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Title

Impact of anti-CD20 tumor-targeting therapeutic monoclonal antibodies on human Natural Killer cell responsiveness and plasticity: relevance of FcγRIIIA/CD16 affinity ligation conditions.

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RESULTS

Summary

My study is focused on understanding the mechanisms underlying the modulation of NK cell responsiveness and plasticity induced by tumor targeting therapeutic anti-CD20 monoclonal antibodies (mAbs) nowadays routinely used in the treatment of B-cell malignancies and autoimmune disorders. Anti-CD20 mAbs are grouped into type I and II subtypes. Type I mAbs induce CD20 redistribution into lipid rafts and display a remarkable ability to activate complement-dependent cytotoxicity (CDC). On the other hand, type II mAbs, which are not able to localize CD20 complexes into lipid rafts and induce weak or no CDC, evoke more homotypic adhesion and direct killing of target cells. Both type I and II mAbs demonstrate efficient phagocytosis and antibody-dependent cytotoxicity (ADCC). Natural Killer (NK) cell-mediated ADCC, based on the recognition of IgG-opsonized targets by the low affinity Fc receptor for IgG FcγRIIIA/CD16, represents one of the main mechanisms by which anti-CD20 mAbs mediate their anti-tumor effects. Besides ADCC, CD16 ligation also results in the production of cytokines such as IFN-γ that plays a key role in the shaping of adaptive immune responses. Rituximab is a chimeric type I anti-CD20 mAb of 1st generation and is considered the reference molecule for the comparison with new generation anti-CD20 mAbs, designed to optimize clinical efficacy. Among them, obinutuzumab is a humanized Fc-glycoengineered type II anti-CD20 mAb of 3rd generation designed to increase the affinity for CD16 receptor and consequently the killing of mAb-opsonized targets. However, the impact of CD16 ligation in optimized affinity conditions on NK functional program is not completely understood. Herein, I demonstrated that CD16 affinity ligation conditions may dictate both the amplitude of NK responsiveness (cytotoxicity and IFN-γ production) as well as the ability to shift the NK functional program. Indeed, I observed that the interaction of NK cells with obinutuzumab-opsonized targets results in enhanced cytotoxicity and IFN-γ production as compared with the parental non-glycoengineered mAb or the reference molecule rituximab, independently from the CD16-158V/F allotype. The affinity ligation conditions also strictly correlate with the ability to induce CD16 surface down-modulation and lysosomal targeting of receptor-coupled signaling elements. Indeed, a preferential degradation of FcεRIγ chain and Syk tyrosine kinase was observed upon obinutuzumab stimulation independently from the CD16-158V/F allotype. Notably, although the down-regulation of FcεRIγ/Syk module hesitates in the impairment of cytotoxic function induced by CD16, NKp46 and NKp30 activating receptors, obinutuzumab-experienced NK cells exhibit an increased ability to produce IFN-γ in

response to cytokines and target stimulation as well as to obinutuzumab-mediated CD16 re-stimulation.

Relying on the observation that obinutuzumab-experienced NK cells, under molecular and functional profile, resemble the distinctive features of the long-lived and highly functional "*memory*" NK cells, a population recently identified in HCMV seropositive individuals, I assessed the capability of anti-CD20 mAbs to affect the expansion as well as the phenotypic and functional properties of the "*memory*" NK subset. My data show that the majority of the analysed healthy donors is HCMV seropositive and exhibits a detectable population of "*memory*" NK cells ($CD3^-$ $CD56^+$ $Fc\varepsilon RI\gamma^-$ $CD16^+$) accounting for 3 to 50% of peripheral blood NK cells. I observed that "*memory*" NK cells selectively undergo 2- to 12-fold expansion upon co-culturing with anti-CD20-opsonized targets; on the opposite, the proliferation of "*conventional*" NK cells ($CD3^-$ $CD56^+$ $Fc\varepsilon RI\gamma^+$ $CD16^+$) is not affected by CD16 stimulation. I also noted that anti-CD20 mAb *in vitro* expanded "*memory*" NK cells show the molecular and functional hallmarks of their freshly isolated counterpart, including the increased expression of NKG2C receptor, the reduced expression of NKp46 receptor associated to an enhanced functional activity in response to CD16 re-stimulation, particularly in terms of IFN-γ production.

Introduction

1. Natural Killer cells

1.1 Morphology and general characteristics

Natural Killer (NK) cells belong to the innate branch of the immune system. They are defined as "*natural killer*" based on their ability to recognize and kill infected or transformed cells without prior immunization (*Whiteside & Heberman, 1994; Lanier, 2005*). Besides immune surveillance against infections and tumors, NK cells may play a role in autoimmunity and hypersensitivity by contributing to the shaping of adaptive immune responses through their ability to secrete cytokines and chemokines and to cooperate with other components of the immune system such as dendritic cells (DCs), macrophages and B or T lymphocytes (*Trinchieri, 1989; Moretta et al., 1994; Raulet, 2004; Newman and Riley, 2007; Caligiuri, 2008; Vivier et al., 2008; Flodstrom-Tullberg et al., 2009; von Bubnoff et al., 2010; Bryceson et al., 2011*). NK cells were identified on purely functional basis and classified as lymphocytes for their origin from a progenitor shared with B and T lymphocytes and for the expression of several lymphoid line markers (*Herbernan et al., 1975; Kiessling et al., 1975; Vivier et al., 2011*). Unlike B and T lymphocytes, NK cells do not express somatically recombined and clonally distributed antigen receptors and interact with target cells by means of a limited pattern of activating and inhibitory germline-encoded receptors (*see below*) (*Lanier, 2005; Long et al., 2013*), thus representing a distinct lymphocyte subset (*Cerwenka and Lanier, 2001*). They account for the 10-15% of circulating lymphocytes in human peripheral blood and are resident in both lymphoid organs and several non-lymphoid tissues including liver, lungs, gut and uterus (*Trinchieri, 1989; Cerwenka & Lanier, 2001; Colucci et al., 2003; Ferlazzo et al., 2004; Freud et al, 2005; Chinen et al., 2007*). NK cells develop and become mature in the bone marrow and, through the action of chemotactic factors, reach lymphoid organs, peripheral tissues as well as sites of inflammation (*Colucci et al., 2003; Carrega & Ferlazzo, 2012*). Although the majority of NK cells is quiescent, they rapidly respond to exogenous stimuli. This makes them efficient in mediating the first line defense against pathogens and tumors and explains the definition of "*ready to go cells*" (*Whiteside & Heberman, 1994; Lanier, 2005*). About morphology, NK cells are characterized by a large cytoplasm enriched in azurophilic granules containing cytolytic mediators (Fig.1).

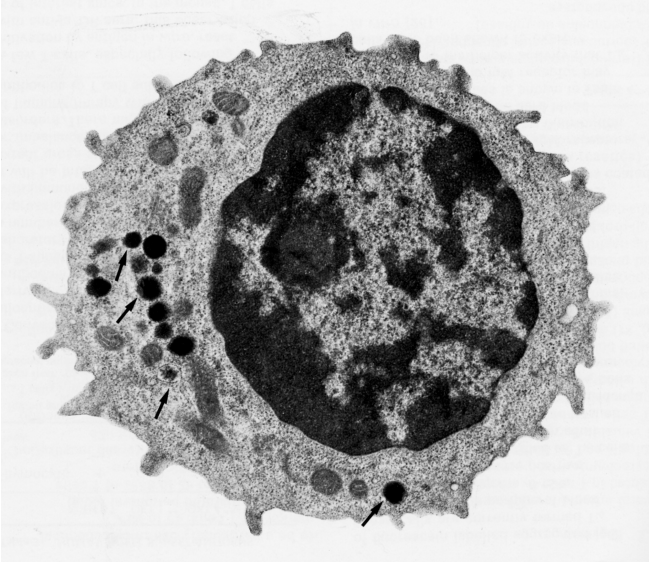


Figure 1. Natural Killer cell examined with Transmission electron microscopy (TEM). Arrows indicate the cytoplasmatic lytic granules containing cytotoxic molecules.

NK cells comprise phenotypically and functionally heterogeneous populations (*Cooper et al., 2001; Caligiuri, 2008; Fauriat et al., 2010; Bryceson et al., 2011; Long et al., 2013*). In humans, they are phenotypically characterized by the absence of CD3, a T-cell co-receptor, by the expression of CD56 (N-CAM-1), a neuronal cell adhesion molecule that participates in homotypic interactions, which may be co-expressed with FcγRIIIA/CD16, the low affinity receptor for the Fc fragment of immunoglobulin (Ig) G. In particular, the 90% of peripheral blood human NK cells expresses low-intermediate CD56 and high CD16 ($CD56^{dim}$ $CD16^{bright}$) while only the 10% of NK cells expresses high CD56 and low or no CD16 ($CD56^{bright}$ $CD16^{dim/-}$) (*Robertson and Ritz, 1990; Cooper et al., 2001; Mavilio et al., 2005*; *Caligiuri, 2008; Di Santo, 2008; Poli et al., 2009*). The $CD56^{dim}$ $CD16^{bright}$ subset, classified as mature NK cells, are potent cytolytic effectors. On the contrary, less mature $CD56^{bright}$ $CD16^{dim/-}$ NK cells have relatively poor cytotoxic activity and predominantly produce inflammatory cytokines and chemokines in response to cytokine stimulation (*Cooper et al., 2001; Caligiuri, 2008; Korbel et al., 2009; Fauriat et al., 2010; De Maria et al., 2011; Long et al., 2013*).

1.2 Functional activity

NK cells serve important functions in immune regulation, in tumor surveillance and in resistance against intracellular pathogens and viruses. Such responses are mediated through two major effector functions: the secretion of several pro-inflammatory and immunoregulatory cytokines and chemokines as well as the killing of sensitive target cells, which can be triggered without requiring any priming, transcription, translation or expansion (*Robertson and Ritz, 1990; Cerwenka & Lanier, 2001; Lanier, 2005; Caligiuri, 2008*). It is well-known that NK cell survival, proliferation, migration and effector functions are influenced by several soluble factors. In response to interleukin (IL)-2, IL-12, IL-15 or IL-18, released by phagocytes and DCs, NK cells produce interferon (IFN)-γ (*Bryceson et al., 2006; Caligiuri, 2008).* IFN-γ, endowed with well-known anti-bacterial/viral effects, promotes the differentiation of T helper (Th) cells in Th1 subset, activates macrophages and stimulates the microbicide activity of phagocytes, thus promoting the killing of phagocytosed microorganisms as well as causes the up-modulation of the major histocompatibility complex (MHC) class I and II and the costimulatory molecules on antigen-presenting cells (APC) (*Mocikat et al., 2003; Bryceson et al., 2006; Filipe Santos et al., 2006; Maher et al., 2007; Fauriat et al., 2010*). By modulating the responses of DCs and T cells, IFN-γ stands as a well-recognized key immunoregulatory factor in the shaping of adaptive immune responses (*Martin-Fontecha et al., 2004; Walzer et al., 2005; Crouse et al., 2015*). Furthermore, in response to IL-2 and IL-12, NK cells produce pro-inflammatory and immunoregulatory cytokines as the tumor necrosis factor (TNF)-α, IL-3 and IL-18, growth factors as the granulocyte macrophage colony-stimulating factor (GM-CSF) and chemokines as the macrophage inflammatory protein (MIP)-1α, MIP-1β and RANTES which in turn recruit other effector cells in the sites of inflammation (*Cerwenka & Lanier, 2001; Raulet, 2004; Bryceson et al., 2006; Caligiuri, 2008; Cerboni et al., 2009; Fauriat et al., 2010; Bryceson et al., 2011*). Besides being an important source of soluble factors, NK cells recognize and kill sensitive target cells (*Bryceson et al., 2009*). The target cell killing may be due to the activation of death domain-containing receptors. Indeed, the TNF family members Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), expressed on NK cells, may recognize the death domain-containing receptors Fas and TRAIL-R expressed on target cells, thus leading to their apoptosis (*Zamai et al., 1998; Screpanti et al., 2005*). However, the primary mechanism by which NK cells mediate target cell killing is the polarized release of the lytic granule content at the effector/target contact area, a highly

specialized structure defined cytolytic synapse. Cytotoxic granules are specialized secretory lysosomes containing lytic mediators such as the Ca^{2+}-dependent pore-forming protein perforin and the apoptosis-inducing serine proteases granzymes, such as granzyme B. To ensure that NK cells do not indiscriminately kill the target cells, the cytotoxic event is a tightly regulated and highly ordered multistep process (Fig.2) (*Rosen et al., 2000; Lieberman, 2003; Stinchcombe & Griffiths, 2007; Orange 2008; Griffiths, 2010; Galandrini et al., 2013*). During the early stages, the NK cell-mediated recognition of a sensitive target leads to the formation of a stable cell conjugate: the effector/target cell contact area undergoes a profound rearrangement leading to the generation of the cytolytic synapse (*Vyas et al., 2002; MacFarlane & Campbell, 2006*). With reference to the effector cell side, the central region of the cytotoxic synapse forms the central supramolecular activation cluster (c-SMAC) which comprises both engaged receptors and signaling molecules. Around c-SMAC structure, it is observed the peripheral SMAC (p-SMAC) in which NK adhesion molecules are recruited (*Vyas et al., 2002*). The assembly of p-SMAC structure both stabilizes the cell-cell adhesion and triggers a localized cytoskeletal remodeling (*Davis, 2002; Vyas et al., 2002; Riteau et al., 2003; MacFarlane & Campbell, 2006; Krzewski & Strominger, 2008*). The polarization step of cytotoxic event includes the reorientation of the microtubule-organizing center (MTOC) that polarizes towards the cytolytic synapse the preformed lytic granules (*Peters et al., 1991*). This process is co-ordinated by effector molecules responsible for actin cytoskeletal changes and for the mobilization of MTOC at cytolytic synapse (*Krzewski et al., 2006; Krzewski et al., 2008*). Later steps include the maturation and Ca^{+2}-dependent exocytosis of lytic granules. During the maturation process, lytic granules are docked to the plasma membrane containing secretory domains (*docking step*) and gain the competence to fuse with them (*priming step)* (*Mènager et al., 2007*) thus leading to the release of their content: perforin and granzymes. Perforin is a homologue of the C9 fraction of complement system and is able to form plasma membrane pores thus altering the osmotic balance and allowing granzyme delivery into cytosol of target cells. Granzymes are enzymes with serine protease activity that cleave proteins at aspartate residues and activate caspases, the cysteine proteases responsible for the programmed cell death or apoptosis (*Davis, 2002; Lieberman, 2003; Krzewski & Strominger, 2008*). Among others, granzyme B plays a role in both the activation of caspase 3 and the cleavage of either cytoplasmic pro-apoptotic BH3-only family members or inhibitors of Caspase-Activated DNase (ICAD) thus promoting apoptosis (*Thomas et al., 2000; Clark and Griffiths, 2003; Lieberman, 2003;*

Bossi and Griffiths, 2005; Waterhouse et al., 2005). Several models have been proposed about the mechanism of synergy by which perforin and granzymes induce target cell death. In the "*plasma membrane pore*" model, perforin undergoes Ca^{+2}-dependent polymerization and, by forming pores in target cell plasma membrane, allows both target cell osmotic lysis and granzymes delivery into the target cell cytosol (*Lowin et al., 1995; Lieberman, 2003; Pipkin & Lieberman, 2007*). The alternative "*endosomolysis*" model instead predicts that, upon binding with the target cell plasma membrane via electrostatic interactions or specific receptors, both perforin and granzymes are internalized into the endosomal compartment and that perforin induces granzyme release into the target cell cytosol by disrupting endosomal structures (*Froelich et al., 1996; Lettau et al., 2007; Pipkin & Lieberman, 2007*). Recently, it has been proposed a hybrid model for which, upon membrane permeabilization, it may occur a Ca^{2+}-mediated membrane repair response, by means of membrane-vesicles patching to the formed pores, or a rapid internalization into endosomes of both perforin and granzymes, followed by perforin-mediated release of granzymes into the target cell cytosol (*Pipkin & Lieberman, 2007*). Other granule-associated molecules take part to the "*lethal hit*" including calreticulin and serglycin as well as the lysosome-associated membrane proteins LAMP1 (CD107a) and LAMP2 (CD107b), cathepsins and CD63 (*Pipkin & Lieberman, 2007*). The cytotoxic event terms with a phase of NK cell inactivity which is characterized by activating receptor down-modulation followed by NK cell detachment from the apoptotic targets. NK cells restore their cytolytic capacity by forming new lytic granules and re-expressing activating receptors (*Orange, 2008*). Due to the recovery of cytolytic potential, NK cells are able to generate a second cytolytic synapse, immediately after dissipation of the first one, through a process defined "*serial killing*" by which an individual NK cell can make serial contacts with multiple targets and several cycles of killing (*Bhat & Watzl, 2007*).

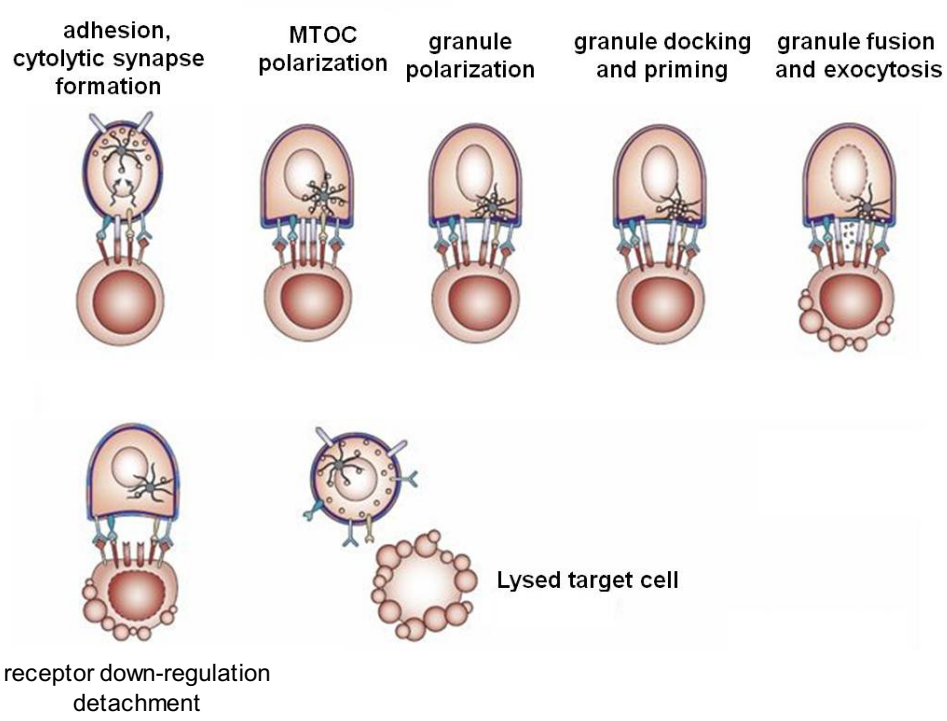


Figure 2. Discrete steps in the activation of NK cytolytic machinery. The activation of cytolytic machinery is a finely regulated multistep process that involves several discrete steps resulting into the secretion of cytotoxic mediators. Target cell recognition is followed by the assembly of a cytotoxic synapse which coordinates granule polarization toward effector/target cell contact area and polarized exocytosis of lytic granule contents (degranulation) at specialized secretory domains within the cytotoxic synapse. Termination step includes the NK cell detachment from the apoptotic target and the recycling of cytolytic activity (*modified from Orange, Nat Rev Immunol 2008*).

1.3 NK-mediated target cell recognition: activating and inhibitory receptors.

The NK-mediated recognition of target cells is characterized by a complex interplay of opposite signals, delivered by inhibitory and activating receptors simultaneously expressed on NK cells and engaged by ligand-bearing targets, and the balance of such opposite signals calibrates NK cell responses toward functional inactivity or activation. In physiological conditions, the signals transduced by activating receptors are balanced by those transduced by inhibitory receptors, which recognize MHC class I molecules expressed by target cells, thus preventing their lysis. By contrast, in non-physiological or stress conditions associated with the down-modulation of MHC class I molecules, the stimulatory signals become unopposed by inhibitory receptors, thus resulting in NK activation and target cell killing (*missing-self recognition*). However, in this scenario the up-regulation of specific molecules which act as ligands for activating receptors generally occurs thus overcoming the action of inhibitory receptors (*induced-self recognition*) (Fig. 3) (*Karre et al., 2002; Natarajan et al., 2002; Lanier, 2005; Raulet & Vance, 2006*).

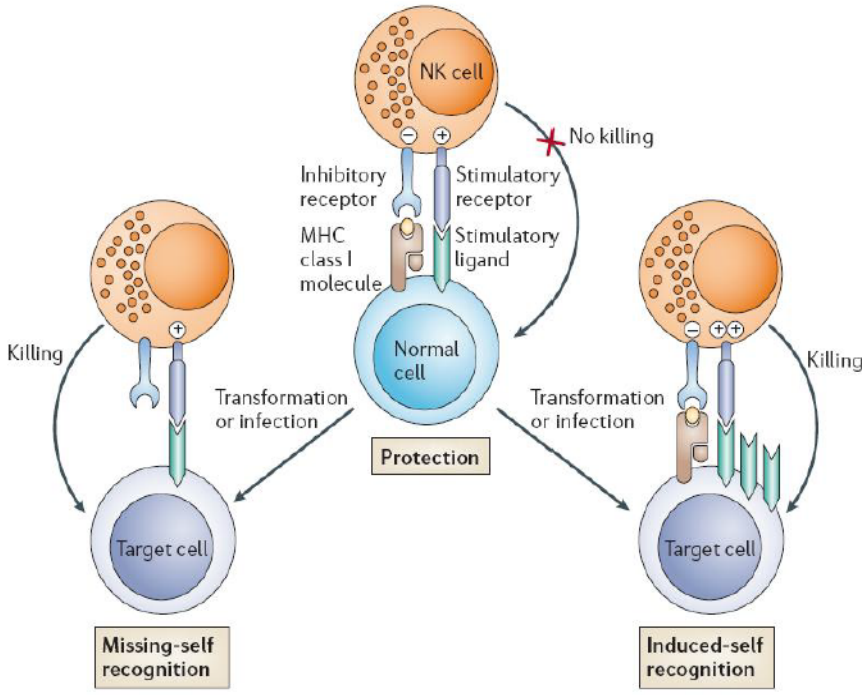
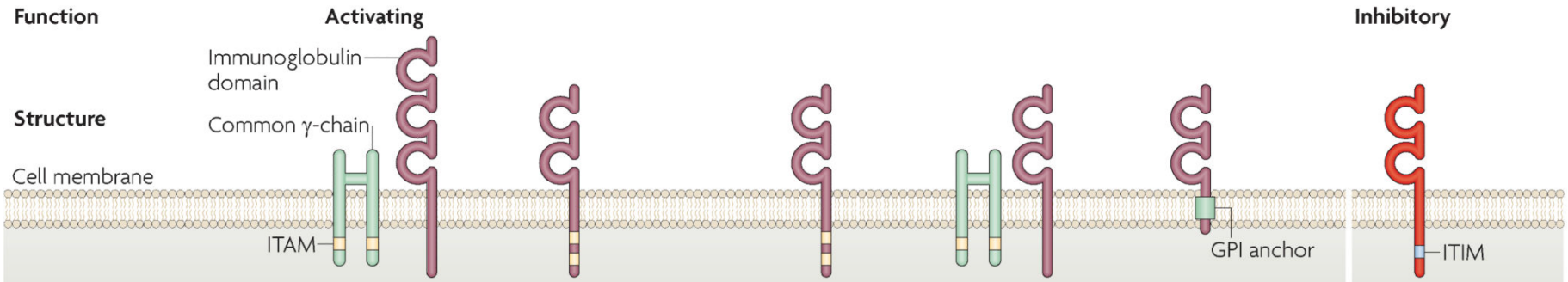


Figure 3. A complex interplay of inhibitory and stimulatory signals calibrates NK cell responses toward functional inactivity or activation. Healthy cells are protected from NK-mediated killing by NK cells. In this scenario the signals transduced by activating receptors are balanced by those transduced by inhibitory receptors which recognize the target-associated MHC class I molecules. The lack of MHC class I molecules results in NK cell activation and target cell killing (*missing-self recognition*). Stress conditions may also up-regulate ligands for activating receptors thus overcoming the action of inhibitory receptors (*induced-self recognition*) (form *Raulet & Vance, Nature Reviews 2006*).

NK-mediated recognition of sensitive target cells may lead to Antibody-Dependent Cellular Cytotoxicity (ADCC) or natural/spontaneous cytotoxicity (natural killing).

ADCC is a key function of NK cells and leads to the lysis of IgG-opsonized targets upon interaction with the low affinity receptor for IgG FcγRIIIA/CD16 (*Trinchieri, 1989; Trinchieri & Valiante, 1993; Perussia, 1998; Cerwenka & Lanier, 2001; Moretta et al., 2001; Bryceson et al., 2009*). FcγRIIIA/CD16 belongs to Fc receptor (FcR) family which include 1. Fc-alpha receptors (FcαRs) that bind IgA 2. Fc-epsilon receptors (FcεRs) that bind IgE 3. Fc-gamma receptors (FcγRs) that bind to IgG isotypes with different affinities and include both activating or inhibitory receptors such as FcγRI (CD64) FcγRIIA (CD32A) FcγRIIB (CD32B) FcγRIIC (CD32C) FcγRIIIA (CD16A) or FcγRIIIB (CD16B) (Fig. 4).



Name		FcγRI	FcγRIIA	FcγRIIC	FcγRIIIA	FcγRIIIB	FcγRIIB
Expression	Lymphoid	Not expressed	Not expressed	NK cell	NK cell	Not expressed	B cell and plasma cell
	Myeloid	Monocyte, DC and macrophage	Monocyte, DC, platelet and macrophage	Not expressed	Monocyte, DC and macrophage	Not expressed	Monocyte, DC and macrophage
	Granulocyte	Neutrophil and eosinophil	Neutrophil	Not expressed	Not expressed	Neutrophil, mast cell and eosinophil	Neutrophil, basophil and mast cell
IgG binding affinity		IgG1>>>IgG2, IgG3 and IgG4	IgG1 and IgG3>> IgG2>IgG4	IgG1>IgG3>> IgG4>IgG2	IgG1>>IgG2 and IgG3 >IgG4	IgG1 and IgG3>> IgG2 and IgG4	IgG1>IgG3>> IgG4>IgG2

Fig. 4. Structure, cellular distribution and affinity for the different IgG isotypes of FcγRs (from *Smith & Clatworthy, Nature Reviews Immunology 2010*).

Regarding the receptor affinity for IgG, it has been described that the FCGR3A gene, which enconds for the FcγRIIIA/CD16 receptor, exhibits a single nucleotide dimorphism (c. 599 G > T p.Phe158Val) that, in turn, encodes for FcγRIIIA/CD16 with either a phenylalanine (F) or a valine (V) at amino acid position 158, thus generating receptor variants with lower (FcγRIIIA -158F) or higher (FcγRIIIA-158V) binding affinity for IgG (*Koene et al., 1997; Rascu et al., 1997; Wu et al., 1997*) (Fig. 5).

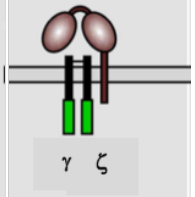
Name	**FcγRIIIA**	
CD	CD16A	
Gene	*FCGR3A*	
		
Alleles	V_{158}	F_{158}
IgG1	$2x10^5$	$1x10^5$
IgG2	$7x10^4$	$3x10^4$
IgG3	**$1x10^7$**	$8x10^6$
IgG4	$2x10^5$	$2x10^5$
Major role	Activation	

Fig. 5. FCGR3A-V158F polimorphism (modified from *Gillis et al., Front Immunol 2014*).

In some individuals, in addition to FcγRIIIA/CD16 receptor, a second FcγR (FcγRIIC/CD32C) has been identified on the surface of NK cells (*Metes et al., 1994; Metes et al., 1998; Metes et al., 1999; Morel et al., 1999; Ernst et al., 2002*). This molecule, which preferentially binds polymeric IgG and has only a low affinity for monomeric IgG (*Ravetch & Kinet, 1991; Van de Winkel & Capel, 1993; Hulett & Hogarth, 1994; Morel et al., 1999*) is an activatory receptor capable of inducing both ADCC and cytokine (IFN-γ, IL-1 and TNF-α) production (*Metes et al., 1994; Metes et al., 1998; Metes et al., 1999; Morel et al., 1999; Ernst et al., 2002*).

