Université de Sherbrooke

Modulation of BLT1 expression in human NK cells by selected cytokines

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ABBREVIATIONS

5-LO: 5-lipoxygenase

ADCC: Antibody-dependent cellular cytotoxicity

BLT1: High affinity LTB_4 receptor

Cys-LTs: Cysteinyl leukotrienes

DC: Dendritic cell

FLAP: Five Lipoxigenase Activating Protein

KIR: Killer-cell immunoglobulin-like receptors

LT: Leukotriene

LTB_4: Leukotriene B_4

NCR: Natural cytotoxicity receptors

NK cell: Natural killer cell

PFP: Perforin

PL: Phospholipases

PPARα: Peroxisome proliferator-activated receptor α

TRAIL: TNF-related apoptosis-inducing ligand

RÉSUMÉ

Leukotriene B_4 (LTB_4) est un dihydroxy acide gras dérivé de l'acide arachidonique par la voie de la 5-lipoxigénase. Deux récepteurs de surface pour le LTB_4 ont été identifiés : un récepteur de haute affinité, le BLT1, et un récepteur de basse affinité, le BLT2. L'expression du récepteur BLT1 par les diverses cellules immunitaires a été l'objet de beaucoup d'études depuis l'identification du récepteur. Dans le sang périphérique, le BLT1 est principalement exprimé sur les granulocytes, les monocytes et, à un moindre degré, sur des sous-population lymphocytaires. Cependant, l'expression sur les cellules *natural killer* (NK) est controversée. Bien qu'un rapport n'ait pas trouvé l'expression BLT1 sur des cellules de NK, plusieurs groupes ont également rapporté que celui les cellules de NK répondent au LTB_4 par cytotoxicité augmentée. Nous avons donc décidé d'étudier l'expression du récepteur BLT1 sur les lymphocytes cytotoxiques et leurs sous-populations. En outre, nous avons cherché à déterminer l'effet de la modulation de l'expression de ce récepteur sur les différentes fonctions effectrices des cellules NK.

Dans cette étude, nous montrons, pour la première fois, en utilisant l'analyse cytofluorimétrie des lymphocytes périphériques frais de sang, que la sous-population de $CD56^+$ (NK) exprime le BLT1. En outre, la stimulation avec des cytokines choisies comme l'IL-2, l'IL-15 et l'IFN-γ pendant 18 heures modulent l'expression du BLT1 de surface dans les lymphocytes humains $CD56^+$. La stimulation avec l'IL-2 et l'IL-15 a eu comme conséquence une augmentation significative de l'expression de surface du BLT1, alors que la stimulation avec l'IFN-γ avait comme conséquence une diminution significative de l'expression du BLT1 sur les cellules NK. Nous nous sommes aussi

intéressés à étudier le rôle du LTB_4 et la modulation de son récepteur dans les différentes fonctions effectrices des cellules NK.

Depuis sa découverte, le LTB_4 est connu en tant que chimioattractant efficace pour des myélocytes, principalement les neutrophiles et à un moindre degré pour les éosinophiles, les monocytes et les macrophages. Récemment, le LTB_4 s'est aussi avéré un chimioattractant efficace pour les cellules T effectrices. Nous prouvons que la sous-population lymphocytaire $CD56^+$ migre en réponse au LTB_4. La stimulation préalable des lymphocytes périphériques frais de sang avec l'IL-15 pendant 18 heures résulte en une migration augmentée des lymphocytes $CD56^+$ en réponse du LTB_4 comparé aux cellules non-stimulées, suggérant que cette réponse accrue provient d'une augmentation de l'expression du BLT1.

Une des fonctions effectrices principales cellules NK est la cytotoxicité. La lyse des cellules cibles est principalement accomplie par la voie d'exocytose de granules par le relâchement de certaines protéines principalement le perforine. Nos résultats ont démontré que l'incubation des lymphocytes frais avec le LTB_4 a eu comme conséquence une diminution de l'expression de la protéine perforine dans des cellules NK. Cela est en accord avec l'effet du LTB_4 sur le processus de dégranulation d'autres types de leucocytes et de son rôle dans l'augmentation de la cytotoxicité des cellules NK. Cependant, d'autres études devraient être faites pour confirmer ces observations.

Les cellules humaines NK forment une population hétérogène qui peut être subdivisée sur la base de la densité d'expression extérieure des molécules CD56 en deux sous-populations principales, soit les $CD56^{dim}$ et $CD56^{bright}$. La majeure partie des analyses phénotypiques et fonctionnelles effectuées sur les sous-populations CD56 prouve qu'elles

sont uniques, avec une expression différentielle des divers antigènes de surface de cellules. Nous étions intéressés d'étudier plus loin l'expression de BLT1 par ces deux sous-population de NK. La sous-population de $CD56^{bright}$ exprimé sensiblement plus de BLT1 par cellule et également un plus grand pourcentage des cellules de cette population exprime le BLT1 comparativement à la sous-population de $CD56^{dim}$. D'ailleurs, les sous-populations de cellules NK ont montré différentes propriétés migratrices, où les cellules $CD56^{bright}$ a montré une meilleure réponse chimiotactique au LTB_4 comparativement aux cellules $CD56^{dim}$, ce qui corrèle avec une expression du BLT1 plus élevée sur les cellules $CD56^{bright}$.

En conclusion, nos résultats suggèrent un rôle possible pour des cellules NK dans la pathogénie des nombreuses conditions inflammatoires où LTB_4 est impliqué, car nous avons prouvé que les cellules de NK expriment le BLT1 et que ces cellules sont capables de migrer en réponse au LTB_4. Cette migration peut être encore réglée en réponse à la modulation du BLT1.

KEY WORDS

LTB_4

BLT1

NK cells

Chemotaxis

Perforin

INTRODUCTION

Part 1. Leukotrienes

1.1. Introduction

Leukotrienes are one of the major constituents of a group of biologically active fatty acids known as eicosanoids (Henderson, 1991). They are involved in the mediation of various inflammatory disorders and have been implicated in inflammatory diseases, such as asthma, psoriasis, rheumatoid arthritis, and inflammatory bowel disease.

"Their role as mediators of inflammation has therefore made them therapeutic targets. Inhibiting the production of Leukotrienes or blocking their receptor sites may decrease the inflammatory response and thereby provide a useful therapeutic modality" as stated by Jafaru I. Abu and Justin C. Konje in 2000.

1.2. History and name

The leukotrienes (LT) refer to a group of highly potent lipid mediators in inflammation and allergy. The name leukotriene was first introduced by Swedish biochemist Bengt Samuelsson in 1979. It came from the words leukocyte, indicating

its source, and triene, indicating the compounds' three conjugated double bonds (Borgeat and Samuelsson, 1979).

1.3. Leukotriene Biosynthesis

The leukotrienes can be divided into two different classes, based upon their chemical structure and biological activity: 1) the dihydroxy-derivative leukotriene B_4 (LTB_4), and 2) the cysteinyl leukotrienes (Cys-LTs), namely leukotriene C_4 (LTC_4), leukotriene D_4 (LTD_4), and leukotriene E_4 (LTE_4), containing different amino acid residues.

Unlike many other biologically active molecules, leukotrienes are not stored preformed but they are synthesized *de novo* from nuclear envelope phospholipids. They are synthesized from arachidonic acid, released from nuclear envelope phospholipids by various phospholipases (PL), mainly PLA_2 (Peters-Golden, 1998). The 5-lipoxygenase (5-LO) enzyme then converts the arachidonic acid to the unstable leukotriene A_4 (5,6-*trans*-oxido-7,9-*trans*-11,14-*cis*-ei-cosatetraenoic acid), the precursor of all the other LTs. This intermediate represents the substrate for two different specific enzymes, namely the leukotriene A_4 hydrolase and the leukotriene C_4 synthase, generating LTB_4 and LTC_4, respectively, as illustrated in figure 1.

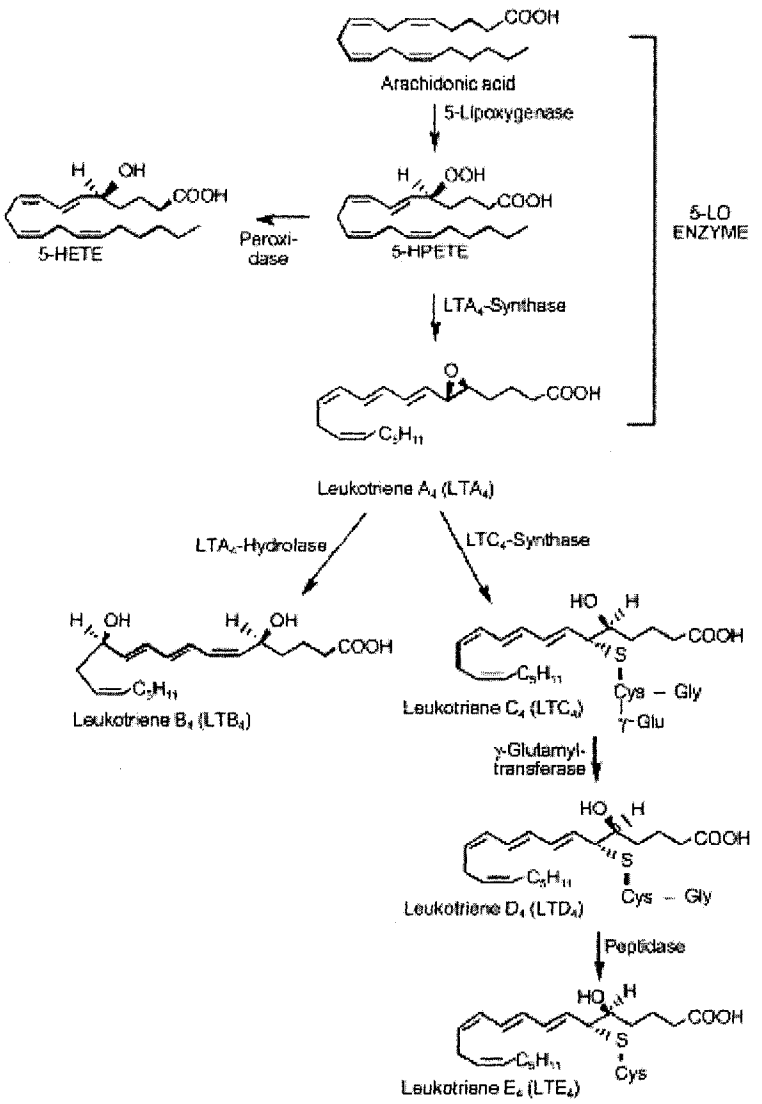

Figure 1. Metabolism of arachidonic acid by the 5-lipoxygenase enzyme and biosynthesis of leukotrienes. 5-HETE, 5-hydroxyeicosatetraenoic acid; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid (Taken from Sala et al 1998).

The 5-LO enzyme is generally located in the soluble fraction, either cytosolic or nuclear, of resting cells, but translocates to the nuclear envelope upon activation (Brock et al, 1995). The translocation requires the presence of Five Lipoxigenase Activating Protein (FLAP) (Dixon et al, 1990) which binds the free arachidonate and presents it to the 5-LO enzyme, enabling the oxygenation reaction to occur. This represents the current model of leukotriene synthesis at the nuclear envelope, as shown in figure 2 (adapted from Peters-Golden, 1998), in contrast to the earlier model that assumed that leukotriene synthesis took place at the plasma membrane.

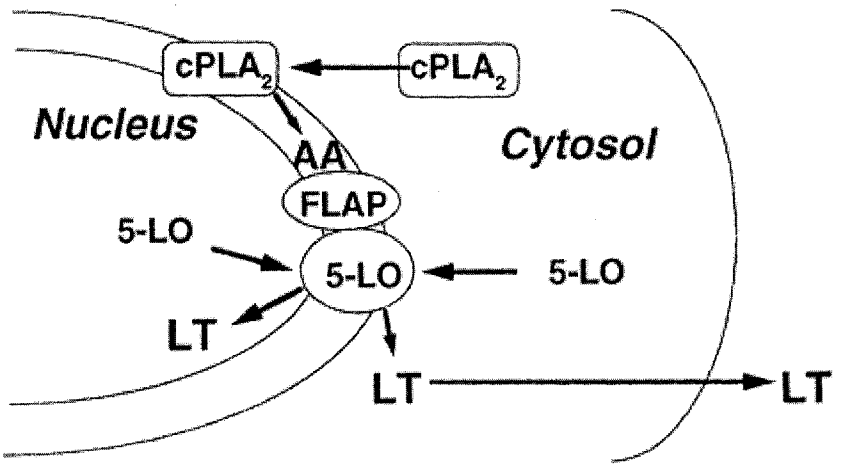

Figure 2. Nuclear envelope as the site of leukotriene synthesis (Adapted from Peters-Golden, 1998).

