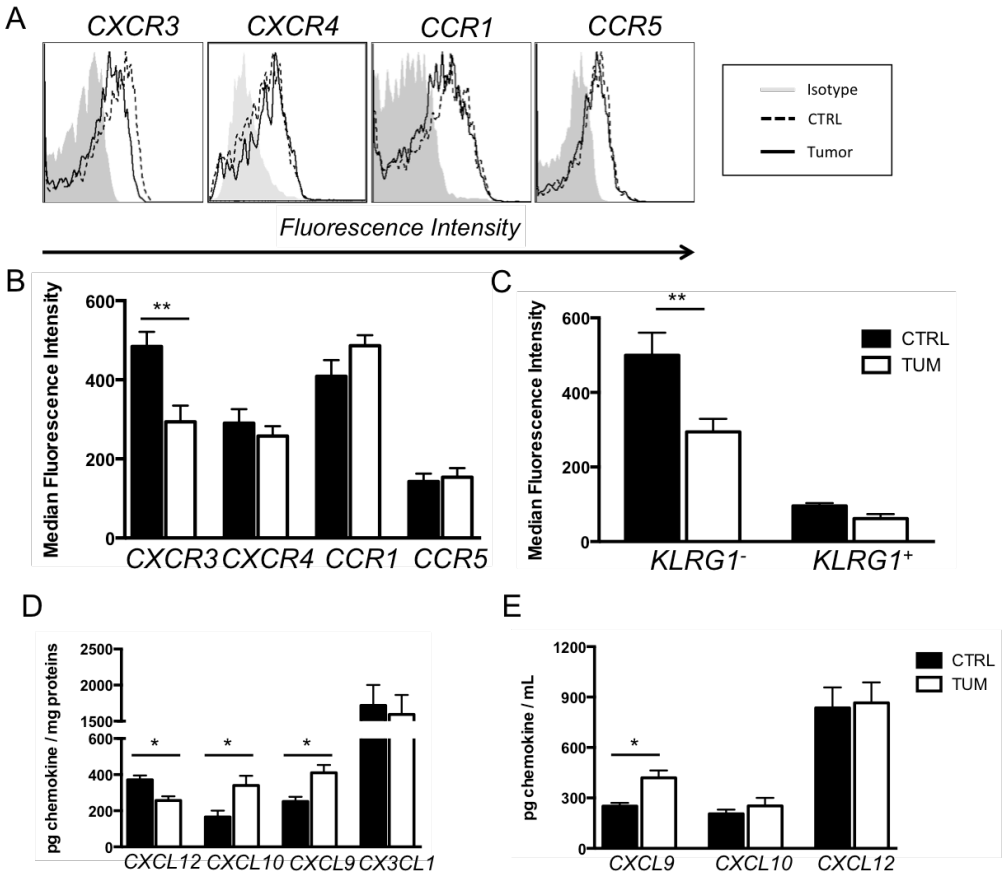


Figure 35 – *MM produces important alterations of CXCR3 and CXCR4 axes within the inflamed BM.* a) Overlay of chemokine receptor expression on BM NK cells from healthy (dotted line) and tumor-bearing (black line). Filled gray line represents isotypic control. b) Quantification of CCR1, CCR5, CXCR3 and CXCR4 expression on total NK cells, represented as mean values of MFI ± SEM. CXCR3 downmodulation was assessed also on KLRG1- and KLRG1+ NK cell subsets, and quantified as MFI ± SEM (c). BM extracellular fluids from healthy and MM-bearing mice were collected and stored as described in Methods. In (d) are displayed the results of ELISA assays for CXCL12, CXCL9, CXCL10 and CX3CL1, normalized for total proteins present

in the extracellular fluid tested. Histograms show the mean values ± SEM. ELISA assays for CX3CL1 (not shown), CXCL9, CXCL10 and CXCL12 were also conducted on serum samples (e), and are displayed as mean values of their concentration ± SEM. At least 8 mice per group, in 3 independent experiments were used for these histograms. * = $p < 0.05$; ** = $p < 0.01$.

4.2.6. Myeloma impairs CXCR3+ NK cell ability to reside and migrate to BM

Chemokine receptor downregulation can be caused by several mechanisms. First, it can be due to an alteration of NK cell maturation (especially as CXCR3 is developmentally modulated); secondly, there could be an alteration in the transcriptional, translational or even post-translational control of the receptor expression; finally, it is possible that CXCR3+ cells are excluded from the analyzed context, reverberating on global CXCR3 expression intensity.

The first case was previously discarded as unlikely (Paragraph 4.2.4) though not formally ruled out, and, moreover, we observed a decrease in frequency of CXCR3-positive cells (not shown), which pointed towards the last hypothesis. Regardless, to hone in on the exact mechanism of CXCR3 downregulation it was important to determine its kinetics: indeed, transcriptional and post-traslational modifications, and developmental alterations would require completely different lapses of time.

To address this issue, a variant of the previously shown adoptive transfer experiment was employed, this time analyzing CXCR3 expression level on transferred splenic NK cells after i.v. injection into control and tumor-bearing mice at different time points (3 and 18 hours). The results represented in Figure 36 reveal that CXCR3 downregulation occurs on engrafted cells already after 3 hours, and persists over 18 hours, thus excluding a long-term mechanism such as transcriptional regulation or alterations of NK cell development.

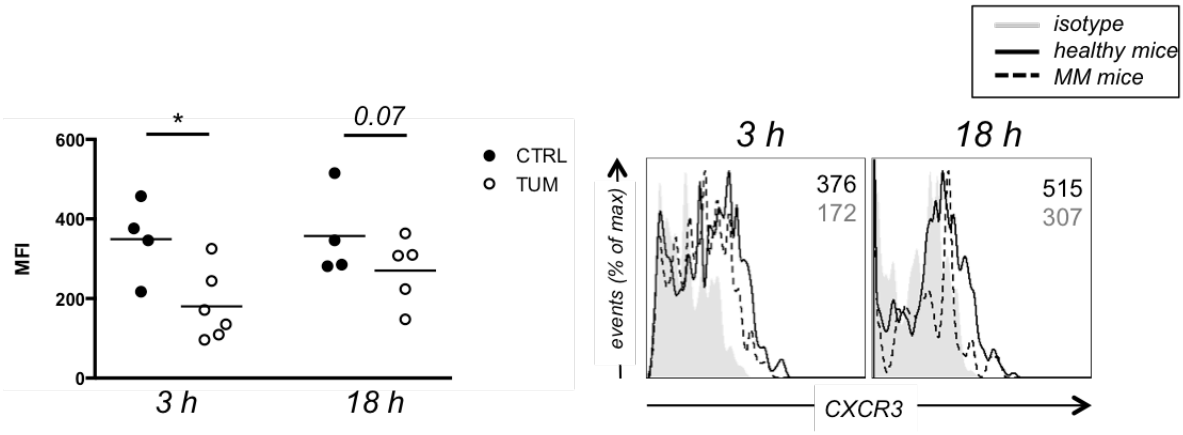


Figure 36 – *CXCR3 downmodulation on BM NK cells from MM-bearing mice takes place also on splenic transferred cells, with a fast kinetics.* Enriched splenic NK cells (≅ 30% of total cells) were previously stained with CFSE and then i.v. transferred into control or tumor-bearing mice after 3 weeks of MM growth. CXCR3 expression level on transferred NK cells was assessed in BM of recipient's mice 3 or 18 hours after injection. On the left graph, the individual values of MFI for each mouse analysed are displayed (horizontal bars represent the mean value), at the time points indicated. At least four transferred mice were used, in two independent experiments. The histogram plots (right) show a representative experiment CXCR3 downmodulation in MM-bearing mice (dotted line) as compared to control mice (black line). The isotypic control is pictured as the filled gray line. Numbers in the plot indicate the MFIs of the representative experiment. * = $p < 0.05$

Nevertheless, it remained to be assessed whether post-translational mechanisms, such as ligand-induced receptor internalization, rather than the direct exclusion of CXCR3+ NK cells, could explain the observed CXCR3 downmodulation. To prove one of these theories, we made use of $CXCR3^{-/-}$ mice, testing the BM trafficking properties of NK cells in absence of this receptor.

Competitive adoptive transfer experiments were performed, in which equal amounts of previously marked WT CD45.1+ C57BL/6J and CD45.2+ $CXCR3^{-/-}$-derived splenocytes were i.v. injected in control and tumor-bearing mice after 3 weeks of MM growth. Cells from recipient organs were collected after 3 and 18 hours, and donor-derived NK cells were distinguished according to their CD45 allelic variant (Figure 37a). Surprisingly, CXCR3-deficient NK cells were more able to traffick to BM than their WT counterpart, at both time points tested, in healthy mice and in the presence of the tumor. An higher amount of transferred CXCR3-deficient cells was also observed in blood, suggesting that

the receptor was needed for NK cells to extravasate to selected peripheral tissues. Indeed, CXCR3 absence determined a reduced migration directed towards liver and spleen, although in the former the differences were more marked than in the latter.

In Figure 37b the data concerning KLRG1- NK cell subset are presented as ratio of WT vs CXCR3$^{-/-}$ cells in each organ. The bar graphs show that the altered migration of KLRG1- NK cells to every organ in absence of CXCR3 was not affected by MM presence, and the same can be said for the BM after 18 hours. Notably, spleen, liver and blood display a different trend at the same time point: in fact, the presence of MM cells seems to significantly reduce CXCR3-sufficient NK cell entry in those organs in favour of their KO counterpart. Notwithstanding this, spleen and liver, but not the blood, are still preferred by WT KLRG1- NK cells.

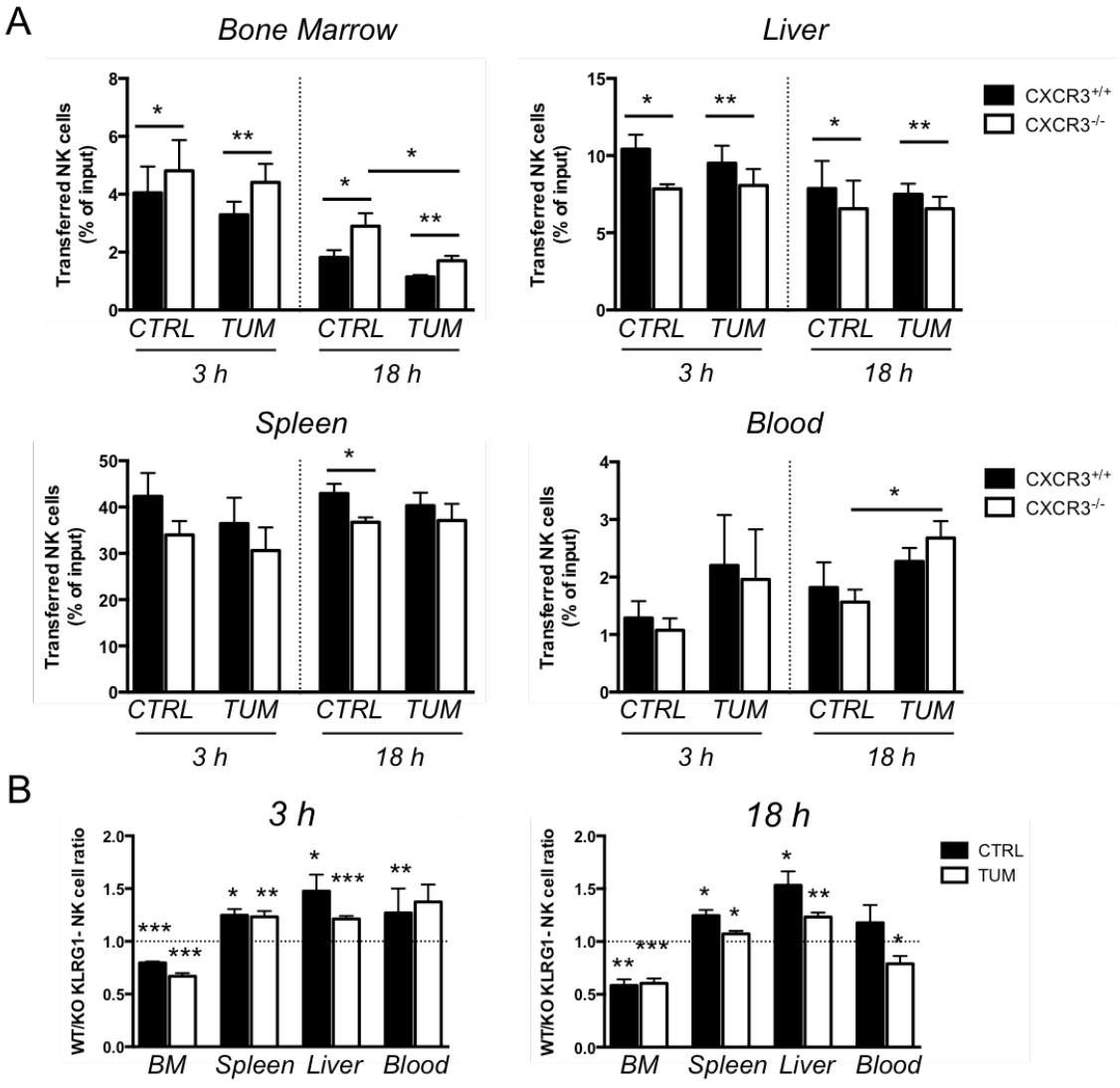


Figure 37 – *CXCR3-deficient KLRG1- NK cells home to the BM better than their WT counterpart.* Splenocytes from CD45.1+ WT and CD45.2+ $CXCR3^{-/-}$ mice were previously stained with CFSE and then adoptively transferred in control or tumor-bearing mice in 1:1 ratio. 3 or 18 hours later, BM, spleen, liver and PBMCs from recipient mice were collected and transferred cells were identified as CFSE+ cells. WT and $CXCR3^{-/-}$ cells were distinguished via their CD45 allelic variant. a) Histograms show the frequency of NK cells from WT and $CXCR3^{-/-}$ in the analysed organs, expressed as percentage of transferred cells in the organ over the total transferred cells in each mouse. In (b) histograms represent the contribution of KLRG1- NK cells of both genotypes, expressed as WT:$CXCR3^{-/-}$ transferred KLRG1- NK cell ratio in the organ at the indicated time. Each value was normalized for the initial WT:$CXCR3^{-/-}$ ratio present in input cells. Horizontal dotted line represents the value at which WT and $CXCR3^{-/-}$ cells are balanced in the organ. At least 5 mice per group, in two independent experiments were used. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Altogether, the competitive adoptive transfer experiments demonstrate that, despite the increase of CXCL9 and CXCL10 concentration observed in Figure 37b, CXCR3 is not requested for BM-directed NK cell migration. On the contrary, it seems to mediate NK cell migration towards peripheral organs, like spleen and liver.