In addition to ADCC, NK cells also carry out an antibody-independent cytotoxic response, the so called "*natural killing*" which leads to the lysis of infected and tumor cells. Natural killing is regulated by a balance of signals derived from the aggregation of multiple inhibitory and activating receptors and requires the simultaneous co-engagement of different activating receptors by means of individual receptor ligands expressed by target cells (*Long and Rajagopalan, 2002; Bryceson et al., 2006; Lodoen, 2006; Bryceson & Ljunggren, 2008; Lanier, 2008; Vivier et al, 2008; Long et al., 2013*). The ligands of activating receptors include both pathogen-derived and *induced-self* molecules which are not or poorly expressed by healthy cells and selectively induced or up-regulated in response to multiple cellular stress conditions, such as heat shock, DNA damage, viral infection or neoplastic transformation (*Long and Rajagopalan, 2002; Bryceson et al., 2006*). Natural killing is mediated by several activating receptor families such as the Natural Cytotoxicity Receptors (NCRs) which are Ig-like and type I transmembrane glycoproteins. NCRs include NKp46, which is selectively expressed on NK cells, NKp30 and NKp44, which are expressed also on T cells (*Moretta et al., 2001; Lanier, 2005; Watzl & Long, 2010*). Although the ligands of NCRs are not fully characterized (*Arnon et al., 2001*; *Mandelboim et al., 2001; Moretta et al., 2001; Arnon et al., 2004; Bryceson & Ljunggren, 2008; Bryceson & Long, 2008; Lanier, 2008*) several studies showed that the ligation of NKp46 by influenza virus-encoded hemagglutinin proteins or by not yet defined tumor-induced molecules leads to the killing of infected or neoplastic cells (*Mandelboim et al., 2001; Jarahian et al., 2011; Tassi et al., 2006; Lanier, 2008*). Ligands of NKp30 have been also identified, such as hemagglutinin proteins, BAT3 protein, BAF3 protein which is implicated in the induction of apoptosis following DNA damage, pp65 protein which is produced by human cytomegalovirus (HCMV) and B7–H6 protein which is a B7 receptor family member expressed on tumor cells (*Tassi et al., 2006; Bryceson & Ljunggren, 2008; Lanier, 2008; Brandt et al., 2009; Watzl & Long, 2010*). Finally, it has been demonstrated that NKp44 binds to hemagglutinin proteins and to the proliferating cell nuclear antigen (PCNA) which is generally expressed by tumor cells (*Rosental et al., 2011*). Other activating receptors involved in natural killing are the Ig-like and type I transmembrane glycoproteins 2B4 (CD244) and leukocyte adhesion molecule DNAX accessory molecule-1 (DNAM-1 or CD226) as well as the C-type lectin-like and type II transmembrane glycoprotein Natural-killer group 2 member D (NKG2D or CD314). 2B4, whose expression is not restricted to NK cells, binds to CD48 ligand, a glycoprotein widely expressed on hematopoietic cells and induced by Epstein-Barr virus (EBV)

(*Lanier, 2008*; *Watzl & Long, 2010*). DNAM-1, which is expressed on NK cells, T cells and monocytes, binds to poliovirus receptor (PVR or CD155) or nectin cell adhesion molecule-2 (Nectin2 or CD112) that are expressed on epithelial and endothelial cells and are up-regulated on certain tumors (*Bottino et al., 2003; Watzl & Long, 2010)*. NKG2D, which is expressed on NK cells, γδ T cells and CD8+ T cells, binds to several MHC class I-homologous ligands such as the MHC class I-chain related molecules (MIC)-A and -B and the UL16-binding family proteins (ULBPs) up-regulated on stressed cells (*Cerwenka & Lanier, 2001; Long & Rajagopalan, 2002; Raulet, 2003; Gasser & Raulet, 2006; Watzl & Long, 2010).*

1.4 Molecular signals mediating NK cell activation: ITAM-dependent or independent signaling pathways.

After recognition of IgG-opsonized targets, the low-affinity receptor for IgG FcγRIIIA/CD16 stimulates several NK responses including ADCC, the transcription of genes encoding cytokines and chemokines as well as the surface expression of activation molecules (*Trinchieri, 1989; Trinchieri and Valiante, 1993; Perussia, 1998*). Notably, FcγRIIIA/CD16 crosslinking is able, on its own, to induce degranulation/cytotoxicity as well as production of pro-inflammatory cytokines and chemokines (IFN-γ, TNF-α, IL-6, GM-CSF and CCL5). Based on this feature, FcγRIIIA/CD16 is referred as the prototype of NK activating receptors (*Trinchieri, 1989; Trinchieri & Valiante, 1993; Perussia, 1998; Bryceson et al., 2005; Bryceson et al., 2011; Long et al., 2013*). FcγRIIIA/CD16, in addition to NK cells, is also expressed by monocytes/macrophages (*Pincetic et al., 2014*) and, along with T cell antigen receptor (TCR) as well as B cell antigen receptor (BCR) and other FcRs, belongs to the family of multi-chain immune recognition receptors (MIRRs), which are composed by Ig-like subunits and signal transducing adaptors (*Langlet et., 2000*). In humans, FcγRIIIA/CD16 is expressed as an oligomeric complex consisting of a Fc-binding α chain non-covalently associated with disulfide-linked homodimers or heterodimers of CD3ζ and FcεRIγ signal trasducing adaptors (*Lanier et al., 1991*; *Perussia, 1998; Bryceson et al., 2009; Bryceson et al., 2011*). In NK cells, FcγRIIIA/CD16 exhibits two extracellular Ig domains, a very short cytoplasmic tail and a transmembrane domain that promotes its association with CD3ζ and FcεRIγ adaptor chains (*Lanier, 1991; Letourneur et al., 1991; Hibbs et al., 1994; Tamm & Schmidt, 1996*). The membrane-proximal Ig domain of FcγRIIIA/CD16 is recognized by the monoclonal antibody (mAb) 3G8 while the distal one is recognized by mAb B73.1

(*Perussia et al., 1984; Perussia & Trinchieri, 1984; Tamm & Schmidt,1996; Grier et al., 2012*). The signaling adaptors CD3ζ and FcεRIγ contain in their cytoplasmic domains the *immunoreceptor tyrosine-based activation motifs* (ITAMs) defined by the sequence (D/E)XXYXX(L/I)X6–8YXX(L/I): FcεRIγ has a single ITAM domain, whereas CD3ζ has three ITAMs (*Reth, 1989; Raulet, 2004; Tassi et al., 2006; Bryceson and Long, 2008; Lanier, 2008*). Upon receptor engagement, the ITAM sequences of CD3ζ and FcεRIγ subunits undergo tyrosine phosphorylation by the Src family member lymphocyte-specific protein kinase Lck, whose catalytic activity increases in response to receptor crosslinking. Phosphorylated ITAMs act as docking sites for SH2 domains of spleen tyrosine kinase (Syk) and ζ-chain-associated protein kinase of 70 kDa (ZAP-70) which, upon recruitment and activation, act on several downstream signaling molecules, thus controlling the signal propagation and the development of functional responses (*Leibson, 1997; Brumbaugh et al., 1998*). Subsequent signaling events include the activation of phosphatidyl-inositol-3-OH kinase (PI3K) which in turn mediates the phosphorylation of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate (PIP3), thus promoting the plasma membrane recruitment of several pleckstrin homology (PH) domain containing molecules, such as phospholipase C (PLC)-γ1, PLC- γ2 and Vav1. PLC-γ exerts its function by hydrolyzing membrane PIP2 into inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG), thus mediating the mobilization of intracellular $Ca^{+2}$and the activation of protein kinase C (PKC). Vav proteins are guanine nucleotide exchange factors (GEFs) for the Rho family GTPases critically involved in both actin cytoskeleton reorganization and polarization of the MTOC-associated lytic granules toward cytolytic synapse (*Azzoni et al., 1992*; *Ting et al., 1992; Leibson, 1997; Lou et al., 2001; Jiang et al., 2000/2002; MacFarlane & Campbell, 2006; Bryceson & Long, 2008; Lanier, 2008; Bryceson et al., 2009; Long et al., 2013; Watzl & Long, 2010*).

As previously described, NK cells express other activating receptors which recognize a multitude of ligands expressed on infected and tumor cells including pathogen-derived and *stress/self-induced* molecules (*Long & Rajagopalan, 2002; Long et al., 2013*). As opposed to FcγRIIIA/CD16, these activating receptors, better classified as "*co-activating*" or "*co-stimulating*", are unable on their own to induce NK activation, but can act synergistically leading to signal complementation and triggering functional responses, such as degranulation and cytokine production (Fig. 6) (*Long & Rajagopalan, 2002; Bryceson et al., 2005; Bryceson et al., 2006; Lodoen, 2006; Bryceson & Ljunggren, 2008;*

Lanier, 2008; Vivier et al., 2008; Bryceson et al., 2009; Kim et al., 2010; Kim & Long, 2012; Long et al., 2013).

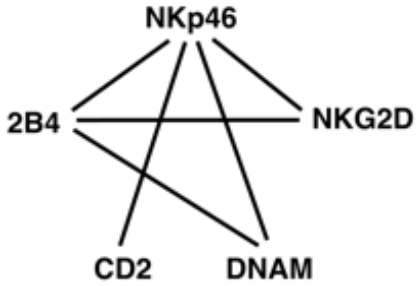


Figure 6. Synergies among co-activating receptors in NK cells. Specific pairwise combinations between NKG2D, 2B4, DNAM-1, NKp46 and CD2 synergize to induce functional responses such as degranulation and cytokine secretion (from *Bryceson et al., Blood 2006*)

Many co-activating NK receptors are multimeric and induce a common signaling pathway, similar to the one triggered by FcγRIIIA/CD16 stimulation, through non-covalently-coupled small adaptor subunits including CD3ζ, FcεRIγ and DNAX activating protein 12 (DAP12) which contain ITAM sequences and act as molecular chaperones essential for the surface expression of their cognate receptors (*Reth, 1989*; *Bryceson & Long, 2008; Lanier, 2008*). These ITAM-dependent receptors include the activating counterparts of KIRs and KLRs as well as NCRs (NKp30, NKp44 and NKp46) (*Kruse et al., 2014*). NCRs are expressed as monomers and contain a positively charged amino acid in their transmembrane domain which, in turn, facilitates the receptor association with the signaling adaptors carrying a negative charge in their transmembrane region as well as extracellular regions consisting of only a few cysteine residues responsible for dimerization (*Bryceson & Long, 2008; Lanier, 2008*; *Watzl & Long, 2010).* NKp30 and NKp46 are coupled with CD3ζ and FcεRIγ chain homodimers or heterodimers, whereas NKp44 associates with the DAP12 homodimer (*Moretta et al., 2001; Lanier, 2003; Bottino et al., 2004; Bryceson et al.,2005; Watzl & Long, 2010).* As described for FcγRIIIA/CD16, NCR engagement results in Src-family kinase-mediated tyrosine phosphorylation of the ITAM sequences. This promotes the recruitment of Syk and ZAP-70 tyrosine kinases which, in turn, act on transmembrane adapter molecules including LAT and NTAL leading to the recruitment, phosphorylation and activation of further downstream signaling complexes such as PI3K, PLC-γ1, PLC-γ 2 and Vav-1,2,3 (*Bryceson & Long, 2008; Lanier, 2008; Watzl & Long, 2010*).

Other co-activating NK receptors induce functional responses through ITAM-independent pathways (Fig. 7) (*Bottino et al., 2003*). This group of receptors includes 2B4, DNAM-1, NKG2D, CD2 and NKp80 (*Bryceson et al., 2005; Bryceson & Ljunggren, 2008*). 2B4 receptor is a member of the signaling lymphocyte activation molecule (SLAM) family (*Claus et al., 2008*). It is expressed as monomer and contains, in its cytoplasmic domain, four *immunoreceptor tyrosine-based switch motifs* (ITSMs). 2B4 has divergent functional properties, and is involved both in NK cell activation and inhibition. In humans, 2B4 acts predominantly as an activating receptor (*Lanier, 2005; MacFarlane & Campbell, 2006; Lanier, 2008; Watzl & Long, 2010*). After receptor crosslinking, the Src-family kinase-mediated ITSM tyrosine phosphorylation induces the recruitment of small SH2-domain containing adaptors, such as the SLAM-associated protein (SAP), which binds to the Src-family tyrosine kinase Fyn that, in turn, can amplify the signal by further phosphorylating 2B4 or act on several downstream effectors (*Latour et al., 2003; Lanier, 2005; McNerney et al., 2005; Tassi et al., 2006; Watzl & Long, 2010)*. 2B4 stimulation induces phosphorylation of LAT, Vav1, PLC-γ, c-Cbl, PI3K and Grb2 *(Trinchieri, 2003; MacFarlane & Campbell, 2006; Tassi et al., 2006; Watzl & Long, 2010).* On the other hand, 2B4 may also suppress NK cell functions *(Sivori et al., 2002; Veillette, 2006; Watzl & Long, 2010).* The inhibitory signaling is promoted by the high expression levels of 2B4, a robust receptor cross-linking and low SAP expression. In this context, phosphorylated 2B4 recruits inhibitory molecules such as the adaptor molecule EAT2, the phosphatases SHP-1, SHP-2 and SHIP as well as the inhibitory kinase Csk (*Chen et al., 2004; Eissmann et al., 2005; Roncagalli et al., 2005; Watzl & Long, 2010*) that compete with SAP for receptor binding. DNAM-1 receptor contains, in its cytoplasmatic domain, three tyrosine residues that can be phosphorylated by Src-family kinases and associates with leukocyte function associated antigen (LFA)-1 integrin (*Shibuya et al., 1998; Shibuya et al., 1999; Bottino et al., 2003; Bryceson et al., 2005; Bryceson & Ljunggren, 2008; Watzl & Long, 2010*). NKG2D receptor is expressed as a disulfide-bonded homodimer and holds an arginine residue in its transmembrane domain which mediates the receptor association with the signaling adaptor DAP10 (*Watzl & Long, 2010*). One homodimer of NKG2D associates with two homodimers of DAP10, thus forming a hexameric complex (*Garrity et al., 2005*). DAP10 contains, in its cytoplasmic domain, the unique tyrosine-based motif YxxM (TyrXXMeth) that undergoes phosphorylation by Src tyrosine kinases and binds either PI3K or the small adaptor molecule Grb2 which is relevant for the recruitment and activation of downstream effector molecules such as Vav1, PLC-γ2 and SH2 domain-

containing leukocyte phosphorylation of 76 kDa (SLP-76). NKG2D signaling is independent of Syk or ZAP70 kinases and does not require the transmembrane adapters LAT and NTAL *(Billadeau et al., 2003; Lanier, 2005; Graham et al., 2006; Upshaw et al., 2006; Watzl & Long, 2010).*

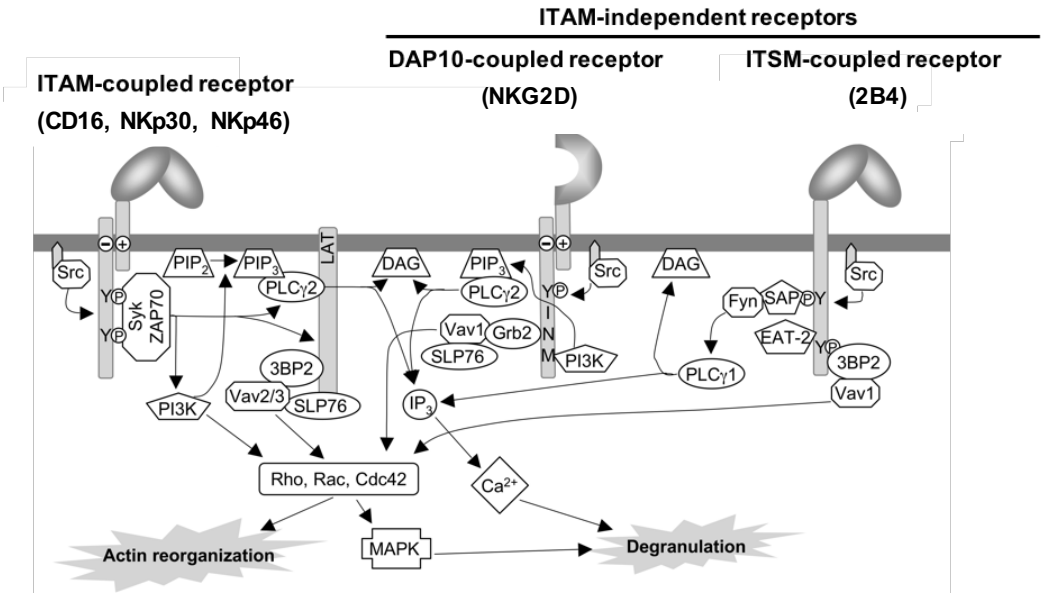


Fig.7 ITAM-dependent or independent signaling pathways (modified from *Watzl & Long, Curr Protoc Immunol 2010*).

1.5 Molecular signals limiting NK cell activation: receptor complex degradation

The extent and duration of activating signals may be counteracted by several mechanisms including the internalization and degradation of engaged receptor complexes and associated protein tyrosine kinases. In this regard, ligand-induced ubiquitin modification of membrane proteins may promote their internalization and delivery to lysosomes or proteasomes for degradation (*Molfetta et al., 2014*). Ubiquitination is a reversible post-translational modification in which the small (*8-kDa*) conserved peptide ubiquitin (Ub) is covalently attached to the ε-amino group of lysine (K) residues of target proteins (*Hochstasser, 1996;* Weissman*, 1997; Ciechanover, 1998; Hershko & Ciechanover, 1998; Laney & Hochstrasser, 1999; Molfetta et al., 2014*). The Ub-conjugating reaction is realized by the combined action of an Ub-activating enzime (E1), an Ub-conjugating enzime (E2) and an Ub protein ligase (E3) (*Molfetta et al., 2014*). Protein acting as E3s include Cbl, which is the product of the protooncogene c-Cbl and a prominent PTK substrate detected in immune receptor-mediated signaling (*Joazeiro et al., 1999; Paolini et al., 2001*). Specifically, ubiquitin modification of target proteins can occur by the

addition of a single molecule of Ub to a single (monoubiquitination) or to different (multiubiquitination) K residues. These modifications regulate several cellular functions including virus budding, nuclear shuttling, transcription and endocytosis (*Mukhopadhyay & Riezman, 2007; Salmena & Pandolfi, 2007: Molfetta et al., 2014*). Moreover, target proteins may also be modified by the addition of polyubiquitin chains (*Pickart & Fushman, 2004; Molfetta et al., 2014*) and different topologies of polyUb chains are associated with diverse biological functions (*Kulathu & Komander, 2012; Molfetta et al., 2014*). Indeed, polyUb chains of at least four Ub molecules linked via K48 direct proteosome degradation of the target protein (*Thrower et al., 2000; Molfetta et al., 2014*) whereas K63-linked chains partecipate in several other cellular processes including DNA damage repair and endocytosis (*Pickart & Fushman, 2004; Woelk et al., 2007; Molfetta et al., 2014*). In regard to ubiquitination as a modification that generates a signal for endocytosis, although monoubiquitination is sufficient for the internalization and endosome-to-lysosome trafficking (*Shih et al., 2000; Haglund et al., 2003; Molfetta et al., 2014*) multiubiquitination and K63-linked polyubiquitination lead to a higher rate of endocytosis/lysosomal transport than monoubiquitination (*Barriere et al., 2006; Lauwers et al., 2009; Haung et al., 2013; Molfetta et al., 2014*). The action of Ub as a signal for delivery along the endosomal compartments, including early endosomes, early intraluminal vesicles (ILVs) of the multivesicular body (MVB) and lysosomes, involves the recognition of ubiquitinated membrane proteins by different endosomal molecular adaptors (*Hicke & Dunn, 2003; Piper & Lehner PJ, 2011; Molfetta et al., 2014*). The best characterized Ub-dependent routes are the clathrin-dependent internalization, which involves several clathrin-binding adaptors that contain Ub-interacting motif (UIM), and the incorporation into ILVs, which fuse with lysosomes, through the action of the endosomal sorting complex required for transport (ESCRT) (*Barriere et al., 2006; Traub & Lukacs GL, 2007; Dikic et al., 2009; Raiborg & Stenmark, 2009; Goh et al., 2010; Bertelsen et al., 2011; Henne et al., 2011; Shields & Piper, 2011; Molfetta et al., 2014).* Among others, Ub act as a sorting signal for several FcRs including the FcγRIIIA/CD16 receptor (*Molfetta et al., 2014*). Indeed, it has been reported that, upon receptor aggregation, the FcγRIIIA/CD16-associated CD3ζ subunit undergoes Ub modification and lysosomal degradation in a tyrosine phophorylation-dependent manner (*Paolini et al., 1999*) and that tyrosine-phophorylated Syk and ZAP-70 kinases may undergo ubiquitin-dependent degradation (*Paolini et al., 2001*).

1.6 "*Adaptive*" or "*memory*" NK cell subpopulation

Deficiency of the transmembrane adaptor protein FcεRIγ, which is important for the signaling transduction of a number of NK cell activating receptors, is the hallmark of the recently described "*adaptive*" or "*memory*" NK subset (*Table 1*). These highly differentiated cells are characterized by a long-term persistence (from 4 months to 1 year) and an enhanced functional responsiveness (*Lee et al., 2015; Schlums et al., 2015; Kovalenko et al., 2017*). The presence of high and variable frequencies of "*memory*" NK cells *in vivo* strikingly correlates with prior exposure to human cytomegalovirus (HCMV) (*Hwang et al., 2012; Zhang et al., 2013*; *Lee et al., 2015, Schlums et al., 2015; Kovalenko et al., 2017*) a common herpesvirus that establishes a life-long latent infection in the majority of human population (*Dowd et al., 2009*). Moreover, such NK subset further expands in individuals experiencing HCMV reactivation including Hematopoietic Cell Transplant (HCT) recipient patients as well as in HCMV-seropositive individuals secondary infected by certain other viruses including hantavirus, HIV-1 and EBV (*Lee et al., 2015; Kovalenko et al., 2017*). It has been also described that "*memory*" NK cells significantly and preferentially expanded *in vitro* upon encounter with HCMV or influenza virus-infected cells only in the presence of virus-specific antibodies (*Lee et al., 2015; Schlums et al., 2015*). The adaptive features along with the loss of FcεRIγ chain of "*memory*" NK cells are acquired and maintained by means of selected stable molecular changes of the transcriptional programs (*Lee et al., 2015; Schlums et al., 2015; Kovalenko et al., 2017*). Besides FcεRIγ chain, other B cell- and myeloid-related proteins may be silenced (*Lee et al., 2015; Schlums et al., 2015; Kovalenko et al., 2017*). The lack of these molecules, which is variegated and stable over time, correlates with epigenetic modifications resulting in DNA hyper-methylation (*Lee et al., 2015; Schlums et al., 2015*). In this regard, it has been described that "*memory*" NK cells are endowed with a specific epigenetic signature and a global DNA methylation pattern which strongly diverges from that of "*conventional*" NK cells being strikingly similar to that of cytotoxic effector T cells (*Schlums et al., 2015*) and which is controlled by transcription factors belonging to the BTB-zinc finger (BTB-ZF) family (*Mathew et al., 2012; Puszyk et al., 2013*) such as ZBTB16 (PLZF) that is transcriptionally down-regulated in "*memory*" NK cells (*Oikawa et al., 2008; Beaulieu et al., 2014; Schlums et al., 2015*). PLZF binds to the promoter sequences of genes encoding for surface receptors and signaling molecules and recruits chromatin-re-modelling co-factors in

order to regulate gene expression (*Lee & Maeda, 2012; Mathew et al., 2012*). Thus, the lack of PLZF results in impaired expression of a group of genes including those encoding for FcεRIγ, Syk and EAT-2 as well as IL-12 (IL12RB2) and IL-18 (IL18RAP) receptor subunits (*Gleimer et al., 2012; Mathew et al., 2012; Schlums et al., 2015*). Moreover, it has been reported that "*memory*" NK cells may be deficient for the zing finger transcription factor HELIOS as well as for the adaptor molecules DAB2 (*Lee et al., 2015; Schlums et al., 2015; Kovalenko et al., 2017*). On the other hand, they express normal levels of the T-box transcription factors T-bet and Eomesodermin (EOMES) and of the other Syk family kinase member ZAP-70 as well as of the T cell-related signaling molecules CD3ζ and SAP (*Hwang et al., 2012; Zhang et al., 2013*; *Lee et al., 2015; Schlums et al., 2015; Zhou et al., 2015; Kovalenko et al., 2017*). From literature it is well-known that "*memory*" NK cells significantly differ from "*conventional*" ones for their receptor repertoire and share many phenotypically characteristics with terminally differentiated NK cells. In fact, they typically lose the expression of NKG2A inhibitory receptor, exhibit increased levels of NKG2C activating receptor and express the maturation marker CD57 as well as the inhibitory KIR2DL1/2/3 or KIR3DL1 and the activating KIR2DS1/2/4 (*Hwang et al., 2012; Zhang et al., 2013*; *Lee et al., 2015; Schlums et al., 2015; Zhou et al., 2015; Kovalenko et al., 2017*). However, unlike mature NK cells, the "*memory*" subset may not lose CD62L expression, a marker that defines poly-functional and less mature NK cells (*Zhou et al., 2015*). Regarding the receptor repertoire, "*memory*" NK cells express reduced levels of FcγRIIIA/CD16 and reduced amounts of the natural cytotoxicity receptors NKp30 and NKp46 (*Hwang et al., 2012; Lee et al., 2015; Schlums et al., 2015; Zhou et al., 2015; Kovalenko et al., 2017*) as well as of the lymphocyte activation marker CD38 (*Zhou et al., 2015*) a NADP glycol hydrolase associated with FcεRIγ and CD3ζ (M*allone et al., 2001; Malavasi et al., 2008*). Conversely, they exhibit normal levels of the FcεRIγ-independent activating receptors such as NKG2D, 2B4, DNAM-1, NTB-A and CRACC and do not express markers that are up-regulated on activated NK cells including CD69, CD25 and NKp44 receptors (*Hwang et al., 2012*). In addition to the phenotypic characteristics, from literature it is also well-known that "*memory*" NK cells significantly differ from "*conventional*" ones for their turnover rates and functional capabilities (*Lee et al., 2015; Schlums et al., 2015; Kovalenko et al., 2017*). In fact, they fail to respond to inflammatory cytokines produced by innate immune cells, such as IL-12 and IL-18, show a poorer reactivity

toward tumor targets in terms of both IFN-γ production and degranulation and may lose the ability to recognize and kill activated immune cells (*Hwang et al., 2012; Schlums et al., 2015*). In fact, they display significantly reduced degranulation in response to activated T cells (*Schlums et al., 2015*). However, "*memory*" NK cells may exhibit enhanced functional activity in response to FcγRIIIA/CD16 stimulation especially in terms of IFN-γ and TNF-α production (*Hwang et al., 2012; Zhang et al., 2013; Lee et al., 2015; Zhou et al., 2015; Kovalenko et al., 2017*) due to hypo-methylation of IFNG and TNF regulatory regions (*Schlums et al., 2015).*

SURFACE	• mature ($CD56^{dim}$ $CD57^{+}$ $NKG2A^{-}$) • $NKG2C^{high}$ • $KIRs^{+}$ • $CD62L^{+}$ • ↓ CD16 and NCRs (NKp30 and NKp46) • normal NKG2D, 2B4, DNAM-1, NTB-A and CRACC • no CD69, CD25 and NKp44
INTRACELLULAR ADAPTORS	• FcεRIγ- • ↓ SYK and EAT-2 • ↓ DAB2 • $ZAP70^{+}$ • normal CD3ζ and SAP
TRANSCRIPTION FACTORS	• $PLZF^{-}$ • $HELIOS^{-}$ • normal Tbet and EOMES
DIFFERENTIATION REQUIREMENTS	• HCMV-driven, no specific antigen • CD16 stimulation • ? inflammatory cytokines
EPIGENETIC CHANGES	• FCER1G (FcεRIγ), SYK, SH2D1B (EAT-2), IL12RB2 and IL18RAP • IFNG and TNF
FUNCTION	• defective response to inflammatory cytokines (IL-12 and IL-18) • poor reactivity toward tumor targets • reduced responsivity to activated immune cells • enhanced functional activity in response to CD16 expecially in terms of IFN-γ and TNF-α production
IN VIVO EXPANSION	• HCMV and other viral infections
IN VITRO EXPANSION	• HCMV or influenza virus-infected cells plus virus-specific Abs

Table 1. Features of "adaptive" or "memory" NK cells (modified from *Fehniger & Cooper, Trends Immunol. 2016*)

2. Anti-CD20 therapeutic monoclonal antibodies

2.1 Mechanism of action of anti-CD20 mAbs

Tumor targeting therapeutic anti-CD20 monoclonal antibodies (mAbs) have revolutionized the treatment of B cell malignancies and autoimmune diseases *(Lim et al., 2010; Weiner et al., 2010; Maloney, 2012*). Anti-CD20-based immunotherapy mediates B cell depletion by targeting CD20 molecule which is a B-cell-specific differentiation antigen expressed on the surface of human mature B cells and in most B cell-lymphomas, but not on early B-cell progenitors or later mature plasma cells (*Tedder et al., 1988; Lenz & Straudt, 2010; van Meerten & Hagenbeekb, 2010; Maloney, 2012*). The CD20 antigen is a non-glycosylated protein (*Gagez & Cartron, 2014; Goede et al., 2015*) with four membrane-spanning hydrophobic domains, two extracellular loops bearing the epitopes recognized by anti-CD20 mAbs and the amino- and the carboxy-terminal domains located within the cytoplasm. Following anti-CD20 mediated aggregation, such cytoplasmatic domains undergo phosphorylation and transduce biochemical signals (*Gagez & Cartron, 2014*). CD20 antigen is organized into complexes comprising dimers or tetramers (*Bubien et al., 1993; Gagez & Cartron, 2014*) closely associated with other proteins, such as Csk-binding protein (Cbp), CD40 and MHC class II (*Gagez & Cartron, 2014*). Anti-CD20 mAbs mediate B cell depletion through different mechanisms (Fig. 8) including 1. Fc-Fcγ receptor (FcγR)-based mechanisms represented by ADCC and antibody-dependent cellular phagocytosis (ADCP) triggered by FcγRs expressed on monocyte/macrophages, neutrophils as well as NK cells (*Cartron et al., 2002; Lefebvre et al., 2006; Jaglowski et al., 2010; Beers et al., 2010; Lim et al., 2010; Taylor & Lindorfer, 2010; Montalvao et al., 2013; Taylor & Lindorfer, 2014*) 2. complement-dependent cytotoxicity (CDC) involving the activation of the classical cascade via C1q component likely as a result of antibody organization into order hexamers, deposition of C3b fragments and generation of the membrane attack complex (MAC) (*Walport, 2001; Gelderman et al., 2004; Wang et al., 2008; Beers et al., 2010; Lim et al., 2010; Taylor & Lindorfer, 2010; Maloney, 2012; Cook et al., 2016*) 3. direct programmed B cell death (PCD) via apoptotic or not apoptotic mechanisms (*Beers et al., 2010; Lim et al., 2010; Taylor & Lindorfer, 2010; van Meerten & Hagenbeek, 2010; Maloney, 2012; Illidge et al., 2014*)

Notably, the relative contribution of each effector mechanism remains controversial. Indeed, while it is widely accepted that Fc-FcγR interactions are critical for their *in vivo* anti-tumor efficacy (*Nimmerjahn & Ravetch, 2007; Lim et al., 2010*), the role of CDC and direct PCD is still disputed (*Cragg et al., 2005; Glennie et al., 2007; Lim et al., 2010*).

