NATURAL KILLER (NK) CELL EDUCATION

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Abstract: Natural Killer (NK) cells are lymphocytes that are critically involved in anti-pathogen and anti-cancer responses. They fulfil these functions through "natural" cytotoxicity, antibody-dependent cellular cytotoxicity (ADCC) and cytokine production. However, before being able to become functionally efficient, they have to go through a process called "education" or "licensing", which might grossly be compared with the positive selection of T cells in the thymus. A lot has been learned about this phenomenon in recent years, and in this review, the most important literature on the topic will be summarized and commented. Several models have been developed to explain the mechanisms of NK cell licensing, for example the "*cis-trans*" model or the rheostat model. Most of the relevant research describes education as a major histocompatibility complex class I (MHC class I)-dependent event, but more recent data shows that education might also occur independently of MHC class I molecules and mediated via other elements of the NK cell microenvironment. Education of NK cells is not only interesting in fundamental immunology but also for immunotherapy. Indeed, more and more groups are using NK cells as weapons against cancer, and in this context, it can be crucial to select the cells expected to be the most efficient ones according to the degree of their licensing. Overall, whereas NK cells were merely considered as killers in the past, it is nowadays recognized that they are a highly sophisticated cell type, which can have beneficial or detrimental effects on the organism, depending on the circumstances.

Keywords: natural killer cells, education, major histocompatibility complex class I, inhibitory receptors

Résumé: Les cellules tueuses naturelles (cellules "Natural Killer" ou cellules NK) sont des lymphocytes qui sont impliqués de façon critique dans la défense anti-infectieuse et anti-cancéreuse. Elles remplissent ces fonctions à travers leur cytotoxicité naturelle, la cytotoxicité dépendante d'anticorps (ADCC pour antibody-dependent cellular cytotoxicity) et la production de cytokines. Cependant, avant d'être en mesure de devenir efficaces sur le plan fonctionnel, elles doivent passer par un processus "d'éducation" ou "d'autorisation" qui peut grossièrement être comparé à la sélection positive des lymphocytes T au niveau du thymus. Beaucoup de détails ont été révélés ces dernières années sur ce phénomène, et dans cette revue, la littérature la plus importante sur le sujet sera résumée et commentée. Plusieurs modèles ont été développés pour essayer d'expliquer les mécanismes de l'éducation des cellules NK, comme par exemple le modèle "*cis-trans*" et le modèle du rhéostat. La plupart des articles scientifiques sur l'éducation la décrivent comme un phénomène dépendant de la classe I du complexe majeur d'histocompatibilité (CMH), mais des données plus récentes démontrent que l'éducation peut également être médiée par des structures indépendantes des molecules de classe I du CMH, mais présentes dans le microenvironnement des cellules NK. L'éducation des cellules NK n'est pas seulement intéressante pour l'immunologie fondamentale mais aussi pour l'immunothérapie. En effet, de plus en plus de groupes utilisent ces cellules comme armes contre le cancer, et dans ce contexte, il peut s'avérer crucial

de sélectionner les cellules NK présumées les plus efficaces sur base de leur degré d'éducation. De manière générale, alors que les cellules NK ont été considérées seulement comme des tueurs par le passé, il s'avère maintenant que ce sont des cellules hautement sophistiquées et qui peuvent avoir, selon les circonstances, des effets bénéfiques ou délétères pour l'organisme.

Mots-clés: cellules tueuses naturelles, éducation, complexe majeur d'histocompatibilité de classe I, récepteurs inhibiteurs

1. Introduction

Natural Killer (NK) cells have first been described in the 1970s as large granular lymphocytes able to kill tumour cell lines without prior stimulation and immunisation, in contrast to cytotoxic T cells. This "natural killing" was not only observed against tumour cells but also against viral-infected cells, and more and more researchers became interested in the elucidation of the mechanisms of the phenomenon. Whereas, according to the PubMed database, seven NK cell papers were published in 1976, there were 2107 during the year 2006 and 2641 during the year 2018. Natural killer cells are not only in the focus of basic research, but become increasingly investigated for their therapeutic potential in cancer (and infectious diseases) with a significant number of finished and ongoing clinical trials (Suen *et al*., 2018). In addition to natural killing, NK cells also perform antibody-dependent cellular cytotoxicity (ADCC) characterized by the crosslinking of a target cell with a NK cell by an antibody whose variable fragment is specific for the target cell while the constant part (Fc fragment) binds to an activating Fc receptor (mostly CD16) on the NK cell. Furthermore, NK cells are important cytokine and chemokine producers, the main cytokine secreted being interferon (IFN)-γ (Fig. 1) (Amand *et al*., 2017).