1.4. Distribution and cellular origins of leukotrienes

Leukotrienes are released from most inflammatory cells by a variety of biological signals, such as antigen/antibody interaction or the activation of a number of receptors.

The sites of synthesis of leukotrienes are determined by the cellular distribution of the enzymes of the biosynthetic pathway. The enzyme 5-LO has a limited cellular distribution and has been found in neutrophils, eosinophils, monocytes, macrophages, mast cells, basophils, and B lymphocytes (Jones et al, 1982; Jakobsson, 1991). These cells produce and secrete LTA_4. However, the enzymes determining the next step in the biosynthetic pathway, LTA_4 hydrolase and LTC_4 synthase, are more widely distributed than 5-LO, and this enables more cells to produce leukotrienes.

LTB_4 is directly synthesized by monocytes, alveolar macrophages and neutrophils (Peters-Golden et al, 2005), while cysteinyl-LTs are formed by eosinophils, basophils, mast cells and alveolar macrophages (Kanaoka and Boyce 2004), under normal conditions. However, during inflammation, LTA_4 translocates from migrating leucocytes to cells of inflamed tissue that have LTA_4 hydrolase activity, allowing them to start synthesis of leukotrienes by a process called 'transcellular synthesis' (Sala et al, 1996).

1.5. Leukotriene B4 receptors

1.5.1. Cell surface receptors for LTB_4

1.5.1.1 Discovery, chromosomal location and structure

LTB_4 produces its biological effects by binding to and activating specific receptors known as the **B** Leukotriene (**BLT**) receptors.

In 1997, Yokomizo and co-workers cloned the first LT receptor, i.e. the BLT1 receptor, from retinoic acid-differentiated HL-60 cells (Yokomizo et al, 1997).
Three years later, the second LTB_4 receptor was identified and cloned by the same group (Yokomizo et al, 2000) as well as by others (Tryselius et al, 2000; Kamohara et al, 2000) and it was given the name BLT2.

The gene for BLT1 is located on chromosome 14, and encodes a cell-surface protein of 352 amino acids belonging to the superfamily of seven transmembrane-spanning domain receptors (7-TMD). Cos-7 cells transfected with the cDNA encoding for the BLT1 receptor showed specific LTB_4 binding with a high affinity Kd of 0.15 nM (Nicosia et al, 2001).

On the other hand, BLT2 binds LTB_4 with significantly less affinity than BLT1; Kd of 61nM. Several eicosanoids other than LTB_4, including 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), 12(S)-hydroperoxyeicosatetraenoic acid

(12(S)-HPETE) and 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE) can also bind BLT2 (Yokomizo et al, 2001) and these are shown in more detail in table 1.

BLT2 gene is also located on chromosome 14, upstream of the gene for BLT1, partially overlapping with it. BLT2 receptor has 7-TMD and shows a high homology with BLT1 (36–45% amino acid identity), however, it is insensitive to several BLT1 antagonists (Yokomizu et al, 2000).

Table 1. Characteristics of BLT1 and BLT2 (Adapted from Tager and Luster, 2003)

	BLT1	BLT2
Affinity for LTB4	Human neutrophils,K_d =0.39-1.5 nM hBLT1 transfected HEK 293 cells, K_d = 1.1 nM mBLT1 transfected CHO cells, K_d = 0.64 nM	hBLT2 transfected HEK 293 cells, K_d = 22.7nM
Affinity for LTB_4 versus other ligands	LTB_4> 20(OH)-LTB_4>>12(R)-HETE	LTB_4>12(S)-HETE>12(S)-HPETE>15(S)-HETE>20(OH)-LTB_4
Expression	Human Leukocytes>>spleen>thymus Mouse Leukocytes>>lung, spleen, lymph nodes	Human Spleen>leukocytes, liver, ovary, pancreas> heart, prostate, testis, small intestine, kidney, lung, colon, thymus, muscle, placenta Mouse No expression in leukocytes, lung, spleen, lymph nodes
Function	Mouse Mediates leukocyte chemotaxis, calcium flux, firm adhesion	No function identified as yet
Pharmacologic inhibitors	CP105696, U75302, CP195543, ONO4057, ZK158252	CP195543, ONO4057, ZK158252, LY244283

1.5.1.2 Expression and regulation

BLT1 expression is highest in peripheral blood leukocytes and is present in much lower amounts in spleen, thymus, bone marrow, lymph nodes, heart, skeletal muscle, brain, and liver. The distribution of BLT2 expression differs significantly from BLT1, as human BLT2 is expressed more ubiquitously with expression being highest in spleen, liver, ovary, and peripheral blood leukocytes (Tager and Luster, 2003).

BLT1 has been reported to be primarily expressed by granulocytes and monocytes (Pettersson et al, 2000; Dasari et al, 2000), by half of B cells (Pettersson et al, 2003), to a lesser extent in antigen primed $CD4^+$ and $CD8^+$ T cells (Islam et al, 2006) and not to be expressed by NK cells as claimed by Pettersson et al in 2003.

1.5.2. Nuclear Receptor for LTB4

Although the classical LTB_4 receptor is the BLT, a cell-surface receptor, yet the first molecule identified as an LTB_4 receptor was the nuclear receptor, peroxisome proliferator-activated receptor α (PPARα) (Devchand et al, 1996).

PPARs belong to the nuclear hormone receptor superfamily. Three isoforms of PPARs have been identified: alpha, beta/delta and gamma, and they are encoded by different genes and distributed in various tissues (Kiec-Wilk et al, 2005).

LTB_4 produced intracellularly binds to PPARα and enhances the transcription of the enzymes involved in LTB_4 inactivation, leading to the clearance of the LTB_4 during inflammation and so acting as a negative feedback by limiting the pro-inflammatory effects of LTB_4 (Yokomizo et al 2000). In other words, pro-inflammatory LTB_4 activities are mediated by BLT, while its inactivation seems to be promoted by PPARalpha.

Consequently, LTB_4 was considered as the first ligand that functions via a dual cell-surface and intranuclear receptor system (Yokomizo et al, 2001; Nicosia et al, 2001).

1.6. Main activities and patho-physiological roles of LTB4

LTB_4 is a potent chemoattractant with diverse effects on several cell types and was found to play a major role in many inflammatory diseases.

1.6.1. Inflammatory cell recruitment

Since the discovery of LTB_4 it has been known as a potent chemoattractant for myelocytes, mainly neutrophils (Ford-Hutchinson et al, 1980; Samuelsson et al, 1987; Yokomizo et al, 1997) and to a lesser extent for eosinophils (Wardlaw et al, 1986). Subsequently, its ability to direct the migration of monocytes and macrophages was established (Migliorisi et al, 1987; Crooks et al, 1998).

In addition, LTB_4 recently has been shown to be a potent chemoattractant for effector T cells (Tager et al, 2003; Goodarzi et al, 2003; Ott et al, 2003).

1.6.2. Cell activation and effector functions

LTB_4 has diverse effects on the function and activities of the immune system, affecting mainly leukocytes. Among these effects, its roles in the function of the following cells are:

Neutrophils:

LTB_4 stimulates neutrophil generation of reactive oxygen species (Sumimoto et al, 1984), release of granular enzymes (Rae and Smith, 1981) and phagocytosis of bacteria (Mancuso et al, 2001)

Monocytes:

In 1991, Poubelle et al demonstrated that LTB_4 upregulates IL-6 production in human peripheral blood monocytes. LTB_4 also augments mouse peritoneal macrophage phagocytosis and killing of bacteria (Demitsu et al, 1989).

Lymphocytes:

LTB_4 has been found to activate and stimulate effector functions of lymphocytes and NK cells. For example, it was found to enhance IL-2 production in $CD4^+$ T cells (Marcinkiewicz et al, 1997), to enhance IL-2Rβ expression in $CD8^+$ T cells (Stankova et al, 1992), to induce the production of IL-5 in human T cells

(Yamaoka and Kolb, 1993) and to enhance activation, proliferation, and immunoglobulin secretion of B cells (Yamaoka and Kolb, 1993).

As for NK cells, it was shown that LTB_4 increases NK cell cytotoxic activity (Jondal, 1985) and it has been suggested that the augmented natural cytotoxicity is possibly a result of enhanced target cell recognition and binding leading to increased lytic efficiency (Gagnon et al, 1987). It has also been demonstrated that LTB_4 increases IL-2Rβ expression, with subsequent increase in sensitivity to IL-2 and increased cytotoxic activity in response to IL-2 (Stankova et al, 1992).

1.6.3. Cell survival

LTB_4 was found to prolong neutrophil survival by preventing apoptosis (Hebert et al, 1996) and up regulation of BLT1 has been demonstrated to be one mechanism through which glucocorticoids prolong neutrophil survival. (Stankova et al, 2002).

1.6.4. Role in inflammatory diseases

LTB_4 and its receptors also participate in the pathogenesis of many inflammatory diseases. For example, significantly elevated levels of LTB_4 have been documented in the sputum, BAL, and/or exhaled breath condensates from patients with asthma (Montuschi and Barnes, 2002) and cystic fibrosis (Konstan et al, 1993). In addition, significantly elevated levels of LTB_4 have been found in the synovial fluid of patients with rheumatoid arthritis (Ahmadzadeh et al, 1991) and in the cerebrospinal fluid of patients with clinically active multiple sclerosis (Neu et al, 1992).

1.7. Assignment of LTB4 activities to BLT1 and BLT2

Two lines of BLT1-deficient mice were generated by targeted gene disruption (Tager et al, 2000; Haribabu et al, 2000), which enabled the studying of the in vivo biological functions and physiological roles of the BLT receptor and determining the potential consequences of chronic BLT receptor deficiency.

In vitro, peritoneal neutrophils from $BLTR^{-/-}$ mice did not respond to LTB_4 in calcium mobilization or chemotaxis but showed normal responses to the inflammatory mediators C5a and platelet-activating factor (PAF). In addition, in vivo, peritoneal neutrophil influx in response to LTB_4 was abolished in $BLTR^{-/-}$ mice but not in control mice, indicating these functions are mediated by BLT1 in these cells and that BLT1 is the sole receptor for LTB_4-induced inflammation in mice (Haribabu et al, 2000). Furthermore, LTB_4-mediated leukocyte calcium flux, chemotaxis, and firm adhesion to endothelium in vivo were abolished in another $BLTR^{-/-}$ mice model (Tager et al, 2000).

These studies led to the conclusion that many of the major activities of LTB_4 are mediated by the BLT1 receptor in mice and also reveal the critical role this receptor plays in acute inflammation and immediate hypersensitivity. However, the activities of LTB_4 that are specifically mediated by BLT2 have not yet been determined.

Part 2. Natural Killer cells

2.1. Introduction

Natural killer cells are cytotoxic lymphocytes which constitute a major component of the innate immune system. They comprise 10 to 15% of human peripheral blood lymphocytes (PBL), ~10% of lymphocytes in the liver, ~5% of splenic lymphocytes and between 0.5-5% of mononuclear cells in lymph nodes and the mucosal-associated lymphoid organs (Ferlazzo and Munz, 2004).

The majority of NK cells have the morphology of large granular lymphocytes. They do not express either of the characteristic T-cell or B-cell surface antigens. Instead, they are defined phenotypically by their expression of CD56 and lack of expression of the pan T cell marker CD3 (Robertson and Ritz, 1990).

2.2. NK cell Ontogeny

Similarly to B- and T-lymphocytes, NK cells derive from $CD34^+$ cell precursors and undergo maturation primarily in the bone marrow (BM). The development of NK cells requires various cytokines, particularly IL-15, released from BM stromal cells (Freud and Caligiuri, 2006).

Initial studies have focused on IL-2, with the demonstration that addition of exogenous IL-2 lead to the differentiation of $CD34^+CD38^-$ hematopoietic progenitors in stromal-free cultures into NK cells, and when added to long-term bone marrow cultures containing stroma, IL-2 induced NK cells with high efficiency (Shibuya et al, 1995). In addition, the in vivo administration of recombinant IL-2 to humans and mice resulted in expansion of NK cell numbers (Caligiuri et al, 1993). However, the physiological significance of these observations was uncertain because IL-2 is produced by activated T lymphocytes in the periphery. Furthermore, NK cells can be found in mice with a genetically disrupted IL-2 gene (Schorle el al, 1991; Willerford et al, 1995), while mice with targeted disruption of the IL-2R γ or β chains lacked NK cells. Collectively, these data suggested that cytokines other than IL-2 utilizing the IL-2R$\beta\gamma$ complex were important in NK cell development (Cao et al, 1995; Suzuki et al, 1997).