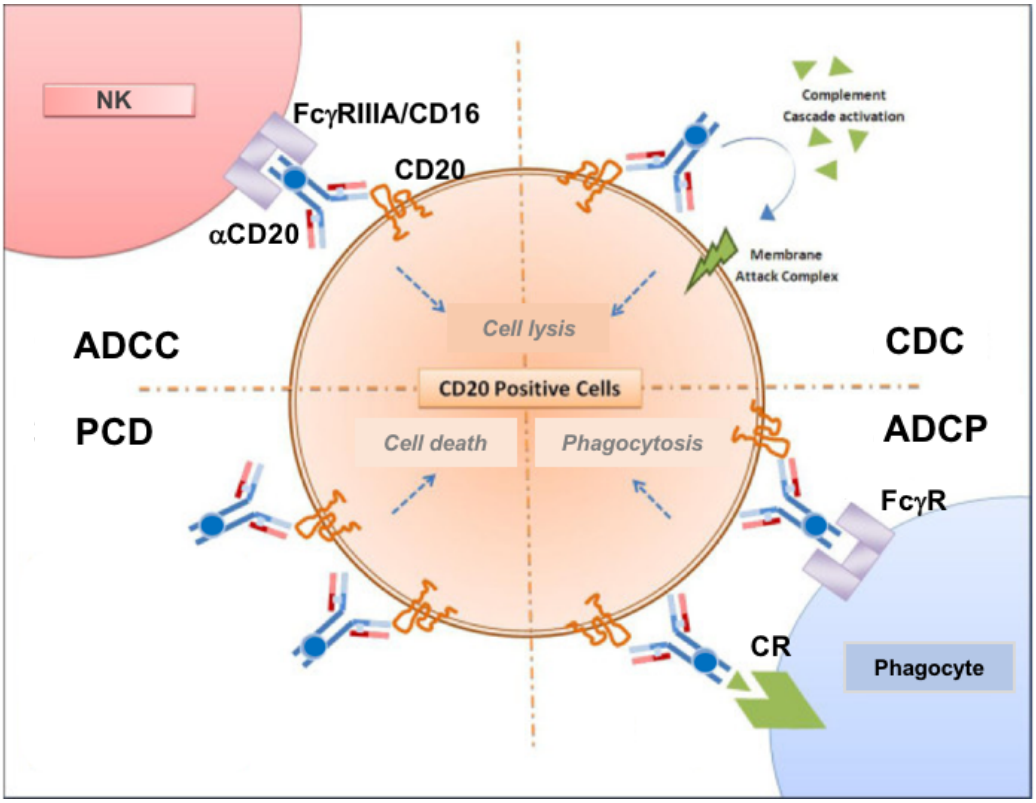


Figure 8. Anti-tumor mechanisms of anti-CD20 mAbs. Anti-CD20 mAbs mediate B cell depletion through different mechanisms of cellular cytotoxicity. CD20 aggregation induced by therapeutic mAbs may induce complement-dependent cytotoxicity (CDC) by the activation of the classical complement pathway leading to the generation of the membrane attack complex (MAC) that mediates B-cell lysis. Besides CDC anti-CD20 mAbs can recruit $Fc\gamma R^+$ immune effector cells such as NK cells, monocytes/macrophages and neutrophils to deplete $CD20^+$ target cells through antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated phagocytosis (ADCP). The interaction with NK cells via FcγRIIIA/CD16 stimulates ADCC. The recognition of the Fc portion of anti-CD20 mAbs as well as the deposited complement fragments by both FcRs and complement receptors on monocytes/macrophages and neutrophils induces both ADCC and ADCP. The CD20 crosslinking by anti-CD20 mAbs may have direct anti-proliferative effects or may induce direct programmed B cell death (PCD) via apoptotic or not apoptotic pathways (modified from *Kasi et al., Critical Care 2012*).

2.2 Type I and II anti-CD20 mAbs

Anti-CD20 mAbs can be classified as "Type I" and "Type II" on the basis of the antigen-binding characteristics and the primary mode of action employed (Fig. 9) (*Chan et al., 2003; Cragg et al., 2003; Cragg et al., 2004; Glennie et al., 2007; Beers et al., 2008; Niederfellner et al., 2011; Klein et al., 2013*). Type I anti-CD20 mAbs bind two CD20 molecules belonging to different CD20 tetramers (*Klein et al., 2013; Gagez & Cartron, 2014; Illidge et al., 2014; Goede et al., 2015*). Such inter-tetrameric binding to CD20 induces a rapid migration of mAb/CD20 complexes into lipid rafts, which are plasma membrane micro domains enriched in cholesterol and sphingolipids and represent a platform for signal transduction allowing the co-localization of receptors and signalling effector molecules (*Cragg et al., 2003; Cragg et al., 2004; Klein et al., 2013; Gagez & Cartron, 2014; Illidge et al., 2015*). The association between CD20 and lipid rafts is dependent on cholesterol and on a short membrane-proximal cytoplasmic domain of CD20 molecule (*Polyak et al., 1998; Gagez & Cartron, 2014*). The translocation of mAb/CD20 complexes into lipid rafts leads to strong CDC and PCD because it allows not only the clustering of the antibody Fc portions resulting in C1q protein recruitment and complement deposition, but also the activation of CD20 calcium channels leading to caspase activation and apoptosis (*Deans et al., 2002; Cragg et al., 2003; Beers et al., 2010; van Marteen & Hagenbeek, 2010; Maloney, 2012*). Conversely, type II anti-CD20 mAbs bind two CD20 molecules within the same tetramer (*Klein et al., 2013; Gagez & Cartron, 2014; Illidge et al., 2014; Goede et al., 2015*). Such intra-tetrameric binding to CD20 does not localize mAb/CD20 complexes into lipid rafts and induce weak or no CDC, but potent PCD because it favours the homotypic B cell adhesion (*Chan et al. 2003; Cragg et al., 2003; Ivanov et al., 2009; van Meerten & Hagenbeek, 2010; Niederfellner et al., 2011; Maloney, 2012; Klein et al., 2013)* that in turn is associated with peripheral re-localization of the cellular actin, the polarization of mitochondria toward the cell-to-cell contact area, the release of lysosomal cathepsin in cytoplasm and in surrounding enviroment and the extra-mitochondrial generation of reactive oxigen species via the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, thus resulting in cell death (*Ivanov et al., 2009; Alduaij & Illidge, 2011; Alduaij et al., 2011; Dalle et al., 2011; Jak et al., 2011; Honeychurch et al., 2012*).

As a result of the different binding topology, type I and type II anti-CD20 mAbs bind to CD20 tetramers in a 2:1 ratio (*Klein et al., 2013; Gagez & Cartron, 2014; Illidge et al., 2014*). Furthermore, type I and type II anti-CD20 mAbs share the same ability to induce ADCC and ADCP by recruiting FcγR^{+} effector cells such as monocytes/macrophages, neutrophils and NK cells (*Cartron et al., 2004; Gagez & Cartron, 2014; Illidge et al., 2015*). Indeed, these properties are related to the Fc portion which does not differ between type I and II mAbs (*Gagez & Cartron, 2014*). Notably, rituximab, ofatumumab, ublituximab, veltuzumab, ocrelizumab, ocaratuzumab, PRO131921 belong to the group of type I anti-CD20 mAbs (*Teeling et al., 2006; Goldenberg et al., 2009; Tobinai et al., 2011; Wierda et al., 2011; Forero-Torres et al., 2012; Gagez & Cartron, 2014; Le Garff-Tavernier et al. 2014*) while tositumumab and obinutuzumab are type II anti-CD20 mAbs (*Zelenetz, 2003; Robak, 2009; Mossner et., 2010; Gagez & Cartron, 2014; Goede et al., 2015; Illidge et al., 2015*).

Type I mAbs	Type II mAbs
CD20 inter-tetrameric binding	CD20 intra-tetrameric binding
CD20 traslocation into lipid rafts	No CD20 traslocation into lipid rafts
Strong CDC	No or weak CDC
No homotypic adhesion Caspases-dependent cell death	Homotypic adhesion Caspases-independent Lysosomes-mediated ROS-dependent cell death
Maximal CD20 binding	Half-maximal CD20 binding
CD20 modulation	No CD20 modulation
ADCC and ADCP	ADCC and ADCP

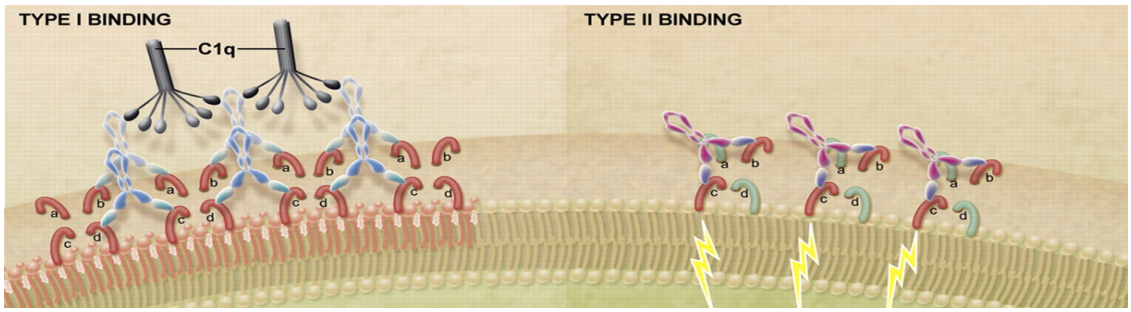


Figure 9. Type I and II anti-CD20 mAbs. Type I mAbs bind two CD20 molecules belonging to different tetramers. Their *inter-tetrameric binding* to CD20 induces rapid traslocation of the mAb/CD20 complex into lipid rafts leading to strong CDC but only weak apoptotic PCD. Type II mAbs bind two CD20 molecules within the same tetramer. Their *intra-tetrameric binding* to CD20 does not localize mAb/CD20 complexes into lipid rafts inducing weak or no CDC but potent non-apoptotic PCD (modified from *Gagez & Cartron, Curr Opin Oncol 2014 and Cragg, Blood 2011).*

2.3 Rituximab and resistance mechanisms

The anti-CD20 mAb of 1st generation rituximab (MABTHERA; RITUXAN) was the first approved in clinical practice and it has substantially improved the clinical outcomes of patients with a broad range of B cell malignancies (*Beers et al., 2010; Lim et al., 2010; van Meerten & Hagenbeek, 2010; Weiner et al., 2010; Maloney et al., 2012*) as well as autoimmune disorders (*Eisenberg & Albert, 2005; Sabahi & Anolik, 2006; Taylor & Lindorfer, 2007*). The use of rituximab, as single agent or in combination with standard chemotherapy/radiotherapy regimens, represents the standard of care in a variety of B-cell malignancies including B cell chronic lymphocytic leukemia (CLL) and non-Hodgkin's lymphomas (NHL), such as diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL) and mantle cell lymphoma (MCL) (*Coiffier et al., 2002; Hiddemann et al., 2005; Pfreundschuh et al., 2006; Glennie et al., 2007; Schulz et al., 2007; Cheson & Leonard, 2008; Marcus et al., 2008; Beers et al., 2010; Gao et al., 2010; Hallek et al., 2010; Lim et al., 2010; van Meerten & Hagenbeek, 2010; Pfreundschuh et al., 2011; Maloney et al., 2012*). Rituximab is a chimeric IgG1κ mAb with murine light- and heavy-chain variable and human constant regions (*Reff et al., 1994; van Meerten & Hagenbeek, 2010).* The infusion of rituximab, by promoting a rapid opsonization of circulating B cells, results in depletion of B cells in peripheral blood, lymph nodes and bone marrow (*Reff et al., 1994; Kennedy et al., 2003; Uchida et al., 2004; Hamaguchi et al., 2006*) and may cause immunodeficiency (*Maloney, 2012*). Infectious complications, such as bacterial, viral and fungal infections, are indeed reported in some patients who are treated with chemoimmunotherapy (*Kimby, 2005, Lau, 2008; Carson et al., 2009*). FcγRIIIA/CD16 receptor plays a relevant role in the anti-tumor activity of rituximab (*Cartron et al 2002; Montalvao et al., 2013; Gagez & Cartron, 2014; Illidge et al., 2014; Taylor & Lindorfer, 2014; Illidge et al., 2015*). In fact, it has been demonstrated that rituximab-based therapy is ineffective in mice devoid of FcRγ chain (*Clynes et al., 1998*) and may be affected by the FCGR3A-V158F single nucleotide polymorphism (*see above*). In this regards, it has been reported that this polymorphism is clinically relevant for rituximab monotherapy in patients with low tumor burden FL. Indeed, the homozygous FcγRIIIA-158V/V patients displayed a better clinical response rate than the low-affinity FcγRIIIA-158F carriers (*Cartron et al., 2002; Weng & Levy, 2003; Weiner et al., 2010;Persky et al., 2012; Gagez & Cartron, 2014; Illidge et al., 2014; Illidge et al., 2015)* likely due to an higher affinity for rituximab as well as an increased antibody-induced FcγRIIIA-bearing cell activation (*Dall'Ozzo et al., 2004; Bowles & Weiner, 2005; Hatjiharissi et al., 2007;Veeramani et*

al., 2011). However, the prognostic significance of the FCGR3A-V158F polymorphism is less clear for rituximab given in combination with chemotherapy in NHLs (*Kim et al., 2006; Carlotti et al., 2007; Mitrovic et al., 2007; Zhang et al., 2010; Ahlgrimm et al., 2011; Fabisiewicz et al., 2011; Prochazka et al., 2011; Ghesquieres et al., 2012; Persky et al., 2012; Varoczy et al., 2012; Illidge et al., 2014; Illidge et al., 2015*) and has not observed in CLL for rituximab monotherapy or immuno-chemotherapy (*Farag et al., 2004; Dornan et al., 2010; Jaglowski et al., 2010; Illidge et al., 2014; Illidge et al., 2015*). Despite the clinical success of rituximab therapy, after a prologed treatment, a relevant fraction of patients develops resistance thus leading to treatment failuire or relapse (*Davis et al., 2000; Hainsworth et al., 2000; Colombat et al., 2001; Maloney, 2005; Martin et al., 2008; Taylor & Lindorfer, 2010; Cartron et al., 2011; Illidge et al., 2014; Goede et al., 2015; Illidge et al., 2015*). Several and currently poorly understood are the mechanisms of resistance to rituximab therapy (*Table 2*) (*Bonavida, 2007; Hiraga et al., 2009; Taylor & Lindorfer, 2010; Alduaij et al., 2011; Mishima et al., 2011; Beurskens et al., 2012; Small et al., 2013; Illidge et al., 2014; Illidge et al., 2015).*

LOSS OF EPITOPE	• **CD20 gene mutation** • **low membrane expression of CD20** • **mAb-mediated CD20 surface down-modulation (e.i internalization/degradation or shaving reaction)**
REDUCED ADCC	• **saturation/exhaustion of ADCC** • **C3b deposition on B cells** • **polymorphisms in effector molecules (e.i. FcγR)** • **FcγR surface down-modulation** • **FcγRIIIA/CD16-driven inhibitory signals (i.e. activation of SHP-1 tyrosine phosphatase)**
REDUCED ADCP	• **saturation/exhaustion of ADCP** • **polymorphisms in effector molecules (e.i. FcgR)** • **FcγR surface down-modulation**
REDUCED CDC	• **saturation/exhaustion of CDC (i.e. consumption of complement components)** • **increased surface expression and activity of complement inhibitory proteins (CRPs) on B cells**
REDUCED PCD	• **increased levels of anti-apoptotic proteins in B cells**

Table 2. Mechanisms of resistance to rituximab therapy (modified from *Taylor & Lindorfer, Semin Hematol. 2010***)**

Tumor cell resistance to rituximab is multifactorial and may be host- or tumor-related (*Taylor & Lindorfer, 2010*). Host-related mechanisms such as the saturation or exhaustion of effector mechanisms responsible for the depletion of anti-CD20-coated targets may reduce, expecially in high tumor burden conditions, therapeutic efficacy by impairing complement activation, macrophage-mediated phagocytosis and NK-mediated ADCC (*Kennedy et al., 2004; Glennie et al., 2007; Taylor & Lindorfer, 2008; Taylor & Lindorfer, 2010; van Meerten and Hagenbeek, 2010*). Defective ADCC may be related to down-modulation of FcγRIIIA/CD16 receptor upon recognition of rituximab-opsonized targets (*Bowles et al., 2006; Taylor & Lindorfer, 2008; Taylor & Lindorfer, 2010; van Meerten & Hagenbeekb, 2010; Veeramani et al., 2011; Zent et al., 2014*) thus impairing the killing of tumor cells until FcγRIIIA/CD16 is re-expressed (*Taylor & Lindorfer, 2010*). Reduced ADCC may be also the conseguence of C3b deposition on opsonized B cells: the binding of C3q to an epitope in the Fc portion which is proximal to that recognized by FcγRIIIA/CD16 may prevent rituximab-induced receptor aggregation (*Wang et al., 2008; Wang et al., 2009; van Meerten & Hagenbeekb, 2010*). Recently, a different mechanism responsible for the exhaustion of NK cytolytic potential has been also described by our group. In particular, the sustained stimulation of FcγRIIIA/CD16 by means of rituximab-coated tumor B cells impairs the cytolytic pontential of NK cells by promoting the long-lasting recruitment of the tyrosine phosphatase SHP-1 to the aggregated receptor complex. Such event leads to the cross-tolerance of several un-releated activating receptors through the de-phosphorylation of signaling molecules critically required for the NK-mediated cytotoxicity such as SLP-76, PLC-γ2 and Vav1 (*Capuano et al., 2015*). Moreover, clinical efficacy of rituximab may be also restricted by the consumption of complement system (*Kennedy et al., 2004*) as well as by polymorphisms in FCGR3A gene encoding for FcγRIIIA/CD16 receptor (*Vance et al., 1993; de Haas et al., 1996; Koene et al., 1997; Wu et al., 1997; Cartron et al., 2002; Hatjiharissi et al., 2007*). Tumor-related resistance mechanisms comprise CD20 gene mutation, low membrane expression of CD20 (*Kennedy et al., 2004; Beum et al., 2006; van Meerten et al., 2006; Beum et al., 2008*) as well as the enhanced surface expression and activity of complement inhibitory proteins (CRPs) such as CD55 and CD59 or of anti-apoptotic proteins in circulating malignant B cells (*Golay et al., 2000; Treon et al., 2001; Kennedy et al., 2003; Czuczman et al., 2008*). The down-regulation of CD20 surface expression, which differs across different tumors, is implicated in imparing the therapeutic efficacy by reducing the antibody *half-life* and

the immune effector cell recruitment and activation (*Beers et al., 2010; Lim et al., 2011; Gagez & Cartron, 2014; Illidge et al., 2014; Goede et al., 2015; Illidge et al., 2015*).
The removal of rituximab/CD20 complexes from B cells in the "*shaving reaction"* may contribute to the loss of CD20 (*Beum et al., 2006; Beum et al., 2008; Taylor & Lindorfer, 2010*). In this antigenic modulation modality Rituximab/CD20 complexes are transferred from opsonized B cells to monocytes/macrophages, which express the high affinity FcγRI, and internalized (*Hudrisier et al., 2007; Beum et al., 2008; Taylor & Lindorfer, 2010; Beum et al., 2011*). Antigenic modulation may be also due to internalization of mAb-CD20 complexes by tumor cells (*Beers et al., 2010*). In order to overcome the resistance mechanisms it has been proposed the combination of rituximab with agents potentiating the NK cell-mediated ADCC including 1. anti-KIR antagonist mAbs, which block inhibitory KIRs (*Kohrt et al., 2014*) 2. immunoregulatory drugs such as lenalidomide and pomalidomide which increase granzyme B and FasL expression (*Wu et al., 2008*) and 3. anti-CD137 agonistic mAbs which up-regulate CD137 surface expression (*Kohrt et al., 2011*). At the same time new different anti-CD20 mAbs have been developed (Fig.10) (*Lim et al., 2010; Taylor & Lindorfer, 2010; van Meerten & Hagenbeek, 2010; Gagez & Cartron, 2015; Illidge et al., 2015*). Such antibodies are endowed with improved properties and enhanced or novel effector mechanisms and are now approching the clinical use as potential competitors of rituximab in first-line therapies or for use in rituximab-refractory diseases (*Teeling et al., 2004; Bowles et al., 2006; Lim et al., 2010; Taylor & Lindorfer, 2010; van Meerten & Hagenbeek, 2010; Bologna et al., 2011; Klein et al., 2013; Gagez & Cartron, 2015*).

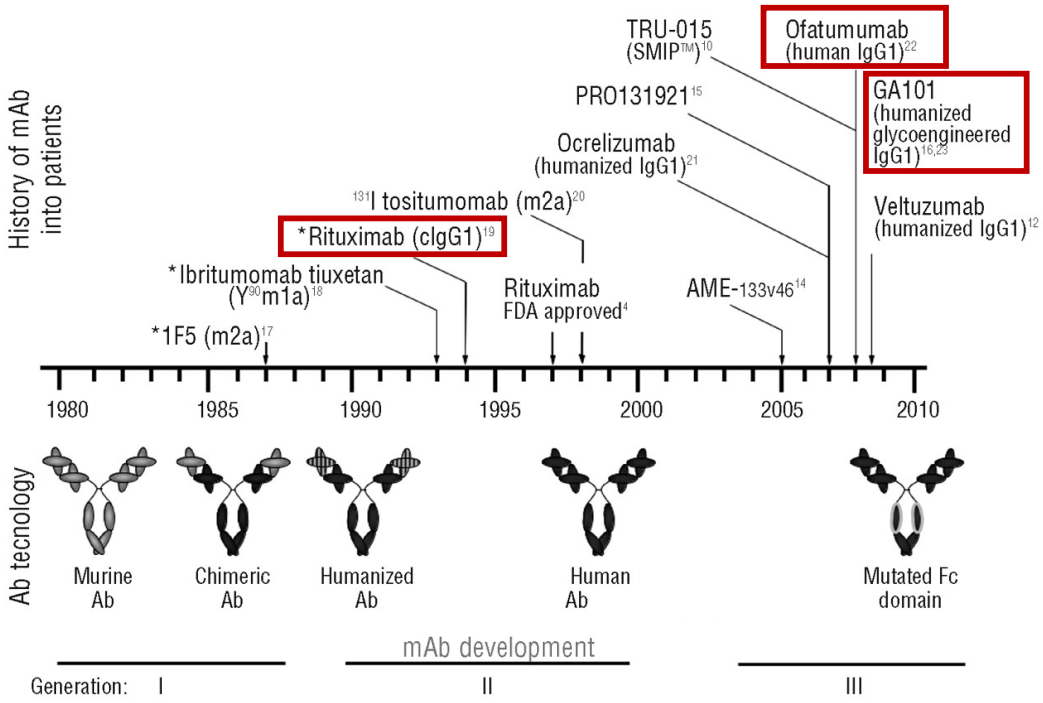


Figure 10. Hystory of anti-CD20 mAbs. The time-line summarizes the chronological introduction over the last three decades of anti-CD20 mAbs in human trials and the corresponding progress in Ab technology from 1st through to 3rd generation reagents: 1st generation mAbs are murine or human/mouse chimeric, 2nd generation mAbs are either humanized or fully human and 3rd generation mAbs have further modifications to the Ab structure e.g. mutation or defucosylation of the Fc domain for enhanced FcR binding profiles (modified from *Lim et al., Hematologica 2010*).

The development of these next-generation anti-CD20 mAbs, classified as 2nd or 3rd generation, has been focused on structural modifications promoting specific cytotoxic mechanisms (*Illidge et al., 2014*). With respect to rituximab, they bind different CD20 epitopes (Fig. 11) and are more efficient in inducing specific effector functions (*Taylor & Lindorfer, 2008; van Meerten & Hagenbeekb, 2010*).

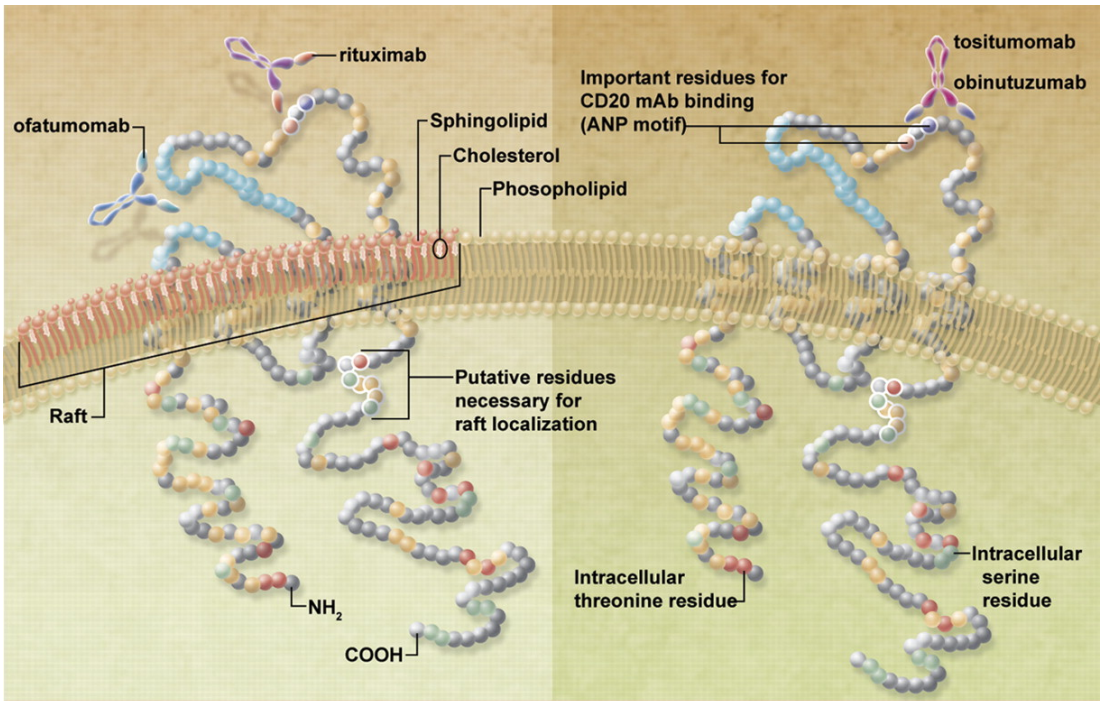


Figure 11. Binding sites of anti-CD20 mAbs. Anti-CD20 mAbs bind to the extracellular domain of CD20 molcule. Most of anti-CD20 mAbs, including rituximab and obinutuzumab, bind to the larger (*large 44-mer*) CD20 extracellular loop around the ANP (alanine – N – proline) motif. By contrast, ofatumumab recognized an epitope including both the smaller (*small 7-mer*) CD20 extracellular loop and the N-terminal region of the larger one (modified from *Cragg, Blood 2011*).

Anti-CD20 mAbs of 2nd generation include humanized or fully human antibodies (*Taylor & Lindorfer, 2008; Van Meerten & Hagenbeekb, 2010*) such as ofatumumab (ARZERRA) which is a IgG1κ type I anti-CD20 mAb approved for the treatment of relapsed CLL in fludarabine- and alemtuzumab-refractory patients (*O' Brien & Osterborg, 2010*). *In vitro* and *in vivo* studies demonstrated that ofatumumab can be successfully used in rituximab-resistant tumors as well as in CLL with low membrane expression of CD20 (*Teeling et al., 2004; Teeling et al., 2006; Coiffier et al., 2008; Hagenbeek et al., 2008; Beers et al., 2010; Jaglowski et al., 2010; van Meerten & Hagenbeekb, 2010*). Unlike rituximab, ofatumumab binds, with high affinity, to a CD20 epitope ("*small 7-mer loop*") which is closer to the plasma membrane, thus showing a greater ability to bind C1q and to induce CDC (*Teeling et al., 2004; Teeling et al., 2006; Beers et al., 2010; Lim et al., 2010; van Meerten & Hagenbeekb, 2010; Wierda et al., 2011; Maloney, 2012*).