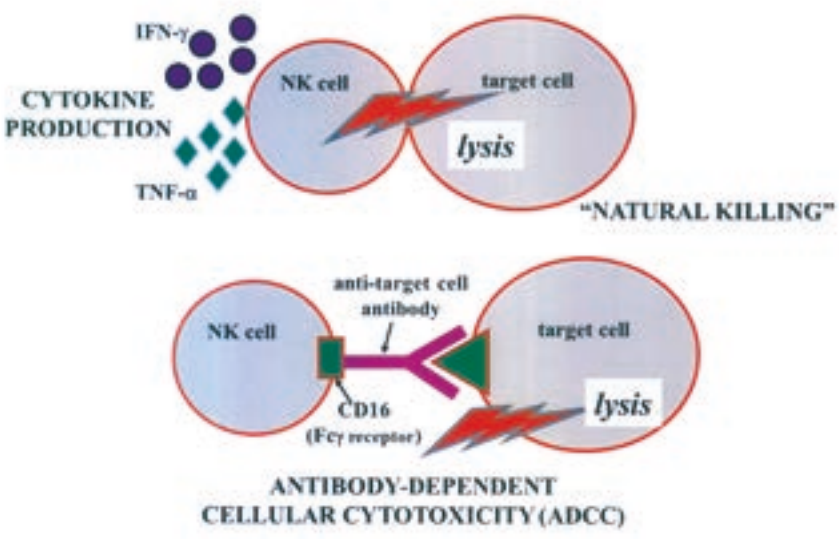

Figure 1: *Properties of NK cells.*

The killing process itself is based on the release of cytolytic granules containing toxic molecules like perforin, granzymes and, in human but not in the mouse, granulysin. Perforin creates pores in the target cell membrane, allowing the entrance of granzymes and granulysin into the cytoplasm where they induce apoptosis (Shifrin *et al*., 2014).

In this regard, NK cells are not very different from cytotoxic CD8+ T lymphocytes. However, the signal triggering this machinery is all but the same between both cell types. Conventional CD8+ T cells express a T cell receptor (TCR) of the αβ type, which recognizes a foreign peptide presented by an autologous MHC class I molecule (recognition of the presence of non-self). In contrast, NK cells preferentially kill targets with a low or absent expression of autologous MHC class I molecules (recognition of the absence of self). In 1990, these observations brought Ljunggren and Kärre to the so-called "missing self hypothesis", implying that NK cells recognize healthy targets via MHC class I-specific inhibitory receptors (IR) (Ljunggren & Kärre, 1990). This was rapidly confirmed with the discovery of the first IR in the mouse and later on in rats and humans.

In the mouse, these IR are members of the Ly49 family. They are stochastically acquired, clonally-expressed C-type lectin-like molecules, which means that each of them is only present on a subpopulation of NK cells, and recognize classical polymorphic mouse H-2 MHC class I molecules (for example Ly49A – H-2D^{d}; Ly49C/I – H-2D^{b} and H-2K^{b}). Functionally, but not structurally, similar receptors in human are members of the immunoglobulin superfamily and recognize classical polymorphic human leukocyte antigen (HLA) class I molecules. They include the KIR (killer immunoglobulin receptors) and the LIR (leukocyte immunoglobulin receptor) CD85j, which are likewise clonally expressed. In both species, the C-type lectin NKG2A, only expressed on the cell surface if bound to the chaperone molecule CD94, is specific for the non-classical (because non polymorphic) MHC class I molecules Qa-1 (mouse) and HLA-E (human). They present peptides derived from the signal sequences of classical MHC class I molecules (Shifrin *et al*., 2014; Boudreau *et al*., 2018).

Whereas the extracellular domains of these IR are structurally quite different between human and mouse, they are all characterized by the presence of one or more intracytoplasmic ITIM motifs, which allow the binding of phosphatases that induce the inhibition of NK cell functions (Shifrin *et al*., 2014). Thus, when these IR are not engaged due to the absence of ligands, the NK cells are not inhibited.

Over the years, additional IR were discovered with ligands different from MHC class I molecules. Whereas the functions of some of them are not well known and/or not well studied, others, like "T-cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domains (TIGIT)", seem to be quite important for NK cell biology (He *et al*., 2017).

After the unravelling of a series of IR came the period of the discovery of activating NK cell receptors (AR) (Shifrin *et al*., 2014; Boudreau *et al*., 2018). Indeed, it became clear that the mere absence of MHC class I molecules might not be sufficient to trigger NK cells. Consequently, an array of AR was described which also belong either to the immunoglobulin or to the C-type lectin superfamily. Some of them are activating isoforms of KIR, Ly49 and NKG2 (Boudreau *et al*., 2018).

The current paradigm is that NK cells sense the surface of target cells with all their IR and AR, and that the balance between the inhibitory and activating signals decides whether the target cell will be killed or not (Fig. 2) (Moretta & Moretta, 2004).