5. Discussion

In the first part of this work it was demonstrated that the expression of the chemokine receptor CX3CR1 identifies two distinct KLRG1+ NK cell subpopulations, differing in effector responses (Figure 14), CXCR4 expression and CXCL12-driven chemotaxis (Figure 13a,b). Besides, KLRG1+CX3CR1- and CX3CR1+ NK cells are developmentally related, marking a further step in the model of NK cell differentiation. The expression analysis of GFP on NK cells and NK cell subsets evidenced a preferential distribution of CX3CR1-expressing NK cells within extra-medullary tissues, particularly spleen, blood and liver, while their frequency was relatively low in the BM. The inverse correlation between CX3CR1 and CXCR4 expression, highlighted on the two KLRG1+ NK cell subsets, recalls the expression pattern described for S1P receptor S1P5 on NK cells[116]. This gradual loss of a BM-tropic receptor fits well in the model of progressive loss of dependence from stromal CXCL12-expressing cells during NK cell development, to approximate the sinusoids, the interface between the BM and the circulation. Indeed, CXCR4/CXCL12 axis has been reported to play an important role not only in NK cell development[114], but also in hematopoietic stem cell, B cell and plasmocytoid DC (pDC) BM retention[115, 334, 335], so the prevalently parenchymal KLRG1- NK cell subset distribution is not surprising, given its CXCR4 expression levels. At the same time, KLRG1+/CX3CR1+ cells are sinusoidal for the 87% (Figure 13d), while KLRG1+/CX3CR1- fraction display an intermediate phenotype (56% of parenchymal cells).

Furthermore, the distinct degree of dependence on CXCR4 shown by KLRG1- NK cells was functionally relevant, as demonstrated by the different outcomes of CXCR4 antagonist AMD-3100 administration on NK cell subset BM retention. Indeed, the KLRG1- fraction was the most affected by CXCR4 blockage, resulting in a significant reduction in their parenchymal cell number, and in an equivalent increase in the sinusoidal compartment (Figure 13g,h) as well as in the peripheral blood (not shown). Conversely, the BM compartmentalization of KLRG1+, both CX3CR1- and CX3CR1+, NK cells was not affected

by AMD-3100. Of note, CXCR4 expression on KLRG1+ subpopulation is only reduced, but not abolished, meaning that CXCR4 might still exert an effect on this subset, other than BM retention.

Considering the most important function of a chemokine receptor, the most logical hypothesis to make for CX3CR1 biological function would be related to cell trafficking: the adoptive transfer experiments shown in Figure 12c were aimed at testing the homing capacity of CX3CR1+ NK cell population, during a mid-term time lapse (18 hours). The preferential localization in peripheral organs such as spleen and liver, might be interpreted differently: on one hand, it would suggest that CX3CR1 directs NK cells towards those districts, implicating the high expression of the ligand CX3CL1 on homeostatic conditions. This interpretation would though contradict a number of papers, where CX3CR1-deficient NK cells were normally represented in spleen and liver[336]. Nevertheless, Yu and colleagues reported that in a different model of CX3CR1-deficient mice, lung-residing NK cells (identified in this work as CD3-/DX5+) were reduced in steady state conditions, if compared to WT controls[336]. In contrast, in our model we did not detect significant differences in pulmonary NK cell number (not shown), but this discrepancy might be due to the different animal models, or to the methods used to identify NK cell population. Another report described that there were no differences in NK cell number within LNs, peripheral blood and spleen between CX3CL1-deficient mice and WT mice[337]. Besides, while CX3CL1 expression has been traced in some hepatic compartments in humans, it appears that its production is notably increased only during inflammatory conditions involving liver, such as cirrhosis or HCV-induced fibrosis[338, 339]. Of note, several publications indicated an important role for CX3CR1/CX3CL1 axis in the lung, since epithelial cells in respiratory airways express high levels of CX3CL1, recruiting CX3CR1+ leukocytes in steady state and inflammatory conditions[336, 340], though we did not assess lung-directed CX3CR1+ NK cell homing in adoptive transfer experiments.

At the same time, it is also true that besides its biological role, our data show that CX3CR1 is a marker of terminal differentiation on NK cells, and thus it is conceivable that mature, CX3CR1+ NK cells would preferentially accumulate in peripheral organs rather than in

BM, mimicking the distribution of endogenous NK cell subset in the different anatomical compartments (Figure 5).

The data obtained from competitive adoptive transfer experiments of $CX3CR1^{+/GFP}$ and $CX3CR1^{GFP/GFP}$ splenocytes shed more light on this issue: in fact, no difference in liver or spleen-directed trafficking was observed at any of the time points analysed, suggesting that, at least during the steady state, CX3CR1 does not play a role in NK cell positioning in these organs (Figure 22b). Conversely, the absence of CX3CL1 receptor entailed an increase of KLRG1+/GFP+ NK cell retention within the BM, and specifically in the BM parenchyma, 36 hours after transplantation. Taken together, the transfer experiments point out a non-redundant role for CX3CR1/CX3CL1 axis in promoting the passage of either developing (BM-derived) or trafficking (blood-derived) KLRG1+ NK cells from BM parenchyma towards the sinusoidal compartment. Considering the lack of effect of AMD-3100 administration on KLRG1+ NK cell BM positioning (Figure 13g,h), it can be assumed that CXCR4 is not involved in this process. This model of CX3CL1-dependent endothelium-directed NK cell migration is in agreement with a previous publication, showing that NK cells, in addition to T cells and monocytes, were captured and activated by CX3CL1 expressed on endothelial cells under physiologic flow conditions[341]. Moreover, the results shown in Figure 22 point out that CX3CR1 does not play a role in NK cell migration towards extramedullary organs in homeostatic conditions. On the other hand, CX3CR1 is abundantly expressed on a significant fraction of peripheral NK cells, but its function in periphery remains to be clarified.

Notwithstanding the evidences obtained so far, the poly(I:C)-induced acute hepatitis model uncovered a link between CX3CR1 on NK cells and their trafficking outside BM, in particular to spleen and liver, in an inflamed context (Figure 25). Some years ago, Wang and colleagues described an increase in hepatic CX3CL1 production after Poly(I:C) injection, and correlated it with the robust NK cell migration to the liver. Accordingly, we found an impairment in KLRG1+ NK cell migration to the liver 18 hours after Poly(I:C) injection, suggesting that CX3CR1, which is abundantly expressed by KLRG1+ splenic NK cell fraction, could actively promote their recruitment to the inflamed liver. In the same

report it was hypothesized that NK cells migrated from the spleen (but not from BM, LNs or thymus) to the liver, as splenectomized mice did not display enhanced hepatic NK cell recruitment[330]. This is in agreement with the significant numeric reduction of splenic NK cells and with the 3-fold increase of liver NK cells observed in WT (and CX3CR1-deficient) mice after 6 and 18 hours in our experimental conditions (Figure 25a). Nevertheless, in contrast with what was reported in that paper, we also detected a significant reduction of BM NK cells after Poly(I:C) injection, pointing out that BM NK cells might replace those migrated from the spleen to the liver.

Besides, the more pronounced NK cell egress from the BM in $CX3CR1^{GFP/GFP}$ mice is complex to decipher. Given the results obtained from the in vitro degranulation assay, CX3CR1-lacking NK cells are more efficiently activated, and this finding is in line with their readier response displayed in case of Poly(I:C)-induced activation. In fact, the effect of CX3CR1-deficiency in maintaining NK cells within BM parenchyma, during steady state conditions, could possibly be overcome by other powerful (chemotactic) stimuli, to which CX3CR1-deficient NK cells might even be more sensitive than WT cells. In this regard, another group reported the involvement of CXCR3 ligands (CXCL9 and CXCL10) as well as CCL5, in NK cell migration from the red pulp to the white pulp area in the spleen, after Poly(I:C) administration[333]: this migration took place early in the inflammation, and peaked after 16 hours, while the initial conditions were restored after about 48 hours. Thus, it is tempting to speculate that the the increased splenic NK cell recruitment observed at 6 and 18 hours might be related to a differential expression of CXCR3 and/or CCR5 between $CX3CR1^{+/GFP}$ and $CX3CR1^{GFP/GFP}$ NK cells in an inflammatory context.

Although in our study we did not investigate NK cell number within the lung, CX3CL1 production in the airways has been described during different inflammatory conditions[340, 342], and moreover pulmonary NK cells are known to contribute via IFNγ production to immune cell recruitment after Poly(I:C) treatment[343], indicating the lung as part of an alternative route in the Poly(I:C)-induced NK cell mobilization.

In CX3CR1-sufficient animals, IFNγ production and YAC-1-induced degranulation after cytokine exposure highlighted significant differences between KLRG1+/CX3CR1- and KLRG1+/CX3CR1+ NK cell subpopulation (Figure 14), implying that the CX3CR1-expressing subset might be less functional, or, rather, more difficultly activated by those stimuli. When the same features were tested in CX3CR1-deficient animals, we did not detect any changes in KLRG1+/GFP± populations, neither in degranulation assay nor in IFNγ production experiments, as in both cases KLRG1+/GFP+ NK cells were less functional than their KLRG1+/GFP- counterpart, in heterozygous as in homozygous mice (Figure 24a,b). Nevertheless, the positivity for the degranulation marker CD107a was increased on total NK cell population in CX3CR1-deficient mice, and was NK cell dependent (Figure 24c), but surprisingly it was mainly due to the KLRG1- contribution, and even more strikingly, to the GFP- fraction of that population. Since it has been previously demonstrated that CX3CL1/CX3CR1 axis does not directly affect NK cell cytotoxicity towards YAC-1 cells in vitro[344], it is possible that this effect could be related to putative maturative alterations linked to CX3CR1 deficiency, which would then interest the whole NK cell population, both GFP- and GFP+.

One of the most relevant features of the chemokine system is its apparent redundancy, or better its resiliency, caused by the overlapping roles played by more than one chemokine or chemokine receptor[124]. This results in the absence of significant alterations when a component of this system is lost, for example in KO mice. There are some exceptions, namely CXCR4 and CXCR7[158, 345, 346], which are involved in organogenesis of the nervous and cardiovascular systems, and incidentally are the most conserved chemokine receptors in vertebrates, highlighting their unicity within the chemokine universe.

In contrast, several receptors were reported to be unrelated with NK cell development, for example CCR1-, CCR2-, CXCR3- and CXCR6-deficient mice displayed normal NK cell number and frequency in peripheral organs, even though the assessment of NK cell maturation was not the primary aim of those papers[347, 348, 349, 350]. CCR5 role has been objectect of debate, as some authors first reported the absence of evident changes[349], while some years later it was described to influence NK cell development[351]. Its absence caused a

drop of NK cell number in BM, spleen and liver, as well as the developmental shift of NK cell subsets towards an immature phenotype. Moreover, later studies demonstrated that CXCR6-deficient mice display an altered NK cell repertoire, although exclusively in the liver[111].

Though it cannot be considered a chemokine, S1P has been demonstrated to be fundamental for NK cell trafficking and maturation[116]. S1P5 is the only S1P receptor expressed on murine NK cells, and $S1P5^{-/-}$ NK cells are reduced in liver, spleen, blood and lung, while their number rises in BM and LNs. Furthermore, these alterations are mostly due to the $CD11b^{high}$/CD27- subpopulation, which mainly expresses S1P5 in WT mice.

Although the phenotype observed in $CX3CR1^{GFP/GFP}$ mice is not really overturned when compared to $CX3CR1^{+/GFP}$ mice, the significant increase of KLRG1+ NK cell subset frequency in BM (Figure 17b), due to the GFP-expressing fraction (which would be CX3CR1+ in WT or heterozygous mice), is reminiscent of what described in Walzer's work[116].

The increase of GFP+, but not of KLRG1+, NK cell population observed in BM reverberates in all the analysed organs, hinting at a role for CX3CR1 in NK cell development, besides the effect in trafficking evidenced with adoptive transfer experiments. It is likely that the two phenomena are related, or even that there is just one biologic effect modulating different aspects of NK cell biology, in this case BM trafficking properties and cell development.

Interestingly, another similarity with the phenotype observed in S1P5-KO mice is the intrinsic effect of the receptor deficiency on the NK cell population, demonstrated through mixed BM chimeras (Figure 20b,c). Nevertheless, given the technical difficulty in determining S1P5 proteic expression level (as of now it can be detected just as mRNA), it is currently unknown whether CX3CR1 and S1P5 are co-expressed on NK cells, and thus cooperate in promoting NK cell exit from BM, or instead are expressed by different cell subpopulations.

The data deriving from this serie of experiments were published in two separate papers, dealing with the characterization of the KLRG1+/CX3CR1+ NK cell population and the definition of the role of CX3CR1 in BM NK cells, respectively[323, 352].

The second part of this thesis work was focused on NK cell trafficking during the progression of Multiple Myeloma, a tumor that grows in and affects mainly the BM. To this aim we made use of a well-known murine model of MM, the 5TMM, which reproduces most of the features of the human pathology[301, 353].
The 5TGM1 cell line we used in our in vivo model was susceptible to NK cell killing, as also assessed in in vitro assays (Figure 27), confirming the results obtained by other groups with human NK cells against U266 human myeloma cell line[281] and with 5T33 murine myelomatous cells[287].

Subsequently, we examined the in vivo model of MM in kinetics. The obtained results, both in terms of BM colonization (Figure 28a) and of macroscopic symptoms such as paraplegia (not shown), were in line with the previous reports on 5TMM[354]. Besides, organ cellularity was analysed in relation to MM growth, not evidencing any alterations either in BM or spleen up to the 3rd week of tumor growth, while after 4 weeks we observed on one hand a strong reduction of BM cell number and on the other a marked splenomegaly (not shown). Thus, similarly to the human disease, it is possible to define an asymptomatic phase of tumor progression (third week), in which hematopoiesis is not affected, and an aggressive phase (fourth week), where the detrimental effects caused by MM progression are evident.

The increased tumor growth highlighted after NK cell depletion demostrates an important role for these lymphocytes in the immune response against MM (Figure 28b). Indeed, the spleen was more robustly affected than BM by NK cell absence, perhaps because of the different composition of NK cell subsets residing in those organs, as splenic NK cells include an higher frequency of the functionally competent, likely licensed and primed,

CD11b^{high} NK cell subset, whereas BM NK cells are substantially represented by the heterogeneous CD11b^{low} NK cell subset.

The MM growth curve over time shows an exponential acceleration between the 3rd and the 4th week, while before its proliferation seemed to be controlled. Interestingly, NK cell number within the BM starts to decrease just at the 3rd week, suggesting a possible contribution in MM restraint after the estabilishment of the disease. Indeed, by depleting NK cells before the 5TGM1 cell injection we were able to evaluate their role in BM colonization of tumor cells and subsequent progression. However, additional experiments with different NK cell depletion protocols would be necessary to determine whether these cells might be needed at specific time points of tumor progression.

Another intriguing aspect is that analysing CD3+ T lymphocyte or CD3+/NK1.1+ NKT cell number in the BM, we did not find any alterations at the 3rd week of tumor growth (not shown), uncovering a selective effect on NK cells, at least regarding their change in cellularity.

The assessment of cell activation state in vivo through CD107a expression analysis denoted the impairment of BM NK cell function, in contrast to what was observed in the spleen (Figure 29). The advantageous ratio (for NK cells) between NK and tumor cells reported in the spleen, but not in the BM, is a possible explanation for the differential activation propensity in the two organs (Figure 31). Furthermore, MM cells produce a great amount of immunosuppressive molecules that inhibit NK cell function, such as TGFβ and IL-10[355, 356, 357], or PD-L1[358], which might overcome the NK cell activating signals deriving from MM presence in the BM. In addition, the recruitment of tolerant cell types such as MDSCs or regulatory T cells might contribute to NK cell functional impairment. Alternatively, the prolonged state of activation induced by tumor cells could gradually affect NK cell efficiency (NK cell exhaustion[359]), particularly at a time in which the generation of new and fresh NK cells is slowed down by tumor growth in BM (as it occurred at the fourth week).

It should also be taken in consideration that the effect of NK cells against the tumor can be exerted through mechanisms different from cytotoxicity (i.e. cytokine or chemokine production, TRAIL/FAS pathways), which were not analysed in this context. It could be possible, then, that BM NK cells are not really functionally impaired after 3 weeks, but just display an anti-tumoral activity other than cell degranulation.