2.4 Third generation of anti-CD20 mAbs: obinutuzumab

Anti-CD20 mAbs of 3rd generation are humanized mAbs with a modified Fc region (*Taylor & Lindorfer, 2008; van Meerten & Hagenbeekb, 2010*). Fc modification strategies include Fc-mutation (*Shields et al., 2001; Gagez & Cartron, 2014*) and Fc-glycoengineering (*Shields et al., 2002; Jefferis, 2009; Gagez & Cartron, 2014; Illidge et al., 2014; Illidge et al., 2015*) and lead to the improvement of ADCC and ADCP by enhancing the binding affinity between the Fc portion of the therapeutic antibody and the FcγRIIIA/B expressed by immune effector cells (*Gagez & Cartron, 2014; Goede et al., 2015; Illidge et al., 2015*). Fc-mutation results in the substitution of Fc amino acid residues involved in Fc-FcγR interaction while Fc- glycoengineering modifies the composition of the two oligosaccharides attached to asparagine (Asn) 297 residues in the CH2 domains of Fc portion (*Shields et al., 2001; Shields et al., 2002; Jefferis, 2009; Gagez & Cartron, 2014; Illidge et al., 2014; Illidge et al., 2015*). The Fc-modified anti-CD20 mAbs tested in clinical trials include the Fc-mutant ocaratuzumab (*Forero-Terres et al., 2012; Gagez & Cartron, 2014*) as well as the Fc-glycoengineered ublituximab (*Cazin et al., 2011; Gagez & Cartron, 2014*) and obinutuzumab (*Mossner et al., 2010; Gagez & Cartron, 2014*). Obinutuzumab (GA101; GAZYVA; GAZYVARO) is a humanized Fc-glycoengineered type II anti-CD20 mAb (IgG1κ isotype) of 3rd generation, developed in an attempt to enhance both the immune effector cell-mediated and the direct killing of cancer cells (*Mossner et al., 2010; Gagez & Cartron, 2014; Illidge et al., 2014; Klein et al., 2014; Goede et al 2015).* Obinutuzumab was derived by humanization of the murine B-Ly1 anti-CD20 mAb and was glycoengineered by defucosylation (*Mossner et al., 2010; Illidge et al., 2014; Klein et al., 2014; Goede et al 2015).* Core fucose residues were removed from the two oligosaccharides attached to *Asn* 297 residues in the CH2 domains of Fc portion thus resulting in a lower amount of fucose than conventional IgG (*Mossner et al., 2010; Illidge et al., 2014; Illidge et al., 2015*). In particular, the addition of fucose to the Fc region carbohydrates was sterically prevented, in the antibody-producing cells, by the insertion of extra N-acetylglucosamine (NAG) groups thus enabling the generation of an antibody glycovariant lacking fucosylation (Fig. 12) (*Mossner et al., 2010; Klein et al., 2014; Goede et al 2015).*

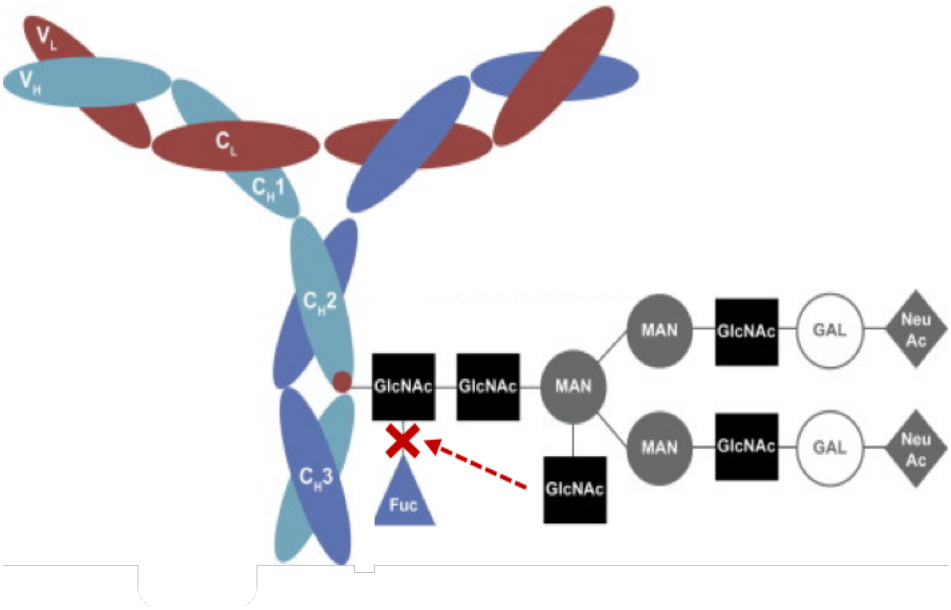


Figure 12. Fc-glycoengineered structure of obinutuzumab. The insertion of extra N-acetylglucosamine (NAG) groups strerically prevents the addition of fucose to the oligosaccharides attached to asparagine (Asn) 297 residues in the CH2 domains of Fc portion (modified from *Illidge et al., Cancer Treat Rev 2015*).

As defucosylated molecule, obinutuzumab exhibits an increased binding affinity for both FcγRIIIA, irrespective to the allotype V or F, and FcγRIIIB, thus improving ADCC and ADCP mediated by monocytes/macrophages and NK cells through FcγRIIIA as well as by neutrophils through FcγRIIIB, which shows high amino acid sequence identity with FcγRIIIA in its extra-cellular binding domain (*Okazaki et al., 2004; Ferrara et al., 2006; Umana et al., 2006; De Romeuf et al., 2008; Jefferis, 2009; Shibata-Koyama et al., 2009; Mossner et al., 2010; Ferrara et al., 2011; Mizushima et al., 2011; Golay et al., 2013; Gagez & Cartron, 2014; Herter et al., 2014; Illidge et al., 2014; Goede et al., 2015; Illidge et al., 2015*). In the induction of these Fc-dependent effector mechanisms, obinutuzumab overcomes the inhibition mediated by KIRs, by complement or by physiological concentrations of competing unspecific endogenous Ig (*Mossner et al., 2010; Kern et al., 2013; Herter et al., 2014; Terszowski et al., 2014; Goede et al., 2015; Illidge et al., 2015*). With respect to rituximab, obinutuzumab recognizes a different but overlapping epitope in the CD20 extracellular domain and binds to the CD20 antigen in an *intra-tetrameric* manner and in a completely different space orientation (*Niederfellner et al., 2011; Klein et al., 2013; Gagez & Cartron, 2014; Illidge et al., 2014; Illidge et al., 2015*). In particular, it appears rotated 90° around its middle axis and also tilted 70° towards the carboxyl terminus of the CD20 epitope (*Goede et al., 2015; Gagez & Cartron, 2014*). These binding properties of obinutuzumab to CD20 are related to a modified *elbow-hinge* sequence with a valine-for-leucine substitution at *Kabat* position 11, arising during the process of

humanization and resulting in a 30° wider elbow angle (*Mossner et al., 2010; Niederfellner et al., 2011; Klein et al., 2013; Gagez & Cartron, 2014; Illidge et al., 2014; Goede et al., 2015)*. These special binding characteristics of obinutuzumab prevent its *cis* interaction with the inhibitory FcγRIIB expressed on B cells, thus preventing the rapid internalization and degradation of mAb/CD20 complex (*Beers et al., 2010; Lim et al., 2011; Klein et al., 2013; Gagez & Cartron, 2014; Illidge et al., 2014; Goede et al., 2015)*. As a consequence, obinutuzumab does not sequester CD20 molecules within lipid rafts and promotes their stable accumulation at sites of homotypic aggregation thus inducing minimal CDC but enhanced PCD via a homotypic adhesion-related, caspase-independent, lysosome/ROS-mediated mechanism (*Mossner et al., 2010; Alduaij et al., 2011; Dalle et al., 2011; Jak et al., 2011; Herter et al., 2013; Gagez & Cartron, 2014; Illidge et al., 2014; Goede et al., 2015; Illidge et al., 2015*). Notably, because of the absence of characteristic hallmarks of apoptosis, this type of B cell death may overcome apoptosis-resistance mechanisms, thus leading to the depletion of malignant B cells which are refractory to conventional chemotherapy or immune-chemotherapy (*Illidge et al., 2014; Goede et al., 2015*). Furthermore, this effector mechanism is induced independently of the Fc-FcγR interactions (*Ivanov et al., 2008; Mossner et al., 2010; Alduaij et al., 2011; Dalle et al., 2011; Honeychurch et al., 2012; Illidge et al., 2014; Goede et al., 2015; Illidge et al., 2015*) and potentially remains active in patients with impaired Fc dependent functions (*Cartron et al., 2002; Glennie et al., 2007; Beurskens et al., 2012; Illidge et al., 2014; Goede et al., 2015*). Notably, in *in vitro* and preclinical studies obinutuzumab was shown to be superior to rituximab (*Gagez & Cartron, 2014; Goede et al., 2015; Illidge et al., 2015*). Obinutuzumab achieved B cell depletion superior to rituximab not only in peripheral blood but also in lymphoid tissues including lymph nodes and spleen (*Mossner et al., 2010; Illidge et al., 2014; Goede et al., 2015; Illidge et al., 2015*). In addition, obinutuzumab demonstrated a superior anti-tumour activity in lymphoma xenograft models, even in rituximab relapsed/refractory tumours (*Mossner et al., 2010; Dalle et al., 2011; Herter et al., 2013; Herting et al., 2014; Illidge et al., 2014; Goede et al., 2015; Illidge et al., 2015*) as well as an enhanced efficacy in combination with chemotherapies such as chlorambucil, fludarabine, bendamustine and cyclophosphamide (*Dalle et al., 2011; Herting et al., 2014; Illidge et al., 2014; Goede et al., 2015*). In clinical studies, obinutuzumab exhibited promising activity in different CD20 diseases as well as an acceptable and manageable safety profile (*Gagez & Cartron, 2014; Goede et al., 2015*). Based on the results of phase III trials, obinutuzumab has been recently approved in

combination with chlorambucil for the treatment of patients with previously untreated B-CLL and co-morbidities, unsuitable for full-dose fludarabine-based therapy (*Goede et al., 2014)* as well as in combination with bendamustine for patients with rituximab-relapsed/refractory indolent B-NHL (*Sehn et al., 2016*). Further clinical studies are planned or ongoing in both untreated and relapsed/refractory patients with B lymphoproliferative disorders (*Gagez & Cartron, 2014; Goede et al., 2015*).

Aim

This study is aimed at understanding the cellular and molecular mechanisms underlying the modulation of NK cell responsiveness and plasticity induced by therapeutic anti-CD20 monoclonal antibodies (mAbs) endowed with different binding affinity for the FcγRIIIA/CD16 receptor. In this regards, in first we addressed whether the CD16 aggregation by the chimeric rituximab or the humanized obinutuzumab, in both its Fc-glycoengineered and non-glycoengineered wild type version, may differently affect the receptor dynamics and NK functions. Then we addressed whether the CD16 affinity aggregation conditions may impact on the capability of anti-CD20 mAbs to support the expansion as well as to affect the phenotypic and functional proprieties of the recently identified long-lived and highly functional "*memory*" NK subset.

Materials and Methods

Antibodies and Reagents

The following anti-CD20 mAbs were used*:* the chimeric IgG1κ type I rituximab, the humanized IgG1κ type II obinutuzumab (GA101) and its non-glycoengineered parental version (wt-GA101) kindly provided by Roche Glycart (Schlieren, Switzerland); the monovalent Fab fragment of obinutuzumab (GA101-Fab) obtained via papain digestion by commercial Fab Preparation kit (Thermo Scientific, code 44985), according to manufacturer's instructions. Anti-CD16 (B73.1, provided by Dr. G. Trinchieri National Cancer Institute, National Institutes of Health, Frederick, USA), anti-2B4 (C1.7, Beckman Coulter), anti-NKp46 (9E2, Biolegend), anti-NKG2D (149810, R&D Systems), anti-NKp30 (210847, R&D Systems), goat-anti-mouse (GAM) $F(ab')_2$ (Jackson Immunoresearch Laboratories). The following fluorochrome-conjugated mAbs for NKG2D (149810, R&D Systems), CD107a (H4A3, BD Biosciences), CD16 (Leu11c, BD Biosciences; MEM-154, Immunological Sciences; 3G8, BD Biosciences; REA423, Miltenyi Biotec), NKp46 (9E2, BD Biosciences; REA808, Miltenyi Biotec), NKp30 (p30-15, BD Biosciences), CD56 (B159, BD Biosciences; REA196, Miltenyi Biotec), CD3 (SK7, BD Biosciences; REA613, Miltenyi Biotec), NKG2C (134591, R&D Systems), IFN-γ (B27, BD Biosciences), FcεRIγ subunit (Millipore), PLZF (Mags.21F7, eBioscience), CD19 (4G7, BD Biosciences) and the goat-anti-human (GAH) κ light chain (Cappel) Ab were used. For immunoblot analysis were used the following Abs: anti-phosphotyrosine (4G10, Millipore), anti-FcεRIγ subunit (Millipore), anti-ZAP-70 (2F3.2, Millipore), anti-ubiquitin (FK2, Enzo Life Sciences), anti-CD3ζ (6B10.2, Santa Cruz Biotechnology Inc), anti-Syk (4D10 Santa Cruz Biotechnology Inc.), anti-β-tubulin (Tub2.1, Sigma-Aldrich), anti-Cbl-b (Santa Cruz Biotechnology) and anti-c-Cbl (Cell Signaling Technology). Monensin, saponin, paraformaldehyde (PFA), poli-L-lysine, brefeldin A (BFA), ammonium chloride (NH_4Cl), protease and phosphatase inhibitors were from Sigma-Aldrich. Nonionic detergent Triton X-100 and glycin were from Biorad.

Cell systems

Peripheral blood mononuclear cells and primary cultured human NK cells

Peripheral blood mononuclear cells (PBMCs) were freshly isolated from whole blood samples of healthy donors of Blood Transfusion Center of Sapienza University of Rome over a Ficoll-Hypaque gradient. The study was conducted according to protocols approved by our local institutional review board and in accordance with Declaration of Helsinky. Primary cultured human NK cells were obtained from 10-day co-cultures of PBMCs with irradiated Epstein-Barr virus positive (EBV^+) RPMI 8866 lymphoblastoid cell line (*Galandrini et al., 1999*). On day 8 to 10, cell population was assayed by cytofluorimetric analysis for CD56, CD16, CD3 expression and the experiments were performed on NK cell populations ($CD3^-CD56^+$) that were more than 80% pure. Primary cultures were maintained in RPMI 1640 medium supplemented with 1% L-glutamine and 10% Fetal Calf Serum (FCS).

Cell lines

The following cell lines were used as targets: the human $CD20^+$ lymphoblastoid Raji provided by Dr. F.D. Batista (Cancer Research UK, London), the murine FcR^+ mastocytoma P815 and the human erytroleukemia K562, all kept in culture for less than two consecutive months in RPMI 1640 medium supplemented with 10% FCS and 1% L-glutamine and regularly checked for mycoplasma negativity.

Anti-CD20-mediated CD16 aggregation

$CD20^+$ Raji cells were opsonized by incubating with saturating dose of rituximab (1 $\mu g/10^6$), obinutuzumab (GA101) (0.1 $\mu g/10^6$), or when indicated, wt-GA101 (0.1 $\mu g/10^6$) or GA101-Fab (1 $\mu g/10^6$) for 20 minutes at room temperature. Not opsonized Raji, used as control, and anti-CD20-opsonized targets were washed and allowed to interact with NK cells at effector (E): target (T) ratio 2:1 for the indicates times. To obtain "anti-CD20 experienced NK cells", Raji cells were biotinylated by loading with 10 μg/ml of EZ-Link Sulfo-NHS-SS-Biotin (*Thermo Fisher Scientific*) for 30 minutes at room temperature, washed twice with Phosphate-Buffered Saline (PBS) supplemented with 1% FCS and then opsonized with rituximab or obinutuzumab as above described. Primary cultured NK cells were mixed (2:1) with biotinylated anti-CD20 opsonized or not opsonized targets and co-cultured for the indicated times.

At the end of stimulation cellular conjugates were mechanically disrupted by washing twice with 5mM ethylenediaminetetraacetic acid (EDTA) containing PBS. In order to immunomagnetically isolate NK populations, conjugates were incubated for 30 minutes at 4 °C with streptavidin-coated magnetic beads (Dynabeads Biotin Binder, Invitrogen, Life Technologies) at beads: cells ratio 4:1. NK populations were recovered by negative selection and then assayed for phenotypic, functional and biochemical analysis using as control population NK cells derived from the co-culture with not opsonized target cells (Fig. 13). For experiments requiring lysosomal inhibitor, 20mM NH_4Cl was added during co-culture. Where indicated, immunomagnetically isolated NK populations were re-plated in culture medium for up to 48 hours.

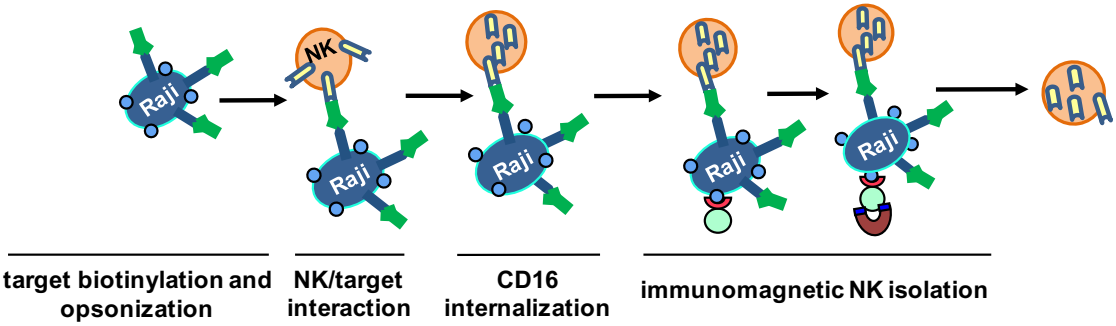


Figure 13. Experimental model to obtain anti-CD20-experienced NK cells.

Anti-CD20-induced *in vitro* expansion of "*memory*" NK subset

Freshly isolated PBMCs from healthy donors were seeded (50.000 cells/well) in round-bottom 96-well plates and cultured for 10 days in RPMI 1640 supplemented with 10% FCS, 1% L-glutamine, 1% Penicillin/Streptomycin plus 100 U/ml of human recombinant IL-2 (R&D system). Two days after cell plating, irradiated Raji cells were added (2:1) in the absence or presence of rituximab or obinutuzumab (10 µg/ml). Half of culture media was removed and replenished with media containing IL-2 every 2-3 days. At the end of the co-culture, cells were harvested from each culture condition and used for phenotypic and functional analysis. For functional assays, 10 ng/ml of IL-15 (Peprotech) were added during the last three days of co-culture (Fig. 14).

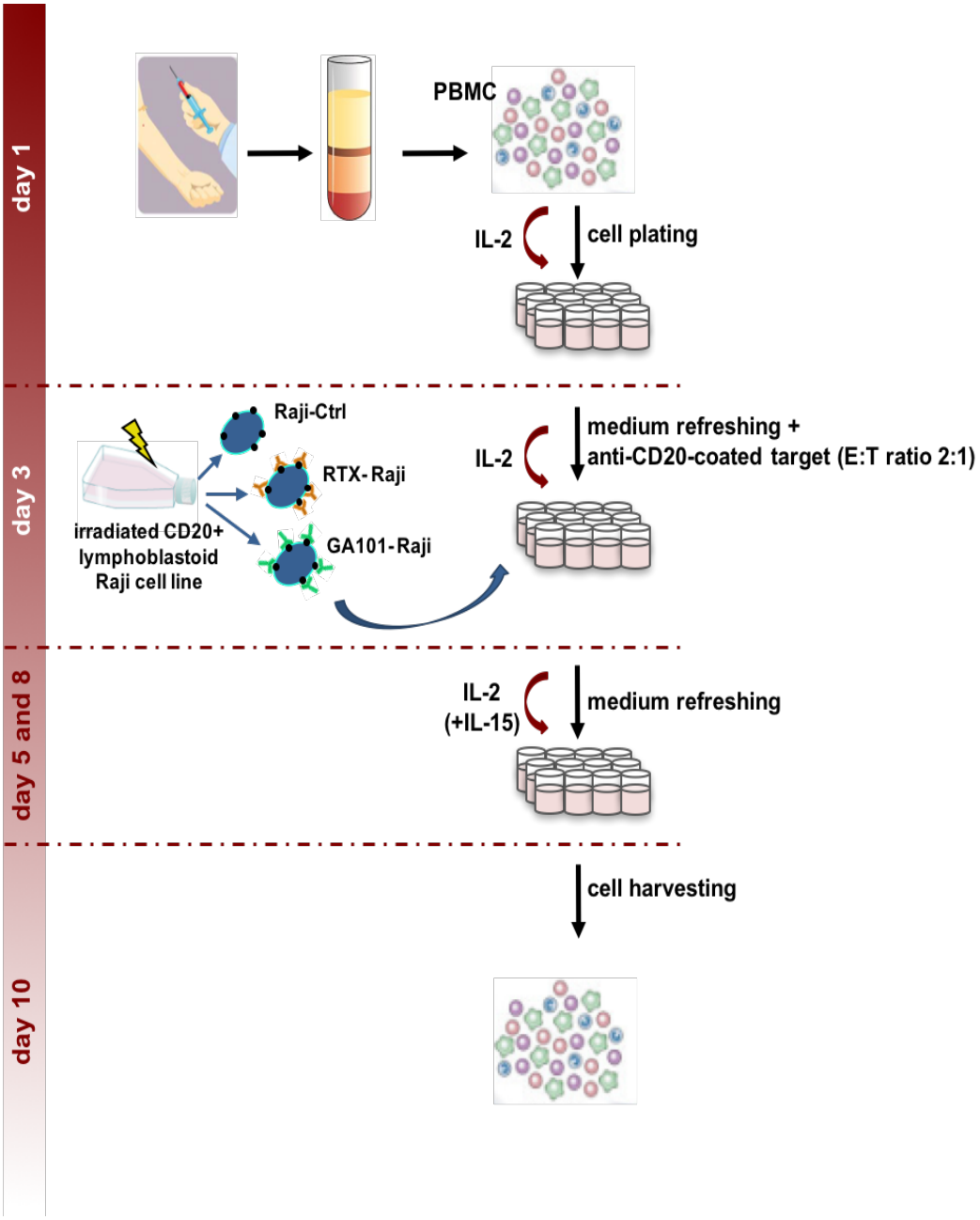


Figure 14. Experimental setting for anti-CD20-induced *in vitro* expansion of "*memory*" NK cells.

Cytofluorimetric analysis

Titration of anti-CD20 antibodies

To determine the minimum saturating doses of anti-CD20 mAbs, Raji cells ($1x10^6$) were incubated with increasing amounts (0.05 μg, 0.1 μg, 1 μg, 10 μg, 100 μg) of rituximab, obinutuzumab (GA101), wt-GA101 or GA101-Fab for 20 minutes at 4°C, washed and then stained with FITC-conjugated GAH κ light chain Ab, that specifically recognizes the κ light chain of immunoglobulins.

Determination of FcγRIIIA-158V/F allotype

To determine FcγRIIIA-158V/F allotype, freshly isolated PBMCs were stained with APC-conjugated anti-CD56 and the following anti-CD16 mAbs: FITC-conjugated 3G8, that binds to a not polymorphic epitope of CD16, or FITC-conjugated MEM-154, whose binding to CD16 is dependent on the presence of valine (*Bottcher et al.,2005*). The ratio between the mean fluorescence intensity (MFI) of MEM-154 and 3G8 allows to classify three different phenotypes: F/F (ratio <0.04), V/V (ratio >0.62), and V/F (ratio between 0.15 and 0.48).

Analysis of activating receptor expression levels

To evaluate CD16 modulation, primary cultured NK cells were mixed (2:1) with anti-CD20 opsonized or not opsonized Raji targets, briefly pelleted for 2 minutes at 600 rpm to allow conjugate formation and incubated for different lengths of time at 37°C. To block the stimulation, samples were treated with 0.1% NaN_3 in cold PBS for 5 minutes on ice and then washed with 5mM EDTA containing PBS to obtain conjugate disruption. After washing, samples were stained with PE-conjugated anti-CD16 (B73.1) and APC-conjugated anti-CD56 mAbs and then fixed for 20 minutes at room temperature with 1% PFA. For each time point, normalized MFI was calculated as follows: (MFI of the stimulated sample/MFI of Ctrl sample) x 100, assuming as 100% the MFI value of NK cells stimulated with not opsonized targets for each time of stimulation. To examine activating receptor expression levels, control and anti-CD20-experienced NK populations were stained with PE-conjugated anti-CD16, anti-NKp46, anti-NKp30 or anti-NKG2D mAbs and then fixed with 1% PFA as above described.

Characterization of "memory" NK cell subset

To identify "*memory*" NK cells, freshly isolated or cultured PBMCs were stained with APC-Vio770-conjugated anti-CD56, PE-Vio 770-conjugated anti-CD16 and PerCP-Vio700-conjugated anti-CD3 mAbs and then fixed for 20 minutes at room temperature with 2% PFA. For the analysis of cell surface markers, samples were stained with APC-conjugated anti-NKp46 and PE-conjugated anti-NKG2C mAbs before fixing. For intracellular FcεRIγ detection, samples were permeabilized by incubating for 30 minutes at room temperature with 0.05% Triton-X in 2mM EDTA and 2% FCS containing PBS, washed and then stained with FITC-conjugated anti-FcεRIγ mAb. The absolute number/10^6 of NK cells was calculated as follows: (percentage of NK cells) x 10^6/100. The absolute number/10^6 of "*conventional*" or "*memory*" NK subset was calculated according to the formula: (percentage of "*conventional*" or "*memory*" NK cells) x (absolute number/10^6 of NK cells)/100.

Functional flow cytometry assays

To determine CD16-mediated CD107a (LAMP-1) surface mobilization and intracellular IFN-γ production, freshly isolated PBMCs or, when specified, primary cultured NK cells were combined (2:1) with not opsonized or anti-CD20-opsonized Raji targets for 6 hours at 37°C in the presence of PE-conjugated anti-CD107a mAb and 50 μM Monensin (Golgi-stop). After the first hour, 10 μg/ml BFA was added. At the end of stimulation, cells were washed with PBS supplemented with 5mM EDTA to promote conjugate disruption and, before fixing for 20 minutes at room temperature with 2% PFA, cells were stained with FITC-conjugated anti-CD56 and PerCP-conjugated anti-CD3 mAbs. For intracellular IFN-γ detection, samples were then permeabilized by incubating for 30 minutes at room temperature with 0.5% saponin in PBS supplemented with 1% FCS, washed and stained with APC-conjugated anti-IFN-γ mAb.

To determine CD107a surface expression in response to CD16 and other NK activating receptors, control and anti-CD20-experienced NK populations were stimulated with 1μg/well plastic-immobilized anti-CD16 (B73.1), anti-NKG2D, anti-NKp46 and anti-2B4 mAbs, alone or in combination and treated as above described.

To assess the functional responses of the "*memory*" NK cell subset, freshly isolated or cultured PBMCs were allowed to interact (2:1) with not opsonized or anti-CD20-opsonized Raji as well as with K562 targets for 6 hours at 37°C as above described.

At the end of stimulation, cells were washed with PBS supplemented with 2% FCS and 2mM EDTA to promote conjugate disruption and, before fixing for 20 minutes at room temperature with 2% PFA, samples were stained with APC-Vio770-conjugated anti-CD56 and PerCP-Vio700-conjugated anti-CD3 mAbs. For intracellular FcεRIγ and IFN-γ detection, samples were then permeabilized by incubating for 30 minutes at room temperature with 0.05% Triton-X in 2% FCS and 2mM EDTA containing PBS, washed and stained with FITC-conjugated anti-FcεRIγ and APC-conjugated anti-IFN-γ mAbs.
All results were analysed using FlowJo version 9.3.2 software (Tree Star, Ashland, OR).

Confocal microscopy

To evaluate the co-localization of CD16 with the lysosomal compartment, primary cultured NK cells were pre-treated for 30 minutes at 37°C with 10 μM LysoTracker Red DND-99 (Life Technologies) and then allowed to interact (2:1) with not opsonized or anti-CD20-opsonized Raji cells previously labeled for 30 minutes at 37°C with 0.5 μM 7-amino-4-cholromethylcoumarin (CMAC) (Life Technologies). Conjugates were gently re-suspended, spun onto multichamber poly-L-lysine-coated glass slides, incubated for 15 minutes at 37°C followed by incubation at 4°C for 10 minutes to block the stimulation and promote spontaneous adhesion to the slide. Cells were fixed for 20 minutes at room temperature with 3.7% PFA, permeabilised for 5 minutes with 0.5% saponin, incubated for 15 minutes with 0.1 M glycin followed by Blocking Buffer (1%FCS, 0.05% saponin) for 10 minutes and stained with anti-CD16 (B73.1) (1 μg/1x10^6) followed by Alexa Fluor 488-conjugated GAM (Invitrogen) Abs. After washing, slides were mounted with SlowFade Gold reagent (Life Technologies). High-resolution images (800 × 800 pixel, 8 μs/pixel) were acquired, on randomly acquired fields, with a IX83 FV1200 laser-scanning confocal microscope (Olympus) with a 60×/1.35 NA UPlanSAPO oil immersion objective. Sequential acquisition was used to avoid crosstalk between different fluorophores. Fluorescence and DIC (Differential Interference Contrast) images were acquired with zoom3. Co-localization was assessed in NK/Raji conjugates (n=50) showing an extensive membrane contact between effector and target cells and expressed as percentage of conjugates containing co-localized CD16 and lysosomes with respect to total conjugates, considering co-localization indexes (Pearson's correlation coefficient >0.2) obtained by FluoView 4.2 software, between two channels on single cells (ROI) after background correction. Images were processed with ImageJ1.41o software.