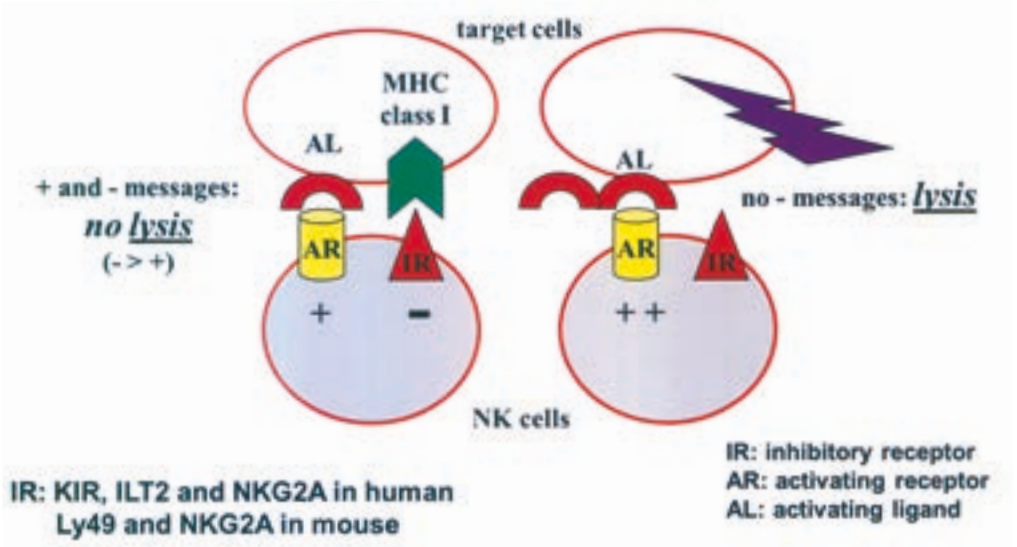

Figure 2: *Balance between inhibitory and activating messages.*

Phenotypically, mouse NK cells are defined as CD3-NK1.1+ or CD3-CD49b+ or CD3-NKp46+ lymphocytes. The T cell receptor (TCR)-associated molecule CD3 is specific for T cells, whereas NK1.1, CD49b and NKp46 are NK cell markers, although their expression is not exclusively restricted to NK cells. In the C57BL/6 (B6) strain, the most frequently used mouse strain for immunological studies, NK1.1 (a strong AR) is present, whereas it is absent in Balb/c mice. However, all current lab mouse strains express CD49b and NKp46 on their NK cells.

Human NK cells are phenotypically characterized as CD3-CD56±CD16± lymphocytes. The relative expression of the adhesion molecule CD56 and the Fcγ receptor CD16, which triggers ADCC, defines actually six different NK cell subsets, each with a distinct phenotype and distinct properties: (1) $CD56^{bright}CD16$-; (2) $CD56^{bright}CD16^{dim}$; (3) $CD56^{dim}CD16$-; (4) $CD56^{dim}CD16^{dim}$; (5) $CD56^{dim}CD16^{bright,}$ and (6) CD56-$CD16^{bright}$ (Fig. 3) (Amand *et al*., 2017; Cooper *et al*., 2001; Michel *et al*., 2016; Poli *et al*., 2009). "Bright" means a strong expression of the marker as assessed by flow cytometry, and "dim" means a weaker but still clearly distinguishable expression level above background. This subset distribution is valid for peripheral blood, where the $CD56^{dim}CD16^{bright}$ cells are numerically largely predominant, whereas in tissues, the proportion of $CD56^{bright}$ cells is much higher.

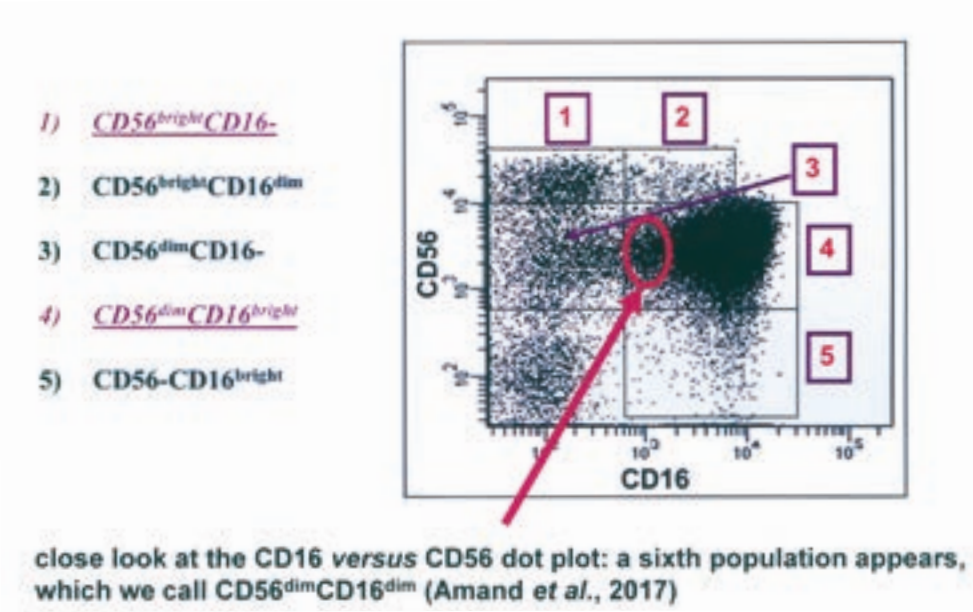

Figure 3: *Human NK cell subpopulations.*

2. Basic principles of NK cell education

It was admitted for a long time that every NK cell had to express at least one self MHC class I-specific IR to avoid a break in self-tolerance (at least one model), and this view was in principle demonstrated by the fact that mRNA for IR could be found in every single NK cell. Immunologists supposed that NK cells without such a receptor, if they existed, would either be anergic or deleted during the differentiation process.