Accordingly, IL-15 protein and transcripts were expressed in primary human bone marrow stromal cells, while expression of IL-2 was lacking (Mrozek et al, 1996). Consequently, it has been confirmed, that IL-15 is the major physiological cytokine present in bone marrow that is important in the development of NK cells (Williams et al, 1998).

Two additional bone marrow stromal factors, c-kit and flt-3 ligands, have been shown to act synergistically with IL-15 in the generation of NK cell progenitors from $CD34^+$

heamatopoietic stem cells (HPCs) through up-regulation of expression of the IL-15R complex (Mrozek et al, 1996; Yu et al, 1998).

Although, it has been generally accepted that NK cell development primarily occurs within the BM (Yokoyama et al, 2004), the possibility that BM-derived pre-NK cells or developmental intermediates may migrate to peripheral tissues to undergo final maturation has not yet been excluded (Santo et al, 2006).

Figure 4 demonstrates the developmental stages through which human NK cells are believed to pass from BM-derived haematopoietic stem cells (HSCs) to stage 5 NK cells. The gray box represents developmental stages that may occur within the secondary lymphoid tissues. As NK cells progress from stage 1 to stage 3, they lose the capacity for T-cell or dendritic cell development and become committed to the NK cell lineage. From stages 3 to 5, it is suggested that NK cells undergo functional maturation, where stage 3 immature NK cells may produce GM-CSF and potentially type 2 cytokines, while stage 4 $CD56^{bright}$ NK cells may preferentially produce IFN-γ, and stage 5 $CD56^{dim}$ NK cells may preferentially mediate cellular cytotoxicity (reviewed in Freud and Caligiuri, 2006).

At present, the relationship between the two populations of NK cells is controversial. Evidence exists that $CD56^{bright}CD16^{-}$ cells are direct precursors of $CD56^{dim}CD16^{+}$ cells; that $CD56^{bright}CD16^{-}$ cells are direct descendents of $CD56^{dim}CD16^{+}$ cells; or

that $CD56^{bright}CD16^{-}$ cells are a separate lineage of NK cells than $CD56^{dim}CD16^{+}$ cells (Berahovich et al, 2006).

The hypothesis that $CD56^{bright}CD16^{-}$ cells are direct precursors of $CD56^{dim}CD16^{+}$ cells is illustrated in figure 4 and has been based on the following observations: (i) blood $CD56^{bright}$ NK cells express CD117 (c-kit), which is expressed by immature HPCs, while CD56 ^{dim} NK cells do not; (ii) some blood $CD56^{bright}$ NK cells are agranular, whereas all blood $CD56^{dim}$ NK cells have a granular morphology; (iii) *in vitro*, IL-2- or IL-15-generated human NK cells from various starting progenitor populations, including cord blood $CD34^{+}$, have a phenotype more similar to peripheral blood $CD56^{bright}$ NK cells, however, these cells do have absent or low expression of other CD56 ^{bright} NK-cell antigens, such as CD2 and (iv) after BM transplantation, the majority of early NK cells are $CD56^{bright}$ but this declines to normal levels by about 4 months post transplantation with concomitant increase in the $CD56^{dim}$ NK cells. This hypothesis has not yet been formally proven and the generation of $CD56^{dim}$ from CD56 ^{bright} NK cells in vitro has not been convincingly generated. It has also been proposed that blood $CD56^{bright}$ NK cells represent the activated, rather than immature, fraction of circulating NK cells (reviewed in Freud and Caligiuri, 2006).

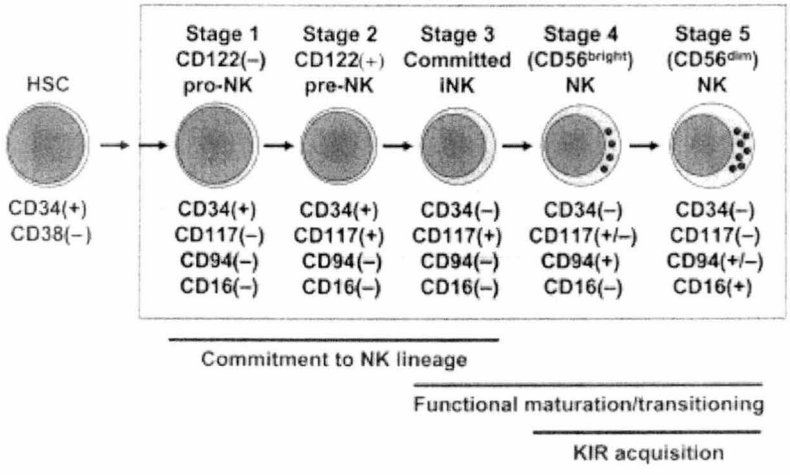

Figure 3. Proposed model of *in vivo* human NK cell development (Taken from Freud and Caligiuri, 2006).

On the other hand, the postulation that $CD56^{bright}$ cells are direct descendents of $CD56^{dim}$ cells was based on the observation that upon entry of $CD56^{dim}$ NK cells into peripheral tissues, CD56 was up-regulated (Robertson, 2002).

However, it is still likely that peripheral blood $CD56^{bright}$ NK cells are a heterogeneous population, potentially containing at least three different subsets: i) immature cells coming from the bone marrow, ii) mature cells activated in the lymph nodes, and iii) $CD56^{dim}$ derived cells returning to the bloodstream after activation in peripheral tissues (Berahovich et al, 2006).

The hypothesis that human $CD56^{bright}$ and $CD56^{dim}$ NK-cell subsets are terminally differentiated lymphocytes, and that they have distinctive pathways of maturation at some point during their development, is supported by many others who regard these

cells as distinctive NK subsets with distinctive biology and function (Cooper et al, 2001).

2.3. Subsets of human mature NK cells

As a heterogeneous population, human NK cells can be divided on the basis of the surface density expression of CD56 molecules into two major subsets, $CD56^{dim}$ and $CD56^{bright}$. The majority (~ 90%) of human NK cells express low density of CD56 ($CD56^{dim}$) but high levels of Fcγ receptor III (FcγRIII, CD16), while a much smaller subset (~10 %) of NK cells express high levels of CD56 molecule on their surface with low or no expression of CD16 and are called $CD56^{bright}$ $CD16^{dim}$ or $CD56^{bright}$ $CD16^{-}$(Cooper et al, 2001). The key differences between the two subsets are well illustrated in figure 4.

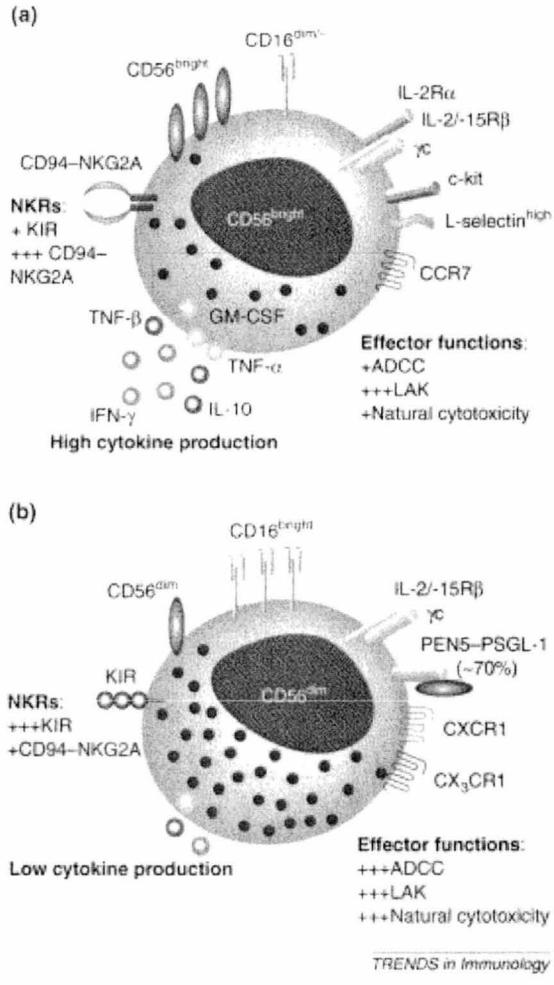

Figure 4. Schema of human natural killer (NK) cell subsets (Taken from Cooper et al, 2001).

Most of the phenotypic and functional analysis carried out on the CD56 subsets show that they are unique subsets, although there is no known direct functional significance of the high or low level of expression of the CD56 molecule (Cooper et al, 2001). Yet, its low or high expression correlates with the expression of other surface markers that confer unique functional properties to the $CD56^{bright}$ and $CD56^{dim}$ subsets (Farag et al, 2006).

Resting $CD56^{dim}$ cells are more granular, more than 95% of these cells express high levels of CD16 and were found to be the more cytotoxic subset (Lanier et al, 1986). On the other hand, most of the $CD56^{bright}$ NK cells (~50–70%) lack expression of CD16, while the remaining cells have low density expression of the receptor. The differences in the level of expression of CD16 by NK-cell subsets have functional consequences for antibody dependent cellular cytotoxicity (ADCC) (Cooper et al, 2001).

In addition, resting $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets show differences in their NK receptor repertoires. $CD56^{dim}$ NK cells express both KIR and C-type lectin-like receptors at high density, while $CD56^{bright}$ cells express high levels of the C-type lectin-like CD94/NKG2 family with only very small fractions expressing killer-cell immunoglobulin-like receptors (KIR) (Cooper et al, 2001).

The differences in antigen expression by the NK subsets are shown in more details in table 2 below.

Table 2. Antigens expressed differentially by resting human NK-cell subsets (Taken from Megan A. Cooper et al, 2001).

	$CD56^{bright}$	$CD56^{dim}$	Refs
CD56	++[b]	(+)[c]	2
CD16	–/+[d]	++	2
NK receptors			
KIR	–/+	++	6
CD94	++	–/+	7
NKG2A	+[e]	–/+	7
ILT-2	–[f]	+	8
Cytokine and chemokine receptors			
IL-2Rαβγ	+	–	9,10
IL-2Rβγ	+	+	9–11
c-kit	+	–	12
IL-1RAcP	+	+	13
IL-1RI	+	–/+	13
IL-18R	+[g]	–/+	14
CCR7	++	–	15
CXCR3	+	–/+	15
CXCR1	–	++	15
CX_3CR1	–	++	15
Adhesion molecules			
CD2	++	+	2
L-selectin (CD62L)	++	–/+	16
PEN5–PSGL-1	–	+	6
LFA-1	(+)	++	16
CD44	++	+	17
CD49e	++	+	17

[a]Abbreviations: CCR7, CC-chemokine receptor 7; CXCR, CX-chemokine receptor; CX_3CR1, CX_3C-chemokine receptor 1; IL, interleukin; IL-1RAcP, interleukin-1 receptor accessory protein; ILT-2, Ig-like transcript 2; KIR, killer Ig-like receptor; LFA-1, leukocyte function-associated antigen 1; PSGL-1, P-selectin glycoprotein ligand 1.

[b]Indicates high-density expression.

[c]Indicates low-density expression.

[d]Indicates variable expression.

[e]Indicates that the majority of cells expresses this molecule.

[f]Not expressed.

[g]Expression of IL-18R is up-regulated in activated $CD56^{bright}$ NK cells.

All NK cells express a functional heterodimeric interleukin-2 receptor (IL-2Rβγ), with intermediate affinity for IL-2. It was shown that the $CD56^{bright}$ NK cell is the only one that constitutively expresses the high-affinity heterotrimeric IL-2R (IL-2Rαβγ) (Nagler et al, 1990) and so can expand in vitro and invivo in response to low (picomolar) concentrations of IL-2 but with little cytotoxic activity (Caligiuri et al, 1993).

The $CD56^{bright}$ NK cell subset also plays an immunomodulatory role by producing significantly more cytokines, including IFN- γ, GM-CSF, IL-10, IL-13, TNF- α and TNF-β compared to $CD56^{dim}$ in response to monocyte/macrophage-derived cytokine stimulation (Cooper et al, 2001).

Currently, in mice, the equivalent of the CD56 molecule which characterizes human NK cells is not known and functional mouse NK cell subsets have not yet been clearly defined.