CD16 polymorphism analysis

Genomic DNA was extracted from PBMCs by commercial Isolate II Genomic DNA kit (Bioline) in a cohort of 125 healthy donors according to manufacturer's instructions. Briefly, samples (up to 10^7 cells) were re-suspended in Lysis Buffer GL (200 μl/sample), incubated at 70°C for 15 minutes with Proteinase K (25 μl/sample) in Lysis Buffer G3 (200 μl/sample) and briefly vortexed. 96-100% ethanol (210 μl/sample) was added to the samples which in turn were vigorously vortexed. Samples were loaded to silica membrane-containing ISOLATE II Genomic DNA spin columns, placed in 2 ml Collection Tubes, and centrifuged 1 minute at 11.000 x g. Wash Buffer GW1 (500 μl/sample) followed by Wash Buffer GW2 (600 μl/sample) were added to the samples which in turn were centrifuged 1 minute at 11.000 x g. Another centrifugation was performed in oder to dry silica membrane. For DNA elution, 70°C-prewarmed Elution Buffer G (100 μl/sample) was added to the samples loaded in ISOLATE II Genomic DNA spin columns placed in 1.5 ml Collection Tubes. Samples were incubated at room temperature for 1 minute and centrifuged 1 minute at 11.000 x g collecting flow-through. The concentration and the quality of the extracted DNA were determined by using NanoDrop 2000 (Thermo Scientific). The genomic region containing the polymorphic c.559 site was amplified by two distinct PCR assays, as described (*Leppers-van de Straat et al., 2000; Quartuccio et al., 2014*). Specifically, for PCR assay 1, PCR primers: forward1 5'-CCCTTCACAAAGCTCTGCACT-3'; reverse1 5-ATTCTGGAGGCTGGTGCTACA-3'; sequencing primer: 5-CCCCAAAAGAATGGACTGAA-3': for PCR assay 2, PCR primers: forward2 5' TGTAAAACGACGGCCAGTTCATCATAATTCTGACCTCT-3', reverse2 5-CAGGAAACAGCTATGACCCTTGAGTGATGGTGATGTTCA-3'; sequencing primers: 21M13 or M13. PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit and a 3130xl Genetic Analyzer (Thermo Fisher Scientific) and analysed by the Sequencing Software Analysis 6 (Applied Biosystems).

Biochemical analysis

Analysis of cellular levels of CD16-coupled signaling elements

Control and anti-CD20 experienced NK cells were lysed 20 minutes at 4°C in 1% Triton-X 100 lysis buffer (50 mM Tris pH7.5, 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid (EGTA) pH8, 1 mM $MgCl_2$ and 50 mM NaF) supplemented with 1mM each of phenylmethanesulfonylfluoride (PMSF) and Na_3VO_4 as well as with 1 μg/ml each of aprotinin and leupeptin. Cell lysates were centrifuged for 20 minutes at 13000 x g at 4°C, supernatants were collected and protein concentration was evaluated by Bradford colorimetric assay (Bio-Rad Laboratories). The absorbance was measured at 620 and 690 nm by using a known concentration curve of bovine serum albumin (BSA) as standard. Equal protein amounts from each sample were diluted in Laemmli Sample Buffer (0.3 M Tris HCl, 10% SDS, 20% glycerol and 2% β-mercaptoethanol) and boiled at 95 °C.

Immunoprecipitation studies

To explore Syk ubiquitination or c-Cbl and Cbl-b tyrosine phosphorylation, CD16 stimulation was obtained by mixing primary cultured NK cells with not opsonized or anti-CD20-opsonized Raji cells for the indicated times at 37°C. Before mixing, targets cells were fixed (0.5% PFA) for 5 minutes at room temperature, washed three times with complete medium, incubated at 37°C for 1 hour and washed once more. At the end of stimulation, samples were lysed in 1% Triton-X 100 lysis buffer (see above) supplemented with 1 mM each of PMSF and Na_3VO_4 as well as with 1 μg/ml each of aprotinin and leupeptin as above described. Samples were pre-cleared and immunoprecipitated with anti-Syk or anti-Cbl-b pre-loaded protein G Sepharose beads (Sigma-Aldrich) or with anti-c-Cbl pre-loaded protein A Sepharose beads (GE Healthcare Life Science) for 2 hours at 4°C. Immunoprecipites were washed five times with lysis buffer supplemented as above described. Proteins were eluted using 50 μl/sample of Laemmli Sample Buffer and boiled at 95 °C.

SDS-polyacrylamide gel electrophoresis (PAGE) and Western Blotting

Depending on the experiment, whole lysates or immunoprecipitated samples were separated by SDS-PAGE. SDS-PAGE maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (disulfide bonds and sulfhydryl groups) thus allowing separation of proteins by their molecular weight. Sample proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of the gel and transferred to a nitrocellulose membrane. Blocking of non-specific binding was achieved by 5% Bovine Serum Albumin (BSA) in TBS-T (20 mM Tris-HCl pH 7.8, 150 mM NaCl, 0.05% Tween-20). Membranes were incubated with the proper primary antibodies, followed by a secondary peroxidase-linked antibody. Enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech) was used to detect the signal.

Cytotoxicity assay

To analyse the cytotoxic response primary cultured or control and anti-CD20 experienced NK populations were assessed in standard 51Cr-release assays. Briefly, target cells were labeled with ^{51}Cr (Amersham) (100 μCi/1x106 cells) for 1 hour at 37°C and then washed twice. In ADCC assays, rituximab- or obinutuzumab-opsonized or not opsonized ^{51}Cr-labeled Raji cells were used as targets. In redirected killing assays ^{51}Cr-labeled FcR^{+} P815 cells were used as targets in the presence of anti-CD16, anti-NKp46, anti- NKp30 or anti-NKG2D mAbs. Serial dilutions of effector cells and 5000/well ^{51}Cr-labelled target cells were plated in round-bottom 96-well plates in a final volume of 200 μl RPMI supplemented with 10% FCS, 1% L-glutamine and 1% Hepes at effector:target (E:T) ratios ranging from 50:1 to 0.04:1. After 4 hours at 37°C, 30 μl of supernatants were collected from each well and counted by a β-counter instrument (TopCount, PerkinElmer Life and Analytical Sciences). Maximal and spontaneous releases were obtained by incubating ^{51}Cr-labeled individual targets, used for each stimulation condition, with SDS 10% or medium alone, respectively. The percentage of specific lysis was calculated according to the formula: (experimental release-spontaneous release) / (maximal release-spontaneous release) x 100. Where indicated, results were reported also as lytic units that have been defined as the number of effector cells required to mediate 10% specific lysis of target cells. Lytic units for 1×10^6 effector cells were calculated as follows: 1×10^6 / (n. of target cells × X), where X is the E:T ratio resulting in 10% specific lysis.

Enzyme-linked immunosorbent assays (ELISA)

IFN-γ release

Control and anti-CD20-experienced NK cells were mixed (2:1) with the indicated targets in the presence or absence of recombinant human IL-12 (10 ng/ml) (Peprotech) or IL-2 (100 U/ml) (R&D Systems) for 18 hours at 37°C. Supernatants were then collected and analysed by a commercial ELISA kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, 50 µl of supernatants were incubated for 2 hours with a biotinylated antibody (Biotinylated Antibody Reagent) in anti-human IFN-γ pre-coated 96-well Stripe Plate. After 3 washes, 100 µl/well of Streptavidin-HRP Solution were added and the plate was incubated for 30 minutes at room temperature. Concentrated chromogen substrate Tetramethylbenzidine (TMB) (100 µl/well) was added to allow color reaction. The reaction was stopped by adding 100 µl/well of Stop Solution and the absorbance was measured on an ELISA plate reader at 450 nm and normalized by using the standard curve of recombinant human IFN-γ.

Serological status for human Cytomegalovirus (HCMV)

The anti-HCMV IgG titre was determined, in donor plasma samples, by using Human Anti-Cytomegalovirus (CMV) IgG ELISA Kit (*Abcam*) according to the manufacturer's instructions. Briefly, 100 µl/well of controls (IgG Positive, IgG Negative and IgG Cut-off) or test samples, diluted 1: 100 with IgG Sample Diluent, were added to a 96-well Stripe Plate pre-coated with HCMV antigens and incubated at 37°C for 1 hour. After three washes, 100 µl/well of horseradish peroxidase (HRP) labelled anti-Human IgG Conjugate, which binds to the immobilized HCMV-specific antibodies, were added to the wells and the plate was incubated for 30 minutes at room temperature. After three washes, 100 µl/well of chromogen substrate Tetramethylbenzidine (TMB) were added to each well and the plate was incubated for 15 minutes at room temperature in the dark to allow color reaction. The reaction was stopped by adding 100 µl/well of Stop Solution and the absorbance was measured on an ELISA plate reader at 450 nm. Samples with an absorbance value greater than 10% over the cut-off control value are considered positive. By contrast, if the absorbance value is lower than 10% below the cut-off control value, samples are considered negative. Finally, samples with an absorbance value of less than 10% above or below the cut-off control value should be considered as inconclusive (grey zone) i.e. neither positive or negative. Results were reported in Standard Units according to the formula: sample absorbance value x 10 / cut-off control value.

Cut-off:	10	Standard Units
Grey zone:	9-11	Standard Units
Negative:	<9	Standard Units
Positive:	>11	Standard Units

Statistical and densitometric analysis

Differences among multiple groups were compared by one-way ANOVA with Tukey post-test correction, by Friedman with Dunn's post-test correction, or by two-way ANOVA with Bonferroni post-test correction, as appropriated. Differences between two groups were determined by performing Wilcoxon matched pairs test or Mann Whitney test. Differences were considered to be statistically significant when *p* value was less than 0.05. Analyses were performed using Prism 5 software (GraphPad). Quantification of specific bands was performed with ImageJ1.41o software (National Institutes of Health, Bethesda, MD).

Results

1. CD16 engagement by obinutuzumab-opsonized targets results in enhanced cytotoxicity and IFN-γ production

It is well-known that obinutuzumab was glyco-engineered by de-fucosylation of its Fc portion and that this modification substantially enhances the antibody binding affinity for CD16 receptor consequently improving the killing of mAb-opsonized targets via both ADCC and ADCP (*Mossner et al., 2010; Bologna et al., 2011; Golay et al., 2013; Herter et al., 2014*). While the increased ability of obinutuzumab to induce NK cell-mediated ADCC has been widely proven (*Bologna et al., 2011; Gagez & Cartron, 2014; Terszowski et al., 2014*), no studies addressed its ability to induce cytokine production by NK cells. In order to investigate the impact of CD16 affinity ligation conditions on the full NK functional program, we compared the ability of rituximab or obinutuzumab, in the defucosylated (GA101) or wild type (wt-GA101) version, to stimulate NK cell degranulation and/or cytokine production. To this end, in peripheral blood NK cells interacting with anti-CD20 opsonized targets we investigated, by multicolor flow cytometry both the intracellular production of IFN-γ and the surface expression of CD107a, which is a useful tool to evaluate lytic granule release since it becomes detectable at plasma membrane upon lytic granule exocytosis (*Alter et al., 2004*). Hereafter, we stimulated CD16 by combining NK cells with $CD20^+$ B cell lymphoma Raji cell line opsonized with anti-CD20 mAbs used at saturating concentrations as illustrated in Fig. 15.

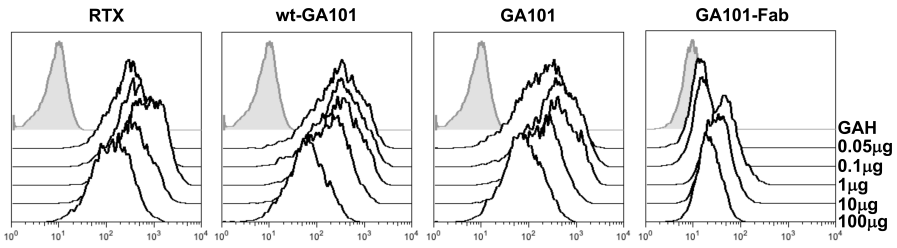


Figure 15. Determination of minimum saturating doses of anti-CD20 monoclonal antibodies on Raji cell line. The $CD20^+$ human lymphoblastoid Raji cells were incubated with the indicated doses of rituximab (RTX), obinutuzumab (GA101) or wt-GA101 followed by PE-conjugated GAH Ab or with the indicated doses of obinutuzumab Fab fragment (GA101-Fab) followed by FITC-conjugated GAH κ light chain Ab for FACS analysis. The overlays of histograms from one representative experiment are shown. Gray histogram represents GAH Ab.

As reported in Fig. 16A and B, while the NK cell interaction with not opsonized targets, that we kept as control population, did not result in significant CD107a surface expression and IFN-γ production, the interaction with rituximab-opsonized Raji promoted both degranulation and IFN-γ production with a higher portion of degranulating than of IFN-γ producing cells, thus confirming the hierarchy existing in NK cell functions (*Fauriat et al., 2010*). Moreover, under such CD16 aggregation conditions only few NK cells acquired the ability to both degranulate and produce IFN-γ. On the other hand, NK cell stimulation with obinutuzumab-opsonised Raji resulted not only in an increased degranulation, as described in literature, but also in an enhanced IFN-γ production with respect to rituximab. Notably, the portion of NK cells that performed both degranulation and IFN-γ production appeared almost twice in response to obinutuzumab- than to rituximab-opsonized targets. It was evident that this increased ability of obinutuzumab to activate NK cells was due to its defucosylated status. In fact, wt-GA101 was less efficient and superimposable to rituximab in stimulating NK responses. Moreover, as shown by the median fluorescence intensity values reported in Fig. 16C, also the IFN-γ amount on a per cell basis appeared enhanced in response to obinutuzumab-opsonized Raji cells.

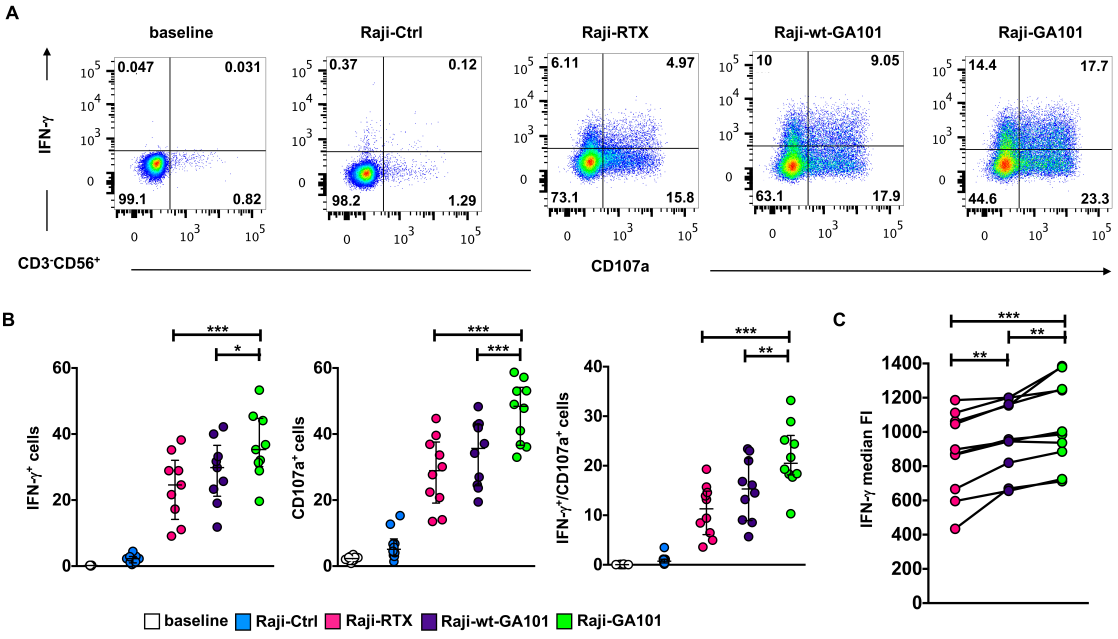


Figure 16. CD16 engagement by obinutuzumab-opsonized targets enhances both CD107a mobilization and IFN-γ production in freshly isolated NK cells. PBMCs were left alone (baseline) or combined (2:1) with rituximab (Raji-RTX)-, obinutuzumab (Raji-GA101)-, wt-GA101 (Raji-wt-GA101)-opsonized or not opsonized Raji (Raji-Ctrl) for 6 hours. The percentages of IFN-γ^+ and CD107a$^+$ cells among NK cells (CD3$^-$CD56$^+$) were analysed by flow cytometry. **(A)** Plots from one representative donor are shown. **(B)** Data (mean ± SEM) from 9 donors are presented as median with the interquartile range. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$. Compared to baseline or Raji-Ctrl samples, all the differences were statistically significant ($p < 0.0001$). **(C)** The median fluorescence intensity (FI) values of IFN-γ^+ NK cells from 9 individuals are reported in the graph. Each line represents a single donor. **$p < 0.01$, ***$p < 0.0001$.

In line with data obtained in peripheral blood NK cells, we observed that in response to obinutuzumab also primary cultured NK cells exhibited an increased killing ability, evaluated by a standard chromium-release based ADCC assay (Fig. 17A), as well as an enhanced IFN-γ producing potential, evaluated by flow cytometry (Fig. 17B).

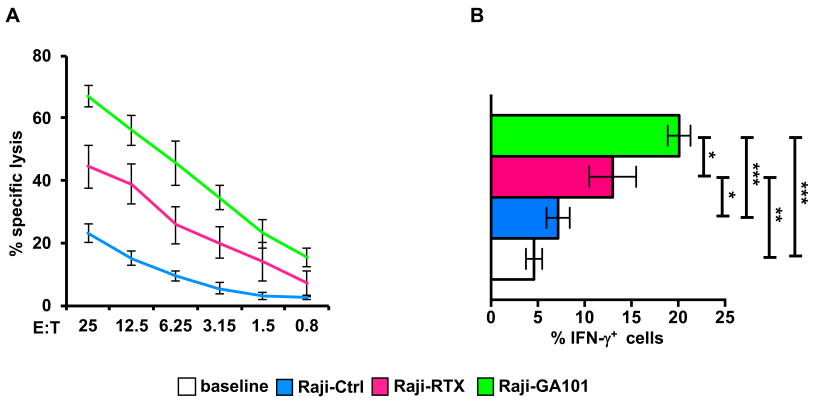


Figure 17. Obinutuzumab-mediated CD16 aggregation induces an enhanced ADCC and IFN-γ production in primary cultured NK cells. (A) Primary cultured NK cells were assessed in a standard ^{51}Cr-release ADCC assay against rituximab (Raji-RTX)-, obinutuzumab (Raji-GA101)-opsonized or not opsonized (Raji-Ctrl) targets. Specific lysis from 4 independent experiments is shown (mean ± SD). **(B)** Primary cultured NK cells were left alone (baseline) or allowed to interact (2:1) with rituximab (Raji-RTX)-, obinutuzumab (Raji-GA101)-opsonized or not opsonized (Raji-Ctrl) targets for 6 hours. The percentage of IFN-γ^+ cells was analysed by flow cytometry gating on CD56$^+$ population. Data from 4 independent experiments (mean ± SEM) are shown. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

Overall our results show that, due to its defucosylation, obinutuzumab improves NK cell functions also inducing multiple effector responses in individual NK cells.

2. CD16 engagement by obinutuzumab-opsonized targets promotes an enhanced receptor down-modulation and lysosomal targeting

Several groups, including ours, demonstrated that upon interaction with anti-CD20 coated targets CD16 receptor undergoes a marked surface down-modulation (*Bowles & Weiner, 2005; Bowles et al., 2006; Veeramani et al., 2011; Zent et al., 2014; Capuano et al 2015*). In order to investigate the impact of CD16 affinity ligation conditions on receptor dynamics, we compared the ability of rituximab or obinutuzumab, in the defucosylated (GA101) or wild type (wt-GA101) version, to induce CD16 surface down-modulation. To this end, by flow cytometry we monitored the CD16 expression levels in primary cultured NK cells interacting, for different lengths of time, with anti-CD20-opsonized Raji cells by using B73.1 mAb, whose binding to CD16 is not affected by Fc masking (*Grier et al., 2012*). As reported in Fig. 18, we observed that, NK cell stimulation with anti-CD20-opsonized targets resulted in a progressive and marked CD16 surface down-modulation with maximal levels between 2 and 18 hours of interaction. However, in response to obinutuzumab-opsonized targets, the down-modulation of CD16 was faster and deeper, leaving almost a 20% of residual receptor surface levels. It was evident that this increased ability of obinutuzumab to induce CD16 surface down-modulation was due to its enhanced CD16 binding affinity obtained by defucosylation. In fact, wt-GA101 was overall less efficient and superimposable to rituximab in inducing receptor down-modulation.

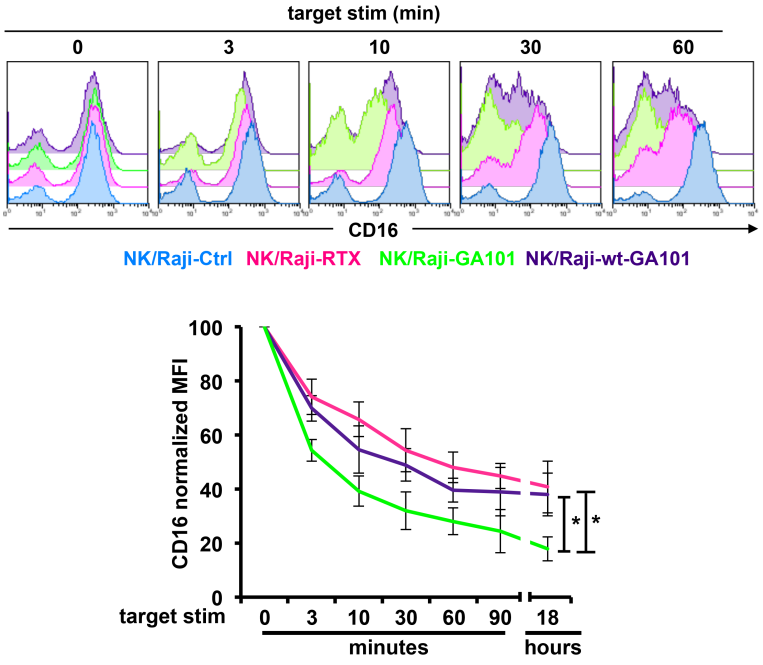


Figure 18. CD16 engagement by anti-CD20-opsonized targets induces receptor down-modulation. Primary cultured NK cells were combined (2:1) for the indicated times with rituximab (Raji-RTX)-, obinutuzumab (Raji-GA101)-, wt-GA101 (Raji-wt-GA101)-opsonized or not opsonized Raji (Raji-Ctrl). CD16 surface expression was evaluated by FACS analysis by anti-CD16 (Leu11c) mAb gating on $CD56^+$ population. (*Upper panels*) The overlays of histograms from one representative experiment are shown. (*Lower panel*) Normalized CD16 MFI was calculated as follows: (MFI of the stimulated sample/MFI of Ctrl sample) x 100, assuming as 100% the MFI value of NK cells co-cultured with not opsonized (Raji-Ctrl) targets for each time of stimulation. Data (mean ± SEM) from 6 independent experiments are shown. *$p < 0.05$.

Based on these data, we then investigated the persistency of the CD16 surface down-modulated status. To this end, by flow cytometry we assessed the CD16 expression levels in primary cultured NK cells immuno-magnetically derived from 18 hour co-cultures with biotinylated anti-CD20-opsonized or not opsonized Raji cells (hereafter defined "experienced" NK cells) and cultured for different lengths of time after target detachment. As shown in Fig. 19, we observed a progressive and almost complete recovery of CD16 expression levels within 48 hours.

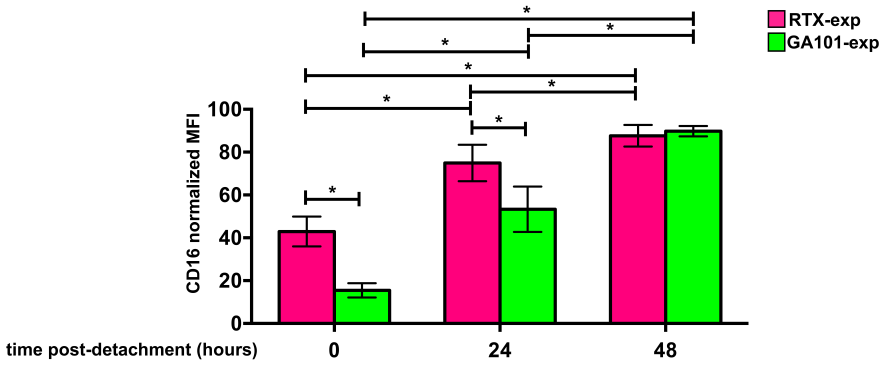


Figure 19. Surface CD16 levels undergo progressive recovery in anti-CD20-experienced NK cells. Primary cultured NK cells were isolated upon 18 hours co-culture (2:1) with biotinylated rituximab (RTX-exp)-, obinutuzumab (GA101-exp)-opsonized or not opsonized Raji (Ctrl-exp), re-plated for the indicated times and analysed for CD16 surface expression by FACS analysis. Bar graph depicts the normalized CD16 MFI calculated for each time point as follows: (MFI of the stimulated sample/MFI of Ctrl sample) x 100, assuming as 100% the MFI value of NK cells isolated from co-culture with not opsonized (Ctrl-exp) targets. Data (mean ± SEM) from 8 independent experiments are reported in bar graph. *$p < 0.05$.

It has been reported that both internalization (*Capuano et al., 2015*) and receptor shedding mediated by metalloproteases such as ADAM-17 (*Romee et al., 2013*) may contribute to anti-CD20 induced CD16 surface down-modulation. By confocal fluorescence microscopy we evaluated the fate of the down-regulated CD16 receptor. With the aim to address CD16 lysosomal targeting we used the lysosome marker Lysotracker. Lysotracker-labeled primary cultured NK cells were stimulated with anti-CD20-opsonized Raji cells for 15 minutes. To discriminate target cells in cellular conjugates, we

took advantage of cell tracker CMAC. As shown in Fig. 20A, with respect to the ring pattern of CD16 receptor observed in unconjugated NK cells, in anti-CD20-stimulated NK cells, CD16 distributed in intracellular dots thus confirming the occurrence of an internalization process, as already demonstrated for other immune-receptors (*Quatrini et al., 2015*). By evaluating E/T conjugates in randomly acquired fields, we observed that a substantial portion of internalized CD16 receptor co-localized with lysosomes (Fig. 20A) and that the percentage of conjugated NK cells with CD16/lysosome co-localization was significantly higher in response to obinutuzumab- than to rituximab- or wt-GA101-opsonized Raji cells (Fig. 20B).

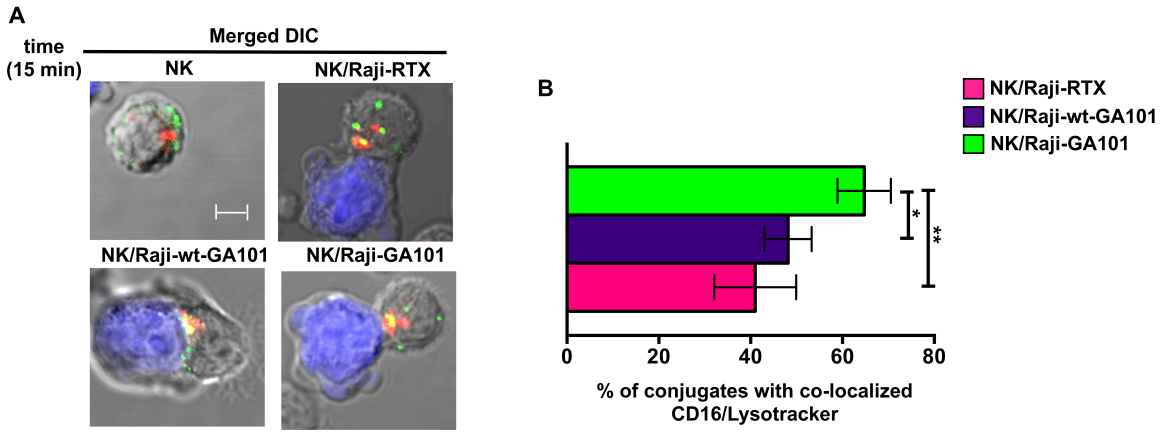


Figure 20. CD16 engagement by anti-CD20-opsonized targets induces receptor lysosomal targeting. (A) LysoTracker-labeled NK cells (red) were combined (2:1) for 15 minutes with CMAC-labeled rituximab (NK/Raji-RTX)-, obinutuzumab (NK/Raji-GA101)- or wt-GA101 (NK/Raji-wt-GA101)-opsonized targets (blue). Cell conjugates were fixed, permeabilized and stained with anti-CD16 (B73.1) followed by Alexa Fluor 488-GAM (green) Abs. A representative image of NK/target conjugate and isolated NK cell are shown. The overlay of the three-color merged image and the differential interference contrast (DIC) is shown. Scale bar, 5μm. **(B)** The percentage of conjugates containing CD16/lysosome co-localization (Pearson's correlation coefficient > 0.2) was analysed on randomly acquired fields of 3 independent experiments (mean ± SEM; n=50 conjugates). *$p < 0.05$, **$p < 0.01$.