Important indications that lead later on to the concept of education were obtained through the study of MHC class I-deficient mouse strains on the B6 background. These strains are knocked out either for (i) β2-microglobulin, the light chain constitutive of MHC class I complexes (together with a heavy chain and a peptide) (Höglund *et al*., 1998), or (ii) for the subunit 1 of the transporter associated with antigen processing (TAP) (Ljunggren *et al*., 1994), which is normally inserted into the membrane of the endoplasmic reticulum (ER) and actively transports endogenous peptides from the cytosol into the lumen of the ER where they are loaded onto newly synthesized MHC class I molecules. In these strains, the surface expression of MHC class I molecules is extremely low to undetectable, and NK have in common that they display a hyporesponsiveness to the stimulation via AR (in terms of cytotoxicity and cytokine production) and do not kill autologous MHC class I-deficient cells (no missing self recognition).

Interestingly, more or less at the same time, De La Salle *et al*. described two human siblings with a negative serotyping for HLA class I molecules and who had chronic bacterial airway infections (De La Salle *et al*., 1994). By flow cytometry, HLA class I molecules could be observed at the cell surface but with a reduction to approximately 1% of normal levels. Patients' NK cells did not kill the totally HLA class I-negative leukemia cell line K562, the usual target to assess NK cell cytotoxic activity (thus: no missing self recognition).

Nevertheless, both mouse and human MHC class I-deficient NK cells express the common IR and even overexpress some of them in terms of percentages of positive cells and of expression levels per cell (mean fluorescence intensity or MFI, one of the parameters that can be measured by flow cytometry). In addition, it was observed that these NK cells could be stimulated via cytokines and feeder cells and then became strongly cytotoxic not only to tumour target cells, but also to autologous B lymphoblastoid cells and fibroblasts (Zimmer *et al*., 1998; Zimmer *et al*., 1999).

A few years later, and in contrast to prior claims, it was demonstrated in the mouse (Fernandez *et al*., 2005) and in human (Anfossi *et al*., 2006) that not all NK cells express self-specific IR but that the cells devoid of them are nevertheless self-tolerant and hyporesponsive, as are the NK cells from MHC class I-deficient individuals, and this despite a normal phenotypic maturation stage and normal expression of AR. They do not lyse MHC class I-negative targets, and stimulation through their AR is inefficient.

In the B6 mouse, three self-specific IR for MHC class I molecules are expressed by NK cells: Ly49I and Ly49C for H-2D^b and H-2K^b and CD94/NKG2A for Qa-1 whose surface expression depends on the signal peptide of H-2b molecules. As IR expression is stochastic, each NK cell has between 0 and 3 of these receptors. Approximately 15 % of splenic B6 NK cells do not display such an IR, thus being hyporesponsive (Fernandez *et al*., 2005).

Also in 2005, a landmark paper by Kim *et al*. in Nature introduced the concept of NK cell licensing. The authors showed that in the mouse system, NK cells expressing the Ly49A IR are much more likely to be functionally competent in terms of IFN-γ production than their Ly49A-counter-parts, provided the cognate Ly49A ligand, the MHC class I molecule H-2D^d, is present in the mouse strain considered (Kim *et al*., 2005). The experimental set-up was quite simple and consisted in the stimulation of splenocytes from various mouse strains with plate-bound anti-NK1.1 antibodies followed by flow cytometry staining and enumeration of IFN-γ+ NK cells distinguished by the presence or absence of Ly49A (and as mentioned above, there were much more IFN-γ+ cells among the Ly49A+ than among the Ly49A- population). Several control experiments confirmed this major finding and it became clear that, somewhat paradoxically, NK cells have to express an IR specific for an autologous MHC class I molecule to become activated and to exert their functions.

What remained unclear however was the mechanism leading to this phenomenon, and, based on these and other previous findings, several models were established to conceptualize NK cell education. Most of them existed before the Kim paper of 2005 because it was clear to the experts that something was going on in the regulation of NK cell functions, although not all details were understood nor all experimental data explained.

3. Different models of NK cell education

1) The disarming model

This hypothesis claims that NK cells receive activating signals form their environment (default state) and that these need to be counterbalanced through inhibitory messages to reach a normal

functional configuration. In contrast, in the absence of the latter, the cells become hyporesponsive (Shifrin *et al*., 2014; He & Tian, 2016).

2) The arming model

This is in a way the reverse hypothesis, as it states that the inhibitory signals received from cells in the environment trigger the adequate threshold for activation in each NK cell except in those that do not receive, for whatever reason, enough inhibitory signalling and therefore remain hyporesponsive (Shifrin *et al*., 2014; He & Tian, 2016).