Conversely, the spleen NK cell/MM cell ratio reaches 0.87 at the 4th week, which, apparently, seems to be still sufficient for the NK cells to be activated and degranulate in vivo.

In other tumor contexts, in particular in solid tumors, such as metastatic melanoma, NK cells have been involved in the control of metastasis[360, 361]: as a result, it is possible that splenic NK cells might react more efficiently against "metastatic" 5TGM1 cells than BM NK cells did, even if this would not explain the increase of CD107a+ cells observed after 2 weeks in BM.

The shift in NK cell subset frequency, more specifically the increase of KLRG1+ fraction observed in the BM at the 3rd week (Figure 32b) recalls what was observed in CX3CR1-deficient mice, in the first part of this work. Nevertheless, in this case this increase was the consequence of an actual numeric reduction of the KLRG1- NK cell subset (Figure 32a). Because there was no difference in survival among NK cell subsets (Figure 34a), we tested the hypothesis of an altered trafficking for the analysed NK cell subsets, in MM-bearing mice, finding a reduced NK cell retention in BM of MM-bearing mice, specifically due to the KLRG1- fraction (Figure 34b). Moreover, no difference in the conversion rate of KLRG1- NK cells to KLRG1+ cells was observed between healthy and tumor-bearing mice (Figure 34c). Together, these two evidences remark that the modulation of the homing properties of NK cell subsets is mainly, if not totally, responsible for the phenotype observed in tumor-bearing mice.

Importantly, no differences were observed at the 3rd week in the spleen, neither for endogenous NK cell subset composition, nor for their retention potential. This might reflect the primary role of BM in MM growth inasmuch as, differently from the spleen, it

provides unique signals (adhesion molecules, cytokines, growth factors, feeder cells, extracellular matrix components and so on). Indeed, the BM microenvironment of C57BL/KaLwRij mice seems to present decisive differences compared to other mouse strains, such as the commonly used C57BL/6, in which the 5TGM1 MM model does not grow. For example, Fowler and colleagues demonstrated that RAG2$^{-/-}$ mice (deficient in T and B cells), but not athymic nude mice (deficient in T cells), were 5TGM1-permissive strains, suggesting that B cells (absent in RAG2$^{-/-}$), and NK cells (more active in athymic mice) might condition MM progression in this mouse strain[362].

The analysis of NK cell chemokine receptor repertoire revealed a significant CXCR3 downregulation (Figure 35b), apperently in contrast with the increase of CXCR3 ligand concentration in the BM extracellular fluids (Figure 35d), although that may condition other CXCR3+ cell types (e.g. activated CD8+ T lymphocytes).

As described in paragraph 1.2.3., there are multiple levels of regulation of chemokine receptor activity: for example, the observed CXCR3 downmodulation on NK cells could be due to the altered NK cell development, to ligand-induced desensitization (especially given the BM increase of CXCL9 and CXCL10), or simply to the impaired CXCR3+ NK cell recruitment to BM in MM-bearing mice.

Having excluded the first of these hypotheses with an adoptive transfer approach (Figure 36), the augmented homing to BM of CXCR3-deficient NK cells, (mainly ascribable to KLRG1- subpopulation) (Figure 37a,b), confirmed the importance of CXCR3 in leading NK cells outside BM, and contextually ruled out the possibility that CXCR3 downmodulation could be due to ligand-induced receptor desensitization.
Taken together, the data obtained from the competitive adoptive transfer experiments demonstrate that CXCR3 drives KLRG1- NK cells outside the BM to other compartments, such as the liver. This finding opposes what recently reported by another group: indeed, CXCR3 was demonstrated to be important for NK cell intratumor infiltration, in a mouse

lymphoma model[363]. Besides, considering the importance of BM microenvironment in MM, this discrepancy is likely related to the different tumor model used by the authors. Interestingly, the advantage in trafficking to the liver displayed by CXCR3-deficient NK cells compared to WT NK cells is significantly weakened in presence of the tumor. This could be related to the enhancement of CXCR3 ligand production from several organs (including liver and spleen), and the consequent increase in their blood concentration. Thus, CXCR3-sufficient NK cells might be more attracted to the peripheral circulation than what was observed in normal conditions; nevertheless, WT NK cell preference in liver-directed homing is maintained also in tumor-bearing mice.

Besides, it is already been described how CXCR3 is important for NK cell migration to the inflamed liver[364, 365, 366, 367], and furthermore the higher sensitivity of BM, compared to splenic, CXCR3+ NK cells to CXCL9 and CXCL10 has been reported[347], probably because of their higher CXCR3 expression level. Interestingly, the same authors described that after IFNγ systemic activation, splenic CXCR3+ NK cells are highly sensitive to CXCL9, and are quickly mobilized to peripheral circulation.

At any rate, with the experimental methods applied here it is complex to determine the directionality of the CXCL9/CXCL10 gradient across the BM/blood interface, since we detected an increase of CXCL9, but not CXCL10, in sera of tumor-bearing mice, as compared to healthy controls. Indeed, the individual contribution of each CXCR3 ligand to cell migration varies depending on the context[368].

We have shown in Figure 26 that the majority of CXCR3+ and CXCR4+ NK cells are also KLRG1-. Therefore we hypothesize that the other axis involved in KLRG1- NK cell impaired BM retention is CXCR4, especially because its ligand CXCL12 was found to be significantly downregulated in BM extracellular fluid of tumor-bearing mice (Figure 35d). Given the importance of CXCL12 within BM microenvironment, this alteration might

likely have considerable biological consequences on tumor progression and immune cell recruitment in the inflamed environment.

Interestingly, we did not detect changes in CXCL12 mRNA (not shown), indicating that post-translational mechanisms, such as shedding by MMPs or citrullination and ubiquitination, could be involved in this reduction. Intriguingly, there are several papers showing the importance of the metalloproteinase-9 (MMP-9) in MM progression and BM colonization[369] of C57BL/KaLwRij mice, and CXCL12 has been demonstrated to be processed by this enzyme (and by many others)[222].

Similarly to our results, CXCL12 downmodulation in MM-bearing mice was recently described, together with upregulation of CXCL9 and CXCL10[370], by van der Voort and colleagues, although in another experimental model. Differently from our observations, the authors reported the reduction of CXCL12 at the mRNA level: this difference with our data could be due to the employed model. In fact, the paper dealt with allogeneic BM transplantation and multiple myeloma, and tumor cells were transplanted along with BM cells into previously irradiated, and thus immunocompromised, mice.

Hence, it is plausible that the kinetics of tumor growth in the two experimental settings are not perfectly matched, and thus CXCL12 expression could be controlled by different factors.

Importantly, the reduction of CXCR4 ligand in BM should not be functionally relevant for KLRG1+ subset, but mainly for KLRG1- cells, since, as shown in Figure 13g,h, KLRG1+ NK cell BM retention is mostly independent by CXCR4 effect.

Indeed, as BM retention of KLRG1+ NK cells is not altered in MM-bearing mice, the absence of differences in CX3CL1 levels in BM extracellular fluids is not surprising, although we cannot exclude changes in S1P/S1P5 axis.

Overall, the data presented in the second part of this thesis uncover a critical role of CXCR3 and, most likely, CXCR4 axes in NK cell retention at the tumor site, at an intermediate phase of MM growth. As KLRG1 expression on mature NK cells has not been formally associated to reduced functionality, it might be incautious to mark the exclusion

of KLRG1- NK cells from the BM as a tumor strategy of immune evasion, since KLRG1- fraction may still play an important role in peripheral tissues. Nevertheless, the reduced number of total NK cells in the BM found in MM-bearing mice is unambiguously a negative sign for the host immune response.

Further efforts, with different experimental approaches, are currently being made to elucidate the biological relevance of CXCL12 reduction.

Moreover, the interference with CXCR3 axis can be hypothesysed in the future, with the aim of increasing BM-directed NK cell migration, to counter MM growth before its exponential proliferative phase, in NK cell-based transfer therapies.

Collectively, the data presented herein contribute to the understanding of the signals that govern NK cell trafficking in BM during normal and pathological conditions, highlighting an important role for specific chemokines-chemokine receptor axes in the NK cell population and within different NK cell subsets.

6. Bibliography

1. Kiessling R, Klein E, Wigzell H. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur J Immunol* 1975, **5**(2)**:** 112-117.

2. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. *International journal of cancer Journal international du cancer* 1975, **16**(2)**:** 216-229.

3. Lanier LL, Phillips JH, Hackett J, Jr., Tutt M, Kumar V. Natural killer cells: definition of a cell type rather than a function. *J Immunol* 1986, **137**(9)**:** 2735-2739.

4. Trinchieri G. Biology of natural killer cells. *Adv Immunol* 1989, **47:** 187-376.

5. Walzer T, Jaeger S, Chaix J, Vivier E. Natural killer cells: from CD3(-)NKp46(+) to post-genomics meta-analyses. *Curr Opin Immunol* 2007, **19**(3)**:** 365-372.

6. Cerwenka A, Lanier LL. Natural killer cells, viruses and cancer. *Nat Rev Immunol* 2001, **1**(1)**:** 41-49.

7. Zinkernagel RM, Doherty PC. Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis. *Nature* 1974, **251**(5475)**:** 547-548.

8. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 1986, **319**(6055)**:** 675-678.

9. Nishimura MI, Stroynowski I, Hood L, Ostrand-Rosenberg S. H-2Kb antigen expression has no effect on natural killer susceptibility and tumorigenicity of a murine hepatoma. *J Immunol* 1988, **141**(12)**:** 4403-4409.

10. Pena J, Alonso C, Solana R, Serrano R, Carracedo J, Ramirez R. Natural killer susceptibility is independent of HLA class I antigen expression on cell lines obtained from human solid tumors. *Eur J Immunol* 1990, **20**(11)**:** 2445-2448.

11. Leiden JM, Karpinski BA, Gottschalk L, Kornbluth J. Susceptibility to natural killer cell-mediated cytolysis is independent of the level of target cell class I HLA expression. *J Immunol* 1989, **142**(6)**:** 2140-2147.

12. Lanier LL. Missing self, NK cells, and The White Album. *J Immunol* 2005, **174**(11)**:** 6565.

13. Lanier LL, Corliss B, Phillips JH. Arousal and inhibition of human NK cells. *Immunol Rev* 1997, **155:** 145-154.

14. Elliott JM, Yokoyama WM. Unifying concepts of MHC-dependent natural killer cell education. *Trends Immunol* 2011, **32**(8)**:** 364-372.

15. Lanier LL. NK cell receptors. *Annu Rev Immunol* 1998, **16:** 359-393.

16. Brown MG, Dokun AO, Heusel JW, Smith HR, Beckman DL, Blattenberger EA*, et al.* Vital involvement of a natural killer cell activation receptor in resistance to viral infection. *Science* 2001, **292**(5518)**:** 934-937.

17. Lee SH, Girard S, Macina D, Busa M, Zafer A, Belouchi A*, et al.* Susceptibility to mouse cytomegalovirus is associated with deletion of an activating natural killer cell receptor of the C-type lectin superfamily. *Nature genetics* 2001, **28**(1)**:** 42-45.

18. Daniels KA, Devora G, Lai WC, O'Donnell CL, Bennett M, Welsh RM. Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49H. *J Exp Med* 2001, **194**(1)**:** 29-44.

19. Lanier LL. NK cell recognition. *Annu Rev Immunol* 2005, **23:** 225-274.

20. Cerwenka A, Bakker AB, McClanahan T, Wagner J, Wu J, Phillips JH*, et al.* Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity* 2000, **12**(6)**:** 721-727.

21. Cerwenka A, Baron JL, Lanier LL. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc Natl Acad Sci U S A* 2001, **98**(20)**:** 11521-11526.

22. Lodoen MB, Lanier LL. Viral modulation of NK cell immunity. *Nature reviews Microbiology* 2005, **3**(1)**:** 59-69.

23. Gasser S, Raulet DH. The DNA damage response arouses the immune system. *Cancer Res* 2006, **66**(8)**:** 3959-3962.

24. Yabe T, McSherry C, Bach FH, Fisch P, Schall RP, Sondel PM*, et al.* A multigene family on human chromosome 12 encodes natural killer-cell lectins. *Immunogenetics* 1993, **37**(6)**:** 455-460.

25. Sivori S, Falco M, Della Chiesa M, Carlomagno S, Vitale M, Moretta L*, et al.* CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. *Proc Natl Acad Sci U S A* 2004, **101**(27)**:** 10116-10121.

26. Gerosa F, Gobbi A, Zorzi P, Burg S, Briere F, Carra G*, et al.* The reciprocal interaction of NK cells with plasmacytoid or myeloid dendritic cells profoundly affects innate resistance functions. *J Immunol* 2005, **174**(2)**:** 727-734.

27. Hart OM, Athie-Morales V, O'Connor GM, Gardiner CM. TLR7/8-mediated activation of human NK cells results in accessory cell-dependent IFN-gamma production. *J Immunol* 2005, **175**(3)**:** 1636-1642.

28. Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC*, et al.* Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu Rev Immunol* 2001, **19:** 197-223.

29. Bezman NA, Kim CC, Sun JC, Min-Oo G, Hendricks DW, Kamimura Y*, et al.* Molecular definition of the identity and activation of natural killer cells. *Nat Immunol* 2012, **13**(10)**:** 1000-1009.

30. Vivier E, Spits H, Cupedo T. Interleukin-22-producing innate immune cells: new players in mucosal immunity and tissue repair? *Nat Rev Immunol* 2009, **9**(4)**:** 229-234.

31. Walzer T, Blery M, Chaix J, Fuseri N, Chasson L, Robbins SH*, et al.* Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. *Proc Natl Acad Sci U S A* 2007, **104**(9)**:** 3384-3389.

32. Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, Bushkin Y*, et al.* Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* 2001, **409**(6823)**:** 1055-1060.

33. Vankayalapati R, Garg A, Porgador A, Griffith DE, Klucar P, Safi H*, et al.* Role of NK cell-activating receptors and their ligands in the lysis of mononuclear phagocytes infected with an intracellular bacterium. *J Immunol* 2005, **175**(7)**:** 4611-4617.

34. Arnon TI, Achdout H, Levi O, Markel G, Saleh N, Katz G*, et al.* Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus. *Nat Immunol* 2005, **6**(5)**:** 515-523.

35. Colonna M, Samaridis J. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science* 1995, **268**(5209)**:** 405-408.

36. Wagtmann N, Biassoni R, Cantoni C, Verdiani S, Malnati MS, Vitale M*, et al.* Molecular clones of the p58 NK cell receptor reveal immunoglobulin-related molecules with diversity in both the extra- and intracellular domains. *Immunity* 1995, **2**(5)**:** 439-449.

37. Bryceson YT, March ME, Ljunggren HG, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* 2006, **107**(1)**:** 159-166.

38. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol* 2008, **9**(5)**:** 495-502.

39. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL*, et al.* Innate or adaptive immunity? The example of natural killer cells. *Science* 2011, **331**(6013)**:** 44-49.

40. Martin-Fontecha A, Thomsen LL, Brett S, Gerard C, Lipp M, Lanzavecchia A*, et al.* Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat Immunol* 2004, **5**(12)**:** 1260-1265.

41. Fauriat C, Long EO, Ljunggren HG, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood* 2010, **115**(11)**:** 2167-2176.

42. Moretta A, Marcenaro E, Sivori S, Della Chiesa M, Vitale M, Moretta L. Early liaisons between cells of the innate immune system in inflamed peripheral tissues. *Trends Immunol* 2005, **26**(12)**:** 668-675.