2.4. NK cell receptors

Natural killer cells have cell surface receptors that, unlike TCRs, can be either immunoglobulin-like or C-type lectin-like in structure. Natural killer cell receptors are expressed in complex patterns of inhibitory and activating isoforms. A fine balance between the signals from activating and inhibitory receptors determines the outcome of NK cell activity.

Some inhibitory receptors recognize MHC class I, which is present on virtually all healthy cells, and so protects these cells from NK cell lysis. Some viral infections or tumor transformations down-regulate the expression of certain MHC class I on the surface of these cells, leading to NK cell activation, as proposed by the 'missing self hypothesis' (Ljunggren and Karre, 1985), provided that an activating receptor is engaged (figure 5). Activating NK receptors (NKRs) bind to host-derived or pathogen-encoded ligands that are up-regulated on 'stressed' or infected cells. NK cells, upon activation, cause lysis of target cells by exocytosis of perforin and granzymes and secretion of cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, and thus mediate their immune response to infection or tumours (Lodoen and Lanier, 2006).

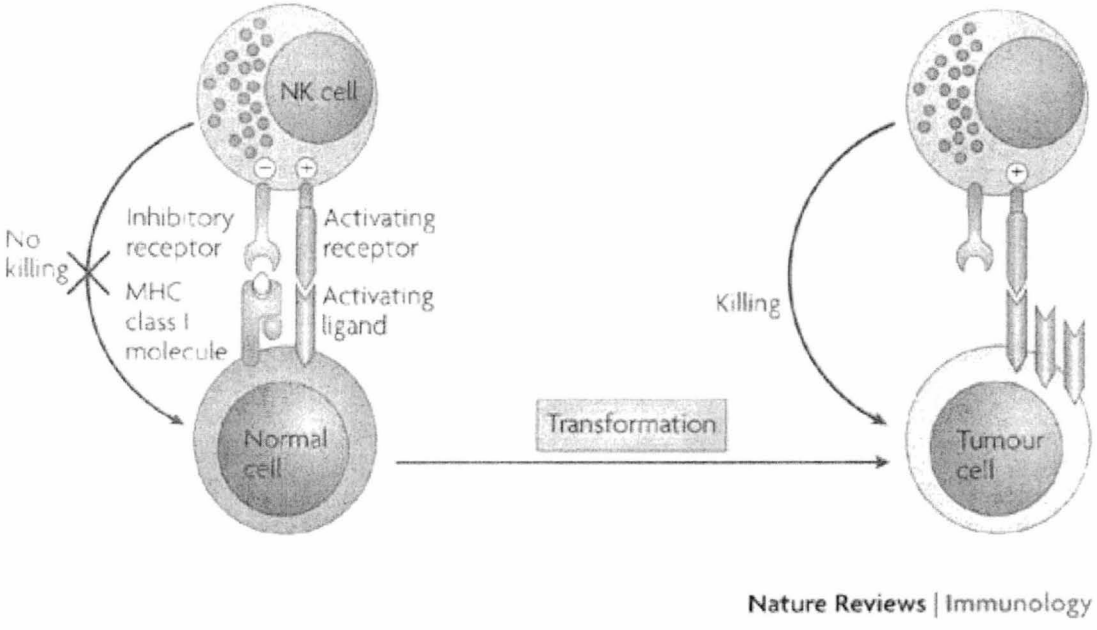

Figure 5. Missing-self recognition of tumour cells (Ljunggren and Malmberg, 2007).

Three major superfamilies of NKR have been described on human NK cells: the killer immunoglobulin (Ig)-like receptor (KIR) superfamily which recognizes classical MHC class I molecules, the C-type lectin superfamily recognizing non-classical MHC class I or class I-like molecules, and the natural cytotoxicity receptors (NCR) whose ligands are still poorly defined. Furthermore, there are other NKR that function primarily as co-receptors, but their significance and ligands remain unknown in some cases. Table 3 summarizes the major NKR and their ligands.

Table 3 Functions and ligands of human Natural Killer (NK) cell receptors (Biassoni et al., 2003).

Receptor name	Other name	Function	Cellular ligand(s)	Viral Ligand(s)
NKp46	Ly94/NCR1	activating	unknown	SV-HA; IV-HA
NKp30	1C7/NCR3	activating	unknown	
NKp44	Ly95/NCR2	activating	unknown	SV-HA; IV-HA
NKG2D	D12S2489E	activating	MICA, MICB, ULBPs	
2B4	CD244	activating*	CD48	
NTB-A	KALI	activating*	unknown	
NKp80	KLRF1	activating	unknown	
CD16	FcRIIIa	activating	IgG	
CD2	LFA-2	activating	CD58; LFA-3	
DNAM-1	CD226	activating	PVR/CD155, Nectin-2/CD112	
p58.1	KIR2DL1	inhibitory	HLA-Cw2, 4, 5, 6	
p58.2	KIR2DL2	inhibitory	HLA-Cw1, 3, 7, 8	
p70	KIR3DL1	inhibitory	HLA-Bw4	
p140	KIR3DL3	inhibitory	HLA-A3, -A11	
p49	KIR2DL4	inhibitory (?)	HLA-G	
LIR1/ILT2	LILRB1	inhibitory	HLA-G, other alleles	HCMV-UL18
LIR2/ILT4	LILRB2	inhibitory (?)	HLA-F	
CD94†	KLRD1	#	HLA-E	
NKG2A†	KLRC1	inhibitory	HLA-E	
p50.1	KIR2DS1	activating	HLA-Cw2, 4, 5, 6	
p50.2	KIR2DS2	activating	HLA-Cw1, 3, 7, 8	
p50.3	KIR2DS4	activating	unknown	
NKG2C†	KLRC2	activating	HLA-E	
NKG2E†	KLRC3	activating	HLA-E	
p40	LAIR1	inhibitory	unknown	
IRC1	IRp60/CMRF35H	inhibitory	unknown	
CMRF35	CMRF35	?	unknown	
p75/AIRM1	Siglec-7	inhibitory	sialylated sugars moieties	

*These molecules have been shown to display inhibitory rather that activating functions in NK cells derived from XLP patients. †CD94 forms heterodimers with NKG2A, NKG2C and NKG2E specific for HLA-E. #CD94 lacks signaling motifs in the cytoplasmic tail.

2.4.1. Killer immunoglobulin-like receptors (KIR)

KIRs belong to the immunoglobulin superfamily. They are located on chromosome 19q13.4 (Wilson et al, 1997) and include 12 members and a number of allelic variants, of which 6 receptors are inhibitory and 6 are activating. KIRs are monomeric (single chain) receptors and are characterized structurally by either 2 (KIR2D) or 3 immunoglobulin like domains (KIR3D) which can specifically recognize MHC class I molecules, including HLA-A, -B, and –C (Farag and Caligiuri, 2006). KIRs can further be classified according to the length of their cytoplasmic tails into long (KIR2DL and KIR3DL) and short (KIR2DS and KIR3DS) cytoplasmic tails. The long-tail KIRs generate an inhibitory signal due to the presence of immunoreceptor tyrosine-based inhibition motifs (ITIM) in their cytoplasmic domains, while the short-tail receptors mediate an activating signal due to their association with adaptor proteins bearing immunoreceptor tyrosine-based activating motifs (ITAM). They exist as sets of paired inhibitory and activating receptors. Each set has an identical extracellular domain and binds to identical ligands. However, because of the differences in their transmembrane and cytoplasmic domains, KIRs can mediate either an inhibitory or an activating response following binding to the identical MHC class I molecules (Biassoni et al, 1996).

The immunoglobulin-like transcripts (ILTs) is another group of inhibitory receptors that belongs to the immunoglobulin superfamily. It is also referred to as leukocyte immunoglobulin-like receptors (LIRs) (Farag et al, 2002). They are immediately centromeric to the KIR loci on human chromosome 19q13.4 (Cosman et al, 1997).

These molecules are primarily expressed on monocytes, macrophages, dendritic cells, and B lymphocytes (Colonna et al, 1997; Chapman et al, 1999). However, ILT-2 (LIR-1) is also expressed on NK cells (Colonna et al, 1997) and interacts directly with a broad spectrum of HLA class I molecules, including HLA-G (Navarro et al, 1999).

2.4.2. Heterodimeric C-type lectin receptors

2.4.2.1. CD94/NKG2 receptor

NK cells express CD94 and NKG2 heterodimeric receptors, which are members of the C-type lectin-like family and recognize the non-classical class I HLA-E molecule (Lee et al, 1998).

They are composed of a common subunit (CD94) covalently bonded to a distinct chain encoded by a gene of the C-type lectin NKG2 family (Lazetic et al, 1996; Perez-Villar et al, 1996). CD94 lacks a cytoplasmic domain for intrinsic signal transduction and the functional specificity of the receptor is determined by the extracellular and cytoplasmic domains of the NKG2 molecules. Four closely related transcripts of the NKG2 gene family have been identified, all located with the CD94 gene on chromosome 12p12.3-13.1, including NKG2A (and its splice variant NKG2B), NKG2C, NKG2E (and its splice variant NKG2H), and NKG2F (Houchins et al, 1991). Of the C-type lectin NK receptors, only CD94/NKG2A (and splice variant NKG2B) is inhibitory, while the other heterodimers are activating receptors.

As with KIR molecules, the NKG2 subunit of the inhibitory receptor CD94/NKG2A has a long intracytoplasmic tail containing an ITIM that mediates an inhibitory signal, while the NKG2 subunits of the activating receptors have only short cytoplasmic tails that associate with adaptor proteins bearing ITAMs. The activating isoforms of both of the human killer cell Ig-like receptors (KIRs) and the CD94/NKG2C receptor associate with the ITAM-containing adaptor protein, DAP12 (Lanier, 2000). DAP12 has a very short extracellular domain, a single ITAM and is expressed as a disulfide-bonded homodimer on the surface of NK cells (Lanier et al., 1998). Also similarly to KIR, the inhibitory receptors have higher binding affinities and the inhibitory signals dominate (O'Connor et al, 2006).

NK clones may selectively bear either inhibitory or activating CD94/NKG2 receptors, yet NKG2A and NKG2C transcripts can both be detected in some NK clones, suggesting that subsets of NK cells may co-express both receptors (Lopez-Botet et al, 1998). Only a minority of NK cell clones express both activating and inhibitory isoforms that recognize the same HLA allotype (Valiante et al, 1997). Each NK cell expressing an activating receptor also co-expresses at least one inhibitory receptor for a different HLA class I allele, to mediate self-tolerance and also to detect altered class I expression on cells (Biassoni et al, 2001). When this inhibitory receptor is engaged with its ligand, it predominates. As a result, the activating receptors may only signal when target cells have lost the expression of an HLA allele that is recognized by the inhibitory receptor (Farag et al, 2002).

2.4.2.2. Human NKG2D receptor

NKG2D is a member of the C-type lectin superfamily. It is constitutively expressed by all NK cells and by most of the cytotoxic T lymphocytes (Bauer et al, 1999). NKG2D gene lies between the CD94 and NKG2A-E genes on chromosome 12p12.3-13.1 (Glienke et al, 1998), but it is only distantly related to the other members of the NKG2 family, as it has little sequence homology (21% homology) with the NKG2A-E subunits and it doesn't associate with CD94, but is expressed as a homodimer (Wu et al, 1999). NKG2D is an activating receptor and it requires the association with DAP10 which recruits phosphatidylinositol (PI)-3 kinase after phosphorylation but doesn't contain a cytoplasmic ITAM (Wu et al, 1999), unlike activating KIR and C-type lectin NKR.

NKG2D recognizes two distinct families of ligands, the MHC class I chain-related (MIC) antigens, MICA and MICB (Bahram, 2000), and the UL16 binding proteins (ULBPs) (Sutherland et al, 2001). MIC antigens are expressed in fibroblast and epithelial cell lines. Induction of MIC expression may occur with cellular stress, viral infection, or neoplastic transformation, which may facilitate an attack by NK cells. NK cell recognition of MIC-positive targets is exclusively mediated by NKG2D and overrides any inhibitory signal delivered at the same time (Farag et al, 2002).

The second family of NKG2D ligands includes human cellular proteins initially identified by their ability to bind the human CMV protein UL16, which is a type I transmembrane protein known to be expressed by CMV-infected cells (Kaye et al, 1992). UL16 binds to MICB and two proteins named ULBP-1 and ULBP-2. ULBPs

have a more ubiquitous expression than MIC proteins, as their transcripts have been detected in heart, lung, testis, brain, lymph nodes, thymus, tonsil, liver, and bone marrow, however, no cell surface expression of ULBPs was detected on these cells (Cosman et al, 2001).