3) The rheostat model

In this concept, a quantitative factor is included in addition to the qualitative one (response versus weak or no response) (Brodin *et al*., 2009a; He & Tian, 2016; Shifrin *et al*., 2014). It is based on experimental observations that have shown a direct correlation between the number of self-specific IR expressed by a NK cell and the degree of its functional responsiveness (in terms of degranulation and/or cytokine production). Thus, the more IR the NK cell is expressing, the more reactive it is to activating stimuli. Likewise, NK cells are more responsive when additional MHC class I alleles are introduced into the environment, or on the contrary less when these alleles are progressively removed (mouse data) (Brodin *et al*., 2009b). In addition, the strength of the affinity between the IR and its ligand also partly determines the outcome of the education process (He & Tian, 2016).

4) The tuning model

It consists in the integration of the arming, disarming and rheostat concepts which are not mutually exclusive, and stipulates that an individual NK cell permanently integrates the inhibitory and activating messages it receives through its IR (particularly via the MHC class I molecules of the environment) and AR, respectively, and adapts its functional behavior to this input (Shifrin *et al*., 2014).

5) The *cis*-*trans* model

Due to my personal contribution to the elaboration of this model, I will describe in more detail how we came to the concept of a role for *cis* interaction between MHC class I molecules and their ligand, the IR Ly49A, on the membrane of a same NK cell, for the education of this cell. The work was performed in the lab of Prof. Werner Held at the Ludwig Institute for Cancer Research in Lausanne, Switzerland. When I integrated his group as a postdoctoral researcher, the so-called "trogocytosis" (Joly & Hudrisier, 2003) was described by several authors, although similar observations had been made decades before but then been forgotten. It consists in the active transfer of cell membrane fragments from one cell to another at the immune synapse in a specific receptor-ligand dependent manner, whereby surrounding membrane molecules are co-transferred. This phenomenon had been shown for B cells (taking up immunoglobulins) (Batista *et al*., 2001) and T cells (acquiring MHC class I or class II molecules from antigen-presenting cells) (Huang *et al*., 1999). The transfer can lead to functional consequences in the recipient cell, such as a more efficient antigen presentation by B cells or T lymphocytes which might present the acquired peptide/MHC complexes to other T cells and induce fratricide.

Our aim was then to verify that NK cells were also able to perform trogocytosis and we logically focused on the interactions between the well-known IR Ly49A and its cognate ligand, the classical mouse MHC class I molecule H-2D^d. B6 mice carry the H-2b haplotype and are therefore H-2D^d-. However, around 20 % of their NK cells express the IR Ly49A (ligand not present). The H-2D^d transgenic mouse B6D^d carries a D^d transgene (ligand present), but is otherwise identical to a B6 mouse. When we now mixed at a 1:1 ratio the total splenocytes from B6 animals with those from B6D^d ones and incubated them at 37°C for two hours, the Ly49A+ NK cells from the former, but importantly not the Ly49A- NK cells, became significantly H-2D^d+. This could be blocked with anti-Ly49A and anti-H-2D^d monoclonal antibodies. As the Ly49A+ NK cells from the B6 mice are genetically H-2D^d-, these molecules can only come from the B6D^d cells in the environment, which demonstrates that NK cells can specifically acquire molecules present exclusively on surrounding cells and thus perform trogocytosis. The phenomenon was confirmed by using non transgenic B10.D2 mice (constitutively H-2D^d+) or B10.BR mice (constitutively positive for another Ly49A ligand, H-2D^k) as donors and MHC class I- β2m-KO animals as recipients (Zimmer *et al*., 2001). Two other groups made similar observations at the same time, one in the mouse (Sjöström *et al*., 2001) and one in humans (Carlin *et al*., 2001) with the KIR/HLA-C receptor-ligand pair.

We went further on in the characterization of these interactions and showed that they also occur *in vivo*. Indeed, in mixed bone marrow chimeras of B6 and B6D^d hematopoietic stem cells injected at a 1:1 ratio into lethally irradiated recipient mice, both types of cells contributed to the reconstitution of the immune system, but specifically the Ly49A+ NK cells from B6 origin were again H-2D^d+.

Things then became even more interesting as we could unravel that the presence of one Ly49A ligand on the Ly49A+ NK cells themselves, as occurs in B10.D2 mice, precluded the uptake of the second ligand, H-2D^k. In other words, Ly49A+ NK cells from B10.BR (H-2k haplotype) mice, who endogenously are H-2D^k+, did not become H-2D^d+ in the presence of H-2D^d+ B10.D2 donor cells, and vice versa, B10.D2 (H-2D^d haplotype) Ly49A+ NK cells did not become H-2D^k+ in the presence of H-2D^k+ B10.BR donor cells (Zimmer *et al*., 2001).