43. Mocikat R, Braumuller H, Gumy A, Egeter O, Ziegler H, Reusch U*, et al.* Natural killer cells activated by MHC class I(low) targets prime dendritic cells to induce protective CD8 T cell responses. *Immunity* 2003, **19**(4)**:** 561-569.

44. Mailliard RB, Son YI, Redlinger R, Coates PT, Giermasz A, Morel PA*, et al.* Dendritic cells mediate NK cell help for Th1 and CTL responses: two-signal requirement for the induction of NK cell helper function. *J Immunol* 2003, **171**(5)**:** 2366-2373.

45. Ferlazzo G, Morandi B, D'Agostino A, Meazza R, Melioli G, Moretta A*, et al.* The interaction between NK cells and dendritic cells in bacterial infections results in rapid induction of NK cell activation and in the lysis of uninfected dendritic cells. *Eur J Immunol* 2003, **33**(2)**:** 306-313.

46. Jinushi M, Takehara T, Tatsumi T, Yamaguchi S, Sakamori R, Hiramatsu N*, et al.* Natural killer cell and hepatic cell interaction via NKG2A leads to dendritic cell-mediated induction of CD4 CD25 T cells with PD-1-dependent regulatory activities. *Immunology* 2007, **120**(1)**:** 73-82.

47. Nocentini G, Ronchetti S, Cuzzocrea S, Riccardi C. GITR/GITRL: more than an effector T cell co-stimulatory system. *Eur J Immunol* 2007, **37**(5)**:** 1165-1169.

48. Gao N, Dang T, Yuan D. IFN-gamma-dependent and -independent initiation of switch recombination by NK cells. *J Immunol* 2001, **167**(4)**:** 2011-2018.

49. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nat Immunol* 2008, **9**(5)**:** 503-510.

50. Paust S, Senman B, von Andrian UH. Adaptive immune responses mediated by natural killer cells. *Immunol Rev* 2010, **235**(1)**:** 286-296.

51. Jamieson AM, Isnard P, Dorfman JR, Coles MC, Raulet DH. Turnover and proliferation of NK cells in steady state and lymphopenic conditions. *J Immunol* 2004, **172**(2)**:** 864-870.

52. Paust S, von Andrian UH. Natural killer cell memory. *Nat Immunol* 2011, **12**(6)**:** 500-508.

53. Bjorkstrom NK, Lindgren T, Stoltz M, Fauriat C, Braun M, Evander M*, et al.* Rapid expansion and long-term persistence of elevated NK cell numbers in humans infected with hantavirus. *J Exp Med* 2011, **208**(1)**:** 13-21.

54. Peritt D, Robertson S, Gri G, Showe L, Aste-Amezaga M, Trinchieri G. Differentiation of human NK cells into NK1 and NK2 subsets. *J Immunol* 1998, **161**(11)**:** 5821-5824.

55. Kimura MY, Nakayama T. Differentiation of NK1 and NK2 cells. *Crit Rev Immunol* 2005, **25**(5)**:** 361-374.

56. Lauwerys BR, Garot N, Renauld JC, Houssiau FA. Cytokine production and killer activity of NK/T-NK cells derived with IL-2, IL-15, or the combination of IL-12 and IL-18. *J Immunol* 2000, **165**(4)**:** 1847-1853.

57. Perussia B, Loza MJ. Linear "2-0-1" lymphocyte development: hypotheses on cellular bases for immunity. *Trends Immunol* 2003, **24**(5)**:** 235-241.

58. Katsumoto T, Kimura M, Yamashita M, Hosokawa H, Hashimoto K, Hasegawa A*, et al.* STAT6-dependent differentiation and production of IL-5 and IL-13 in murine NK2 cells. *J Immunol* 2004, **173**(8)**:** 4967-4975.

59. Mehrotra PT, Donnelly RP, Wong S, Kanegane H, Geremew A, Mostowski HS*, et al.* Production of IL-10 by human natural killer cells stimulated with IL-2 and/or IL-12. *J Immunol* 1998, **160**(6)**:** 2637-2644.

60. Moretta A, Sivori S, Vitale M, Pende D, Morelli L, Augugliaro R*, et al.* Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. *J Exp Med* 1995, **182**(3)**:** 875-884.

61. Stewart CA, Laugier-Anfossi F, Vely F, Saulquin X, Riedmuller J, Tisserant A*, et al.* Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors. *Proc Natl Acad Sci U S A* 2005, **102**(37)**:** 13224-13229.

62. Fernandez NC, Treiner E, Vance RE, Jamieson AM, Lemieux S, Raulet DH. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood* 2005, **105**(11)**:** 4416-4423.

63. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song YJ, Yang L*, et al.* Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* 2005, **436**(7051)**:** 709-713.

64. Furukawa H, Yabe T, Watanabe K, Miyamoto R, Miki A, Akaza T*, et al.* Tolerance of NK and LAK activity for HLA class I-deficient targets in a TAP1-deficient patient (bare lymphocyte syndrome type I). *Human immunology* 1999, **60**(1)**:** 32-40.

65. Liao NS, Bix M, Zijlstra M, Jaenisch R, Raulet D. MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. *Science* 1991, **253**(5016)**:** 199-202.

66. Anfossi N, Andre P, Guia S, Falk CS, Roetynck S, Stewart CA*, et al.* Human NK cell education by inhibitory receptors for MHC class I. *Immunity* 2006, **25**(2)**:** 331-342.

67. Kim S, Sunwoo JB, Yang L, Choi T, Song YJ, French AR*, et al.* HLA alleles determine differences in human natural killer cell responsiveness and potency. *Proc Natl Acad Sci U S A* 2008, **105**(8)**:** 3053-3058.

68. Gasser S, Raulet DH. Activation and self-tolerance of natural killer cells. *Immunol Rev* 2006, **214:** 130-142.

69. Raulet DH, Vance RE. Self-tolerance of natural killer cells. *Nat Rev Immunol* 2006, **6**(7)**:** 520-531.

70. Brodin P, Karre K, Hoglund P. NK cell education: not an on-off switch but a tunable rheostat. *Trends Immunol* 2009, **30**(4)**:** 143-149.

71. Joncker NT, Fernandez NC, Treiner E, Vivier E, Raulet DH. NK cell responsiveness is tuned commensurate with the number of inhibitory receptors for self-MHC class I: the rheostat model. *J Immunol* 2009, **182**(8)**:** 4572-4580.

72. Orr MT, Lanier LL. Natural killer cell education and tolerance. *Cell* 2010, **142**(6)**:** 847-856.

73. Boos MD, Ramirez K, Kee BL. Extrinsic and intrinsic regulation of early natural killer cell development. *Immunol Res* 2008, **40**(3)**:** 193-207.

74. Suzuki R, Nakamura S. Malignancies of natural killer (NK) cell precursor: myeloid/NK cell precursor acute leukemia and blastic NK cell lymphoma/leukemia. *Leukemia research* 1999, **23**(7)**:** 615-624.

75. Suzuki R, Suzumiya J, Nakamura S, Aoki S, Notoya A, Ozaki S*, et al.* Aggressive natural killer-cell leukemia revisited: large granular lymphocyte leukemia of cytotoxic NK cells. *Leukemia* 2004, **18**(4)**:** 763-770.

76. Orange JS. Human natural killer cell deficiencies. *Current opinion in allergy and clinical immunology* 2006, **6**(6)**:** 399-409.

77. Kumar V, Ben-Ezra J, Bennett M, Sonnenfeld G. Natural killer cells in mice treated with 89strontium: normal target-binding cell numbers but inability to kill even after interferon administration. *J Immunol* 1979, **123**(4)**:** 1832-1838.

78. Seaman WE, Blackman MA, Gindhart TD, Roubinian JR, Loeb JM, Talal N. beta-Estradiol reduces natural killer cells in mice. *J Immunol* 1978, **121**(6)**:** 2193-2198.

79. Di Santo JP. Natural killer cell developmental pathways: a question of balance. *Annu Rev Immunol* 2006, **24:** 257-286.

80. Igarashi H, Gregory SC, Yokota T, Sakaguchi N, Kincade PW. Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. *Immunity* 2002, **17**(2)**:** 117-130.

81. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* 1997, **91**(5)**:** 661-672.

82. Michie AM, Carlyle JR, Schmitt TM, Ljutic B, Cho SK, Fong Q*, et al.* Clonal characterization of a bipotent T cell and NK cell progenitor in the mouse fetal thymus. *J Immunol* 2000, **164**(4)**:** 1730-1733.

83. Douagi I, Colucci F, Di Santo JP, Cumano A. Identification of the earliest prethymic bipotent T/NK progenitor in murine fetal liver. *Blood* 2002, **99**(2)**:** 463-471.

84. Jaleco AC, Blom B, Res P, Weijer K, Lanier LL, Phillips JH*, et al.* Fetal liver contains committed NK progenitors, but is not a site for development of CD34+ cells into T cells. *J Immunol* 1997, **159**(2)**:** 694-702.

85. Rosmaraki EE, Douagi I, Roth C, Colucci F, Cumano A, Di Santo JP. Identification of committed NK cell progenitors in adult murine bone marrow. *Eur J Immunol* 2001, **31**(6)**:** 1900-1909.

86. Kim S, Iizuka K, Kang HS, Dokun A, French AR, Greco S*, et al.* In vivo developmental stages in murine natural killer cell maturation. *Nat Immunol* 2002, **3**(6)**:** 523-528.

87. Yoshizawa K, Nakajima S, Notake T, Miyagawa S, Hida S, Taki S. IL-15-high-responder developing NK cells bearing Ly49 receptors in IL-15-/- mice. *J Immunol* 2011, **187**(10)**:** 5162-5169.

88. Vosshenrich CA, Ranson T, Samson SI, Corcuff E, Colucci F, Rosmaraki EE*, et al.* Roles for common cytokine receptor gamma-chain-dependent cytokines in the generation, differentiation, and maturation of NK cell precursors and peripheral NK cells in vivo. *J Immunol* 2005, **174**(3)**:** 1213-1221.

89. Hochweller K, Striegler J, Hammerling GJ, Garbi N. A novel CD11c.DTR transgenic mouse for depletion of dendritic cells reveals their requirement for homeostatic proliferation of natural killer cells. *Eur J Immunol* 2008, **38**(10)**:** 2776-2783.

90. Ikawa T, Fujimoto S, Kawamoto H, Katsura Y, Yokota Y. Commitment to natural killer cells requires the helix-loop-helix inhibitor Id2. *Proc Natl Acad Sci U S A* 2001, **98**(9)**:** 5164-5169.

91. Freud AG, Becknell B, Roychowdhury S, Mao HC, Ferketich AK, Nuovo GJ*, et al.* A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells. *Immunity* 2005, **22**(3)**:** 295-304.

92. Barton K, Muthusamy N, Fischer C, Ting CN, Walunas TL, Lanier LL*, et al.* The Ets-1 transcription factor is required for the development of natural killer cells in mice. *Immunity* 1998, **9**(4)**:** 555-563.

93. Colucci F, Samson SI, DeKoter RP, Lantz O, Singh H, Di Santo JP. Differential requirement for the transcription factor PU.1 in the generation of natural killer cells versus B and T cells. *Blood* 2001, **97**(9)**:** 2625-2632.

94. Lacorazza HD, Miyazaki Y, Di Cristofano A, Deblasio A, Hedvat C, Zhang J*, et al.* The ETS protein MEF plays a critical role in perforin gene expression and the development of natural killer and NK-T cells. *Immunity* 2002, **17**(4)**:** 437-449.

95. Gascoyne DM, Long E, Veiga-Fernandes H, de Boer J, Williams O, Seddon B*, et al.* The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development. *Nat Immunol* 2009, **10**(10)**:** 1118-1124.

96. Yokota Y, Mansouri A, Mori S, Sugawara S, Adachi S, Nishikawa S*, et al.* Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature* 1999, **397**(6721)**:** 702-706.

97. Takeda K, Cretney E, Hayakawa Y, Ota T, Akiba H, Ogasawara K*, et al.* TRAIL identifies immature natural killer cells in newborn mice and adult mouse liver. *Blood* 2005, **105**(5)**:** 2082-2089.

98. Peng H, Jiang X, Chen Y, Sojka DK, Wei H, Gao X*, et al.* Liver-resident NK cells confer adaptive immunity in skin-contact inflammation. *J Clin Invest* 2013, **123**(4)**:** 1444-1456.

99. Wu Q, Sun Y, Wang J, Lin X, Wang Y, Pegg LE*, et al.* Signal via lymphotoxin-beta R on bone marrow stromal cells is required for an early checkpoint of NK cell development. *J Immunol* 2001, **166**(3)**:** 1684-1689.

100. Lian RH, Chin RK, Nemeth HE, Libby SL, Fu YX, Kumar V. A role for lymphotoxin in the acquisition of Ly49 receptors during NK cell development. *Eur J Immunol* 2004, **34**(10)**:** 2699-2707.

101. Prlic M, Blazar BR, Farrar MA, Jameson SC. In vivo survival and homeostatic proliferation of natural killer cells. *J Exp Med* 2003, **197**(8)**:** 967-976.

102. Ranson T, Vosshenrich CA, Corcuff E, Richard O, Muller W, Di Santo JP. IL-15 is an essential mediator of peripheral NK-cell homeostasis. *Blood* 2003, **101**(12)**:** 4887-4893.

103. Laouar Y, Sutterwala FS, Gorelik L, Flavell RA. Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma. *Nat Immunol* 2005, **6**(6)**:** 600-607.

104. Vosshenrich CA, Garcia-Ojeda ME, Samson-Villeger SI, Pasqualetto V, Enault L, Richard-Le Goff O*, et al.* A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. *Nat Immunol* 2006, **7**(11)**:** 1217-1224.

105. Lanier LL, Le AM, Phillips JH, Warner NL, Babcock GF. Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. *J Immunol* 1983, **131**(4)**:** 1789-1796.

106. Lanier LL, Le AM, Civin CI, Loken MR, Phillips JH. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J Immunol* 1986, **136**(12)**:** 4480-4486.

107. Hayakawa Y, Smyth MJ. CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. *J Immunol* 2006, **176**(3)**:** 1517-1524.

108. Chiossone L, Chaix J, Fuseri N, Roth C, Vivier E, Walzer T. Maturation of mouse NK cells is a 4-stage developmental program. *Blood* 2009, **113**(22)**:** 5488-5496.

109. Silva A, Andrews DM, Brooks AG, Smyth MJ, Hayakawa Y. Application of CD27 as a marker for distinguishing human NK cell subsets. *International immunology* 2008, **20**(4)**:** 625-630.

110. Huntington ND, Tabarias H, Fairfax K, Brady J, Hayakawa Y, Degli-Esposti MA*, et al.* NK cell maturation and peripheral homeostasis is associated with KLRG1 up-regulation. *J Immunol* 2007, **178**(8)**:** 4764-4770.

111. Paust S, Gill HS, Wang BZ, Flynn MP, Moseman EA, Senman B*, et al.* Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. *Nat Immunol* 2010, **11**(12)**:** 1127-1135.

112. Walzer T, Vivier E. G-protein-coupled receptors in control of natural killer cell migration. *Trends Immunol* 2011, **32**(10)**:** 486-492.

113. Bernardini G, Sciume G, Bosisio D, Morrone S, Sozzani S, Santoni A. CCL3 and CXCL12 regulate trafficking of mouse bone marrow NK cell subsets. *Blood* 2008, **111**(7)**:** 3626-3634.