Overall our data indicate that obinutuzumab induces a more rapid and profound CD16 surface down-modulation as well as an enhanced co-localization of the internalized receptor with lysosomes.

3. Preferential FcεRIγ degradation induced by obinutuzumab: impact of FCGR3A-V158F polymorphism

Several studies demonstrated that polymorphisms exist in the gene FCGR3A encoding for CD16 receptor and that the single nucleotide polymorphism (FCGR3A c.559G > T) resulting in valine (V) or phenylalanine (F) expression at aminoacid position 158 (FCGR3A-V158F) influences CD16 binding affinity for the Fc portion of IgG by generating a lower (FcγRIIIA-158F) or higher (FcγRIIIA-158V) affinity receptor variants. In particular, it is known that the clinical response rate to rituximab therapy may be affected by the different CD16 allotypes. In fact, it has been observed that individuals homozygous for phenylalanine (F/F) showed a worse response rate to rituximab therapy due to a reduced CD16 binding affinity for the antibody Fc portion (*Rascu et al., 1997; Cartron et al., 2002; Persky et al., 2012*). In this regards, we investigated the impact of FCGR3A-V158F polimorphism on CD16 aggregation and dependent functions induced by anti-CD20 mAbs. To this end, we typed healthy donors by cytofluorimetric analysis (*Bottcher et al., 2005*) by staining donor-derived PBMCs with two different anti-CD16 mAbs: 3G8 and MEM-154, that specifically recognizes residues of valine at CD16 aminoacid position 158. Based on the MEM/3G8 MFI ratio, we classified the donors in low (F/F)-, intermediate (V/F)- or high (V/V)-affinity (Fig. 21A lower panels) and data obtained were compared and confirmed by sequencing approaches (Fig. 21A upper panels) as described in literature (*Leppers-van de Straat et al., 2000; Quartuccio et al., 2014*). Among our cohort of 125 donors, 21 and 34 were V/V and F/F homozygous respectively while 70 were V/F heterozygous thus confirming a higher frequency of the intermediate (V/F) or high (V/V) affinity individuals (Fig. 21B). As shown in Fig. 21C, when we compared the ability of rituximab and obinutuzumab to induce CD107a surface mobilization on peripheral blood NK cells among different CD16 genotypes, we noted that, in low (F/F) affinity donors, NK cells degranulated better in response to obinutuzumab than to rituximab. This was in line with the widely described ability of obinutuzumab to promote an increased ADCC regardeless of the CD16 genotype, thus overcoming the individual heterogeneity in therapy response (*Terszowski et al., 2014; Herter et al., 2013*).

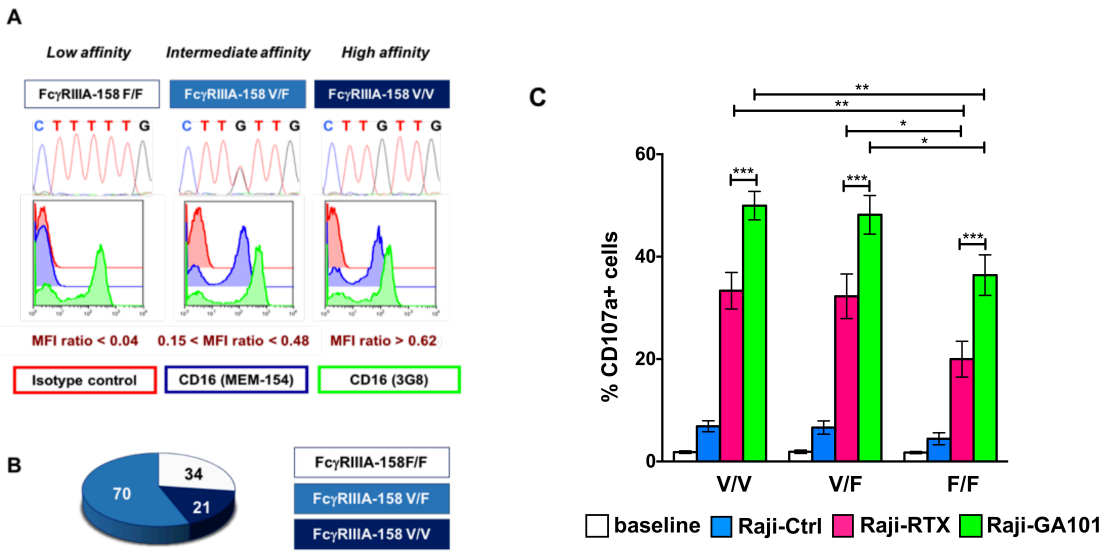


Figure 21. CD16 engagement by obinituzumab-opsonized targets results in enhanced degranulation irrespective of FCGR3A-V158F polymorphism. (A) The low (F/F)-, intermediate (V/F)- and high (V/V)-affinity variants of FcγRIIIA/CD16 were assessed by sequencing and cytofluorimetric approaches. (*Upper panels*) Genomic DNA from PBMCs was amplified by PCR and sequenced. *(Lower panels)* PBMCs were evaluated by flow cytometry upon staining with the FITC-conjugated anti-CD16 antibody 3G8 or MEM-154 and donors were typed on the basis of the MEM/3G8 MFI ratio. **(B)** The distribution of the CD16 polymorphic variants observed in a cohort of 125 donors is reported in the pie chart. **(C)** PBMCs were left alone (baseline) or allowed to interact (2:1) with rituximab (Raji-RTX)-, obinutuzumab (Raji-GA101)-opsonized or not opsonized (Raji-Ctrl) Raji cells for 6 hours. The percentage of $CD107a^+$ cells was evaluated by FACS analysis gating on NK population ($CD3^-CD56^+$) in individuals grouped by FCGR3A genotype (high-affinity V, low-affinity F; V/V, n=6; V/F, n=6; F/F, n=6). Data are presented as mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. For each group, compared to baseline or Raji-Ctrl samples, all the differences were statistically significant ($p < 0.001$).

Furthermore, we also investigated the impact of FCGR3A-V158F polymorphism on CD16 dynamics induced by anti-CD20 mAbs. To this purpose, we compared the ability of rituximab and obinutuzumab to induce CD16 surface down-modulation among different CD16 genotypes evaluating by flow cytometry the receptor expression levels in experienced NK cells. As shown in Fig. 22A, we found that, in low (F/F) affinity donors, only obinutuzumab induced a significant CD16 surface down-modulation. Indeed, while rituximab-induced CD16 down-modulation was less marked in F/F donors than in V/F or V/V donors, in response to obinutuzumab receptor down-modulation was enhanced and not substantially affected by the CD16 genotype.

It is well-established that, in NK cells, CD16 receptor is associated with homo or heterodimers of CD3ζ and/or FcεRIγ signaling chains (*Letourneur et al., 1991*).

In experienced NK cells, we evaluated by biochemical approaches the cellular levels of both CD3ζ and FcεRIγ chains among different CD16 genotypes. Data obtained highlighted that in low (F/F) affinity donors only obinutuzumab induced a selective and marked decrease of FcεRIγ chain, being CD3ζ chain only marginally affected (Fig. 22B and C). Conversely, as reported in Fig. 22D and 22E, we noted that both rituximab-and obinutuzumab-mediated CD16 aggregation promoted FcεRIγ and, to a lesser extent, CD3ζ chain down-modulation in intermediate (V/F) and high (V/V) affinity donors. Moreover, as shown in Fig. 22B to E, we also found that the down-regulation of FcεRIγ and CD3ζ chains was in part due to lysosomal degradation since it was partially, but significantly, reverted in the presence of the lysosome inhibitor ammonium chloride (NH_4Cl).

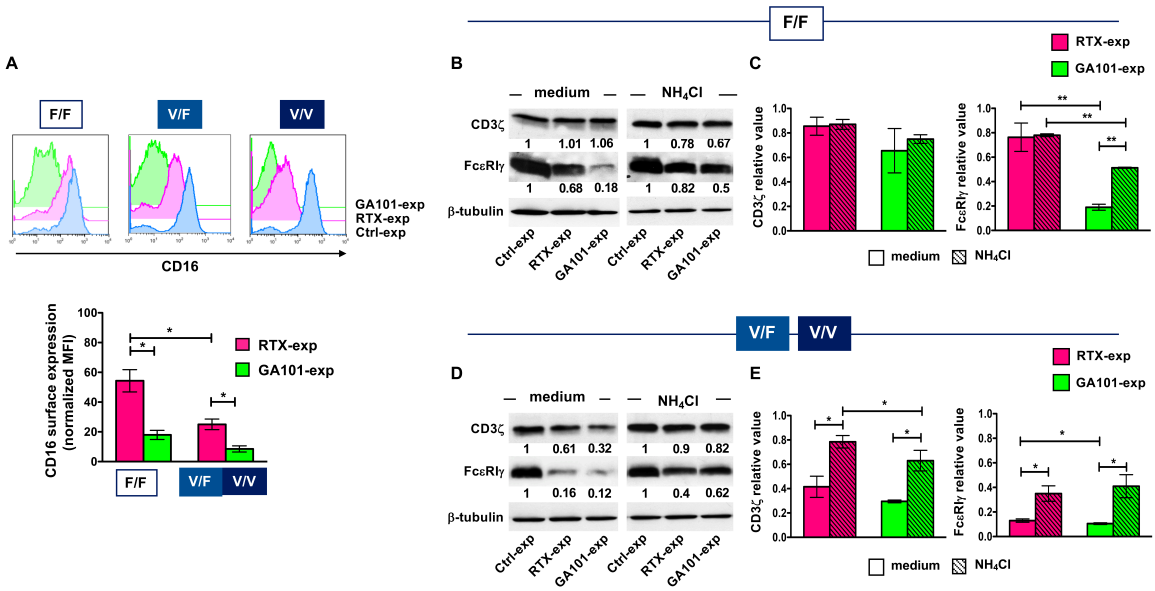


Figure 22. CD16 engagement by obinituzumab-opsonized targets results in enhanced FcεRIγ lysosomal degradation irrespective of FCGR3A-V158F polymorphism. Primary cultured NK cells (n=5/genotype) were isolated upon 18 hours co-culture (2:1) with biotinylated rituximab (RTX-exp)-, obinutuzumab (GA101-exp)-opsonized or not opsonized Raji (Ctrl-exp) in the presence of medium alone (medium) or, when indicated, with 20mM NH_4Cl. **(A)** CD16 surface expression was evaluated by FACS analysis by anti-CD16 (Leu11c) mAb in individuals grouped by FCGR3A genotype. (*Upper panels*) Histogram overlay of one representative donor/genotype is shown and (*lower panel*) normalized CD16 MFI values calculated as described in Fig. 2A are depicted in bar graph (mean ± SEM). *$p < 0.05$. **(B,D)** An equal amount of proteins from whole cell lysates was immunoblotted as indicated. The same membrane was immunoblotted with anti-FcεRIγ and, after stripping, with anti-CD3ζ Abs followed by anti-β-tubulin for sample normalization. Membranes containing untreated- (medium) or NH_4Cl-treated samples were developed in the same film. The black vertical lines indicate that intervening lanes were sliced out. The numbers represent the relative protein amount of the indicated proteins obtained by normalizing to the level of β-tubulin and expressed as fold change respect to Ctrl-exp samples (arbitrarily set to 1). One representative experiment is shown. **(C,E)** The relative values (mean ± SEM) of CD3ζ or FcεRIγ chains from 5 independent experiments are depicted in bar graphs. *$p < 0.05$, **$p < 0.01$.

Then we focused our analysis on intermediate (V/F) and high (V/V) affinity donors in which both rituximab and obinutuzumab were able to induce lysosomal degradation of FcεRIγ and, to a lesser extent, of CD3ζ chains. As shown in Fig. 23A, we analysed the cellular levels of CD16-dependent tyrosine kinases. Data obtained by biochemical approaches showed that the cellular levels of Syk, but not of ZAP-70, kinase were markedly down-regulated in anti-CD20-experienced NK cells. It has been reported a key role of ubiquitin modification in Syk degradation in response to CD16 stimulation (*Paolini et al., 2001*). With the aim to investigate whether the anti-CD20-mediated receptor aggregation could promote Syk ubiquinitination, primary cultured NK cells were allowed to interact with fixed anti-CD20 opsonized or not opsonized Raji cells. In Syk immunoprecipitated samples, we evaluated the levels of tyrosine phosphorylation and ubiquitination, using as negative control the obinutuzumab monovalent Fab fragment which does not bind to CD16. As illustrated in Fig. 23B, the immunoblot analysis of Syk immunoprecipitates revealed that CD16 stimulation by anti-CD20 mAbs induced a rapid tyrosine phosphorylation of Syk with a pattern consisting of multiple molecular species with a regular increase of the molecular weight, thus suggesting that ubiquitination process has occured. Indeed, the immunoblotting with anti-ubiquitin Ab visualized multiple bands in the region of phosphorylated Syk. Interestingly, Syk ubiquitin modification resulted more persistent and stronger in NK cells interacting with obinutuzumab- than with rituximab-opsonized targets.

Cbl proteins are known to act, upon tyrosin phosphorylation, as ubiquitin ligases driving the ubiquitination of several molecules and their sorting to lysosomes for degradation (Molfetta et al., 2014). Based on these evidences, we focused our interest on Cbl ligases. The analysis of c-Cbl and Cbl-b tyrosine phosphorylation levels did not reveal major differences in rituximab- and obinutuzumab-stimulated NK cells (Fig.23C).

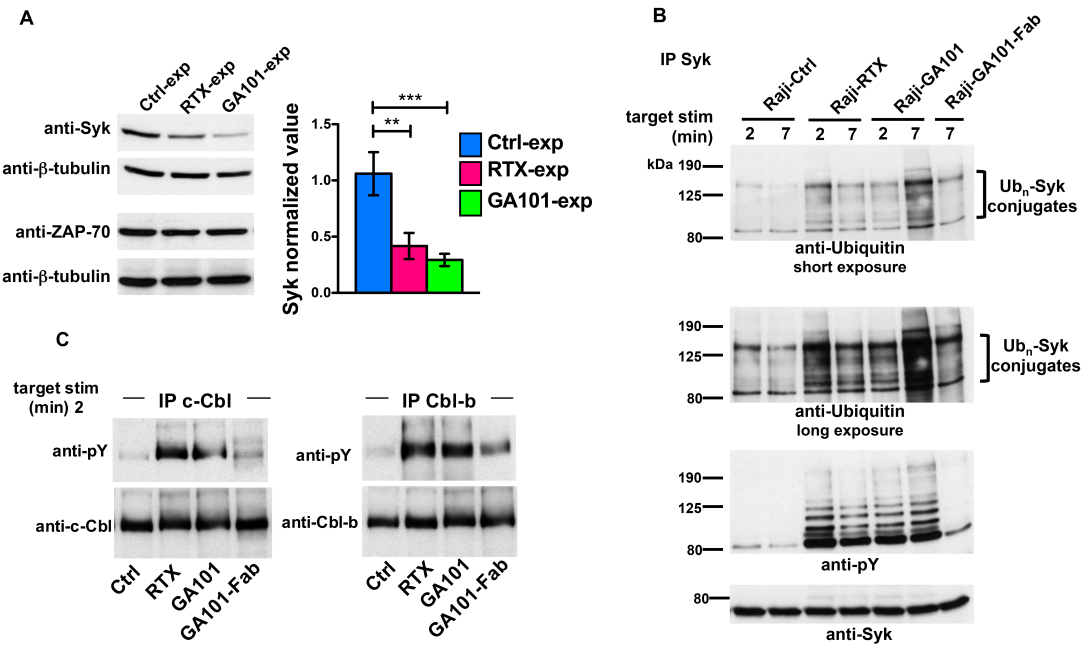


Figure 23. CD16 engagement by anti-CD20-opsonized targets promotes Syk kinase ubiquitination and the tyrosine phosphorylation of c-Cbl and Cbl-b ligases. **(A)** Primary cultured NK cells from V/F and V/V individuals were isolated upon 18 hours co-culture (2:1) with biotinylated rituximab (RTX-exp)-, obinutuzumab (GA101-exp)-opsonized or not opsonized Raji (Ctrl-exp). (*Left panel*) An equal amount of proteins from whole cell lysates was immunoblotted with anti-Syk or anti-ZAP-70 followed by anti-β-tubulin mAbs as indicated. One representative experiment is shown. (*Right panel*) Syk protein amounts were calculated by normalizing to the levels of β-tubulin. Data (mean ± SEM) from 5 independent experiments are depicted in bar graph. $^{**}p < 0.005$, $^{***}p < 0.0005$. **(B,C)** Primary cultured NK cells from V/F individuals were allowed to interact (2:1) with rituximab (Raji-RTX)-, obinutuzumab (Raji-GA101)-, obinutuzumab Fab fragment (Raji-GA101-Fab)-opsonized or not opsonized (Raji-Ctrl) fixed Raji for the indicated times. **(B)** Syk immunoprecipitates were run on the 8% SDS-PAGE and sequentially immunoblotted with anti-Syk followed by anti-ubiquitin, and, after stripping, with anti-phosphotyrosine (pY) mAbs, as indicated. The position of molecular weight markers is reported. For ubiquitin analysis, different times of exposure of the same membrane are shown. One representative of 3 independent experiments is shown. **(C)** Cell lysates were splitted and immunoprecipitated with anti-c-Cbl or anti-Cbl-b Abs. Immunoprecipitates were run on 8% SDS-PAGE and immunoblotted with the indicated Abs. One representative of 3 independent experiments is shown.

Overall our results demonstrate that the affinity ligation conditions dictate the ability of anti-CD20 mAbs to induce CD16 internalization and lysosomal degradation of receptor-associated signaling molecules with FcεRIγ chain and Syk kinase being more susceptible to degradative event. Further, while in intermediate/high affinity donors both rituximab and obinutuzumab behave similarly in inducing the degradation of FcεRIγ and, at lower degree, of CD3ζ chain and Syk kinase, in low affinity donors only obinutuzumab activates a degradative pathway selectively involving FcεRIγ chain.

4. NK cell interaction with anti-CD20-opsonized targets results in the impairment of NKp46 and NKp30-dependent cytotoxic response

Several evidences demonstrated that the exhaustion of the NK cell cytolytic potential may contribute to the resistance to anti-CD20 mAb-based immunotherapy, thus limiting the clinical efficacy in patients with hematological malignancies (*Berdeja et al., 2007; Hatjiharissi et al., 2007; Taylor & Lindorfer, 2010; Veeramani et al., 2011; Capuano et al., 2015*). In this regards, we assessed whether the anti-CD20-induced CD16 receptor complex internalization and degradation, leading to reduced cellular levels of FcεRIγ, CD3ζ chains and Syk kinase, could impact on NK cytolytic potential. Besides ADCC, NK cells also mediate the spontaneous/natural cytotoxicity which is regulated by a balance of signals derived from the aggregation of multiple inhibitory and activating receptors and which requires the simultaneous engagement of different activating receptors by the ligands expressed by target cells (*Lanier, 2008; Long et al., 2013*). With the aim to explore the ability of ITAM-dependent or -independent activating receptors to induce cytotoxicity, experienced NK cells, derived from intermediate (V/F) or high (V/V) affinity donors, were tested in a standard chromium-release based redirected killing assay in the presence of anti-CD16, anti-NKp46, anti-NKp30 or anti-NKG2D mAbs. As control population we used NK cells isolated from not opsonized targets. As expected, on the basis of the reduced expression levels of CD16, we observed that in anti-CD20 experienced NK cells CD16-dependent killing (reverse ADCC) was strongly compromised (Fig. 24A). Moreover, we also noted that both rituximab- and obinutuzumab-experienced NK cells exhibited a significant impairment of the killing activity induced by NKp46 and NKp30 (Fig. 24A), which share with CD16 the CD3ζ and FcεRIγ chains (*Kruse et al., 2014*). Conversely, the statistical analysis of lytic units (LU) reported in Fig. 24A showed that in anti-CD20 experienced NK cells the killing activity induced by NKG2D, which signals through DAP10 molecular adaptor (*Long et al., 2013*), was not affected. Finally, data obtained by cytofluorimetric analysis highlighted a partial decrease of NKp46 surface levels in anti-CD20 experienced NK cells (Fig. 24A), thus confirming a non-redundant role for CD3ζ and FcεRIγ chains as molecular chaperones for the surface expression of this receptor.

To confirm these functional data, we explored the ability of experienced NK cells to secrete lytic granules in response to activating receptor stimulation. To this end, we evaluated, by flow cytometry, the CD107a surface expression levels in experienced NK cells stimulated for 4 hours with plastic-immobilized mAbs specific for different activating receptors, alone or in combination. As shown in Fig. 24B, we found that the degranulation induced by CD16 or NKp46 is also strongly compromised in anti-CD20 experienced NK cells. No differences with respect to the control population were observed following stimulation of NKG2D and 2B4, all CD3ζ- and FcεRIγ-independent activating receptors, which are able to induce degranulation only when engaged in paired combinations (*Bryceson et al., 2006*).

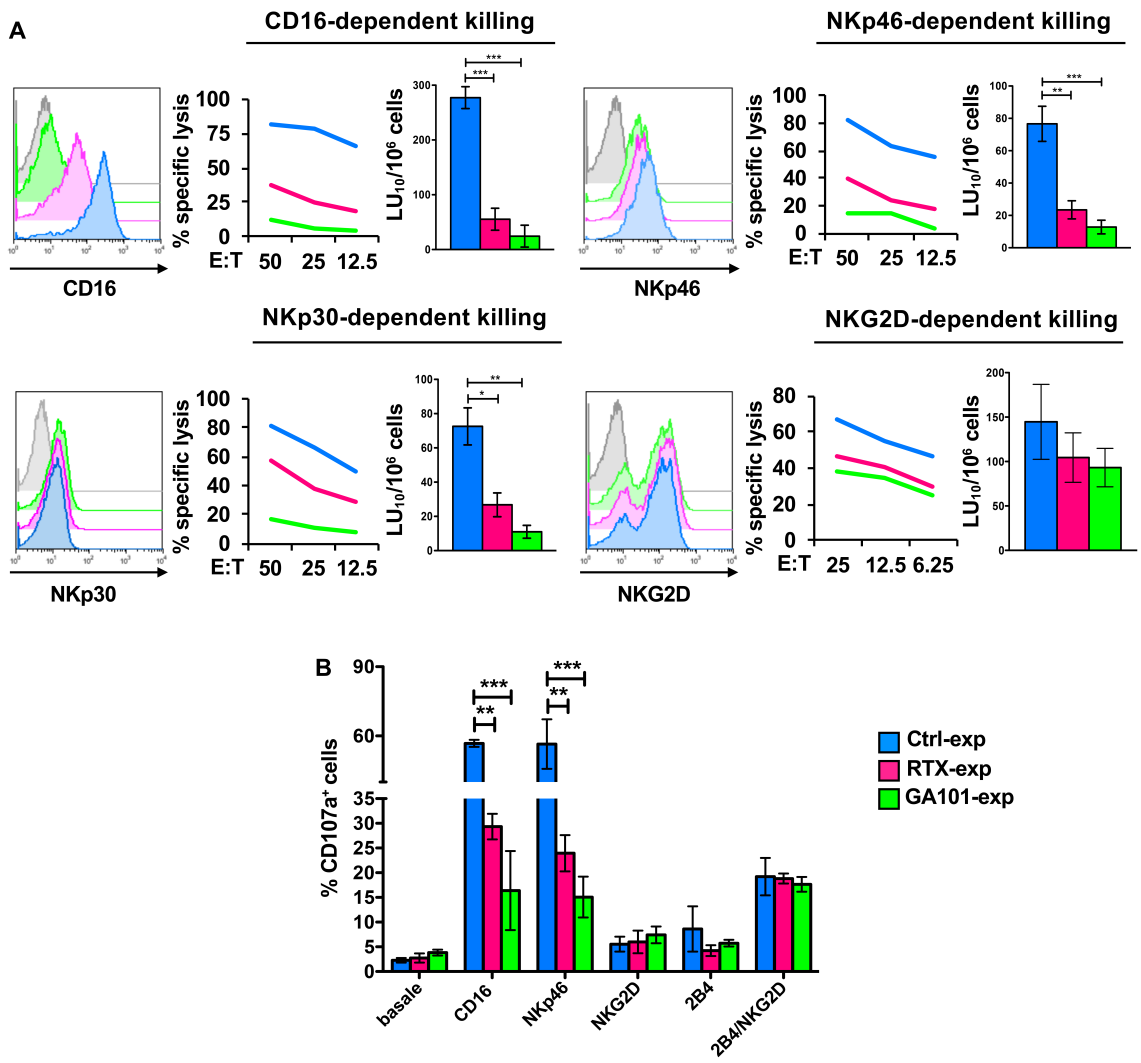


Figure 24. CD16 engagement by anti-CD20-opsonized targets results in the impairment of FcεRIγ- and CD3ζ-dependent NKp46- and NKp30-mediated killing. (A) Primary cultured NK cells from V/F and V/V individuals (n=5) were isolated upon 18 hours co-culture (2:1) with biotinylated rituximab (RTX-exp)-, obinutuzumab (GA101-exp)-opsonized or not opsonized Raji (Ctrl-exp). Cells were stained as indicated for FACS analysis and tested in ^{51}Cr-release redirected killing assays toward P815 FcR^{+} cells in presence of Abs for the indicated receptors. Histogram overlays and the specific lysis from one representative experiment of 5 performed are shown. Grey histogram represents isotype control Ab. Lytic units (LU) from 5 independent experiments are shown. Bar graphs depict mean ± SEM. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$. **(B)** NK cells as in **A** were stimulated for 4 hours with the indicated plastic-immobilized mAbs. The percentage of CD107a^{+} cells was evaluated by FACS analysis gating on CD56^{+} cells. Data (mean ± SEM) from 3 independent experiments are shown. **$p < 0.01$, ***$p < 0.001$.

We also assessed the cytotoxic activity of experienced NK cells derived from low (FF) affinity donors. In line with the lack of CD16 adaptor degradation in response to rituximab reported in Fig. 8B and C, we observed that the killing activity induced by NKp46 (Fig. 25A) and NKp30 (Fig. 25B) was less compromised in rituximab- than in obinutuzumuab-experienced NK cells.

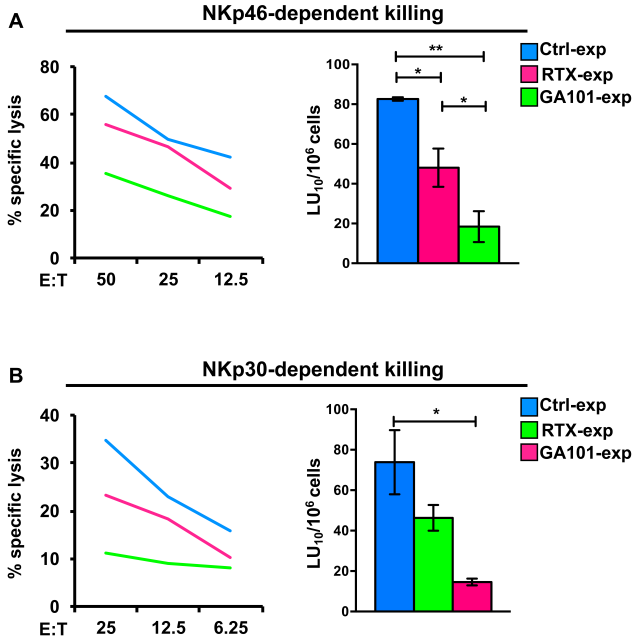


Figure 25. NKp46- and NKp30-mediated killing in anti-CD20 experienced cells from F/F individuals. Primary cultured NK cells from F/F individuals were isolated upon 18 hours co-culture (2:1) with biotinylated rituximab (RTX-exp)-, obinutuzumab (GA101-exp)-opsonized or not opsonized Raji (Ctrl-exp). Cells were tested in ^{51}Cr-release redirected killing assays toward P815 FcR^{+} cells in presence of anti-NKp46 (A) or anti-NKp30 (B) mAbs. (*Left panels*) The percentage of specific lysis from one representative experiment of 3 performed is shown. (*Right panels*) Lytic units (LU) from 3 independent experiments are shown. Bar graphs depict mean ± SEM. *$p < 0.05$, **$p < 0.005$.

Taken together these data provide evidences that anti-CD20-induced CD16 receptor complex internalization and degradation promotes a hyporesponsive status of NK cells by inducing cross-tolerance of the CD3ζ- and FcεRIγ-dependent, but not independent, activating receptors.