The Held lab also housed B6 mice that were transgenic for the IR Ly49A, as well as B6 mice double transgenic for H-2D^d and Ly49A. When we studied the cytotoxic activity of activated NK cells from both of these strains, they similarly killed the H-2b tumour target cell line C1498. However, in the case of the H-2D^d transfected variant of C1498 (C1498D^d), the NK cells from the Ly49A single transgenic mouse strain were strongly inhibited and did not kill the target efficiently, whereas the NK cells from the double transgenic mouse were almost not inhibited. This suggests that Ly49A is normally functional and inhibits NK cell cytotoxicity when its ligand H-2D^d is present in the environment (for example on tumour target cells), but that the inhibitory function is very strongly affected (reduced) when H-2D^d is also expressed by the NK cells themselves. Nevertheless, these cells do not lyse normal syngeneic cells like activated T lymphocytes from B6D^d animals, so that self-tolerance to healthy targets is preserved (Zimmer *et al*., 2001).

Besides cytotoxicity, cytokine release is the second major NK cell function. Thus, we investigated IFN-γ production in Ly49A+ compared to Ly49A- NK cells and found that approximately 25% of B6 as well as B6D^d Ly49A- NK cells produced IFN-γ in response to C1498. In contrast, the Ly49A+ NK cells of the B6 mice (no H-2D^d expression by themselves) were almost completely inhibited by C1498D^d, whereas the Ly49A+ NK cells from the B6D^d animals (H-2D^d expression by themselves) continued to produce IFN-γ in the presence of C1498D^d (Ioannidis *et al*., 2001). This shows once again that Ly49A does not efficiently inhibit NK cells in the presence of its ligand on the NK cells themselves.

Our findings brought us to the hypothesis that, in addition to the conventional interaction of Ly49A on NK cells with H-2D^d ligands on target cells in *trans* (interaction between a receptor and its ligand located on two different cells), Ly49A might also interact with H-2D^d in *cis* (interaction between a receptor and its ligand located in the plane of the same membrane, i.e. on the same cell).

We then of course had to demonstrate our idea, and this was done in a complicated series of experiments and together with other colleagues. It was notably possible to co-immunoprecipitate Ly49A and H-2D^d (Doucey *et al*., 2004), so that we established the model of *cis-trans* interaction. If H-2D^d is absent from the NK cell, Ly49A is fully available for *trans* interaction and efficiently inhibits NK cell functions. However, when H-2D^d is also present on the NK cell, which corresponds in fact to the physiological situation, Ly49A is partially engaged in *cis*, so that less Ly49A is available for the interaction in *trans*. Consequently, the receptor is less accessible and the threshold for NK cell activation is lowered (as there is less inhibition). According to the concept of the balance between activating and inhibitory signalling, this allows the fine-tuning of NK cell responses and a great sensitivity to modifications of H-2D^d levels in the environment.

After I left the lab, Held *et al*. published several follow-up papers that detailed and complemented the initial model (Back *et al*., 2011; Back *et al*., 2007; Chalifour *et al*., 2009). Overall it was well received (Shifrin *et al*., 2014), although some criticisms remain, such as the argument that the relative contribution of *cis* and *trans* binding still has to be clarified (Claus *et al*., 2019; He & Tian, 2016) or the observation that *cis* interactions for human KIR and NKG2A IR with their ligands have not been demonstrated (Claus *et al*., 2019). Indeed, the *cis-trans* model in this aspect seems to be applicable to the mouse system only.

Interestingly however, Masuda and colleagues could demonstrate *cis* interaction between HLA class I molecules and the IR LILRB2 (or ILT4) which is not expressed by NK cells but by human mast cells and which regulates the activation of the latter (Masuda *et al*., 2007).

In addition, some signaling lymphocyte activation molecule (SLAM) family receptors, which can also be involved in NK cell education, undergo *cis* interactions on the NK cell surface, at least under certain conditions (Claus *et al*., 2019).

6) The confining model

This hypothesis includes data demonstrating that at the immunological synapse, where the close contact between the NK cell and the target cell takes place, there is a clustering of IR and AR. Signalling through adhesion molecules stabilizes the conjugate and the NK cell re-organizes its actin meshwork. Activating receptors are localized in micro-clusters in educated NK cells, whereas they are dispersed in unlicensed cells. The model takes into account the role of adhesion molecules and receptor "confinement" at the immunological synapse, in addition to the mere presence of the IR and AR (He & Tian, 2016).

7) Other aspects of NK cell education

It is important to emphasize that NK cell education is not a final and fixed process but remains plastic to some extent. Thus, when hyporesponsive MHC class I-deficient NK cells are adoptively transferred into normal recipient mice, they acquire functional competence, and vice versa, normal NK cells become hyporesponsive in an MHC class I-deficient environment (Elliott *et al*., 2010; Joncker *et al*., 2010; Shifrin *et al*., 2014). These results are very well compatible with the tuning model and suggest that NK cells permanently adapt to their environment to remain self-tolerant and at the same time ready to exert their functions in case of need.