114. Noda M, Omatsu Y, Sugiyama T, Oishi S, Fujii N, Nagasawa T. CXCL12-CXCR4 chemokine signaling is essential for NK-cell development in adult mice. *Blood* 2011, **117**(2)**:** 451-458.

115. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 2006, **25**(6)**:** 977-988.

116. Walzer T, Chiossone L, Chaix J, Calver A, Carozzo C, Garrigue-Antar L*, et al.* Natural killer cell trafficking in vivo requires a dedicated sphingosine 1-phosphate receptor. *Nat Immunol* 2007, **8**(12)**:** 1337-1344.

117. Gregoire C, Chasson L, Luci C, Tomasello E, Geissmann F, Vivier E*, et al.* The trafficking of natural killer cells. *Immunol Rev* 2007, **220:** 169-182.

118. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu Rev Immunol* 2005, **23:** 127-159.

119. Bachelerie F, Ben-Baruch A, Burkhardt AM, Combadiere C, Farber JM, Graham GJ*, et al.* International Union of Pharmacology. LXXXIX. Update on the Extended Family of Chemokine Receptors and Introducing a New Nomenclature for Atypical Chemokine Receptors. *Pharmacological reviews* 2014, **66**(1)**:** 1-79.

120. Yoshimura T, Matsushima K, Tanaka S, Robinson EA, Appella E, Oppenheim JJ*, et al.* Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proc Natl Acad Sci U S A* 1987, **84**(24)**:** 9233-9237.

121. Rollins BJ. Chemokines. *Blood* 1997, **90**(3)**:** 909-928.

122. Strieter RM, Burdick MD, Gomperts BN, Belperio JA, Keane MP. CXC chemokines in angiogenesis. *Cytokine Growth Factor Rev* 2005, **16**(6)**:** 593-609.

123. Bajetto A, Bonavia R, Barbero S, Florio T, Schettini G. Chemokines and their receptors in the central nervous system. *Frontiers in neuroendocrinology* 2001, **22**(3)**:** 147-184.

124. Rossi D, Zlotnik A. The biology of chemokines and their receptors. *Annu Rev Immunol* 2000, **18:** 217-242.

125. Mantovani A. The chemokine system: redundancy for robust outputs. *Immunol Today* 1999, **20**(6)**:** 254-257.

126. Moser B. Chemokines: role in immune cell traffic. *European cytokine network* 2003, **14**(4)**:** 204-210.

127. Kinashi T. Intracellular signalling controlling integrin activation in lymphocytes. *Nat Rev Immunol* 2005, **5**(7)**:** 546-559.

128. Thelen M, Stein JV. How chemokines invite leukocytes to dance. *Nat Immunol* 2008, **9**(9)**:** 953-959.

129. Rot A, von Andrian UH. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. *Annu Rev Immunol* 2004, **22:** 891-928.

130. Aragay AM, Mellado M, Frade JM, Martin AM, Jimenez-Sainz MC, Martinez AC*, et al.* Monocyte chemoattractant protein-1-induced CCR2B receptor desensitization mediated by the G protein-coupled receptor kinase 2. *Proc Natl Acad Sci U S A* 1998, **95**(6)**:** 2985-2990.

131. Barlic J, Khandaker MH, Mahon E, Andrews J, DeVries ME, Mitchell GB*, et al.* beta-arrestins regulate interleukin-8-induced CXCR1 internalization. *J Biol Chem* 1999, **274**(23)**:** 16287-16294.

132. Borroni EM, Mantovani A, Locati M, Bonecchi R. Chemokine receptors intracellular trafficking. *Pharmacology & therapeutics* 2010, **127**(1)**:** 1-8.

133. Fan GH, Yang W, Wang XJ, Qian Q, Richmond A. Identification of a motif in the carboxyl terminus of CXCR2 that is involved in adaptin 2 binding and receptor internalization. *Biochemistry* 2001, **40**(3)**:** 791-800.

134. Allen SJ, Crown SE, Handel TM. Chemokine: receptor structure, interactions, and antagonism. *Annu Rev Immunol* 2007, **25:** 787-820.

135. Sadir R, Imberty A, Baleux F, Lortat-Jacob H. Heparan sulfate/heparin oligosaccharides protect stromal cell-derived factor-1 (SDF-1)/CXCL12 against proteolysis induced by CD26/dipeptidyl peptidase IV. *J Biol Chem* 2004, **279**(42)**:** 43854-43860.

136. Gough PJ, Garton KJ, Wille PT, Rychlewski M, Dempsey PJ, Raines EW. A disintegrin and metalloproteinase 10-mediated cleavage and shedding regulates the cell surface expression of CXC chemokine ligand 16. *J Immunol* 2004, **172**(6)**:** 3678-3685.

137. Garton KJ, Gough PJ, Blobel CP, Murphy G, Greaves DR, Dempsey PJ*, et al.* Tumor necrosis factor-alpha-converting enzyme (ADAM17) mediates the cleavage and shedding of fractalkine (CX3CL1). *J Biol Chem* 2001, **276**(41)**:** 37993-38001.

138. Johnson Z, Proudfoot AE, Handel TM. Interaction of chemokines and glycosaminoglycans: a new twist in the regulation of chemokine function with opportunities for therapeutic intervention. *Cytokine Growth Factor Rev* 2005, **16**(6)**:** 625-636.

139. Handel TM, Johnson Z, Crown SE, Lau EK, Proudfoot AE. Regulation of protein function by glycosaminoglycans--as exemplified by chemokines. *Annual review of biochemistry* 2005, **74:** 385-410.

140. Lau EK, Paavola CD, Johnson Z, Gaudry JP, Geretti E, Borlat F*, et al.* Identification of the glycosaminoglycan binding site of the CC chemokine, MCP-1: implications for structure and function in vivo. *J Biol Chem* 2004, **279**(21)**:** 22294-22305.

141. Proudfoot AE, Handel TM, Johnson Z, Lau EK, LiWang P, Clark-Lewis I*, et al.* Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc Natl Acad Sci U S A* 2003, **100**(4)**:** 1885-1890.

142. Campanella GS, Grimm J, Manice LA, Colvin RA, Medoff BD, Wojtkiewicz GR*, et al.* Oligomerization of CXCL10 is necessary for endothelial cell presentation and in vivo activity. *J Immunol* 2006, **177**(10)**:** 6991-6998.

143. Paoletti S, Petkovic V, Sebastiani S, Danelon MG, Uguccioni M, Gerber BO. A rich chemokine environment strongly enhances leukocyte migration and activities. *Blood* 2005, **105**(9)**:** 3405-3412.

144. Sebastiani S, Danelon G, Gerber B, Uguccioni M. CCL22-induced responses are powerfully enhanced by synergy inducing chemokines via CCR4: evidence for the involvement of first beta-strand of chemokine. *Eur J Immunol* 2005, **35**(3)**:** 746-756.

145. Gouwy M, Struyf S, Catusse J, Proost P, Van Damme J. Synergy between proinflammatory ligands of G protein-coupled receptors in neutrophil activation and migration. *J Leukoc Biol* 2004, **76**(1)**:** 185-194.

146. Milligan G, Smith NJ. Allosteric modulation of heterodimeric G-protein-coupled receptors. *Trends in pharmacological sciences* 2007, **28**(12)**:** 615-620.

147. Moser B, Wolf M, Walz A, Loetscher P. Chemokines: multiple levels of leukocyte migration control. *Trends Immunol* 2004, **25**(2)**:** 75-84.

148. Proost P, Schutyser E, Menten P, Struyf S, Wuyts A, Opdenakker G*, et al.* Amino-terminal truncation of CXCR3 agonists impairs receptor signaling and lymphocyte chemotaxis, while preserving antiangiogenic properties. *Blood* 2001, **98**(13)**:** 3554-3561.

149. Berahovich RD, Miao Z, Wang Y, Premack B, Howard MC, Schall TJ. Proteolytic activation of alternative CCR1 ligands in inflammation. *J Immunol* 2005, **174**(11)**:** 7341-7351.

150. Guan E, Wang J, Norcross MA. Amino-terminal processing of MIP-1beta/CCL4 by CD26/dipeptidyl-peptidase IV. *Journal of cellular biochemistry* 2004, **92**(1)**:** 53-64.

151. Borroni EM, Cancellieri C, Vacchini A, Benureau Y, Lagane B, Bachelerie F*, et al.* beta-arrestin-dependent activation of the cofilin pathway is required for the scavenging activity of the atypical chemokine receptor D6. *Science signaling* 2013, **6**(273)**:** ra30 31-11, S31-33.

152. Pruenster M, Mudde L, Bombosi P, Dimitrova S, Zsak M, Middleton J*, et al.* The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity. *Nat Immunol* 2009, **10**(1)**:** 101-108.

153. Chaudhuri A, Nielsen S, Elkjaer ML, Zbrzezna V, Fang F, Pogo AO. Detection of Duffy antigen in the plasma membranes and caveolae of vascular endothelial and epithelial cells of nonerythroid organs. *Blood* 1997, **89**(2)**:** 701-712.

154. Ikin EW, Mourant AE, Pettenkofer HJ, Blumenthal G. Discovery of the expected haemagglutinin, anti-Fyb. *Nature* 1951, **168**(4288)**:** 1077-1078.

155. Horuk R, Wang ZX, Peiper SC, Hesselgesser J. Identification and characterization of a promiscuous chemokine-binding protein in a human erythroleukemic cell line. *J Biol Chem* 1994, **269**(26)**:** 17730-17733.

156. Gardner L, Patterson AM, Ashton BA, Stone MA, Middleton J. The human Duffy antigen binds selected inflammatory but not homeostatic chemokines. *Biochemical and biophysical research communications* 2004, **321**(2)**:** 306-312.

157. Gosling J, Dairaghi DJ, Wang Y, Hanley M, Talbot D, Miao Z*, et al.* Cutting edge: identification of a novel chemokine receptor that binds dendritic cell- and T cell-active chemokines including ELC, SLC, and TECK. *J Immunol* 2000, **164**(6)**:** 2851-2856.

158. Sierro F, Biben C, Martinez-Munoz L, Mellado M, Ransohoff RM, Li M*, et al.* Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proc Natl Acad Sci U S A* 2007, **104**(37)**:** 14759-14764.

159. Dambly-Chaudiere C, Cubedo N, Ghysen A. Control of cell migration in the development of the posterior lateral line: antagonistic interactions between the chemokine receptors CXCR4 and CXCR7/RDC1. *BMC developmental biology* 2007, **7:** 23.

160. Levoye A, Balabanian K, Baleux F, Bachelerie F, Lagane B. CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. *Blood* 2009, **113**(24)**:** 6085-6093.

161. Comerford I, Nibbs RJ, Litchfield W, Bunting M, Harata-Lee Y, Haylock-Jacobs S*, et al.* The atypical chemokine receptor CCX-CKR scavenges homeostatic chemokines in

circulation and tissues and suppresses Th17 responses. *Blood* 2010, **116**(20)**:** 4130-4140.

162. Heinzel K, Benz C, Bleul CC. A silent chemokine receptor regulates steady-state leukocyte homing in vivo. *Proc Natl Acad Sci U S A* 2007, **104**(20)**:** 8421-8426.

163. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* 2008, **454**(7203)**:** 436-444.

164. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002, **420**(6917)**:** 860-867.

165. Mantovani A. Cancer: Inflaming metastasis. *Nature* 2009, **457**(7225)**:** 36-37.

166. Mantovani A, Savino B, Locati M, Zammataro L, Allavena P, Bonecchi R. The chemokine system in cancer biology and therapy. *Cytokine Growth Factor Rev* 2010, **21**(1)**:** 27-39.

167. Zlotnik A. Chemokines and cancer. *International journal of cancer Journal international du cancer* 2006, **119**(9)**:** 2026-2029.

168. Nibbs RJ, Gilchrist DS, King V, Ferra A, Forrow S, Hunter KD*, et al.* The atypical chemokine receptor D6 suppresses the development of chemically induced skin tumors. *J Clin Invest* 2007, **117**(7)**:** 1884-1892.

169. Vetrano S, Borroni EM, Sarukhan A, Savino B, Bonecchi R, Correale C*, et al.* The lymphatic system controls intestinal inflammation and inflammation-associated Colon Cancer through the chemokine decoy receptor D6. *Gut* 2010, **59**(2)**:** 197-206.

170. Borrello MG, Alberti L, Fischer A, Degl'innocenti D, Ferrario C, Gariboldi M*, et al.* Induction of a proinflammatory program in normal human thyrocytes by the RET/PTC1 oncogene. *Proc Natl Acad Sci U S A* 2005, **102**(41)**:** 14825-14830.

171. Mestdagt M, Polette M, Buttice G, Noel A, Ueda A, Foidart JM*, et al.* Transactivation of MCP-1/CCL2 by beta-catenin/TCF-4 in human breast cancer cells. *International journal of cancer Journal international du cancer* 2006, **118**(1)**:** 35-42.

172. Sparmann A, Bar-Sagi D. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer cell* 2004, **6**(5)**:** 447-458.

173. Yan W, Chen X. Identification of GRO1 as a critical determinant for mutant p53 gain of function. *J Biol Chem* 2009, **284**(18)**:** 12178-12187.

174. Soucek L, Lawlor ER, Soto D, Shchors K, Swigart LB, Evan GI. Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. *Nat Med* 2007, **13**(10)**:** 1211-1218.

175. Buonamici S, Trimarchi T, Ruocco MG, Reavie L, Cathelin S, Mar BG*, et al.* CCR7 signalling as an essential regulator of CNS infiltration in T-cell leukaemia. *Nature* 2009, **459**(7249)**:** 1000-1004.

176. Schwieger M, Schuler A, Forster M, Engelmann A, Arnold MA, Delwel R*, et al.* Homing and invasiveness of MLL/ENL leukemic cells is regulated by MEF2C. *Blood* 2009, **114**(12)**:** 2476-2488.

177. Crowther-Swanepoel D, Qureshi M, Dyer MJ, Matutes E, Dearden C, Catovsky D*, et al.* Genetic variation in CXCR4 and risk of chronic lymphocytic leukemia. *Blood* 2009, **114**(23)**:** 4843-4846.

178. Bierie B, Chung CH, Parker JS, Stover DG, Cheng N, Chytil A*, et al.* Abrogation of TGF-beta signaling enhances chemokine production and correlates with prognosis in human breast cancer. *J Clin Invest* 2009, **119**(6)**:** 1571-1582.

179. Yang L, Huang J, Ren X, Gorska AE, Chytil A, Aakre M*, et al.* Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. *Cancer cell* 2008, **13**(1)**:** 23-35.

180. Strieter RM, Burdick MD, Mestas J, Gomperts B, Keane MP, Belperio JA. Cancer CXC chemokine networks and tumour angiogenesis. *European journal of cancer* 2006, **42**(6)**:** 768-778.

181. Li A, Varney ML, Valasek J, Godfrey M, Dave BJ, Singh RK. Autocrine role of interleukin-8 in induction of endothelial cell proliferation, survival, migration and MMP-2 production and angiogenesis. *Angiogenesis* 2005, **8**(1)**:** 63-71.

182. Kryczek I, Lange A, Mottram P, Alvarez X, Cheng P, Hogan M*, et al.* CXCL12 and vascular endothelial growth factor synergistically induce neoangiogenesis in human ovarian cancers. *Cancer Res* 2005, **65**(2)**:** 465-472.

183. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R*, et al.* Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 2005, **121**(3)**:** 335-348.