5. Obinutuzumab-experienced NK cells are primed for IFN-γ production

Several studies demonstrated that cytotoxic activity and cytokine production can be uncoupled in NK cells (*Rajasekaran et al., 2013*). In this regards, we explored whether anti-CD20-induced internalization and degradation of CD16 receptor complex could impact on IFN-γ producing potential. To this end, we evaluated the ability of experienced NK cells, from intermediate (VF) or high (VV) affinity donors, to secrete IFN-γ in response to different stimuli. Experienced NK cells, which were maintained in culture for 12 hours upon target detachment to allow a partial CD16 re-expression, were stimulated for 18 hours. As control population we used NK cells isolated from not opsonized targets. In spite of the lower CD16 expression levels observed in obinutuzumab-experienced NK cells (Fig. 26A, yellow highlight) we found that they efficiently released IFN-γ in response to obinutuzumab re-stimulation (Fig. 26B, blue highlight) behaving similarly to control and rituximab-experienced NK cells. On the opposite CD16 re-stimulation by rituximab was ineffective in inducing IFN-γ release in anti-CD20 experienced NK cells (Fig. 26B). Importantly, when we evaluated the ability of experienced NK cells to repond to cytokine stimulation, we observed that IFN-γ production induced by IL-12 or IL-2 treatment was substantially higher in obinutuzumab- with respect to control or rituximab-experienced NK cells (Fig. 26C, red highlight). Further, when we evaluated the ability of experienced NK cells to respond to target cells, we found that IFN-γ production induced by Raji cells was increased in obinutuzumab- but not in rituximab-experienced NK cells, with respect to the control population (Fig. 26B, green highlight), thus indicating that obinutuzumab-experienced NK cells were more prone to respond to the activating receptors engaged by target-expressed ligands (*Joyce et al., 2011*).

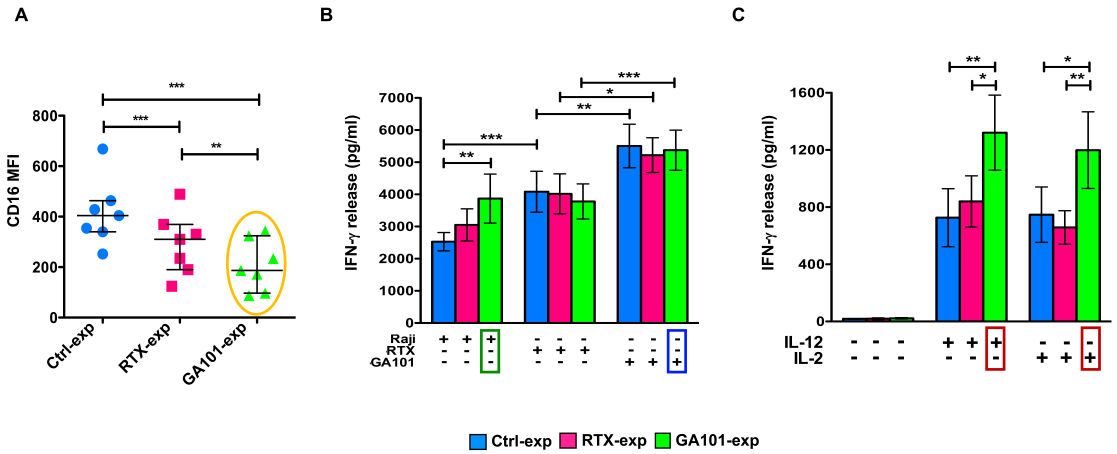


Figure 26. Obinutuzumab-experienced NK cells exhibit an enhanced IFN-γ production in response to cytokines, targets or obinutuzumab re-stimulation. Primary cultured NK cells were isolated upon 90 minute co-culture (2:1) with biotinylated rituximab (RTX-exp)-, obinutuzumab (GA101-exp)-opsonized or not opsonized Raji (Ctrl-exp) and re-plated for 12 hours. **(A)** NK cells were stained with anti-CD16 (Leu11c) mAb for FACS analysis. Graph depicts CD16 MFI and data are presented as median with the interquartile range. $**p < 0.01$, $***p < 0.0005$. NK cells were **(B)** re-stimulated (2:1) with not opsonized targets (Raji), rituximab-opsonized (Raji-RTX) or obinutuzumab-opsonized (Raji-GA101) target cells in the presence of IL-12 as well as **(C)** were left alone (baseline) or treated with IL-2 (100 U/ml) or IL-12 (10 ng/ml). After 18 hours, supernatants were collected and assessed for IFN-γ levels. Data are presented as mean ± SEM of 7 independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.0001$. Compared to baseline or Raji-Ctrl samples, all the differences were statistically significant ($p < 0.001$).

Overall these results highlight that obinutuzumab-experienced NK cells exhibit an enhanced IFN-γ production in response to cytokines or Raji target cells as well as in response to obinutuzumab-mediated CD16 re-stimulation.

6. Identification of "*memory*" NK cells in individuals grouped by HCMV serological status

Data reported here have shown that the sustained interaction of NK cells with anti-CD20 opsonized targets leads to the down-regulation of FcεRIγ chain and Syk kinase that may be associated with an enhanced ability to produce IFN-γ in response to CD16 re-stimulation. Relaying on the observation that such molecular and functional characteristics resemble the distinctive features of the recently identified long-lived and highly functional "*memory*" NK cells (*Zhang et al., 2013; Lee et al., 2015; Schlums et al., 2015; Cerwenka & Lanier, 2016*) we assessed the capability of tumor targeting anti-CD20 mAbs to drive the expansion and to affect the phenotypic and functional properties of this specific NK subset. We firstly evaluated the frequency (%) of "*memory*" NK cells in a cohort of 218 healthy donors. As illustrated in Fig. 27A, "*memory*" NK cells were identified by multicolour flow cytometry as $CD3^{-}CD56^{+}Fc\varepsilon RI\gamma^{-}CD16^{+}$ lymphocytes. As reported in Fig. 27B, we observed that 112 out of 218 analysed donors exhibited a detectable population of "*memory*" NK cells accounting for 3 to 50% of peripheral blood NK cells.

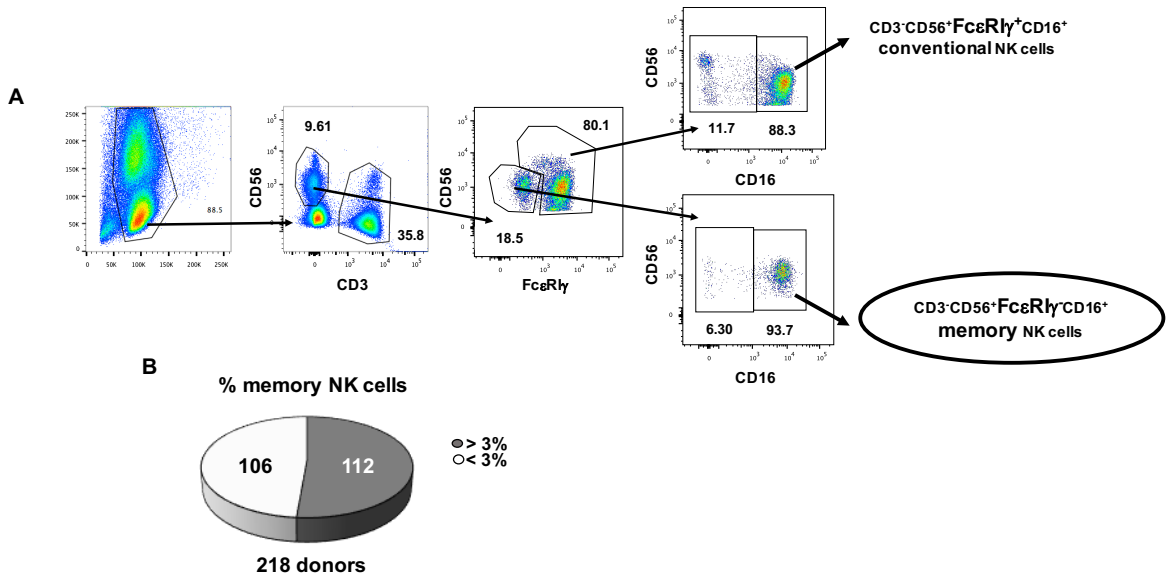


Fig. 27. Identification of "*memory*" NK cells in the peripheral blood of healthy donors. The frequency (%) of $Fc\varepsilon RI\gamma^{-}$ $CD16^{+}$ NK cells was evaluated by multicolour flow cytometry gating on $CD3^{-}CD56^{+}$ population in freshly isolated PBMCs from 218 healthy donors. **(A)** Plots from one representative donor and the gating strategy used for determining the proportion of "*memory*" ($CD3^{-}CD56^{+}Fc\varepsilon RI\gamma^{-}CD16^{+}$) NK cells are shown. **(B)** The distribution of the individuals with a detectable "*memory*" NK population accounting for more than 3% of peripheral blood NK cells is depicted in the pie chart.

Several studies demonstrated a strong correlation between "*memory*" NK cells and prior exposure to human cytomegalovirus (HCMV) (*Hwang et al., 2012; Zhang et al., 2013*; *Lee et al., 2015, Schlums et al., 2015; Zhou et al., 2015; Kovalenko et al., 2017*). By evaluating the HCMV seropositivity within a cohort of healthy donors, we observed that 87 out of 118 analysed donors were seropositive for IgG anti-HCMV (Fig. 28A) and that 63.5% of HCMV seropositive individuals exhibited a detectable "*memory*" NK population while almost none (0.06%) of seronegative donors showed such population (Fig. 28B). By contrast, the HCMV serological status did not affect the frequency (%) of NK cells, identified as $CD3^{-}CD56^{+}$ lymphocytes (Fig. 28B).

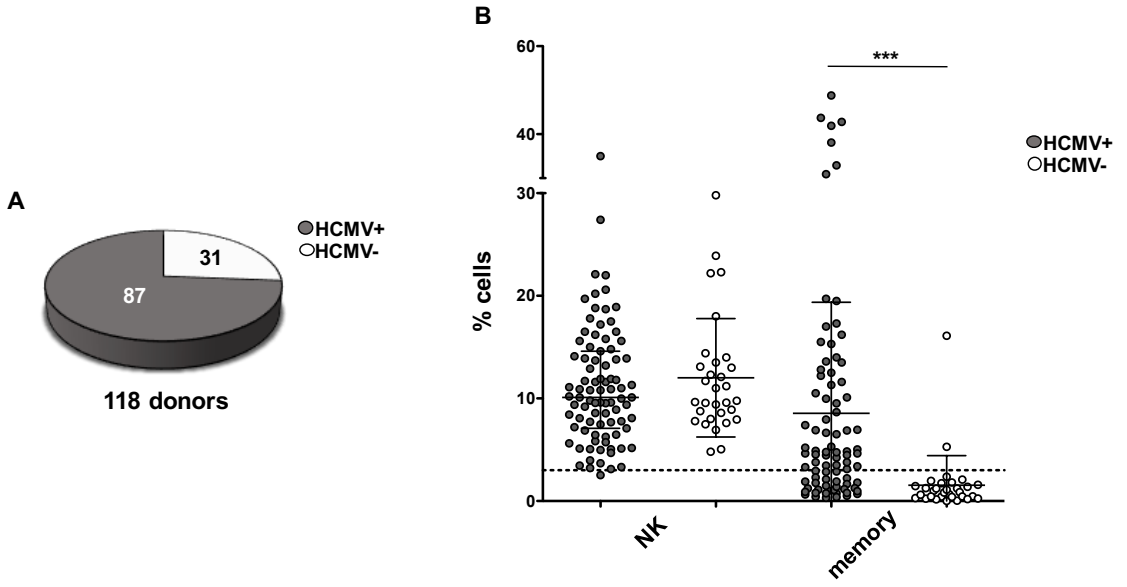


Fig. 28. Only HCMV seropositive individuals exhibit *ex vivo* a "*memory*" NK cell population. A cohort of 118 healthy donors was screened for the serological status for HCMV. **(A)** The distribution of the HCMV seropositive or seronegative individuals, observed in our cohort, is depicted in the pie chart. **(B)** The frequency (%) of $CD3^{-}CD56^{+}$ (NK) and $CD3^{-}CD56^{+}FcεRIγ^{-}CD16^{+}$ (*memory*) cells was evaluated by multicolour flow cytometry in freshly isolated PBMCs. Data are presented as median with interquartile range. Each point represents a single donor. The dashed line indicates the arbitrary cut-off value of 3%. ***$p < 0.0001$.

7. Anti-CD20-dependent *in vitro* expansion of "*memory*" NK cells

It has been reported that "*memory*" NK cells may be expanded *in vitro* in an antibody-dependent manner in response to HCMV or influenza virus-infected cells (*Lee et al., 2015; Schlums et al., 2015*). Based on such evidences, we addressed whether the stimulation of NK cells by means of rituximab- or obinutuzumab-coated tumor B cells may support the in *vitro* expansion of "*memory*" ($CD3^-CD56^+Fc\varepsilon RI\gamma^-CD16^+$) or "*conventional*" ($CD3^-CD56^+Fc\varepsilon RI\gamma^+CD16^+$) NK subsets. To this end, we set up the experimental system illustrated in Fig. 29, that we used for all the experiments described below. Freshly isolated PBMCs from $HCMV^+$ donors were screened, by multicolour flow cytometry, to select individuals with a detectable and well-defined $Fc\varepsilon RI\gamma^-$ $CD16^+$ population among $CD3^-$ $CD56^+$ lymphocytes. PBMCs were cultured for 10 days in the presence of IL-2. At day 3, irradiated Raji cells were added (2:1) in the absence or presence of rituximab or obinutuzumab. At the end of the co-cultures (day 10), cells were analysed by multicolour flow cytometry.

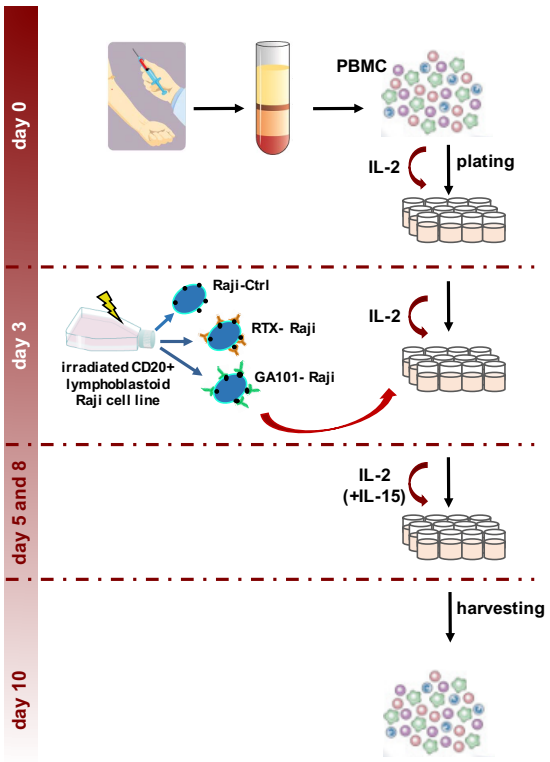


Fig. 29. Experimental setting for anti-CD20-induced *in vitro* expansion of "*memory*" NK subset.

As reported in Fig. 30, we observed that the frequency (%) and the absolute number of NK ($CD3^-CD56^+$) cells increased at the end of 10-day co-cultures, thus indicating that our experimental conditions were efficient in driving the selective expansion of NK cells. In particular, NK cells significantly and comparably expanded in response to not opsonized or anti-CD20 opsonized targets, thus demonstrating that it was largely independent from anti-CD20-mediated CD16 stimulation.

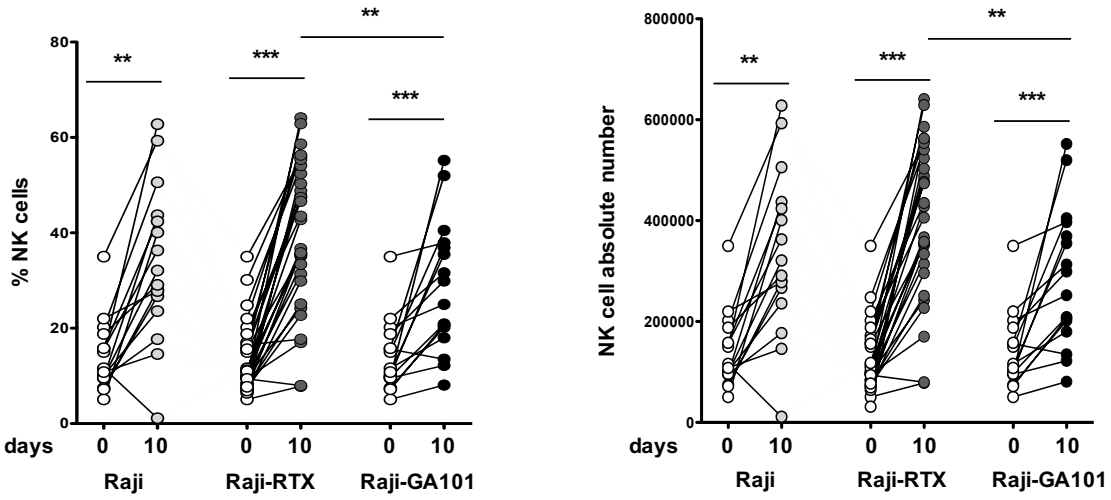


Fig. 30. 10 day PBMC co-colture with Raji cells promotes NK cell expansion in an antibody-independent manner. Freshly isolated PBMCs were seeded in 96-well plates, in the presence of 100 U/ml IL-2, and co-cultured for 10 days with irradiated rituximab- (Raji-RTX), obinutuzumab (Raji-GA101)-opsonized or not opsonized (Raji) target cells (E:T ratio 2:1). The frequency (%) of NK cells ($CD3^-CD56^+$) was assessed by multicolour flow cytometry before (day 0) and at the end (day 10) of co-coltures. For each time point, the absolute number/10^6 of NK cells was calculated as follows: (percentage of NK cells) x $10^6/100$. Data from 30 different donors are shown. Each line represents a single donor. *$p < 0.005$, ***$p < 0.001$.

When we analysed "*memory*" vs "*conventional*" NK cells, we observed that "*memory*" NK cells underwent a significant expansion only in response to anti-CD20-mediated CD16 aggregation (Fig. 31C). In fact, only in the presence of anti-CD20 mAbs we observed a marked increase of their absolute number. We also noted that rituximab was more efficient in driving "*memory*" NK expansion than obinutuzumab (Fig. 31C).
By contrast, the expansion of "*conventional*" NK cells was largely independent from anti-CD20-mediated CD16 aggregation (Fig. 31B).

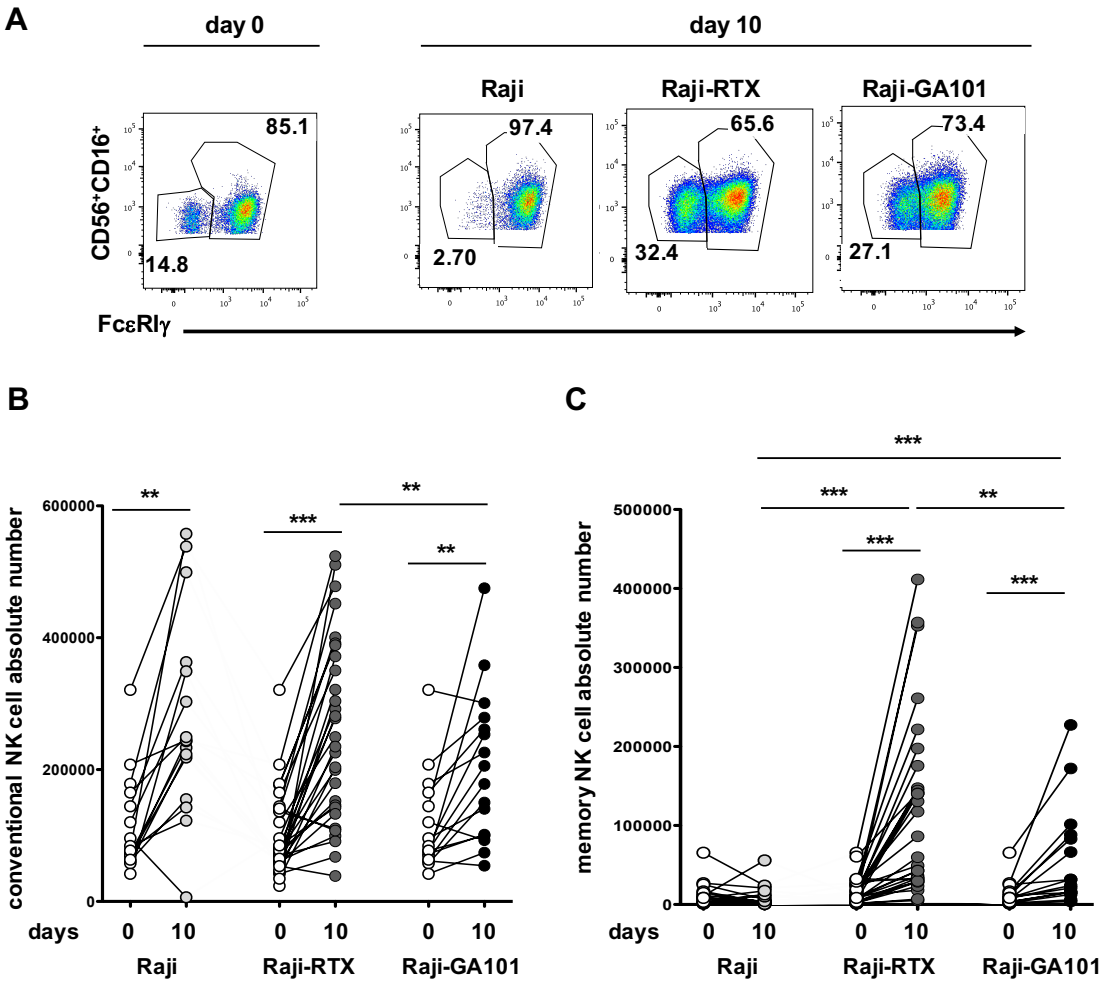


Fig. 31. "*Memory*" but not "*conventional*" NK cells undergo *in vitro* expansion in an anti-CD20-dependent manner. Freshly isolated PBMCs were seeded in 96-well plates, in the presence of 100 U/ml IL-2, and co-cultured for 10 days with irradiated rituximab- (Raji-RTX), obinutuzumab (Raji-GA101)-opsonized or not opsonized (Raji) target cells (E:T ratio 2:1). Cells were assessed by multicolour flow cytometry before (day 0) and at the end (day 10) of co-coltures. **(A)** Plots from one representative donor are shown. **(B-C)** Graphs depict the absolute number/10^6 of **(B)** "*conventional*" ($CD3^-CD56^+Fc\varepsilon RI\gamma^+CD16^+$) and **(C)** "*memory*" ($CD3^-CD56^+Fc\varepsilon RI\gamma^-CD16^+$) NK subsets calculated as follows: (percentage of "*conventional*" or "*memory*" NK cells) x (absolute number/10^6 of NK cells)/100. Data from 30 different donors are shown. Each line represents a single donor. $^{**}p < 0.005$, $^{***}p < 0.0005$.

Based on these results, we investigated whether anti-CD20-dependent expansion of "*memory*" NK cells may also occur in HCMV seronegative donors. To this end, PBMCs derived from HCMV$^-$ individuals, in which as shown in Fig. 28 the frequency (%) of "*memory*" NK cells was lower than 3%, were co-cultured as above described and analysed by multicolour flow cytometry upon 10 days. At the end of co-culture, no expansion of "*memory*" NK cells was detected (Fig. 32B), in front of a marked proliferation of "*conventional*" NK cells (Fig. 32A), thus suggesting that only HCMV *in vivo* primed "*memory*" NK cells may undergo anti-CD20-dependent *in vitro* expansion.

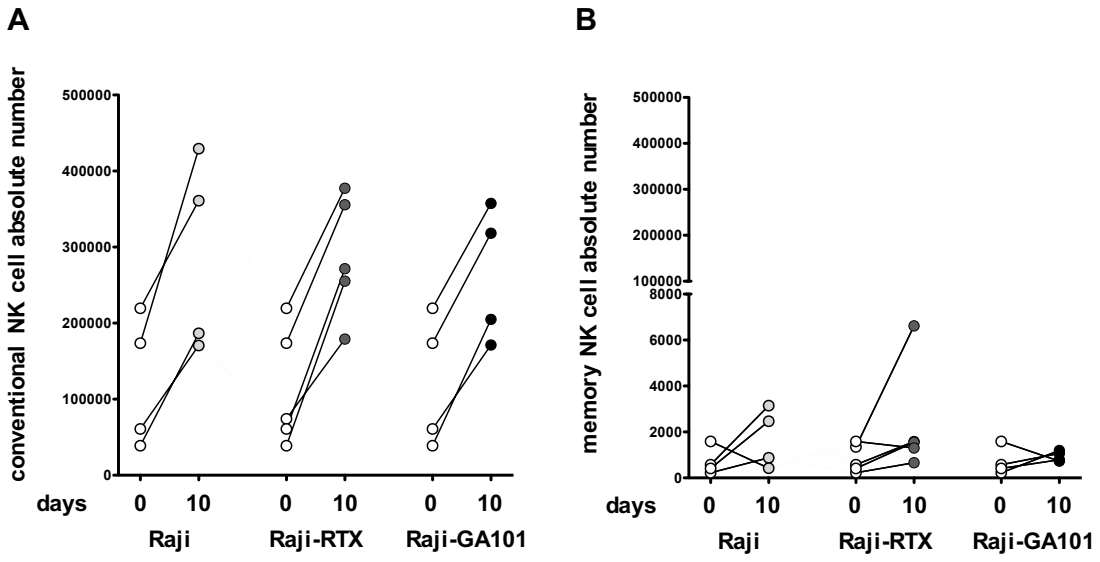


Fig. 32. Anti-CD20-driven *in vitro* expansion of "*memory*" NK cells may require the *in vivo* priming induced by HCMV infection. PBMC suspensions, freshly isolated from HCMV seronegative donors, were seeded in 96-well plates, in the presence of 100 U/ml IL-2, and co-cultured for 10 days with irradiated rituximab- (Raji-RTX), obinutuzumab (Raji-GA101)-opsonized or not opsonized (Raji) target cells (E:T ratio 2:1). Cells were assessed by multicolour flow cytometry before (day 0) and at the end (day 10) of co-cultures. Graphs depict the absolute number/10^6 of (**A**) "*conventional*" ($CD3^-CD56^+Fc\varepsilon RI\gamma^+CD16^+$) and (**B***)* "*memory*" ($CD3^-CD56^+Fc\varepsilon RI\gamma^-CD16^+$) NK subsets calculated as follows: (percentage of "*conventional*" or "*memory*" NK cells) x (absolute number/10^6 of NK cells) /100. Data from different donors are shown. Each line represents a single donor.

8. *In vitro* expanded "*memory*" NK cells maintain the phenotypic profile of their freshly isolated counterpart

It has been reported that "*memory*" NK cells display a unique receptor repertoire (*Hwang et al., 2012; Zhang et al., 2013*; *Lee et al., 2015; Schlums et al., 2015; Zhou et al., 2015; Kovalenko et al., 2017*). Herein, we analysed by multicolour flow cytometry the phenotypic profile of "*conventional*" and "*memory*" NK cells in both *ex vivo* (Fig.33A) and *in vitro* expanded (Fig.33B) settings. In line with literature, we observed that freshly isolated "*memory*" NK cells exhibited a higher frequency of NKG2C receptor along with reduced levels of CD16 and lower frequency of NKp46 receptor, with respect to "*conventional*" ones. Of note, such phenotypical features were maintained in *in vitro* expanded populations.