In addition, hyporesponsive NK cells may become functional particularly in the case of infections, where an inflammatory environment rich in stimulating cytokines and chemokines may activate these cells (Fernandez *et al*., 2005). Furthermore, NK cells from MHC class I-deficient mice and humans become cytotoxic after a short culture period in the presence of interleukin (IL)-2 (Salcedo *et al*., 1998; Vitale *et al*., 2002; Zimmer *et al*., 1998) and produce IFN-γ when stimulated with IL-12 and IL-15 or IL-12 and IL-18 (our unpublished results). It has even been reported that during the infection with the mouse cytomegalovirus (MCMV), uneducated NK cells respond better than educated ones, presumably because the activity of the former is not blocked by self-specific IR (Orr *et al*., 2010; Shifrin *et al*., 2014). In fact, one study using both influenza virus and MCMV found that unlicensed NK cells accumulated in the draining lymph nodes where they produced the cytokine granulocyte macrophage colony-stimulating factor (GM-CSF) which might have contributed to the maturation of dendritic cells and antigen-specific responses of CD8+ T lymphocytes. In contrast, educated NK cells preferentially located in infected tissues where they produced the effector cytokine IFN-γ (Zamora *et al*., 2017). All these findings suggest that, although not endowed with classical NK cell effector functions, the unlicensed NK cells nevertheless have specific biological roles and are not simply useless bystanders. By the way, human TAP-deficient individuals do not develop especially severe viral infections but are prone to chronic bacterial infections, which they cannot clear (Zimmer *et al*., 1998; Zimmer *et al*., 2005).

In a complex set of experiments with fetal liver chimeras and adoptive transfers, the Raulet group revealed a difference between the educating capacities of MHC class I molecules on hematopoietic versus nonhematopoietic cells from the NK cell environment. Their data suggests a predominant role of the latter in imprinting a normal responsiveness of NK cells to stimulation via AR (Shifrin *et al*., 2016). However, Ebihara *et al*. found the exact contrary, namely that the hematopoietic cell-expressed MHC class I molecules are the most important for NK cell education, via the use of an inducible MHC class I transgene model (Ebihara *et al*., 2013).

The integration of *cis* interactions in a more global model has been recently proposed (Boudreau *et al*., 2016) and commented (Cooper, 2016). It shows, based on a humanized mouse model transgenic for a HLA class I molecule, that both *cis* and *trans* interactions of NK cell IR with their ligand are required for education, but that the *cis* interactions are more important for its maintenance. Furthermore, by performing adoptive transfer experiments, the authors observed that educated NK cells remained educated even in a non-transgenic environment (which is opposed to the findings in a pure mouse system where the educated NK cells rapidly loose functionality in an MHC class I-deficient context, see above) and that uneducated NK cells became functional in transgene+ mice. As to the elucidation of the mechanism, the human IR were able to acquire their HLA class I ligands shed from surrounding cells, with presumably a *cis* interaction and an increased responsiveness as consequence.

Whereas NK cell education has predominantly been considered by many authors as a process governed by interactions between Ly49 (mouse) and KIR (human) IR on the one hand and classical polymorphic MHC class I molecules on the other hand, the concept has in the meantime been extended to IR specific for non-classical MHC class I molecules, non MHC molecules and AR, even if some of these observations remain controversial. He and Tian (He & Tian, 2016) thus distinguish between (i) classical NK cell education or NK cell licensing, (ii) non-classical MHC class I-dependent NK cell education and (iii) MHC class I-independent NK cell education.

The first item has been extensively discussed above. The second one is based on the fact that NKG2A+ NK cells are better educated, and consequently more functional, than NKG2A- ones (Meyer *et al*., 2017). A very large analysis of human IR and HLA class I haplotypes concluded

on the existence of two different "schools" of HLA haplotypes in the human population: one provides predominantly ligands for the KIR, the other one predominantly ligands for NKG2A, and the NK cells are educated accordingly (Horowitz *et al*., 2016). The non-classical MHC class I molecule H2-M3 (via Ly49A) and the MHC class I-like molecule CD1d, the restriction element for conventional NKT cells (via an unknown IR), are likewise described as having an educating effect on NK cells (He & Tian, 2016).

Regarding the non MHC class I-dependent NK cell education, several receptor-ligand pairs have been found playing a role (He & Tian, 2016): 2B4 – CD48, SLAMF6 – SLAMF6 (homotypic interaction), NKRP1B – Clr-b (a pair of C-type lectin receptors), and the IR TIGIT – CD155 (or poliovirus receptor). In the latter case, studies were performed with TIGIT+ wildtype mice compared to their TIGIT- counterparts, which revealed that the ligand CD155 acted like an MHC class I molecule in the sense of a missing-self indicator. TIGIT+ NK cells were shown to be more functional than TIGIT- ones, but TIGIT+ NK cells from CD155-KO mice were hyporesponsive (He *et al*., 2017).