184. Romagnani P, Annunziato F, Lasagni L, Lazzeri E, Beltrame C, Francalanci M*, et al.* Cell cycle-dependent expression of CXC chemokine receptor 3 by endothelial cells mediates angiostatic activity. *J Clin Invest* 2001, **107**(1)**:** 53-63.

185. Tazzyman S, Lewis CE, Murdoch C. Neutrophils: key mediators of tumour angiogenesis. *International journal of experimental pathology* 2009, **90**(3)**:** 222-231.

186. Sozzani S, Rusnati M, Riboldi E, Mitola S, Presta M. Dendritic cell-endothelial cell cross-talk in angiogenesis. *Trends Immunol* 2007, **28**(9)**:** 385-392.

187. Mantovani A, Schioppa T, Porta C, Allavena P, Sica A. Role of tumor-associated macrophages in tumor progression and invasion. *Cancer metastasis reviews* 2006, **25**(3)**:** 315-322.

188. Schioppa T, Uranchimeg B, Saccani A, Biswas SK, Doni A, Rapisarda A*, et al.* Regulation of the chemokine receptor CXCR4 by hypoxia. *J Exp Med* 2003, **198**(9)**:** 1391-1402.

189. Staller P, Sulitkova J, Lisztwan J, Moch H, Oakeley EJ, Krek W. Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. *Nature* 2003, **425**(6955)**:** 307-311.

190. Facciabene A, Peng X, Hagemann IS, Balint K, Barchetti A, Wang LP*, et al.* Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T(reg) cells. *Nature* 2011, **475**(7355)**:** 226-230.

191. Zlotnik A, Burkhardt AM, Homey B. Homeostatic chemokine receptors and organ-specific metastasis. *Nat Rev Immunol* 2011, **11**(9)**:** 597-606.

192. Balkwill FR. The chemokine system and cancer. *J Pathol* 2012, **226**(2)**:** 148-157.

193. Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME*, et al.* Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001, **410**(6824)**:** 50-56.

194. Ehtesham M, Mapara KY, Stevenson CB, Thompson RC. CXCR4 mediates the proliferation of glioblastoma progenitor cells. *Cancer Lett* 2009, **274**(2)**:** 305-312.

195. Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M*, et al.* Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell stem cell* 2007, **1**(3)**:** 313-323.

196. Wendt MK, Cooper AN, Dwinell MB. Epigenetic silencing of CXCL12 increases the metastatic potential of mammary carcinoma cells. *Oncogene* 2008, **27**(10)**:** 1461-1471.

197. Wendt MK, Johanesen PA, Kang-Decker N, Binion DG, Shah V, Dwinell MB. Silencing of epithelial CXCL12 expression by DNA hypermethylation promotes colonic carcinoma metastasis. *Oncogene* 2006, **25**(36)**:** 4986-4997.

198. Hattermann K, Held-Feindt J, Lucius R, Muerkoster SS, Penfold ME, Schall TJ*, et al.* The chemokine receptor CXCR7 is highly expressed in human glioma cells and mediates antiapoptotic effects. *Cancer Res* 2010, **70**(8)**:** 3299-3308.

199. Varney ML, Johansson SL, Singh RK. Distinct expression of CXCL8 and its receptors CXCR1 and CXCR2 and their association with vessel density and aggressiveness in malignant melanoma. *American journal of clinical pathology* 2006, **125**(2)**:** 209-216.

200. Jones D, Benjamin RJ, Shahsafaei A, Dorfman DM. The chemokine receptor CXCR3 is expressed in a subset of B-cell lymphomas and is a marker of B-cell chronic lymphocytic leukemia. *Blood* 2000, **95**(2)**:** 627-632.

201. Meijer J, Zeelenberg IS, Sipos B, Roos E. The CXCR5 chemokine receptor is expressed by carcinoma cells and promotes growth of colon carcinoma in the liver. *Cancer Res* 2006, **66**(19)**:** 9576-9582.

202. Darash-Yahana M, Gillespie JW, Hewitt SM, Chen YY, Maeda S, Stein I*, et al.* The chemokine CXCL16 and its receptor, CXCR6, as markers and promoters of inflammation-associated cancers. *PloS one* 2009, **4**(8)**:** e6695.

203. Manes S, Mira E, Colomer R, Montero S, Real LM, Gomez-Mouton C*, et al.* CCR5 expression influences the progression of human breast cancer in a p53-dependent manner. *J Exp Med* 2003, **198**(9)**:** 1381-1389.

204. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nature reviews Cancer* 2004, **4**(1)**:** 71-78.

205. Dwyer RM, Potter-Beirne SM, Harrington KA, Lowery AJ, Hennessy E, Murphy JM*, et al.* Monocyte chemotactic protein-1 secreted by primary breast tumors stimulates migration of mesenchymal stem cells. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2007, **13**(17)**:** 5020-5027.

206. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P*, et al.* Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004, **10**(9)**:** 942-949.

207. Liu J, Zhang N, Li Q, Zhang W, Ke F, Leng Q*, et al.* Tumor-associated macrophages recruit CCR6+ regulatory T cells and promote the development of colorectal cancer via enhancing CCL20 production in mice. *PloS one* 2011, **6**(4)**:** e19495.

208. Iellem A, Mariani M, Lang R, Recalde H, Panina-Bordignon P, Sinigaglia F*, et al.* Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells. *J Exp Med* 2001, **194**(6)**:** 847-853.

209. Lesokhin AM, Hohl TM, Kitano S, Cortez C, Hirschhorn-Cymerman D, Avogadri F*, et al.* Monocytic CCR2(+) myeloid-derived suppressor cells promote immune escape by limiting activated CD8 T-cell infiltration into the tumor microenvironment. *Cancer Res* 2012, **72**(4)**:** 876-886.

210. Viola A, Sarukhan A, Bronte V, Molon B. The pros and cons of chemokines in tumor immunology. *Trends Immunol* 2012, **33**(10)**:** 496-504.

211. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L*, et al.* Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer cell* 2009, **16**(3)**:** 183-194.

212. Lee LF, Hellendall RP, Wang Y, Haskill JS, Mukaida N, Matsushima K*, et al.* IL-8 reduced tumorigenicity of human ovarian cancer in vivo due to neutrophil infiltration. *J Immunol* 2000, **164**(5)**:** 2769-2775.

213. Ohta M, Tanaka F, Yamaguchi H, Sadanaga N, Inoue H, Mori M. The high expression of Fractalkine results in a better prognosis for colorectal cancer patients. *International journal of oncology* 2005, **26**(1)**:** 41-47.

214. Gutwein P, Schramme A, Sinke N, Abdel-Bakky MS, Voss B, Obermuller N*, et al.* Tumoural CXCL16 expression is a novel prognostic marker of longer survival times in renal cell cancer patients. *European journal of cancer* 2009, **45**(3)**:** 478-489.

215. Dieu-Nosjean MC, Antoine M, Danel C, Heudes D, Wislez M, Poulot V*, et al.* Long-term survival for patients with non-small-cell lung cancer with intratumoral lymphoid

structures. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2008, **26**(27)**:** 4410-4417.

216. Mangalmurti NS, Xiong Z, Hulver M, Ranganathan M, Liu XH, Oriss T*, et al.* Loss of red cell chemokine scavenging promotes transfusion-related lung inflammation. *Blood* 2009, **113**(5)**:** 1158-1166.

217. Horton LW, Yu Y, Zaja-Milatovic S, Strieter RM, Richmond A. Opposing roles of murine duffy antigen receptor for chemokine and murine CXC chemokine receptor-2 receptors in murine melanoma tumor growth. *Cancer Res* 2007, **67**(20)**:** 9791-9799.

218. Bandyopadhyay S, Zhan R, Chaudhuri A, Watabe M, Pai SK, Hirota S*, et al.* Interaction of KAI1 on tumor cells with DARC on vascular endothelium leads to metastasis suppression. *Nat Med* 2006, **12**(8)**:** 933-938.

219. Wu FY, Ou ZL, Feng LY, Luo JM, Wang LP, Shen ZZ*, et al.* Chemokine decoy receptor d6 plays a negative role in human breast cancer. *Molecular cancer research : MCR* 2008, **6**(8)**:** 1276-1288.

220. Wang J, Shiozawa Y, Wang J, Wang Y, Jung Y, Pienta KJ*, et al.* The role of CXCR7/RDC1 as a chemokine receptor for CXCL12/SDF-1 in prostate cancer. *J Biol Chem* 2008, **283**(7)**:** 4283-4294.

221. Van Rechem C, Rood BR, Touka M, Pinte S, Jenal M, Guerardel C*, et al.* Scavenger chemokine (CXC motif) receptor 7 (CXCR7) is a direct target gene of HIC1 (hypermethylated in cancer 1). *J Biol Chem* 2009, **284**(31)**:** 20927-20935.

222. Mortier A, Gouwy M, Van Damme J, Proost P. Effect of posttranslational processing on the in vitro and in vivo activity of chemokines. *Experimental cell research* 2011, **317**(5)**:** 642-654.

223. Meijer J, Ogink J, Kreike B, Nuyten D, de Visser KE, Roos E. The chemokine receptor CXCR6 and its ligand CXCL16 are expressed in carcinomas and inhibit proliferation. *Cancer Res* 2008, **68**(12)**:** 4701-4708.

224. Molon B, Ugel S, Del Pozzo F, Soldani C, Zilio S, Avella D*, et al.* Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. *J Exp Med* 2011, **208**(10)**:** 1949-1962.

225. Sozzani S, Luini W, Bianchi G, Allavena P, Wells TN, Napolitano M*, et al.* The viral chemokine macrophage inflammatory protein-II is a selective Th2 chemoattractant. *Blood* 1998, **92**(11)**:** 4036-4039.

226. De Clercq E. The AMD3100 story: the path to the discovery of a stem cell mobilizer (Mozobil). *Biochemical pharmacology* 2009, **77**(11)**:** 1655-1664.

227. Li X, Loberg R, Liao J, Ying C, Snyder LA, Pienta KJ*, et al.* A destructive cascade mediated by CCL2 facilitates prostate cancer growth in bone. *Cancer Res* 2009, **69**(4)**:** 1685-1692.

228. Palumbo A, Anderson K. Multiple myeloma. *N Engl J Med* 2011, **364**(11)**:** 1046-1060.

229. Brenner H, Gondos A, Pulte D. Recent major improvement in long-term survival of younger patients with multiple myeloma. *Blood* 2008, **111**(5)**:** 2521-2526.

230. Kyle RA, Durie BG, Rajkumar SV, Landgren O, Blade J, Merlini G*, et al.* Monoclonal gammopathy of undetermined significance (MGUS) and smoldering (asymptomatic) multiple myeloma: IMWG consensus perspectives risk factors for progression and guidelines for monitoring and management. *Leukemia* 2010, **24**(6)**:** 1121-1127.

231. Kyle RA, Therneau TM, Rajkumar SV, Offord JR, Larson DR, Plevak MF*, et al.* A long-term study of prognosis in monoclonal gammopathy of undetermined significance. *N Engl J Med* 2002, **346**(8)**:** 564-569.

232. Kuehl WM, Bergsagel PL. Multiple myeloma: evolving genetic events and host interactions. *Nature reviews Cancer* 2002, **2**(3)**:** 175-187.

233. Bergsagel PL, Kuehl WM, Zhan F, Sawyer J, Barlogie B, Shaughnessy J, Jr. Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma. *Blood* 2005, **106**(1)**:** 296-303.

234. Zhan F, Huang Y, Colla S, Stewart JP, Hanamura I, Gupta S*, et al.* The molecular classification of multiple myeloma. *Blood* 2006, **108**(6)**:** 2020-2028.

235. Abe M. Targeting the interplay between myeloma cells and the bone marrow microenvironment in myeloma. *International journal of hematology* 2011, **94**(4)**:** 334-343.

236. Teoh G, Anderson KC. Interaction of tumor and host cells with adhesion and extracellular matrix molecules in the development of multiple myeloma. *Hematology/oncology clinics of North America* 1997, **11**(1)**:** 27-42.

237. Hideshima T, Bergsagel PL, Kuehl WM, Anderson KC. Advances in biology of multiple myeloma: clinical applications. *Blood* 2004, **104**(3)**:** 607-618.

238. Kumar S, Witzig TE, Timm M, Haug J, Wellik L, Fonseca R*, et al.* Expression of VEGF and its receptors by myeloma cells. *Leukemia* 2003, **17**(10)**:** 2025-2031.

239. Radtke F, Raj K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nature reviews Cancer* 2003, **3**(10)**:** 756-767.

240. Gunn WG, Conley A, Deininger L, Olson SD, Prockop DJ, Gregory CA. A crosstalk between myeloma cells and marrow stromal cells stimulates production of DKK1 and interleukin-6: a potential role in the development of lytic bone disease and tumor progression in multiple myeloma. *Stem cells* 2006, **24**(4)**:** 986-991.

241. Noonan K, Borrello I. The immune microenvironment of myeloma. *Cancer microenvironment : official journal of the International Cancer Microenvironment Society* 2011, **4**(3)**:** 313-323.

242. Manier S, Sacco A, Leleu X, Ghobrial IM, Roccaro AM. Bone marrow microenvironment in multiple myeloma progression. *Journal of biomedicine & biotechnology* 2012, **2012:** 157496.

243. Giuliani N, Colla S, Rizzoli V. New insight in the mechanism of osteoclast activation and formation in multiple myeloma: focus on the receptor activator of NF-kappaB ligand (RANKL). *Exp Hematol* 2004, **32**(8)**:** 685-691.

244. Sezer O, Heider U, Jakob C, Zavrski I, Eucker J, Possinger K*, et al.* Immunocytochemistry reveals RANKL expression of myeloma cells. *Blood* 2002, **99**(12)**:** 4646-4647; author reply 4647.

245. Choi SJ, Cruz JC, Craig F, Chung H, Devlin RD, Roodman GD*, et al.* Macrophage inflammatory protein 1-alpha is a potential osteoclast stimulatory factor in multiple myeloma. *Blood* 2000, **96**(2)**:** 671-675.

246. Han JH, Choi SJ, Kurihara N, Koide M, Oba Y, Roodman GD. Macrophage inflammatory protein-1alpha is an osteoclastogenic factor in myeloma that is independent of receptor activator of nuclear factor kappaB ligand. *Blood* 2001, **97**(11)**:** 3349-3353.

247. Kim MS, Magno CL, Day CJ, Morrison NA. Induction of chemokines and chemokine receptors CCR2b and CCR4 in authentic human osteoclasts differentiated with RANKL and osteoclast like cells differentiated by MCP-1 and RANTES. *Journal of cellular biochemistry* 2006, **97**(3)**:** 512-518.

248. Giuliani N, Bataille R, Mancini C, Lazzaretti M, Barille S. Myeloma cells induce imbalance in the osteoprotegerin/osteoprotegerin ligand system in the human bone marrow environment. *Blood* 2001, **98**(13)**:** 3527-3533.

249. Ehrlich LA, Chung HY, Ghobrial I, Choi SJ, Morandi F, Colla S*, et al.* IL-3 is a potential inhibitor of osteoblast differentiation in multiple myeloma. *Blood* 2005, **106**(4)**:** 1407-1414.

250. Zannettino AC, Farrugia AN, Kortesidis A, Manavis J, To LB, Martin SK*, et al.* Elevated serum levels of stromal-derived factor-1alpha are associated with increased osteoclast activity and osteolytic bone disease in multiple myeloma patients. *Cancer Res* 2005, **65**(5)**:** 1700-1709.