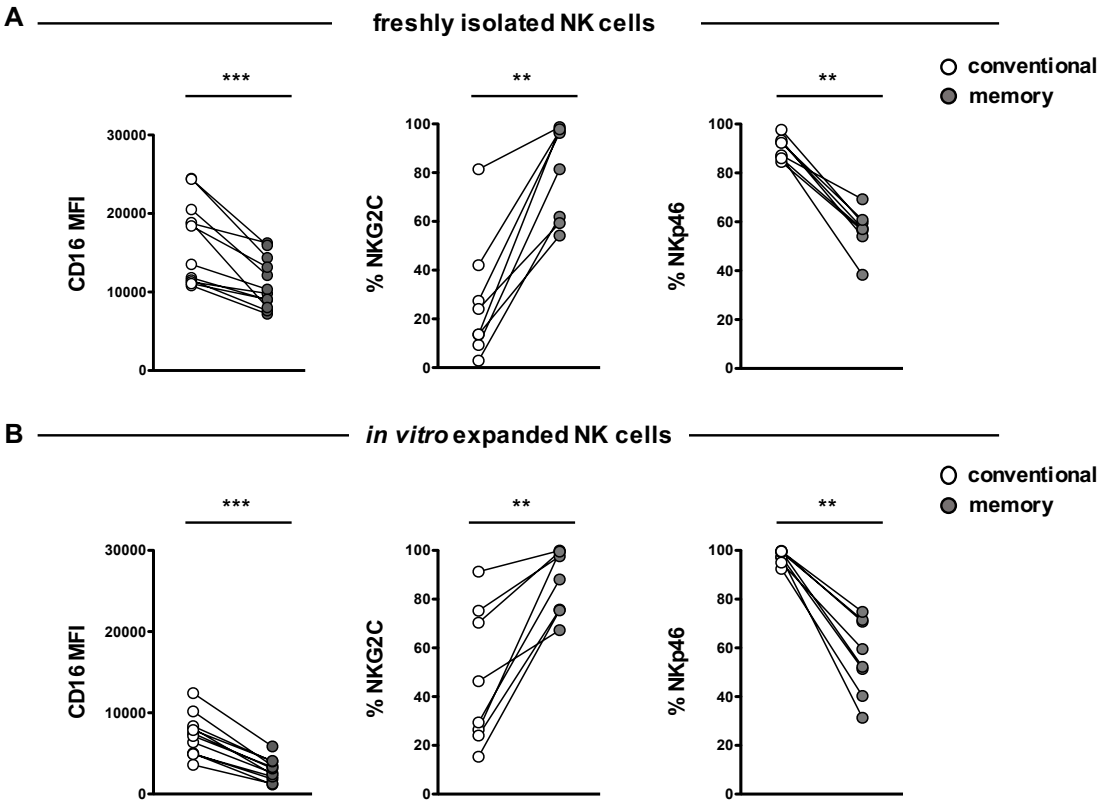


Fig. 33. Phenotypic profile of freshly isolated and *in vitro* expanded "*conventional*" and "*memory*" NK cells. Freshly isolated PBMCs were seeded in 96-well plates, in the presence of 100 U/ml IL-2, and co-cultured for 10 days with irradiated rituximab-opsonized (Raji-RTX) target cells (E:T ratio 2:1). The surface levels of CD16, NKG2A or NKp46 were assessed by multicolour flow cytometry on "*conventional*" ($CD3^-CD56^+Fc\varepsilon RI\gamma^+CD16^+$) and "*memory*" ($CD3^-CD56^+Fc\varepsilon RI\gamma^-CD16^+$) NK cell subsets **(A)** before and **(B)** at the end of co-coltures. Data from different donors are shown. Each line represents a single donor. $^{**}p < 0.01$, $^{***}p < 0.0005$.

9. *In vitro* expanded "*memory*" NK cells exhibit enhanced functional responses to CD16 re-stimulation

It has been reported that "*memory*" NK cells significantly differ from "*conventional*" ones for their functional capabilities (*Lee et al., 2015; Schlums et al., 2015; Kovalenko et al., 2017*). In fact, recent reports demonstrated that, despite a poor responsivity toward tumor targets, "*memory*" NK cells exhibit an enhanced functional activity in response to CD16 stimulation, particularly in terms of IFN-γ and TNF-α production (*Hwang et al., 2012; Zhang et al., 2013; Lee et al., 2015; Zhou et al., 2015; Kovalenko et al., 2017*). Herein, we characterized the functional profile of *in vitro* expanded "*memory*" NK cells with respect to "*conventional*" ones as well as to their freshly isolated counterpart. To this end, freshly isolated (Fig. 34A-B) or 10-day co-cultured (Fig. 34C-D) NK cells were stimulated with anti-CD20 opsonized or not opsonized Raji as well as with K562 targets and assessed by multicolour flow cytometry for IFN-γ production or degranulation.

By evaluating the CD16 responsiveness, we observed that "*memory*" NK cells exhibited an enhanced IFN-γ production, with respect to "*conventional*" ones, in response to both rituximab- and obinutuzumab-opsonized targets in freshly isolated (Fig. 34A-B) as well as *in vitro* expanded (Fig. 34C-D) settings. On the other hand, regarding degranulation, while freshly isolated "*memory*" NK cells showed an increased functional activity in response to both rituximab- and obinutuzumab-coated targets (Fig. 34A-B) *in vitro* expanded "*memory*" NK cells respond better to obinutuzumab (Fig. 34C-D).

By evaluating the direct responsiveness to MHC-I positive Raji tumor target, which is almost resistant to the direct NK lysis, we observed that freshly isolated "*memory*" NK cells exhibited an enhanced ability to degranulate, but not to produce IFN-γ, with respect to "*conventional*" ones (FIG. 34A-B). On the other hand, we noted that *in vitro* expanded "*conventional*" and "*memory*" NK subsets comparably respond to Raji cells (Fig. 34C-D). Our data also revealed a reduced ability of both freshly isolated (Fig. 34A-B) and *in vitro* expanded (Fig. 34C-D) "*memory*" NK cells to respond to K562 tumor target which, being MHC-I negative, efficiently activates functional responses in "*conventional*" NK cells.

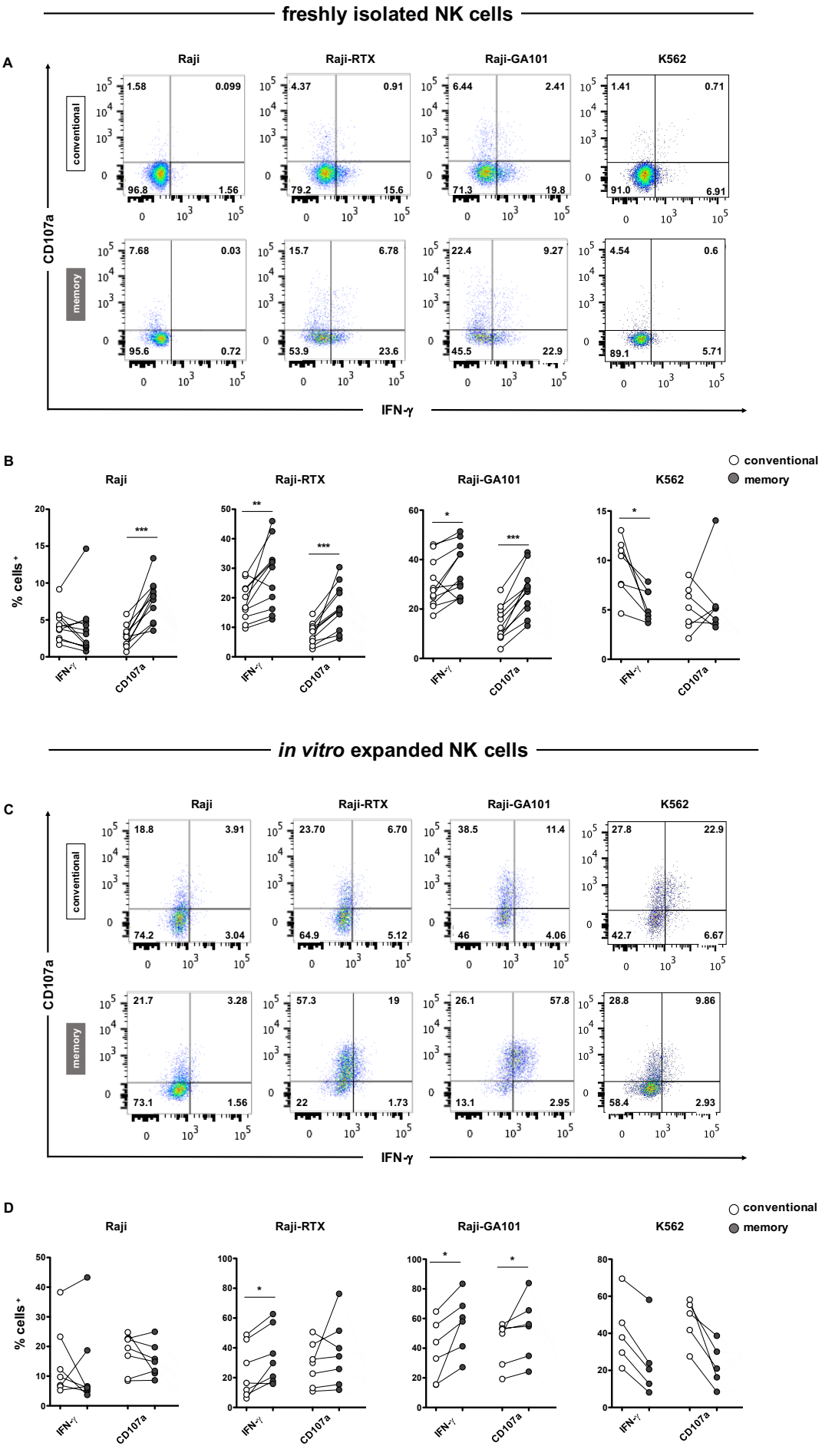

freshly isolated NK cells
A
Raji
Raji-RTX
Raji-GA101
K562
conventional
memory
CD107a
IFN-γ
1.58
0.099
96.8
1.56
4.37
0.91
79.2
15.6
6.44
2.41
71.3
19.8
1.41
0.71
91.0
6.91
7.68
0.03
95.6
0.72
15.7
6.78
53.9
23.6
22.4
9.27
45.5
22.9
4.54
0.6
89.1
5.71
B
conventional
memory
% cells+
IFN-γ
CD107a
*
**
***
in vitro expanded NK cells
C
18.8
3.91
74.2
3.04
23.70
6.70
64.9
5.12
38.5
11.4
46
4.06
27.8
22.9
42.7
6.67
21.7
3.28
73.1
1.56
57.3
19
22
1.73
26.1
57.8
13.1
2.95
28.8
9.86
58.4
2.93
D

Fig. 34. *In vitro* expanded "*memory*" NK cells exhibit an enhanced functional activity in response to CD16 re-stimulation. PBMC suspensions **(A-B)** freshly isolated or **(C-D)** recovered from 10-day co-coltures with irradiated rituximab-opsonized (Raji-RTX) target cells (E:T ratio 2:1) in presence of 100 U/ml IL-2 and 10 ng/ml IL-15 were combined (2:1) with not opsonized or anti-CD20-opsonized Raji cells as well as K562 targets. The percentage (%) of IFN-γ^+ or CD107a$^+$ cells were analysed by multicolour flow cytometry among "*conventional*" ($CD3^-CD56^+Fc\varepsilon RI\gamma^+$) and "*memory*" ($CD3^-CD56^+Fc\varepsilon RI\gamma^-$) NK subsets. **(A, C)** Plots from representative donors are shown. **(B, D)** Graphs depict data from independent experiments. Each line represents a single donor. **p< 0.05, **p< 0.01, ***p< 0.001.*

Looking at the differences between the two anti-CD20 mAbs, we noted that obinutuzumab is more active than rituximab in enhancing IFN-γ production, degranulation as well as multifunctional responses from both "*conventional*" and "*memory*" NK cells in freshly isolated (Fig. 35A) and *in vitro* expanded (Fig. 35B) cellular subsets.

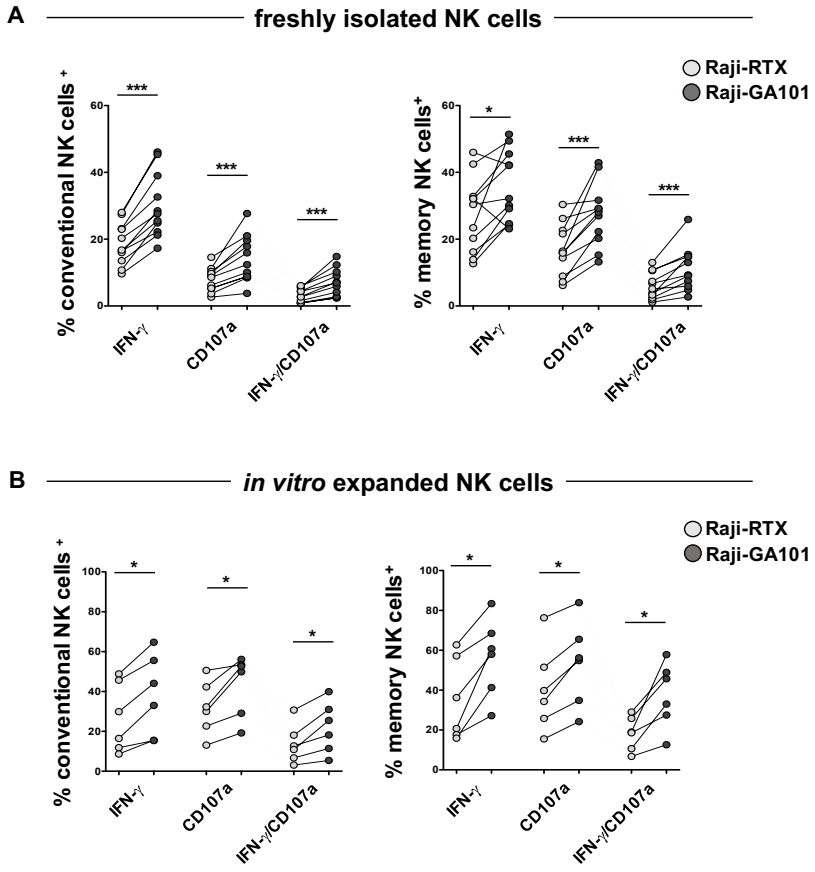


Fig. 35. "*Memory*" NK cells exhibit enhanced functional responses to obinutuzumab with respect to rituximab stimulation. PBMC suspensions **(A)** freshly isolated or **(B)** recovered from 10-day co-coltures with irradiated rituximab-opsonized (Raji-RTX) target cells (E:T ratio 2:1) in presence of 100 U/ml IL-2 and 10 ng/ml IL-15 were combined (2:1) with anti-CD20-opsonized Raji cells. The percentage (%) of IFN-γ^+ and/or CD107a$^+$ cells were analysed by multicolour flow cytometry among "*conventional*" ($CD3^-CD56^+Fc\varepsilon RI\gamma^+$) and "*memory*" ($CD3^-CD56^+Fc\varepsilon RI\gamma^-$) NK subsets. Graphs depict data from independent experiments. Each line represents a single donor. **p< 0.05, ***p< 0.001.*

Taken together these data demonstrate that "*memory*" NK subset may selectively expand *in vitro* in response to anti-CD20-opsonized tumor B cells and that such *in vitro* expanded population shows the phenotypic and functional hallmarks of their freshly isolated counterpart.

Discussion

Anti-CD20 mAbs are widely used in the treatment of B-cell malignancies and autoimmune disorders. Despite anti-CD20-based therapy has substantially changed the natural history of B-cell malignancies a relevant fraction of patients still relapses and becomes resistant to the therapy. For this reason, different strategies, including Fc-mutation or Fc-glycoengeneering, have been designed to enhance the clinical efficacy of anti-CD20 mAbs. Such Fc optimised mAbs include obinutuzumab which is, in fact, the first glycongineered anti-CD20 mAb approved for clinical use (*Klein et al., 2013; Goede et al., 2014*). The defucosylated Fc domain of this mAb substantially increases the affinity for FcγRIIIA/CD16 receptor, thus improving the capability to stimulate NK cells. Of note, although some studies demonstrated that obinutuzumab is more efficient in stimulating NK cell-mediated tumor target cell killing with respect to the reference mAb rituximab (*Mossner et al., 2010; Bologna et al., 2011; Pincetic et al., 2014*), the impact of mAb defucosylation on the ability to induce cytokine production in NK cells remained unexplored. Herein, by comparing the ability of rituximab and obinutuzumab to induce the activation of NK cells, we demonstrated that obinutuzumab, used at ten times lower concentration than rituximab, stimulates NK cell-mediated IFN-γ production more efficiently than rituximab by enhancing both the portion of IFN-γ producing cells and the amount of IFN-γ on a per cell basis. On the other hand, in line with literature, obinutuzumab is more potent than rituximab in stimulating NK degranulation/cytotoxicity. Such increased efficacy of obinutuzumab is attributable to glycoengineering since the non-defucosylated wild type (wt) mAb is less efficient in inducing NK functions. The observation that the wt version has enhanced activity with respect to rituximab indicates that the superiority of obinutuzumab is not exclusively due to differences in the Fc domain, but also to other features that may impact on CD16 stimulation such as the missed ability to induce CD20 surface down-modulation (*Herter et al., 2013; Klein et al., 2013*). It is well-known that the interaction of NK cells with opsonized targets results in both cytotoxicity and cytokine production with a higher activation threshold required for cytokine secretion (*Fauriat et al., 2010; Long et al., 2013*). In line with this notion, we observed that in response to rituximab or obinutuzumab the portion of NK cells that degranulate is higher than the portion of NK cells that produce IFN-γ. Notably, we found that the fraction of NK cells performing both effector functions is higher following obinutuzumab than rituximab stimulation, thus suggesting that the increased binding affinity of obinutuzumab for CD16 allows more cells to reach the

signaling threshold required for cytokine production. Such enhanced IFN-γ production in response to obinutuzumab may explain the cytokine release syndrome observed in treated patients, characterized by high levels of circulating IFN-γ (*Freeman et al., 2015*). Several studies, including ours, reported that CD16 undergoes surface down-modulation in response to anti-CD20-opsonized targets (*Veeramani et al., 2011; Capuano et al., 2015; Cox et al., 2015*) which may result from either receptor shedding (*Romee et al., 2013*) or internalization (*Capuano et al., 2015*). When we compared the ability of rituximab and obinutuzumab to induce CD16 surface down-modulation, we noted that in response to obinutuzumab CD16 undergoes a more rapid, marked and progressive surface down-modulation reaching, upon 18 hours of stimulation, almost 17% of initial levels (vs 40 % in rituximab-stimulated cells). It has been reported that obinutuzumab is endowed with a higher affinity for CD16 regardless from receptor allotype (*Mossner et al., 2010; Herter et al., 2013*). In line with this notion, we found that the interaction with obinutuzumab-opsonized targets leads to a stronger CD16 down-modulation accross all allotypes, whereas rituximab induces only a marginal receptor down-modulation in donors bearing the low affinity CD16 allotype. In low affinity (F/F) donors, obinutuzumab is able to induce a robust degranulation, thus confirming the correlation existing between affinity aggregation conditions, receptor down-modulation and functional responses. The stronger CD16 surface down-modulation observed in obinutuzumab stimulated cells is not in conflict with the enhanced functional responses. To this purpose, it has been recently reported that internalized activating receptors in NK cells can still transduce signals from intracellular compartments before their lysosomal targeting (*Quatrini et al., 2015*). In this regard, we noted that upon interaction with anti-CD20-opsonized targets a fraction of CD16 receptor internalizes and co-localizes with lysosomes. Moreover, we observed that the stimulation of NK cells with anti-CD20-opsonized targets leads to the lysosomal degradation of CD16-coupled signaling molecules with affinity aggregation conditions affecting the strength and the quality of the response. In particular, we found that the cellular levels of CD16-associated FcεRIγ chain are significantly reduced in response to obinutuzumab in all CD16 allotypes, whereas rituximab-mediated stimulation induces FcεRIγ degradation only in intermediate/high affinity donors. On the other hand, CD3ζ chain is more marginally affected by degradation. Such different behaviour between CD16-associated chains may be due to the greater amount of γ/γ homodimers than γ/ζ heterodimers or ζ/ζ homodimers coupled to CD16 receptor in human NK cells (*Letourneur et al., 1991*). Since it is well-known that upon receptor engagement the ITAM

sequences of FcεRIγ and CD3ζ chains undergo tyrosine phosphorylation followed by the recruitment and activation of Syk and ZAP-70 tyrosine kinases, we investigated whether anti-CD20-activated degradative pathway could involve other CD16-coupled signaling molecules. We observed that in intermediate/high affinity donors the stimulation of NK cells with anti-CD20-opsonized targets leads to the selective degradation of Syk kinase, likely by an ubiquitin-dependent pathway. The reduced cellular levels of FcεRIγ and CD3ζ chains as well as of Syk kinase, heavily impact on NK functions. Indeed, we found a significant impairment of NK cell ability to kill targets via CD16 as well as NKp46 and NKp30 activating receptors, which share FcεRIγ and CD3ζ chains with CD16 (*Kruse et al., 2014*). The impaired cytotoxic activity of both rituximab- and obinutuzumab-experienced NK cells shows that the residual levels of CD3ζ chain are not sufficient to allow the optimal signals required for degranulation. Indeed, the defective cytotoxicity observed in F/F donors, where obinutuzumab selectively induces the degradation of FcεRIγ, indicates a non-redundant role for such molecule in ITAM receptor-dependent killing which may be explained by a different coupling of CD3ζ and FcεRIγ chains to downstream signaling elements (*Galandrini et al., 1997*). The observation that in F/F donors rituximab-experienced NK cells exhibit a significant defect of NKp46- and NKp30-mediated cytotoxicity despite the lack of CD16 adaptor degradation is not very surprising; in fact, in such low affinity aggregation conditions receptor-driven inhibitory signals may occur. In this regard, we have recently demonstrated that aggregation of CD16 in low affinity conditions, obtained by rituximab, results in NK cell hyporesponsiveness due to the activation of Shp-1 tyrosine phosphatase (*Capuano et al., 2015*). Such observation is in line with the emerging paradigm considering that the targeting of some classically activating ITAM-bearing FcRs with low affinity ligands parodoxically trasmits inhibitory signals through the recruitment of Shp-1 leading to the inhibition of a number of innate immune responses (*Aloulou et al., 2012*). On the other hand, the down-regulation of CD16-coupled signaling elements in obinutuzumab-experienced NK cells is associated with an enhanced ability to produce IFN-γ thus indicating that the aggregation of CD16 in high affinity conditions may prime NK cells to a reduced signaling threshold leading to enhanced effector functions. Moreover, we observed that, while anti-CD20-experienced NK cells appears desensitized to a subsequent rituximab-mediated CD16 re-stimulation, they efficiently respond to obinutuzumab re-stimulation. Such response is also observed in obinutuzumab-experienced NK cells despite the lower residual CD16 surface expression. Such enhanced response, independent from CD16 levels, may be likely due

to the favoured coupling of CD16 receptor to CD3ζ chain that leads to more robust and efficient biochemical signals given the quantitative differences in ITAM motifs (3 ITAM in CD3ζ vs 1 ITAM in FcεRIγ). Moreover, the residual CD3ζ levels observed in anti-CD20 experienced NK cells may also preserve the co-stimulatory activity of CD2/CD58 interaction during target cell contact (*Grier et al., 2012*). Indeed, obinutuzumab-experienced NK cells exhibit an enhanced IFN-γ production in response to Raji cell stimulation. The identification of the molecular mechanisms underlying the enhanced IFN-γ production observed in obinutuzumab-experienced NK cells requires further investigation. In particular, we are currently evaluating whether post-trascriptional as well as epigenetic or signaling modifications may account for the primed status of NK cells dictated by the high affinity CD16 ligation conditions. Overall, our data demonstrate that CD16 ligation in optimised affinity aggregation conditions, by means of the Fc-glycoengineered anti-CD20 mAb obinutuzumab, may shift NK functional program toward cytokine production, thus potentially affecting the clinical response and immuno-competence of anti-CD20 treated patients. Interestingly, it has been recently described the capability of mAb-based therapy to favour a long-lasting protective anti-tumor response through the generation of effector/memory T lymphocytes, the so-called "*vaccinal effect*" (*Abès et al., 2010; DiLillo & Ravetech, 2015*). In this scenario, the role of NK-derived IFN-γ in promoting DC maturation/antigen-presentation functions and the development of adaptive responses has been demonstrated (*Martin-Fontecha et al., 2004; Walzer et al., 2005; Crouse et al., 2015*) thus indicating that, besides short-term cytotoxic properties, NK cells may be relevant actors in promoting a long-term anti-tumor protective immunity in response to mAb-based therapeutic strategies. Our data also highlight that obinutuzumab-experienced NK cells are reminescent, under molecular and functional profile, of the recently identified long-lived and highly fuctional NK population called "*memory*" NK cells, which is in fact defined by the lack of expression of FcεRIγ chain and the ability to produce high amounts of IFN-γ (*Zhang et al., 2013; Lee et al., 2015; Schlums et al., 2015; Cerwenka & Lanier, 2016*). It is known that the presence of "*memory*" NK cells in the peripheral blood of healthy donors is associated with a prior exposure to human cytomegalovirus (HCMV) (*Hwang et al., 2012; Zhang et al., 2013; Lee et al., 2015; Schlums et al., 2015; Kovalenko et al., 2017*) a herpesvirus that establishes a life-long latent infection in the majority of the human population (*Dowd et al., 2009*). In line with this notion, by evaluating the presence of the "*memory*" ($CD3^-CD56^+Fc\varepsilon RI\gamma^-CD16^+$) NK subset in a cohort of healthy individuals, we observed that the

vast majority of HCMV seropositive individuals exhibits a detectable population of "*memory*" NK cells accounting for 3 to 50% of peripheral blood NK cells. Several recent studies demonstrated that the CD16 receptor may play a key role in driving the *in vitro* expansion of the "*memory*" NK subset. Indeed, it has been reported that "*memory*" NK cells may be expanded *in vitro* upon encounter with HCMV or influenza virus-infected cells only in the presence of virus-specific antibodies (*Lee et al., 2015; Schlums et al., 2015*). Based on such evidences, we assessed the capability of tumor targeting anti-CD20 mAbs to affect the expansion as well as the phenotypic and functional properties of "*memory*" NK subset. Herein, we observed that "*memory*" NK cells udergo a significant expansion only in response to anti-CD20-mediated CD16 aggregation. On the opposite the expansion of "*conventional*" ($CD3^-CD56^+Fc\varepsilon RI\gamma^+CD16^+$) NK cells is not affected by CD16 stimulation since they comparably proliferate in response to anti-CD20 opsonized or not opsonized targets in the presence of IL-2. Looking at the differences between the two anti-CD20 mAbs, we also noted that rituximab is more efficient in driving "*memory*" NK cell expansion with respect to obinutuzumab. It remains to establish whether such different efficiency may be due to the CD16 affinity aggregation conditions and/or to the different CD20 epitope bound on tumor cells. Furthermore, the observation that the *in vitro* culture with anti-CD20-coated targets fails to induce the expansion of the "*memory*" NK subset in HCMV seronegative donors confirms that mAb-dependent *in vitro* expansion of "*memory*" NK cells may require an *in vivo* priming likely mediated by HCMV. Because of a specific epigenetic signature, "*memory*" NK cells significantly differ from "*conventional*" ones for their receptor repertoire. Indeed, they express increased levels of the activating receptor NKG2C as well as lower amounts of CD16 and of the natural cytotoxicity receptors NKp30 and NKp46 (*Hwang et al., 2012; Zhang et al., 2013; Lee et al., 2015; Schlums et al., 2015; Zhou et al., 2015; Kovalenko et al., 2017*). We reported here that the phenotypic profile of *in vitro* expanded "*memory*" NK cells recapitulates their freshly isolated counterpart. In addition, "*memory*" NK cells significantly differ from "*conventional*" ones for their functional capabilities (*Lee et al., 2015; Schlums et al., 2015; Kovalenko et al., 2017*). In fact, despite a poor reactivity toward tumor targets, "*memory*" NK cells exhibit an enhanced CD16 responsiveness particularly in terms of IFN-γ and TNF-α production (*Hwang et al., 2012; Zhang et al., 2013; Lee et al., 2015; Zhou et al., 2015; Kovalenko et al., 2017*). On this purpose, in line with literature we found a superior capacity of freshly isolated "*memory*" NK cells to produce IFN-γ and to degranulate with respect to "*conventional*" NK cells. The

observation that such enhancement is less evident in anti-CD20-expandent "*memory*" NK cells, especially in terms of degranulation, may be explained by the increased responsiveness of "*conventional*" NK cells induced by IL-2 plus IL-15 exposure. Further, both freshly isolated and cultured "*memory*" NK cells exhibit a reduced functional activity in response to K562 stimulation. These data are consistent with the prominent role of NKp46 receptor in K562 NK-mediated recognition (*Sivori et al., 1999; Hwang et al., 2012*). Obinutuzumab exhibits an enhanced ability to activate both "*conventional*" and "*memory*" NK cells by increasing IFN-γ production and/or degranulation in freshly isolated as well as in *in vitro* expanded cellular subsets. Such enhanced functional activation, which is in contrast to what we have observed for the cellular expansion, led us to hypothesize that the molecular signals governing proliferation vs functional activation downstream CD16 may be uncoupled. With the aim to improve the clinical response rate to mAb-based therapy scientific interest is now focused in exploring the possibility to arm the host immune system in order to mediate a long-lasting effect. In this context, it is tempting to speculate that, due to their long persistance and peculiar functional capabilities (i.e. enhanced ability to kill and expecially to produce IFN-γ in response to CD16 stimulation), anti-CD20-expanded memory NK cells may be highly relevant for the promotion and maintenance of a long-term protection in treated patients thus ultimately reducing the relapsing rate after treatment. Indeed, given the well-recognized role of IFN-γ in the shaping of adaptive immunity by modulating the responses of both dendritic and T cells, it is tempting to speculate that anti-CD20-expanded memory NK cells may represent a potentially very actrative tool to be exploited for the potentiation of the "*vaccinal effect*" in patients after anti-CD20-based therapy. In this regard, our next goal will be to explore the impact of anti-CD20-based therapeutic regimens on in vivo dynamics and anti-tumor relevance of "memory" NK cells in the mAb-induced "*vaccinal effect*".

In conclusion, our data highlight a new aspect of the interplay between anti-CD20 therapeutic mAbs and NK cell functions and plasticity and provide advancements in the knowledge of NK cell anti-tumor functions. Our data may also offer potential tools for the clinical exploitation of NK effector functions in the context of novel therapy protocols aimed at optimizing the efficacy of therapeutic mAbs.

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