2.4.3. Natural cytotoxicity receptors (NCRs)

The NCRs are Ig-like activating receptors that include NKp46, NKp44 and NKp30, they possess limited homology with other known human molecules and no homology to each other (Moretta et al, 2000). NKp46 and NKp30 are constitutively expressed on all peripheral blood NK cells (Sivori et al, 1999 ; Pende et al, 1999) while the NKp44, is expressed only on IL-2-activated NK cells as well as a proportion of $\gamma\delta$T cells (Cantoni et al, 1999; Vitale et al, 1998). This might explain, at least in part, the higher levels of cytolytic activity of IL-2 activated NK cells.

The ligands recognized by NCRs are still not molecularly defined and their roles are still uncertain. However, they have been implicated in the recognition and lysis of tumor cells by human NK cells (Farag and Caligiuri, 2006). Monoclonal antibodies to NCR may block the NK cell-mediated lysis of many tumors, to a varying extent, however, the simultaneous masking of the three NCR can virtually abrogate the lysis of certain tumors (Pende et al, 1999). Furthermore, tumor cells appear to differ significantly in their expression of the relevant ligands which are absent on normal cells (Pende et al, 2001). It is also possible that certain tumor cells may down-regulate NCR expression on NK cells or that they may acquire expression of these NCR

ligands (Biassoni et al., 2003). NKp46 and NKp44 (but not NKp30) can also bind viral hemagglutinins, suggesting that the NCR may also play an important role in the recognition of virus-infected cells (Arnon et al, 2001; Mandelboim et al, 2001).

2.5. NK cell recruitment to target sites

Most NK cells are found in the blood and spleen, but they are also present in the lung, uterus, and intestinal mucosa (Trinchieri, 1989). In certain pathological conditions NK cells migrate rapidly to target areas, especially during viral and bacterial infections (Su et al, 1993, Holmberg et al, 1981). Moreover, NK cells were found in the infiltrate of allograft tissues during the early phase of rejection (Nemlander et al, 1983). However, the molecular basis responsible for NK cell recruitment into those tissues is still poorly understood. Nevertheless, the chemokine repertoire of NK cells has been the focus of many studies. It was shown that NK cells express a number of chemokine receptors including CXC chemokine receptor (CXCR) 1 (Qin et al, 1996), CCR2 and CCR5 (Nieto et al, 1998), CCR7 (Kim et al, 1999), and CX3CR1 (Imai et al, 1997).

NK cells can also migrate in response to some chemokines, including MIP-lα, IP-10, as well as MIP-lβ, RANTES, MCP-1, MCP-2 and MCP-3 (Taub et al, 1995) in addition to classical chemotactic factors as casein and C5a ((Bottazzi et al, 1985), to certain cytokines e.g. TNF-α, IL-2, and IL-12 (Maghazachi, 1991; Allavena et al, 1994)..

2.6. Effector functions mediated by NK cells:

2.6.1. Cytotoxicity

Cytotoxicity is the major effector function of NK cells. NK cells were originally defined as lymphocytes that were able to lyse certain tumor targets such as YAC-1 (a Moloney tumor virus-induced lymphoma from A/Sn mice) and K562 (a cell line derived from a patient with chronic myelogenous leukaemia), when they were freshly isolated and without prior activation (Kiessling et al, 1975; Ortaldo et al, 1977). Yet the mechanisms and molecular basis involved in NK cell-target recognition and interactions are still poorly understood.

However, it is believed that lysis of target cells by NK cells involves several steps that occur in the following sequence: i) recognition of potential target cells; ii) conjugate formation between NK and target cells; iii) NK cell activation, resulting in the redistribution and release of cytotoxic granules (degranulation) and the transcription and secretion of cytokines; and iv) injury or lysis of target cells (Whiteside and Herberman, 1994).

NK cells have "natural killer" i.e. spontaneous antibody-independent cytotoxic activity against some virus-infected, leukemic, and other tumor cells and also mediate antibody-dependent cellular cytotoxicity (ADCC) through the engagement and activation of the FcγRII (CD16), a receptor that specifically binds the Fc part of an antibody (Perussia, 1998).

Once NK cells come in contact with and recognize their targets, NK mediated lysis can be accomplished by one of two major pathways. The first and principal way is through the granule exocytosis pathway (Trapani et al, 2000; Trapani and Smyth, 2002) and the second is through the death receptor pathway (Nagata and Golstein, 1995; Smyth et al, 2003).

2.6.1.1. The granule exocytosis pathway

The granule exocytosis pathway involves the release of cytotoxic cytoplasmic granules containing a number of proteins, such as perforin and granzymes, which cause target cell lysis.

Perforin is constitutively expressed in NK cells and γδT cells and can be detected in cytotoxic T-lymphocytes (CTL) following activation (Nakata et al, 1990; Muller et al, 1989). It is a 70 KDa calcium-dependent pore-forming protein. It represents an important component of cell-mediated cytotoxicity and owes its name to the fact that it can 'perforate' target membranes by forming transmembrane pores (Liu et al, 1995). Perforin is released as a monomer which polymerizes in the presence of calcium and forms pore-like structures analogous to the C9 component of complement (Catalfamo and Pierre, 2003). Through these pores and by endocytosis, Granzymes released from the NK cell are delivered into the target cell, where their principal role (mainly by Granzyme B) is to cleave the pro-apoptotic, Bcl-2-like molecule Bid. Truncated Bid disrupts the mitochondria and results in the release of

pro-apoptotic mediators. These mediators induce efficient caspase processing and caspase-mediated cell death (Smyth et al., 2001). Cell death can also occur without efficient caspase activation through a non-nuclear mechanism, which is poorly understood. However, it may involve the cleavage of cytoskeletal elements (Heibein et al., 1999). Perforin-mediated cytotoxicity is well documented in NK cell-mediated defense against tumour cells as well as host cells infected with several types of viruses, bacteria, and parasites (Ma et al, 2004).

2.6.1.2. Death Receptor-Mediated Apoptosis

The second way through which NK cells can mediate target cell lysis is through "Death Receptor-Mediated Apoptosis". The key apoptosis-inducing members of this family 'TNF-family ligands' are Fas ligand (FasL), TNF- α, and TNF related apoptosis-inducing ligand (TRAIL).

In 1995, Montel and his coworkers have shown that fresh human NK cells can use the FAS lytic pathway, and later it was demonstrated that NK cells expressing FasL kill tumor cells constitutively expressing Fas (Bradely et al, 1998). NK cells have also been shown to cause the induction of Fas expression on Fas-negative targets with the subsequent induction of apoptosis through this receptor. The role played by FasL in the cytotoxicity mediated by NK cells is now well established, where the FasL expressed on NK cells interacts with Fas on target cells and results in apoptosis and target cell death.

In addition, TRAIL has been shown to be involved in the cytotoxic activity of NK cells against TRAIL-sensitive tumor cells without much effect on normal cells (Srivastava, 2001) and to play an important role in the surveillance against tumour metastasis. This was shown by the administration of neutralizing monoclonal antibody against TRAIL which resulted in a significant increase in the experimental liver metastases of several TRAIL-sensitive tumor cell lines (Takeda K et al, 2001).

2.6.2. Cytokines

NK cells secrete a number of TH1 and TH2 effector cytokines. Loza, et al (2002) proposed that NK-cell production of these cytokines occurs at different stages during NK-cell maturation and that the functionally mature NK cells lose the ability to produce TH2 cytokines and acquire the capacity to produce interferon gamma (IFN-γ).

Many studies have found that NK cell-produced IFNγ plays an important role in suppression of bacterial and viral infections (Brion et al, 1999). IFN-γ produced by NK cells was also seen to act as a self-activating molecule, since the impairment of NK cell activation was correlated with the reduction of IFN-γ secretion and limited NK cell mediated killing (as reviewed in Papamichail et al, 2004)

In addition to IFN-γ, NK cells, mostly $CD56^{bright}$ NK cells, were found to produce a series of immunoregulatory cytokines. Among these, TNF-α was found to directly promote NK cell-mediated killing (Lee et al, 1996), while GM-CSF enhanced lymphokine-activated killer cell function synergistically with other cytokines

(Baxevanis et al, 1995). IL-10 production by NK cells may also have a regulatory role by inhibiting IFN-γ production or enhancing IFN-γ secretion in synergy with IL-18 (Goodier and Londei, 2000; Cai et al, 1999).

It has also been proposed that NK cells participate in the regulation of humoral immune responses, and play a significant role in asthma-associated eosinophilia through the production of IL-5, and possibly other factors (Reviewed by Loza et al, 2002).

2.6.3. Link between innate and adaptive immunity

Several studies have shown evidence for NK- dendritic cell (DC) crosstalk resulting in cellular activation, maturation and even death. Early work by Fernandez and his coworkers has demonstrated DC-dependent activation of NK cell anti-tumor effects, thus providing the first evidence of NK-DC interactions in vivo (Fernandez et al, 1999). Subsequently, the importance of NK-DC interactions was demonstrated during in vivo viral infections as well as during anti-tumor immunity (Andrews et al, 2003; Kalinski et al, 2005).

Early, at the onset of an inflammatory reaction, and following encounter with target cells, immature DCs (iDCs) are activated. Maturing DCs release a variety of cytokines such as TNF-α, IL-2, IL-15, IL-12 or IL-18 which could then act on NK cells recruited from the periphery by inflammatory signals and/or DC-derived chemokines. Through cytokines and cell-contact signals, mature DCs (mDCs) could induce the maturation and activation of resting NK-cells (Moretta, 2002). Activated

NK cells cause the lysis of tumour cells and provide antigenic cellular debris that can be internalized by mature DCs to present to T cells in lymph nodes (Pardoll, 2001). In this way, NK cells participate in DC-mediated cognate T-cell responses (Papamichail et al 2004; Cooper et al 2004).

On the other hand, NK cells may also terminate such responses, at a later stage of the immune response where NK cells overwhelm and lyse surrounding DCs, leading to inhibition of antigen presentation (Ferlazzo et al., 2003).

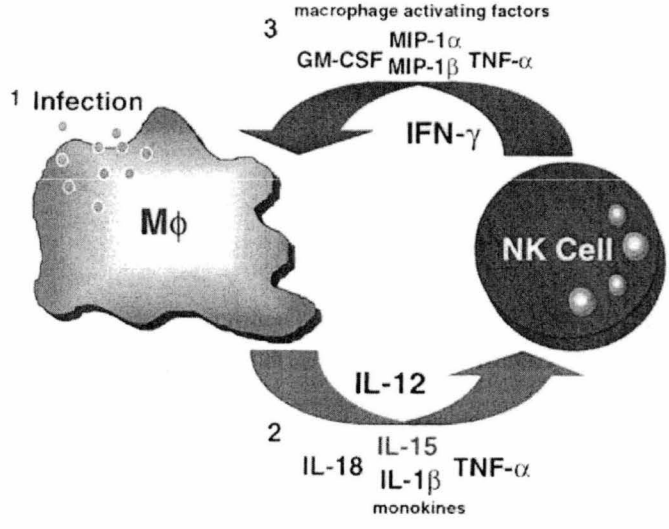

Figure 6. Innate immune cross-talk between activated monocytes/macrophages and NK cells. (Taken from Fehniger and Caligiuri, 2001)

2.7. Cytokine regulation of NK cells

A series of cytokines were found to have many effects on the behavior of NK cells. For example, some cytokines were found to induce the production of IFN-γ by NK cells, such as type I interferons (IFN-α and IFN-β), IL-1α and IL-1β (Hunter, 1997), IL-18 (Okamura, 1995) and IL-10 when combined with IL-18 (Cai et al, 1999). TNF-

α and IL-12 can induce the production of IFN-γ by NK cells in SCID mice with listeriosis (Tripp et al, 1993).

In addition to its role in enhancing IFN-γ production, IL-10, when combined with IL-18, can also enhance NK-cell proliferation and cytotoxicity (Cai et al, 1999). Both IL-12 and IL-18, alone or synergistically, can also induce NK cell activation and cytotoxicity (Smyth et al, 2000). However, the two cytokines which play an important role in NK cell development, homing, proliferation, and survival are IL-2 and IL-15. In addition, exogenous IL-15 was found to significantly induce proliferation, survival, and effector functions of resting NK cells (Carson et al, 1994; Carson et al, 1997). Also in synergism with IL-12, IL-15 or IL-2 can induce the production of various cytokines and chemokines, including IFN-γ, TNF-α, and MIP1-α (Fehniger et al, 2002).