251. Hjertner O, Torgersen ML, Seidel C, Hjorth-Hansen H, Waage A, Borset M*, et al.* Hepatocyte growth factor (HGF) induces interleukin-11 secretion from osteoblasts: a possible role for HGF in myeloma-associated osteolytic bone disease. *Blood* 1999, **94**(11)**:** 3883-3888.

252. Lee JW, Chung HY, Ehrlich LA, Jelinek DF, Callander NS, Roodman GD*, et al.* IL-3 expression by myeloma cells increases both osteoclast formation and growth of myeloma cells. *Blood* 2004, **103**(6)**:** 2308-2315.

253. Shipman CM, Croucher PI. Osteoprotegerin is a soluble decoy receptor for tumor necrosis factor-related apoptosis-inducing ligand/Apo2 ligand and can function as a paracrine survival factor for human myeloma cells. *Cancer Res* 2003, **63**(5)**:** 912-916.

254. Oshima T, Abe M, Asano J, Hara T, Kitazoe K, Sekimoto E*, et al.* Myeloma cells suppress bone formation by secreting a soluble Wnt inhibitor, sFRP-2. *Blood* 2005, **106**(9)**:** 3160-3165.

255. Giuliani N, Morandi F, Tagliaferri S, Lazzaretti M, Donofrio G, Bonomini S*, et al.* Production of Wnt inhibitors by myeloma cells: potential effects on canonical Wnt pathway in the bone microenvironment. *Cancer Res* 2007, **67**(16)**:** 7665-7674.

256. Tian E, Zhan F, Walker R, Rasmussen E, Ma Y, Barlogie B*, et al.* The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. *N Engl J Med* 2003, **349**(26)**:** 2483-2494.

257. Colucci S, Brunetti G, Oranger A, Mori G, Sardone F, Specchia G*, et al.* Myeloma cells suppress osteoblasts through sclerostin secretion. *Blood cancer journal* 2011, **1**(6)**:** e27.

258. Feliers D, Woodruff K, Abboud S. Potential role of insulin-like growth factor binding protein-4 in the uncoupling of bone turnover in multiple myeloma. *British journal of haematology* 1999, **104**(4)**:** 715-722.

259. Giuliani N, Colla S, Morandi F, Lazzaretti M, Sala R, Bonomini S*, et al.* Myeloma cells block RUNX2/CBFA1 activity in human bone marrow osteoblast progenitors and inhibit osteoblast formation and differentiation. *Blood* 2005, **106**(7)**:** 2472-2483.

260. Takeuchi K, Abe M, Hiasa M, Oda A, Amou H, Kido S*, et al.* Tgf-Beta inhibition restores terminal osteoblast differentiation to suppress myeloma growth. *PloS one* 2010, **5**(3)**:** e9870.

261. Vallet S, Mukherjee S, Vaghela N, Hideshima T, Fulciniti M, Pozzi S*, et al.* Activin A promotes multiple myeloma-induced osteolysis and is a promising target for myeloma bone disease. *Proc Natl Acad Sci U S A* 2010, **107**(11)**:** 5124-5129.

262. Roodman GD. Targeting the bone microenvironment in multiple myeloma. *Journal of bone and mineral metabolism* 2010, **28**(3)**:** 244-250.

263. Dhodapkar MV, Krasovsky J, Olson K. T cells from the tumor microenvironment of patients with progressive myeloma can generate strong, tumor-specific cytolytic responses to autologous, tumor-loaded dendritic cells. *Proc Natl Acad Sci U S A* 2002, **99**(20)**:** 13009-13013.

264. Noonan K, Marchionni L, Anderson J, Pardoll D, Roodman GD, Borrello I. A novel role of IL-17-producing lymphocytes in mediating lytic bone disease in multiple myeloma. *Blood* 2010, **116**(18)**:** 3554-3563.

265. Noonan K, Matsui W, Serafini P, Carbley R, Tan G, Khalili J*, et al.* Activated marrow-infiltrating lymphocytes effectively target plasma cells and their clonogenic precursors. *Cancer Res* 2005, **65**(5)**:** 2026-2034.

266. Mills KH, Cawley JC. Abnormal monoclonal antibody-defined helper/suppressor T-cell subpopulations in multiple myeloma: relationship to treatment and clinical stage. *British journal of haematology* 1983, **53**(2)**:** 271-275.

267. San Miguel JF, Gonzalez M, Gascon A, Moro MJ, Hernandez JM, Ortega F*, et al.* Lymphoid subsets and prognostic factors in multiple myeloma. Cooperative Group for the Study of Monoclonal Gammopathies. *British journal of haematology* 1992, **80**(3)**:** 305-309.

268. Ogawara H, Handa H, Yamazaki T, Toda T, Yoshida K, Nishimoto N*, et al.* High Th1/Th2 ratio in patients with multiple myeloma. *Leukemia research* 2005, **29**(2)**:** 135-140.

269. Thomas DA, Massague J. TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer cell* 2005, **8**(5)**:** 369-380.

270. Mozaffari F, Hansson L, Kiaii S, Ju X, Rossmann ED, Rabbani H*, et al.* Signalling molecules and cytokine production in T cells of multiple myeloma-increased abnormalities with advancing stage. *British journal of haematology* 2004, **124**(3)**:** 315-324.

271. Dhodapkar KM, Barbuto S, Matthews P, Kukreja A, Mazumder A, Vesole D*, et al.* Dendritic cells mediate the induction of polyfunctional human IL17-producing cells (Th17-1 cells) enriched in the bone marrow of patients with myeloma. *Blood* 2008, **112**(7)**:** 2878-2885.

272. Gupta R, Ganeshan P, Hakim M, Verma R, Sharma A, Kumar L. Significantly reduced regulatory T cell population in patients with untreated multiple myeloma. *Leukemia research* 2011, **35**(7)**:** 874-878.

273. Feyler S, von Lilienfeld-Toal M, Jarmin S, Marles L, Rawstron A, Ashcroft AJ*, et al.* CD4(+)CD25(+)FoxP3(+) regulatory T cells are increased whilst CD3(+)CD4(-)CD8(-)alphabetaTCR(+) Double Negative T cells are decreased in the peripheral blood of patients with multiple myeloma which correlates with disease burden. *British journal of haematology* 2009, **144**(5)**:** 686-695.

274. Feyler S, Scott GB, Parrish C, Jarmin S, Evans P, Short M*, et al.* Tumour cell generation of inducible regulatory T-cells in multiple myeloma is contact-dependent and antigen-presenting cell-independent. *PloS one* 2012, **7**(5)**:** e35981.

275. Fiore F, Nuschak B, Peola S, Mariani S, Muraro M, Foglietta M*, et al.* Exposure to myeloma cell lysates affects the immune competence of dendritic cells and favors the induction of Tr1-like regulatory T cells. *Eur J Immunol* 2005, **35**(4)**:** 1155-1163.

276. Ratta M, Fagnoni F, Curti A, Vescovini R, Sansoni P, Oliviero B*, et al.* Dendritic cells are functionally defective in multiple myeloma: the role of interleukin-6. *Blood* 2002, **100**(1)**:** 230-237.

277. Brown RD, Pope B, Murray A, Esdale W, Sze DM, Gibson J*, et al.* Dendritic cells from patients with myeloma are numerically normal but functionally defective as they fail to up-regulate CD80 (B7-1) expression after huCD40LT stimulation because of inhibition

by transforming growth factor-beta1 and interleukin-10. *Blood* 2001, **98**(10)**:** 2992-2998.

278. Hwang W, Jung K, Jeon Y, Yun S, Kim TW, Choi I. Knockdown of the interleukin-6 receptor alpha chain of dendritic cell vaccines enhances the therapeutic potential against IL-6 producing tumors. *Vaccine* 2010, **29**(1)**:** 34-44.

279. Wang S, Yang J, Qian J, Wezeman M, Kwak LW, Yi Q. Tumor evasion of the immune system: inhibiting p38 MAPK signaling restores the function of dendritic cells in multiple myeloma. *Blood* 2006, **107**(6)**:** 2432-2439.

280. Feyler S, Selby PJ, Cook G. Regulating the regulators in cancer-immunosuppression in multiple myeloma (MM). *Blood reviews* 2013, **27**(3)**:** 155-164.

281. Frohn C, Hoppner M, Schlenke P, Kirchner H, Koritke P, Luhm J. Anti-myeloma activity of natural killer lymphocytes. *British journal of haematology* 2002, **119**(3)**:** 660-664.

282. Carbone E, Neri P, Mesuraca M, Fulciniti MT, Otsuki T, Pende D*, et al.* HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells. *Blood* 2005, **105**(1)**:** 251-258.

283. El-Sherbiny YM, Meade JL, Holmes TD, McGonagle D, Mackie SL, Morgan AW*, et al.* The requirement for DNAM-1, NKG2D, and NKp46 in the natural killer cell-mediated killing of myeloma cells. *Cancer Res* 2007, **67**(18)**:** 8444-8449.

284. Jinushi M, Vanneman M, Munshi NC, Tai YT, Prabhala RH, Ritz J*, et al.* MHC class I chain-related protein A antibodies and shedding are associated with the progression of multiple myeloma. *Proc Natl Acad Sci U S A* 2008, **105**(4)**:** 1285-1290.

285. Fionda C, Malgarini G, Soriani A, Zingoni A, Cecere F, Iannitto ML*, et al.* Inhibition of glycogen synthase kinase-3 increases NKG2D ligand MICA expression and sensitivity to NK cell-mediated cytotoxicity in multiple myeloma cells: role of STAT3. *J Immunol* 2013, **190**(12)**:** 6662-6672.

286. Soriani A, Zingoni A, Cerboni C, Iannitto ML, Ricciardi MR, Di Gialleonardo V*, et al.* ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype. *Blood* 2009, **113**(15)**:** 3503-3511.

287. Alici E, Konstantinidis KV, Sutlu T, Aints A, Gahrton G, Ljunggren HG*, et al.* Anti-myeloma activity of endogenous and adoptively transferred activated natural killer cells in experimental multiple myeloma model. *Exp Hematol* 2007, **35**(12)**:** 1839-1846.

288. Alici E, Sutlu T, Bjorkstrand B, Gilljam M, Stellan B, Nahi H*, et al.* Autologous antitumor activity by NK cells expanded from myeloma patients using GMP-compliant components. *Blood* 2008, **111**(6)**:** 3155-3162.

289. Garg TK, Szmania SM, Khan JA, Hoering A, Malbrough PA, Moreno-Bost A*, et al.* Highly activated and expanded natural killer cells for multiple myeloma immunotherapy. *Haematologica* 2012, **97**(9)**:** 1348-1356.

290. Shi J, Tricot G, Szmania S, Rosen N, Garg TK, Malaviarachchi PA*, et al.* Infusion of haplo-identical killer immunoglobulin-like receptor ligand mismatched NK cells for relapsed myeloma in the setting of autologous stem cell transplantation. *British journal of haematology* 2008, **143**(5)**:** 641-653.

291. Mitsiades CS, Mitsiades NS, Bronson RT, Chauhan D, Munshi N, Treon SP*, et al.* Fluorescence imaging of multiple myeloma cells in a clinically relevant SCID/NOD in vivo model: biologic and clinical implications. *Cancer Res* 2003, **63**(20)**:** 6689-6696.

292. Tassone P, Neri P, Carrasco DR, Burger R, Goldmacher VS, Fram R*, et al.* A clinically relevant SCID-hu in vivo model of human multiple myeloma. *Blood* 2005, **106**(2)**:** 713-716.

293. Pilarski LM, Hipperson G, Seeberger K, Pruski E, Coupland RW, Belch AR. Myeloma progenitors in the blood of patients with aggressive or minimal disease: engraftment and self-renewal of primary human myeloma in the bone marrow of NOD SCID mice. *Blood* 2000, **95**(3)**:** 1056-1065.

294. Yata K, Yaccoby S. The SCID-rab model: a novel in vivo system for primary human myeloma demonstrating growth of CD138-expressing malignant cells. *Leukemia* 2004, **18**(11)**:** 1891-1897.

295. Calimeri T, Battista E, Conforti F, Neri P, Di Martino MT, Rossi M*, et al.* A unique three-dimensional SCID-polymeric scaffold (SCID-synth-hu) model for in vivo expansion of human primary multiple myeloma cells. *Leukemia* 2011, **25**(4)**:** 707-711.

296. Morito N, Yoh K, Maeda A, Nakano T, Fujita A, Kusakabe M*, et al.* A novel transgenic mouse model of the human multiple myeloma chromosomal translocation t(14;16)(q32;q23). *Cancer Res* 2011, **71**(2)**:** 339-348.

297. Carrasco DR, Sukhdeo K, Protopopova M, Sinha R, Enos M, Carrasco DE*, et al.* The differentiation and stress response factor XBP-1 drives multiple myeloma pathogenesis. *Cancer cell* 2007, **11**(4)**:** 349-360.

298. Chesi M, Robbiani DF, Sebag M, Chng WJ, Affer M, Tiedemann R*, et al.* AID-dependent activation of a MYC transgene induces multiple myeloma in a conditional mouse model of post-germinal center malignancies. *Cancer cell* 2008, **13**(2)**:** 167-180.

299. Radl J, De Glopper ED, Schuit HR, Zurcher C. Idiopathic paraproteinemia. II. Transplantation of the paraprotein-producing clone from old to young C57BL/KaLwRij mice. *J Immunol* 1979, **122**(2)**:** 609-613.

300. Avet-Loiseau H, Facon T, Grosbois B, Magrangeas F, Rapp MJ, Harousseau JL*, et al.* Oncogenesis of multiple myeloma: 14q32 and 13q chromosomal abnormalities are not randomly distributed, but correlate with natural history, immunological features, and clinical presentation. *Blood* 2002, **99**(6)**:** 2185-2191.

301. Vanderkerken K, Asosingh K, Croucher P, Van Camp B. Multiple myeloma biology: lessons from the 5TMM models. *Immunol Rev* 2003, **194:** 196-206.

302. Asosingh K, Radl J, Van Riet I, Van Camp B, Vanderkerken K. The 5TMM series: a useful in vivo mouse model of human multiple myeloma. *The hematology journal : the official journal of the European Haematology Association / EHA* 2000, **1**(5)**:** 351-356.

303. Vanderkerken K, De Raeve H, Goes E, Van Meirvenne S, Radl J, Van Riet I*, et al.* Organ involvement and phenotypic adhesion profile of 5T2 and 5T33 myeloma cells in the C57BL/KaLwRij mouse. *British journal of cancer* 1997, **76**(4)**:** 451-460.

304. Croucher PI, Shipman CM, Lippitt J, Perry M, Asosingh K, Hijzen A*, et al.* Osteoprotegerin inhibits the development of osteolytic bone disease in multiple myeloma. *Blood* 2001, **98**(13)**:** 3534-3540.

305. Van Valckenborgh E, De Raeve H, Devy L, Blacher S, Munaut C, Noel A*, et al.* Murine 5T multiple myeloma cells induce angiogenesis in vitro and in vivo. *British journal of cancer* 2002, **86**(5)**:** 796-802.

306. Dallas SL, Garrett IR, Oyajobi BO, Dallas MR, Boyce BF, Bauss F*, et al.* Ibandronate reduces osteolytic lesions but not tumor burden in a murine model of myeloma bone disease. *Blood* 1999, **93**(5)**:** 1697-1706.

307. Vanderkerken K, De Greef C, Asosingh K, Arteta B, De Veerman M, Vande Broek I*, et al.* Selective initial in vivo homing pattern of 5T2 multiple myeloma cells in the C57BL/KalwRij mouse. *British journal of cancer* 2000, **82**(4)**:** 953-959.