4. Recent developments

Currently, immunometabolism, investigating the biochemical events associated with the function of immune cells, is a hot topic. Thus, it is not surprising that the metabolism of NK cells is likewise on the agenda. A detailed description would go beyond the aim of the present work, but the recent overview by Kobayashi and Mattarollo gives a solid introduction into this field (Kobayashi & Mattarollo, 2019). Regarding the relationship between NK cell metabolism and education, it has been shown that the higher functional activity of licensed compared to unlicensed NK cells is essentially based on an enhanced glycolysis in the former (Schafer *et al*., 2019). Natural killer cell education is also governed by the mTOR/AKT pathway, which is more active in licensed cells and up-regulates the signalling through AR. This phenomenon can be reversed by pharmacological inhibition of mTOR, whereas stimulating cytokines can rescue the uneducated phenotype of hyporesponsive NK cells (Marçais *et al*., 2017). These reports are quite important, as they resolve, at least in part, the longstanding questions about the molecular mechanisms of NK cell education and hyporesponsiveness.

Another aspect that raises increasing interest is the contribution of AR to NK cell licensing. Several years ago, a role for the activating isoforms of KIR in this regard has been demonstrated (Fauriat *et al*., 2010). More recently, the SLAM family, whose members are solely expressed by hematopoietic cells and in most cases self-binding, was shown to likewise intervene in this process (Chen *et al*., 2016; Cooper, 2016). Finally, Jelencic *et al*. demonstrated that the AR NKG2D, expressed early during NK cell ontogenesis, sets the activation threshold for another AR, NKp46, with the outcome that NK cells from NKG2D-KO mice display increased functional responses towards tumours and during MCMV infection. The mechanism of this phenomenon is based on quite complex up- and down-regulations of intracellular signalling molecules (Jelencic *et al*., 2018; Veillette & Yu, 2018; Wensween *et al*., 2018).

The AR DNAX accessory molecule-1 (DNAM-1) or CD226, whose ligands are CD112 and CD155, is closely associated to, but not absolutely required, for NK cell education. It confers enhanced effector functions to NK cells and its expression is linked to the one of MHC class I-specific IR (KIR and NKG2A). Moreover, upon stimulation of educated NK cells, a change in the conformational state of the adhesion molecule LFA-1 (CD11a/CD18) occurs and it co-localizes with DNAM-1 at the immunological synapse, favouring granule polarization and cytotoxicity (Enqvist *et al*., 2015; Wagner *et al*., 2017).

Human immunodeficiency virus (HIV) infection is one of the most intensively studied infectious diseases, and a role for NK cells is known for quite some time. Although they are directly affected in this pathology, particularly due to the expansion of a hyporesponsive CD56-CD16^{bright} subset which is numerically minor in healthy donors, they might also play a role in the anti-HIV immune response. Interestingly, HIV-infected cells down-regulate HLA-A and -B molecules but maintain their expression of HLA-C and -E, which should theoretically lead to their escape from both cytotoxic CD8+ T cells and NK cells. Lisovsky *et al*. have determined that the functional response (degranulation, cytokine and chemokine production) to autologous infected CD4+ T lymphocytes is dominated by NKG2A+ NK cells, independently of the expression of the HLA-Bw4 specific IR KIR3DL1, whereas in the presence of HLA class I- target cells, the NKG2A+ KIR3DL1+ cells were, as expected, the most functional (Lisovsky *et al*., 2015), in accordance with the rheostat and the tuning models. Thus, NKG2A seems to have a special role in anti-HIV defense mediated by NK cells. Natural killer cell education likewise intervenes in the regulation of anti-HIV antibody activity, particularly in ADCC (Bernard *et al*., 2017; Kristensen *et al*., 2018).

5. Concluding remarks

After this short visit of the topic of NK cell education, which is rather fundamental in nature, what could be the sense and the translational value of such studies? As previously mentioned, NK cell immunotherapy of cancer, that has been in the clinics already for many years (Suen *et al*., 2018), can now be considered as coming of age (Rezvani *et al*., 2017; Souza-Fonseca-Guimaraes *et al*., 2019; Barrow & Colonna, 2019). However, not all problems are resolved, like for example the treatment of solid tumours as opposed to those of haematological origin. In addition, although they are somewhat traditionally considered as less toxic and less dangerous than activated T cells, NK cells might sometimes have negative side effects (Martín-Antonio *et al*., 2017; Poli *et al*., 2018). Nevertheless, the balance is overall clearly positive and the coming years will most likely witness an increased use of NK cells in cancer immunotherapy and maybe even in anti-infectious approaches. In order to reduce the costs and the time from engineering to administration to the patient, NK cells might be generated in the future from induced pluripotent stem cells (which are of adult origin, usually from fibroblasts that are de-differentiated and then re-directed in vitro to become NK cells and thus do not give rise to the major ethical problems caused by the use of embryonic stem cells). This would represent an off-the-shelf therapeutic option for cancer patients (Saetersmoen *et al*., 2018). Furthermore, Wang *et al*. have shown that after expansion of NK cells with the HLA class I- feeder cells K562 transfected with 4.1BB ligand and membrane IL-15 (a currently used method to generate high numbers of activated NK cells, whereby the technique works even better if IL-15 is replaced by IL-21), NK cells keep their initial educational characteristics (Wang *et al*., 2016). This means practically that it might be not only possible, but also advantageous, to select the NK cell subsets that are likely to be most efficient in a given clinical situation.

6. References

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