Both IL-2 and IL-15 belong to the four-α-helix bundle cytokine family which utilize heterotrimeric receptors that include the cytokine-specific receptors IL-2R alpha and IL-15R alpha, as well as the two receptor elements that they share, IL-2Rbeta and gamma chains (Tagaya et al, 1996). Signal transduction mediated by these two cytokines involves common JAK (Janus kinase) and STAT (signal transducer and activator of transcription) signaling molecules (Waldmann et al, 2001). IL-15 and IL-2 are produced by different cell types. IL-15 is secreted by monocyte/macrophages, epithelial and fibroblast cell lines, while IL-2 is produced by activated T cells (Grabstein et al, 1994; Tagaya et al, 1996).

Among the many biologic effects of IL-2 and IL-15, is the induction of NK cell activation as shown by enhanced NK cytotoxic activity, antibody-dependent cellular cytotoxicity, and NK cell production of IFNγ, TNFα, and GM-CSF (Tagaya, et al 1996). Although several biological activities of IL-2 and IL-15 are overlapping, some differences have also been reported. For example, IL-2 is a stronger stimulus for GM-CSF production by NK cells (Carson et al, 1994), in addition, NK (CD56 bright) and T lymphocytes were found to have distinct proliferative responses to IL-2 and IL-15 (Carson et al, 1995).

IFN-γ is a pleiotropic cytokine, produced primarily by NK and T cells. Beside its important roles in activating macrophages (Johnston, 1988), in antiviral and antibacterial immunity and in tumour surveillance (Kaplan et al, 1998), it was also found to enhance NK cytotoxicity (Robertson and Ritz, 1990).

The functional IFN-γ receptor (IFNGR) is composed of two ligand-binding IFNGR1 chains associated with two signal-transducing IFNGR2 chains and related signaling machinery. IFNGR1 and IFNGR2 chains belong to class II cytokine receptor family (Schroder et al, 2004).

HYPOTHESIS

The expression of the BLT1 receptor by the various myeloid and lymphoid cells has been the focus of many studies since the discovery of the receptor. However, little is known about the expression of this receptor on NK cell, as it is either not studied, or has been shown not to be expressed by this cell (Pettersson et al, 2003), although it has been shown by work from our laboratory as well as by others, that LTB4 augments NK cell cytotoxicity (Stankova et al, 1992; Rola-Pleszczynski et al, 1983).

OBJECTIVE

We therefore decided to investigate the expression of BLT1 receptor on this important subset of lymphocytes, the NK cells, and their subsets and to determine the effect of the modulation of this receptor on the different functions of NK cells.

MATERIALS AND METHODS

1. Reagents

- IL-15, IL-2 and IFN-γ were obtained from PeproTech, Rocky Hill, NJ, USA.
- LTB_4 and purified rabbit anti-BLT1 polyclonal Ab were obtained from Cayman Chemical, Ann Arbor, Michigan, USA.
- Purified mouse anti-PFP monoclonal Ab and anti-CD56-PE antibody were obtained from BD biosciences, Mississauga, ON, Canada.
- Goat Anti-Rabbit FITC and Anti-Mouse FITC were obtained from Jackson ImmunoResearch, West Grove, PA, USA.
- Chemotaxis 5-μm pore size polycarbonate filters were obtained from GE Osmonics, Minnetonka, MN, Canada.
- RPMI 1640 was obtained from Gibco, Burlington, ON, Canada.
- Fetal Bovine serum (FBS) was obtained from Sigma-Aldrich, Oakville, ON, Canada.

2. Cells

Peripheral blood lymphocytes (PBL) were obtained from the peripheral blood of healthy medication-free volunteers, following informed consent in accordance with an Internal Review Board-approved protocol. Peripheral blood leukocytes were enriched

by dextran sedimentation, layered over a Ficoll-Hypaque cushion, and centrifuged at 1400 rpm for 20 min. Mononuclear leukocytes were collected at the interface and washed twice with PBS and resuspended in RPMI 1640 supplemented with 10% heat-inactivated FBS 100, U/ml penicillin (Novopharm, Toronto, ON, Canada) and 100 μg/ml streptomycin (Sigma-Aldrich, Oakville, ON, Canada) in a humidified atmosphere with 5% carbon dioxide at 37°C. Monocytes were then depleted by adherence (60 min, 37°C) on plastic Petri dishes coated with defibrinized autologous serum. Monocyte-depleted PBL were then collected and resuspended in RPMI 1640 5% FBS at 2 x 10^6 cells/ml before stimulation with the appropriate stimuli.

3. Stimulation of PBL

Two million cells/ml resuspended in RPMI supplemented with 2% Fetal Bovine Serum (FBS) were incubated in a humidified atmosphere with 5% carbon dioxide at 37 °C with either IL-15 (10ng/ml), IL-2 (50ng/ml) or IFN-γ (400U/ml) for 18 hours or LTB_4 (10^{-8} M) for 72 hours or the appropriate control vehicle .

4. Flow cytometry

Cells were washed with Phosphate Buffered Saline (PBS) 1X and fixed with 2% paraformaldehyde (Sigma-Aldrich, Oakville, ON, Canada) for 15 min at room temperature, followed by permeabilization with 0.1% saponin (Sigma-Aldrich,

Oakville, ON, Canada) for an additional 20 min at room temperature. Cells were washed and resuspended in presence of human IgG to block non-specific sites, then resuspended in PBS 2% BSA and labeled with appropriate antibodies

4.1. Identification of NK cells

PBL were labeled for 30 min with directly conjugated anti-CD56-phycoerytherin (PE) Ab in the dark, at room temperature.

4.2. Expression of BLT1 on NK cells

The expression of BLT1 on NK cells was assessed using a polyclonal anti-BLT1 Ab directed against the carboxyl-terminal portion of the receptor. A 1/2000 dilution of the anti-BLT1 Ab was used in all cytometry studies. PBL were labeled in the presence or absence of anti-BLT1 Ab for 30 min at room temperature. Cells were then washed with PBS 1X and incubated for 30 min, in the dark, at room temperature, with FITC-conjugated goat anti-rabbit IgG, diluted 1/1000 in PBS 2% BSA.

4.3. Expression of perforin (PFP) in NK cells

The expression of PFP in NK cells was assessed using a 1/2000 dilution of purified mouse anti-human PFP monoclonal antibody. PBL were labeled with/without anti-PFP Ab for 30 min at room temperature. Cells were then washed with PBS 1X and incubated for 30 min, in the dark, at room temperature, with FITC-conjugated goat anti-mouse IgG, diluted 1/1000 in PBS 2% BSA.

Cells were washed and resuspended in PBS 1X before double-color immunofluorescence analysis. Fifty thousand events were analyzed on FACScalibur flow cytometer (BD Biosciences, CA) using CellQuest software.

5. Chemotaxis assay

PBL were labeled for 30 min with anti-CD56-PE Ab in the dark, at room temperature. Cells were then washed with PBS 1X and resuspended in RPMI 1640 supplemented with 2% BSA. Natural killer cell chemotactic activity was performed with Boyden chambers using a modified Boyden chamber chemotaxis assay. A volume of 200 µl of cells (5×10^5) in RPMI 1640 supplemented with 2% BSA was added to the upper chamber. A volume of 200 µl of 100nM of LTB_4 or vehicle (EtOH) diluted in RPMI 1640 supplemented with 2% BSA was added to the lower chamber. The two chambers were separated by a 5-µm pore size polycarbonate filter. After incubation for 3h at 37°C in 5% CO_2, the chambers were disassembled and the upper side of the filter was scraped free of cells. Cells on the lower side were removed with 5 mM EDTA together with cells from the lower chamber, centrifuged and resuspended in PBS 1X. The number of $CD56^+$ cells acquired in 20 seconds by the FACScalibur was analyzed. Data is expressed in terms of migration index.

$$\text{Migration index (MI)} = \frac{\text{the number of } CD56^+ \text{ cells that migrated in response to } LTB_4}{\text{the number of } CD56^+ \text{ cells that migrated in response to EtOH}}$$

6. Statistical analyses

Data were analyzed for statistical significance using Student's paired *t* test. Differences were considered significant at $p < 0.05$.

RESULTS

Part 1 Expression of BLT1 by NK cell and its subsets

1.1. Expression of BLT1 by NK cells

To investigate the expression of BLT1 on NK cells, freshly isolated lymphocytes were double labeled for CD56 and BLT1 expression, and then analyzed by flow cytometry. A dot plot set for the standard side and forward scatters shows the peripheral blood lymphocytes with the gate set on living lymphocytes as shown in figure 7A. Then with a dot plot of forward scatter versus CD56-PE, we gated on the $CD56^+$ lymphocytes which shows a typical representation of the percentage of NK cells in peripheral blood lymphocytes (12%) (Figure 7B).

We then examined the expression of BLT1 on $CD56^+$ lymphocytes and figure 7C shows a representative histogram of one donor from a total of 7 different donors, showing that 45% of NK cells express BLT1, while the mean for all donors was 38% ± 11% SD for the expression of BLT1 by NK cells. The geomean for expression of BLT1 by NK cells for this representative donor was 3.02 and the mean of the geomean for the 7 donors was 2.23 ± 0.81 SD, where the geomean is representative of the average BLT1 expression per NK cell.

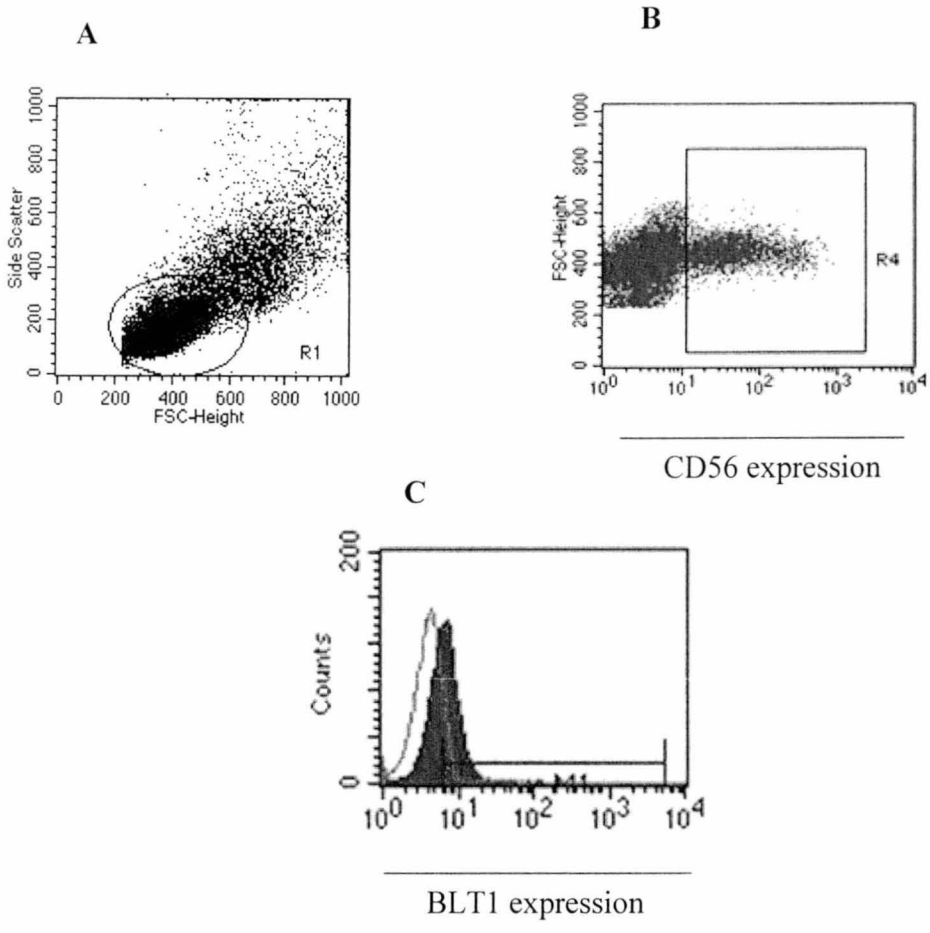

Figure 7. Expression of BLT1 by NK cells.

Peripheral blood lymphocytes were isolated as described in the Materials and Methods. After isolation, cells were labeled with anti-CD56 and anti-BLT1 antibodies.(A) A dot plot set for the standard side and forward scatters showing peripheral blood lymphocytes, the gate is set on living lymphocytes. (B) Then with a dot plot of forward scatter versus FL2-height representing CD56-PE, we gated on the $CD56^+$ lymphocytes. (C) Represents BLT1 expression (dark histogram) on $CD56^+$ lymphocytes, while light histogram represents the negative control.

A representative experiment of one donor from a total of 7 healthy donors is shown.