308. Vanderkerken K, Vande Broek I, Eizirik DL, Van Valckenborgh E, Asosingh K, Van Riet I*, et al.* Monocyte chemoattractant protein-1 (MCP-1), secreted by bone marrow endothelial cells, induces chemoattraction of 5T multiple myeloma cells. *Clinical & experimental metastasis* 2002, **19**(1)**:** 87-90.

309. Menu E, Asosingh K, Indraccolo S, De Raeve H, Van Riet I, Van Valckenborgh E*, et al.* The involvement of stromal derived factor 1alpha in homing and progression of multiple myeloma in the 5TMM model. *Haematologica* 2006, **91**(5)**:** 605-612.

310. Vande Broek I, Vanderkerken K, Van Camp B, Van Riet I. Extravasation and homing mechanisms in multiple myeloma. *Clinical & experimental metastasis* 2008, **25**(4)**:** 325-334.

311. Vanderkerken K, Asosingh K, Braet F, Van Riet I, Van Camp B. Insulin-like growth factor-1 acts as a chemoattractant factor for 5T2 multiple myeloma cells. *Blood* 1999, **93**(1)**:** 235-241.

312. Asosingh K, Gunthert U, De Raeve H, Van Riet I, Van Camp B, Vanderkerken K. A unique pathway in the homing of murine multiple myeloma cells: CD44v10 mediates binding to bone marrow endothelium. *Cancer Res* 2001, **61**(7)**:** 2862-2865.

313. Bernardini G, Gismondi A, Santoni A. Chemokines and NK cells: regulators of development, trafficking and functions. *Immunol Lett* 2012, **145**(1-2)**:** 39-46.

314. Huang D, Shi FD, Jung S, Pien GC, Wang J, Salazar-Mather TP*, et al.* The neuronal chemokine CX3CL1/fractalkine selectively recruits NK cells that modify experimental autoimmune encephalomyelitis within the central nervous system. *FASEB J* 2006, **20**(7)**:** 896-905.

315. Bolovan-Fritts CA, Spector SA. Endothelial damage from cytomegalovirus-specific host immune response can be prevented by targeted disruption of fractalkine-CX3CR1 interaction. *Blood* 2008, **111**(1)**:** 175-182.

316. Yoneda O, Imai T, Goda S, Inoue H, Yamauchi A, Okazaki T*, et al.* Fractalkine-mediated endothelial cell injury by NK cells. *J Immunol* 2000, **164**(8)**:** 4055-4062.

317. Guo J, Chen T, Wang B, Zhang M, An H, Guo Z*, et al.* Chemoattraction, adhesion and activation of natural killer cells are involved in the antitumor immune response induced by fractalkine/CX3CL1. *Immunol Lett* 2003, **89**(1)**:** 1-7.

318. Robinson LA, Nataraj C, Thomas DW, Howell DN, Griffiths R, Bautch V*, et al.* A role for fractalkine and its receptor (CX3CR1) in cardiac allograft rejection. *J Immunol* 2000, **165**(11)**:** 6067-6072.

319. Pallandre JR, Krzewski K, Bedel R, Ryffel B, Caignard A, Rohrlich PS*, et al.* Dendritic cell and natural killer cell cross-talk: a pivotal role of CX3CL1 in NK cytoskeleton organization and activation. *Blood* 2008, **112**(12)**:** 4420-4424.

320. Lyszkiewicz M, Witzlau K, Pommerencke J, Krueger A. Chemokine receptor CX3CR1 promotes dendritic cell development under steady-state conditions. *Eur J Immunol* 2011, **41**(5)**:** 1256-1265.

321. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 2003, **19**(1)**:** 71-82.

322. Auffray C, Fogg DK, Narni-Mancinelli E, Senechal B, Trouillet C, Saederup N*, et al.* CX3CR1+ CD115+ CD135+ common macrophage/DC precursors and the role of CX3CR1 in their response to inflammation. *J Exp Med* 2009, **206**(3)**:** 595-606.

323. Sciume G, De Angelis G, Benigni G, Ponzetta A, Morrone S, Santoni A*, et al.* CX3CR1 expression defines 2 KLRG1+ mouse NK-cell subsets with distinct functional properties and positioning in the bone marrow. *Blood* 2011, **117**(17)**:** 4467-4475.

324. Pappa CA, Tsirakis G, Kanellou P, Kaparou M, Stratinaki M, Xekalou A*, et al.* Monitoring serum levels ELR+ CXC chemokines and the relationship between microvessel density and angiogenic growth factors in multiple myeloma. *Cytokine* 2011, **56**(3)**:** 616-620.

325. Dairaghi DJ, Oyajobi BO, Gupta A, McCluskey B, Miao S, Powers JP*, et al.* CCR1 blockade reduces tumor burden and osteolysis in vivo in a mouse model of myeloma bone disease. *Blood* 2012, **120**(7)**:** 1449-1457.

326. Pereira JP, An J, Xu Y, Huang Y, Cyster JG. Cannabinoid receptor 2 mediates the retention of immature B cells in bone marrow sinusoids. *Nat Immunol* 2009, **10**(4)**:** 403-411.

327. Jung S, Aliberti J, Graemmel P, Sunshine MJ, Kreutzberg GW, Sher A*, et al.* Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol* 2000, **20**(11)**:** 4106-4114.

328. Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA*, et al.* CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* 2005, **307**(5707)**:** 254-258.

329. Imai T, Hieshima K, Haskell C, Baba M, Nagira M, Nishimura M*, et al.* Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell* 1997, **91**(4)**:** 521-530.

330. Wang J, Xu J, Zhang W, Wei H, Tian Z. TLR3 ligand-induced accumulation of activated splenic natural killer cells into liver. *Cellular & molecular immunology* 2005, **2**(6)**:** 449-453.

331. Hayakawa Y, Huntington ND, Nutt SL, Smyth MJ. Functional subsets of mouse natural killer cells. *Immunol Rev* 2006, **214:** 47-55.

332. Berahovich RD, Lai NL, Wei Z, Lanier LL, Schall TJ. Evidence for NK cell subsets based on chemokine receptor expression. *J Immunol* 2006, **177**(11)**:** 7833-7840.

333. Gregoire C, Cognet C, Chasson L, Coupet CA, Dalod M, Reboldi A*, et al.* Intrasplenic trafficking of natural killer cells is redirected by chemokines upon inflammation. *Eur J Immunol* 2008, **38**(8)**:** 2076-2084.

334. Nagasawa T. Microenvironmental niches in the bone marrow required for B-cell development. *Nat Rev Immunol* 2006, **6**(2)**:** 107-116.

335. Kohara H, Omatsu Y, Sugiyama T, Noda M, Fujii N, Nagasawa T. Development of plasmacytoid dendritic cells in bone marrow stromal cell niches requires CXCL12-CXCR4 chemokine signaling. *Blood* 2007, **110**(13)**:** 4153-4160.

336. Yu YR, Fong AM, Combadiere C, Gao JL, Murphy PM, Patel DD. Defective antitumor responses in CX3CR1-deficient mice. *International journal of cancer Journal international du cancer* 2007, **121**(2)**:** 316-322.

337. Cook DN, Chen SC, Sullivan LM, Manfra DJ, Wiekowski MT, Prosser DM*, et al.* Generation and analysis of mice lacking the chemokine fractalkine. *Mol Cell Biol* 2001, **21**(9)**:** 3159-3165.

338. Shimoda S, Selmi C, Gershwin ME. Fractalkine and other chemokines in primary biliary cirrhosis. *International journal of hepatology* 2012, **2012:** 102839.

339. Wasmuth HE, Zaldivar MM, Berres ML, Werth A, Scholten D, Hillebrandt S*, et al.* The fractalkine receptor CX3CR1 is involved in liver fibrosis due to chronic hepatitis C infection. *Journal of hepatology* 2008, **48**(2)**:** 208-215.

340. Mionnet C, Buatois V, Kanda A, Milcent V, Fleury S, Lair D*, et al.* CX3CR1 is required for airway inflammation by promoting T helper cell survival and maintenance in inflamed lung. *Nat Med* 2010, **16**(11)**:** 1305-1312.

341. Fong AM, Robinson LA, Steeber DA, Tedder TF, Yoshie O, Imai T*, et al.* Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. *J Exp Med* 1998, **188**(8)**:** 1413-1419.

342. Nakano M, Fujii T, Hashimoto M, Yukawa N, Yoshifuji H, Ohmura K*, et al.* Type I interferon induces CX3CL1 (fractalkine) and CCL5 (RANTES) production in human pulmonary vascular endothelial cells. *Clinical and experimental immunology* 2012, **170**(1)**:** 94-100.

343. Jin JO, Yu Q. Systemic administration of TLR3 agonist induces IL-7 expression and IL-7-dependent CXCR3 ligand production in the lung. *J Leukoc Biol* 2013, **93**(3)**:** 413-425.

344. Robinson LA, Nataraj C, Thomas DW, Cosby JM, Griffiths R, Bautch VL*, et al.* The chemokine CX3CL1 regulates NK cell activity in vivo. *Cell Immunol* 2003, **225**(2)**:** 122-130.

345. Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y*, et al.* The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature* 1998, **393**(6685)**:** 591-594.

346. Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* 1998, **393**(6685)**:** 595-599.

347. Wald O, Weiss ID, Wald H, Shoham H, Bar-Shavit Y, Beider K*, et al.* IFN-gamma acts on T cells to induce NK cell mobilization and accumulation in target organs. *J Immunol* 2006, **176**(8)**:** 4716-4729.

348. Hokeness KL, Kuziel WA, Biron CA, Salazar-Mather TP. Monocyte chemoattractant protein-1 and CCR2 interactions are required for IFN-alpha/beta-induced inflammatory responses and antiviral defense in liver. *J Immunol* 2005, **174**(3)**:** 1549-1556.

349. Khan IA, Thomas SY, Moretto MM, Lee FS, Islam SA, Combe C*, et al.* CCR5 is essential for NK cell trafficking and host survival following Toxoplasma gondii infection. *PLoS pathogens* 2006, **2**(6)**:** e49.

350. Geissmann F, Cameron TO, Sidobre S, Manlongat N, Kronenberg M, Briskin MJ*, et al.* Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. *PLoS biology* 2005, **3**(4)**:** e113.

351. Weiss ID, Shoham H, Wald O, Wald H, Beider K, Abraham M*, et al.* Ccr5 deficiency regulates the proliferation and trafficking of natural killer cells under physiological conditions. *Cytokine* 2011, **54**(3)**:** 249-257.

352. Ponzetta A, Sciume G, Benigni G, Antonangeli F, Morrone S, Santoni A*, et al.* CX3CR1 regulates the maintenance of KLRG1+ NK cells into the bone marrow by promoting their entry into circulation. *J Immunol* 2013, **191**(11)**:** 5684-5694.

353. Garrett IR, Dallas S, Radl J, Mundy GR. A murine model of human myeloma bone disease. *Bone* 1997, **20**(6)**:** 515-520.

354. Asosingh K, Menu E, Van Valckenborgh E, Vande Broek I, Van Riet I, Van Camp B*, et al.* Mechanisms involved in the differential bone marrow homing of CD45 subsets in 5T murine models of myeloma. *Clinical & experimental metastasis* 2002, **19**(7)**:** 583-591.

355. Kim R, Emi M, Tanabe K. Cancer immunosuppression and autoimmune disease: beyond immunosuppressive networks for tumour immunity. *Immunology* 2006, **119**(2)**:** 254-264.

356. Cook G, Campbell JD. Immune regulation in multiple myeloma: the host-tumour conflict. *Blood reviews* 1999, **13**(3)**:** 151-162.

357. Cook G, Campbell JD, Carr CE, Boyd KS, Franklin IM. Transforming growth factor beta from multiple myeloma cells inhibits proliferation and IL-2 responsiveness in T lymphocytes. *J Leukoc Biol* 1999, **66**(6)**:** 981-988.

358. Benson DM, Jr., Bakan CE, Mishra A, Hofmeister CC, Efebera Y, Becknell B*, et al.* The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood* 2010, **116**(13)**:** 2286-2294.

359. Gill S, Vasey AE, De Souza A, Baker J, Smith AT, Kohrt HE*, et al.* Rapid development of exhaustion and down-regulation of eomesodermin limit the antitumor activity of adoptively transferred murine natural killer cells. *Blood* 2012, **119**(24)**:** 5758-5768.

360. Takeda K, Nakayama M, Sakaki M, Hayakawa Y, Imawari M, Ogasawara K*, et al.* IFN-gamma production by lung NK cells is critical for the natural resistance to pulmonary metastasis of B16 melanoma in mice. *J Leukoc Biol* 2011, **90**(4)**:** 777-785.

361. Ballas ZK, Buchta CM, Rosean TR, Heusel JW, Shey MR. Role of NK cell subsets in organ-specific murine melanoma metastasis. *PloS one* 2013, **8**(6)**:** e65599.

362. Fowler JA, Mundy GR, Lwin ST, Lynch CC, Edwards CM. A murine model of myeloma that allows genetic manipulation of the host microenvironment. *Disease models & mechanisms* 2009, **2**(11-12)**:** 604-611.

363. Wendel M, Galani IE, Suri-Payer E, Cerwenka A. Natural killer cell accumulation in tumors is dependent on IFN-gamma and CXCR3 ligands. *Cancer Res* 2008, **68**(20)**:** 8437-8445.

364. Salazar-Mather TP, Orange JS, Biron CA. Early murine cytomegalovirus (MCMV) infection induces liver natural killer (NK) cell inflammation and protection through macrophage inflammatory protein 1alpha (MIP-1alpha)-dependent pathways. *J Exp Med* 1998, **187**(1)**:** 1-14.

365. Salazar-Mather TP, Hamilton TA, Biron CA. A chemokine-to-cytokine-to-chemokine cascade critical in antiviral defense. *J Clin Invest* 2000, **105**(7)**:** 985-993.

366. Salazar-Mather TP, Lewis CA, Biron CA. Type I interferons regulate inflammatory cell trafficking and macrophage inflammatory protein 1alpha delivery to the liver. *J Clin Invest* 2002, **110**(3)**:** 321-330.

367. Orange JS, Wang B, Terhorst C, Biron CA. Requirement for natural killer cell-produced interferon gamma in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. *J Exp Med* 1995, **182**(4)**:** 1045-1056.

368. Groom JR, Luster AD. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol Cell Biol* 2011, **89**(2)**:** 207-215.

369. Van Valckenborgh E, Mincher D, Di Salvo A, Van Riet I, Young L, Van Camp B*, et al.* Targeting an MMP-9-activated prodrug to multiple myeloma-diseased bone marrow: a proof of principle in the 5T33MM mouse model. *Leukemia* 2005, **19**(9)**:** 1628-1633.

370. van der Voort R, Volman TJ, Verweij V, Linssen PC, Maas F, Hebeda KM*, et al.* Homing characteristics of donor T cells after experimental allogeneic bone marrow transplantation and posttransplantation therapy for multiple myeloma. *Biol Blood Marrow Transplant* 2013, **19**(3)**:** 378-386.

7. Acknowledgements

I would like to express my gratitude to Giovanni Bernardini, who introduced me to the study of immunology and NK cell biology, guiding me since I joined the lab.

My scientific and human growth lies also on the several meetings and productive discussions I had with Angela Santoni, and all the other members of the lab.

Finally, I cannot immagine these three years without the help, the support and the friendship of Giorgia, and of the other members of my research unit, Fabrizio and Emilio.