1.2. Modulation of BLT1 in NK cells by selected cytokines

Given that we have shown that NK cells do express the BLT1 protein, the next question we asked was whether this receptor can be modulated by cytokines which activate NK cells. Freshly isolated lymphocytes ($2x10^6$cells/ml) were incubated with IL-15 (10ng/ml), IL-2 (50ng/ml) or IFN-γ (400U/ml) for 18 hours. Cells were then fixed, permeabilized and double labeled for CD56 and BLT1 and then analyzed by flow cytometry. The results are expressed as the percentage of the NK cells expressing BLT1 which is the $CD56^+BLT1^+$ population and in terms of the geomean which is representative of the average BLT1 expression per NK cell.

Treatment with IL-15 or IL-2 for 18 hours significantly induced the expression of BLT1 in NK cells compared to unstimulated lymphocytes. It resulted in an increase in the percentage of NK cells expressing BLT1 by 38.37% ± 24% SD for lymphocytes treated with IL-15 and 36.19% ± 16% SD for lymphocytes treated with IL-2 (figures 8A and C respectively) compared to unstimulated cells. This increase was also accompanied by upregulation of the BLT1 expression per NK cell as shown by the geomean, where IL-15 resulted in 46.11% ± 39% SD increase in BLT1 expression per NK cell, whereas IL-2 resulted in 41.93% ± 19% SD increase in BLT1 expression per NK cell (figures 8B and D respectively) and it reached up to 2 fold increase in some donors for IL-15.

On the other hand, treatment with IFN-γ, for 18 hours, resulted in a small but significant down regulation of the BLT1 receptor in terms of the percentage of NK

cells expressing BLT1 and the expression of BLT1 per NK cell, where 14% ± 6.7% SD less NK cells expressed BLT1 (figure 8E), while expression of BLT1 per NK cell was reduced by 17% ± 3.5% SD (figure 8F).

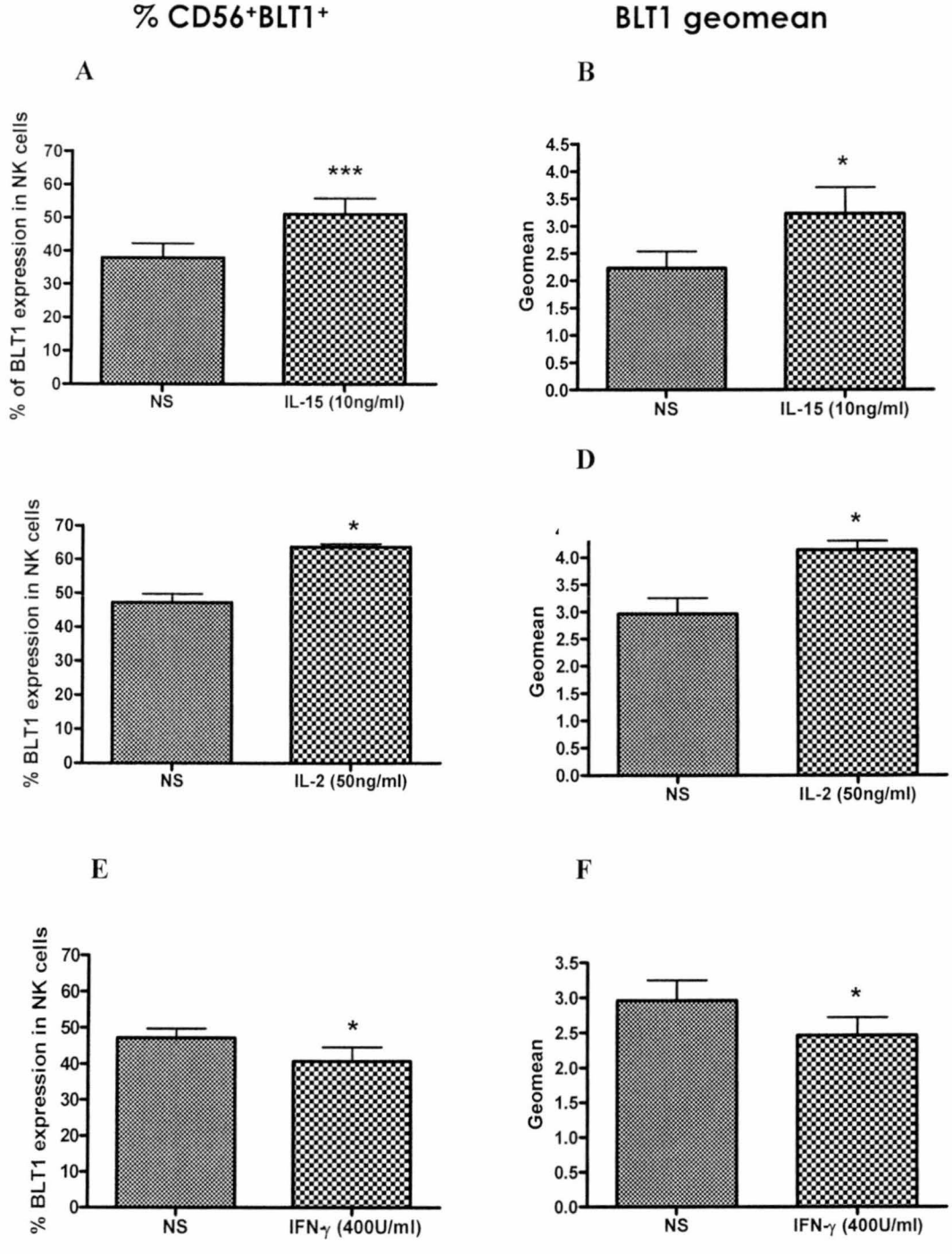

Figure 8. Modulation of BLT1 by selected cytokines. Flow cytometric analysis of $CD56^+$ cells. Freshly isolated lymphocytes were incubated with IL-15, IL-2, IFN-γ or medium (NS) at the indicated concentrations for 18 hours at 37°C, 5%CO_2. Cells

were then fixed, permeabilized and double labeled with anti-CD56 and anti-BLT1 antibodies and analyzed by flow cytometry. The percentage of $CD56^+$ cells expressing BLT1 after stimulation with (A) IL-15, (C) IL-2 or (E) IFN-γ (left panels), and the BLT1 geomean on $CD56^+$ cells after stimulation with (B) IL-15, (D) IL-2 or (F) IFN-γ (right panels) are shown as bar charts comparing stimulated to unstimulated NK cells. The bar charts represent the mean and the standard deviation for 7 separate donors and experiments for IL-15 and 3 for each of IL-2 and IFN-γ. * p <0.05; ** p <0.01; *** p <0.005

1.3. Expression of BLT1 by NK cell subsets

NK cells can be divided into two distinct subsets based on the density of cell-surface expression of CD56 molecules. The majority of NK cells express low levels of CD56 molecules and they are called $CD56^{dim}$, while a minority expresses high levels of CD56 molecules and they are called $CD56^{bright}$. The two subsets show different patterns of expression of cytokines and cytotoxicity receptors as well as chemokine production and receptor expression. We therefore examined the BLT1 expression by the two NK subsets.

After gating on the living lymphocytes using the standard side and forward scatters (figure 9A), the NK cells can be divided into two distinct subsets based on the CD56 density expression using the dot plot of the forward scatter versus the FL2-height for CD56-PE.

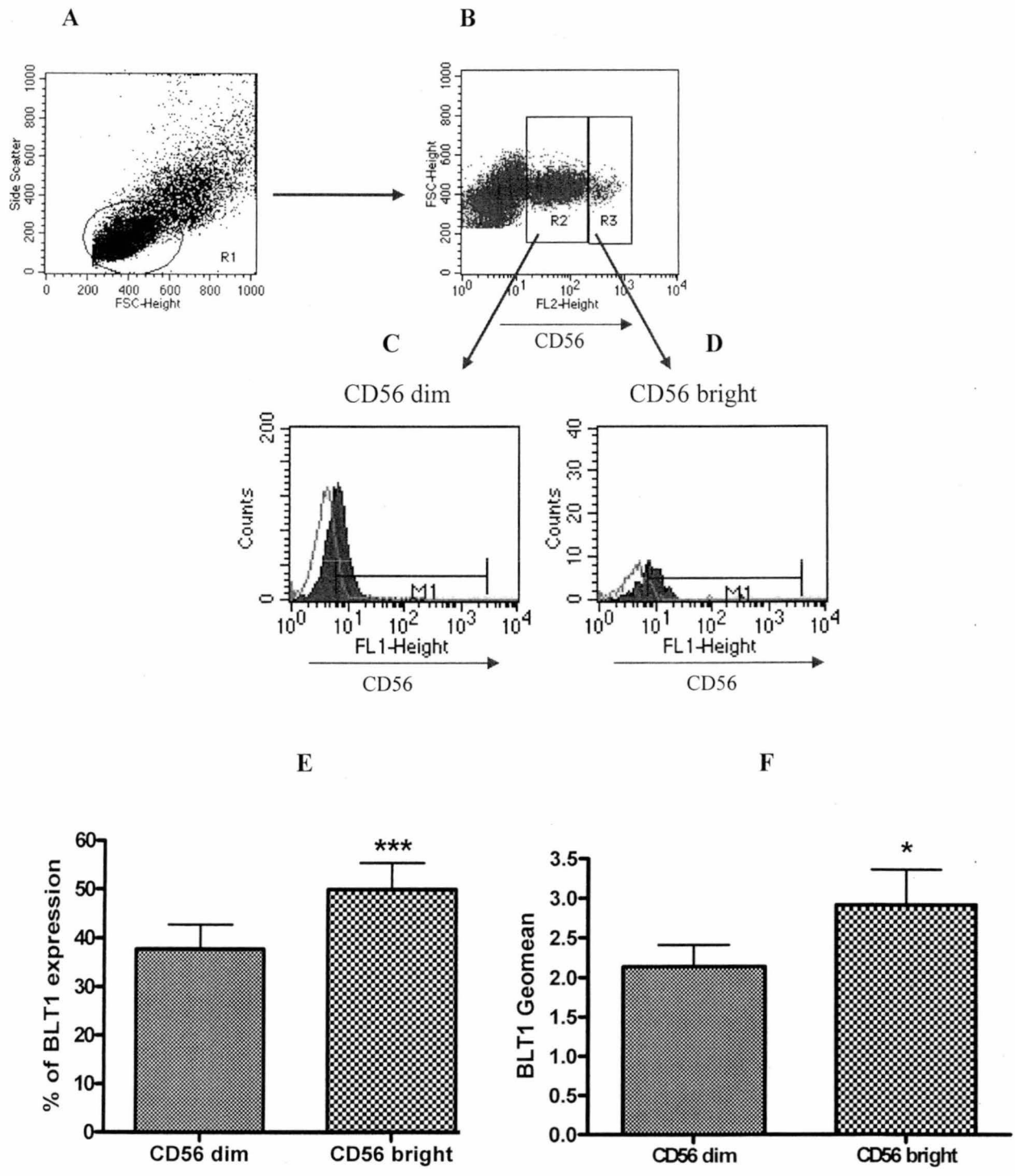

Figure 9. Expression of BLT1 by the CD56$^+$ subsets.

Freshly isolated lymphocytes were labeled with anti-CD56 and anti-BLT1 antibodies as described in the Materials and Methods. (A) A dot plot set for the standard side and forward scatters showing peripheral blood lymphocytes, the gate is set on living lymphocytes. (B) Then with a dot plot of forward scatter versus FL2-height representing CD56-PE, we gated on each of the $CD56^{+}$ lymphocyte subsets ($CD56^{dim}$ and $CD56^{bright}$) according to surface density expression of CD56 molecules. The dark histogram represents BLT1 expression on (C) $CD56^{dim}$ and (D) $CD56^{bright}$ lymphocytes while light histogram represents the isotype control. A-D show a representative experiment of one donor from a total of 7 healthy donors. (E) Bar chart showing the percentage expression of BLT1 by the $CD56^{dim}$ and $CD56^{bright}$ subsets. (F) Bar chart showing the BLT1 geomean expressed by the $CD56^{dim}$ and $CD56^{bright}$ subsets. * $p < 0.05$; *** $p < 0.005$. n=7

As shown in Figure 9B, $CD56^{dim}$ are represented by R2 while $CD56^{bright}$ by R3. Figure 9B is a representative plot showing a typical profile of the two subsets of $CD56^{+}$ lymphocytes, where the $CD56^{bright}$ subset comprises approximately 10% of the $CD56^{+}$ population.

Figure 9C is a representative histogram showing the expression of BLT1 by the $CD56^{dim}$ subset. It shows that 44% of this subset expresses BLT1, and the geomean of BLT1 in this subset is 3.06. On the other hand, 57% of the $CD56^{bright}$ subset express BLT1 with a geomean of 4.85 (figure 9D).

Compilation of data from seven donors demonstrated a statistical difference of BLT1 expression between the two subsets (p = 0.0003), where 37% ± 4.9% SEM of $CD56^{dim}$ subset express BLT1 while 50% ± 5.4% SEM of $CD56^{bright}$ express the receptor as shown by the bar chart (figure 10E).

We also found statistically significant differences in the expression of BLT1 per NK cell by each of the two subsets (p = 0.0174), where the $CD56^{bright}$ expressed more BLT1 per cell (3 ± 0.5 SEM) compared to the $CD56^{dim}$ (2 ± 0.3 SEM) as shown by the geomean bar chart (figure 9F).

1.4. Modulation of BLT1 in $CD56^{+}$ subsets by cytokines

Given that the two subsets express different levels of BLT1, we sought to determine whether the two subsets respond differently by modulating BLT1 expression in response to the selected cytokines. Following stimulation of lymphocytes with either of IL-15 or IL-2 for 18 hours, both $CD56^{+}$ subsets responded by an increase in the percentage of cells expressing BLT1, with a statistically significant difference between the two subsets for each of IL-15 (p=0.0088) and IL-2 (p=0.0066) (figure 10A). However, $CD56^{dim}$ subset showed a slightly higher response to both cytokines in terms of up regulation of BLT1 expression as shown by comparing the fold induction of each subset in response to each cytokine. $CD56^{dim}$ showed upregulation of BLT1 by 1.38 fold ± 0.11 SEM, while $CD56^{bright}$ showed upregulation of BLT1 by 1.26 fold ± 0.07 SEM in response to IL-15 stimulation. Likewise, $CD56^{dim}$ showed upregulation of BLT1 by 1.30 fold ± 0.11 SEM, while $CD56^{bright}$ showed

upregulation of BLT1 by 1.19 fold ± 0.09 SEM in response to IL-2 stimulation as shown in figure 10B.

Conversely, in response to IFN-γ the $CD56^{brigh}$ subset (0.84 fold ±0.05 SEM) showed more down regulation of BLT1 expression compared to the $CD56^{dim}$ subset (0.26 fold ± 0.03 SEM) with a statistically significant difference between the two subsets of p=0.0435.

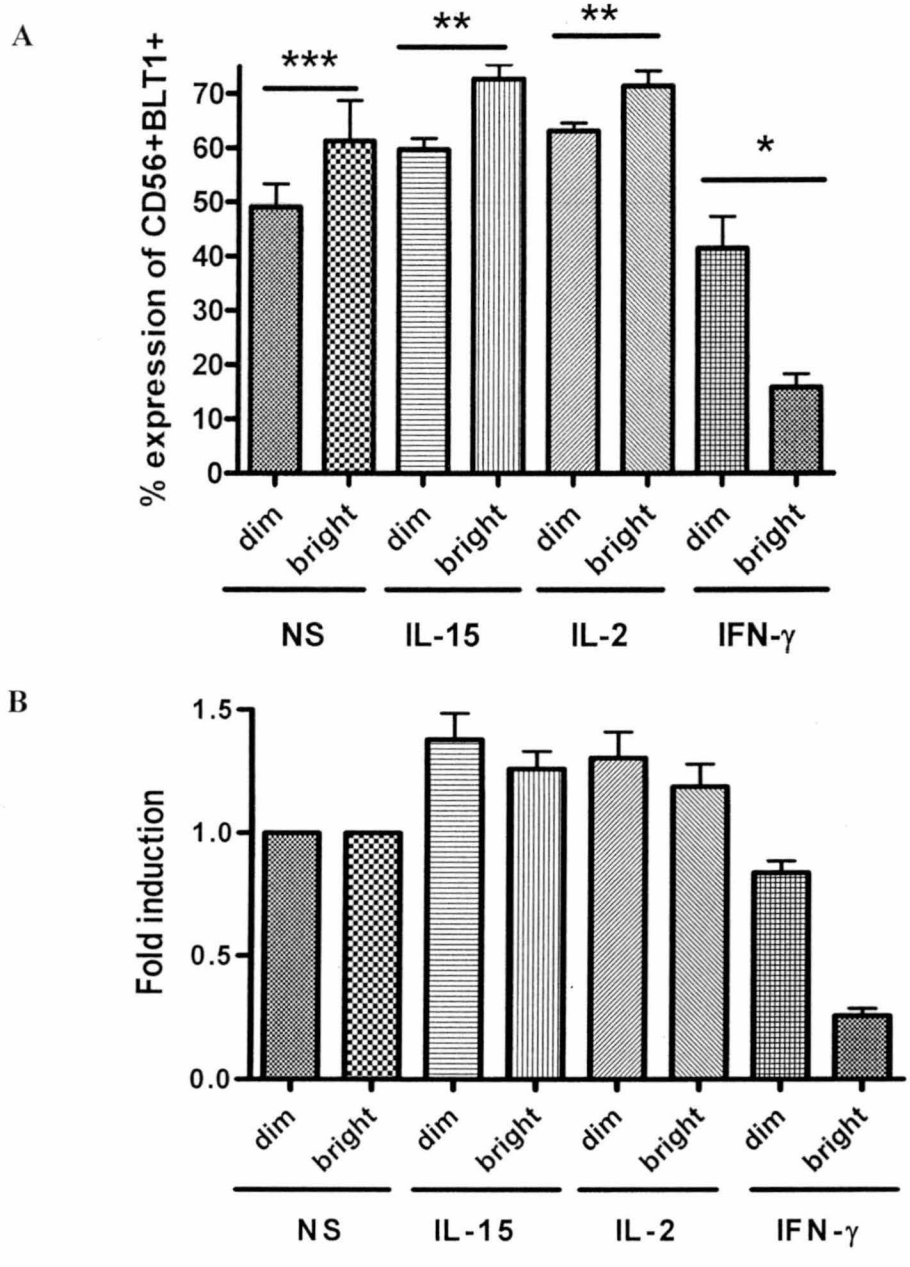

Figure 10. Modulation of BLT1 in the CD56$^+$ subsets by different cytokines. Freshly isolated lymphocytes were stimulated by the indicated cytokines, then labeled with anti-CD56 and anti-BLT1 antibodies as described in the Materials and Methods. (A) Bar chart showing the percentages of CD56$^+$ lymphocyte subsets expressing

BLT1 after 18 hours of stimulation with of IL-15, IL-2, IFN-γ or medium (NS). (B) Bar chart of fold induction of BLT1 expression by the $CD56^+$ lymphocyte subsets in response to 18 hours stimulation with IL-15, IL-2 and IFN-γ after setting the unstimulated control (NS) to one.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$, for 3-7 different donors

Part 2 Natural Killer cell chemotaxis in response to LTB_4

2.1. Natural Killer cell migration in response to LTB_4

LTB_4 is known as a potent chemoattractant for various cell types. We have shown that NK cells do express BLT1 and we therefore sought to determine if BLT1 expression by these cells correlated with their ability to migrate in response to LTB_4.

Freshly isolated lymphocytes were labeled with anti-CD56-PE then allowed to migrate in response to LTB_4 or its vehicle (EtOH) using the Boyden chamber technique. After allowing the cells to migrate for three hours, cells that migrated to the lower chamber together with cells from the under side of the filter were collected then analyzed using flow cytometry.

Migration assays done for fresh peripheral blood NK cells from seven different donors and experiments showed a significant migration of NK cells in response to LTB_4 compared to EtOH. NK cells showed 75.5 % ± 36.57 SEM more migration to

LTB_4 and the migration index reached up to two fold that of the migration in response to vehicle (figure 11).

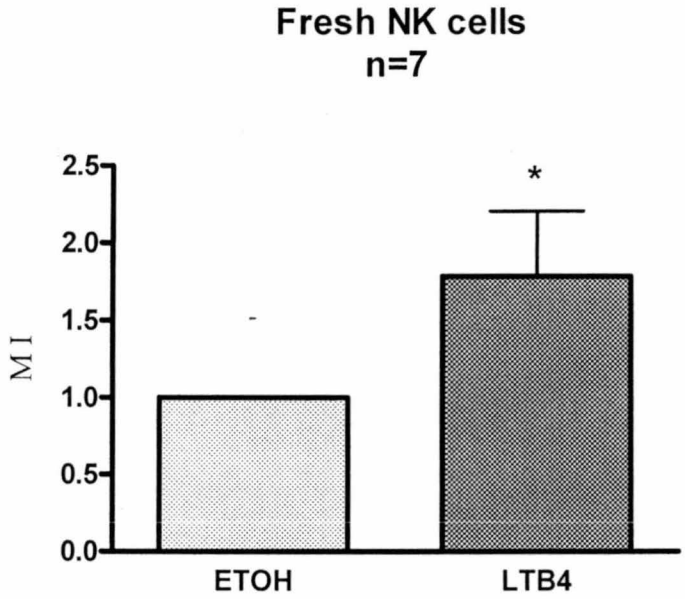

Figure 11. Migration of NK cells to LTB_4. Fresh PBL were labeled with anti-CD56-PE, then allowed to migrate for 3 hours to LTB_4 (100nM) or its vehicle (EtOH), using the Boyden chamber technique. Cells were then collected and analyzed using flow cytometry. The bar chart shows the migration index (MI) (see Materials and Methods) of fresh NK cells to LTB_4. This bar chart shows the mean and the standard error of the migration index for 7 individual donors. $P = 0.0224$

2.2. Effect of BLT1 modulation on NK cell migration

After showing that NK cells migration is enhanced by LTB_4 (figure 12), we sought to investigate the effect of BLT1 modulation on NK cell migration. Freshly isolated

lymphocytes were incubated overnight in the presence or absence of IL-15, a cytokine which up regulates BLT1 (figure 8A and B). PBL were then labeled with anti-CD56-PE and allowed to migrate to LTB_4 or its vehicle for three hours using the Boyden chamber technique. Cells were then collected from the lower chambers together with cells from the under side of the filters and analyzed by flow cytometry.

NK cells, that had been preincubated overnight in medium, showed a significantly enhanced migration (* p=0.0281) to LTB_4 compared to vehicle (EtOH) with 22.78 % ± 7.56 SEM more migration to LTB_4. However, preincubation with IL-15 resulted in more NK cells migrating in response to LTB_4 compared to vehicle (EtOH) (54.62 % ± 13.19 SEM ** p=0.0042).

Interestingly, we also noticed that NK cells that were incubated overnight (22.78 % ± 7.56 SEM) showed less migration in response to LTB_4 compared to fresh NK cells (75.5 % ± 36.57 SEM).

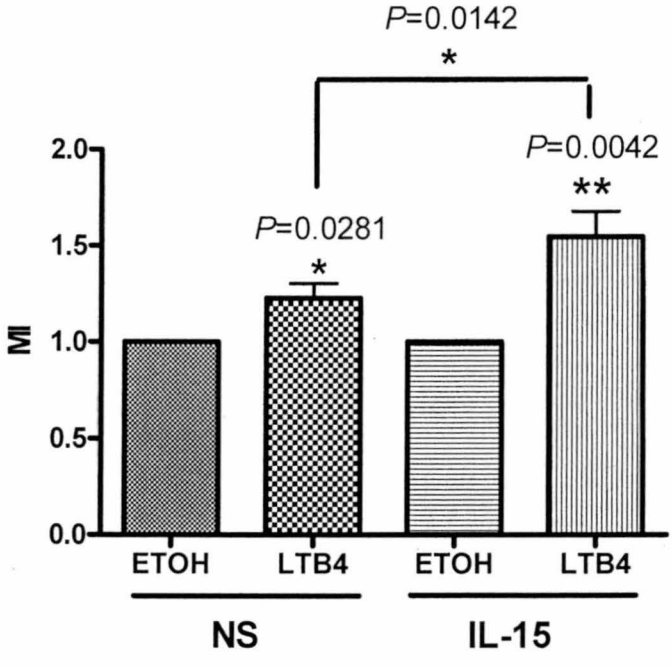

Figure 12. Effect of preincubation with IL-15 on the migration of NK cells in response to LTB_4. Fresh PBL were incubated with IL-15 (10ng/ml) or medium (NS) for 18 hours. PBL were then washed and labeled with anti-CD56-PE, and allowed to migrate for 3 hours to LTB_4 or its vehicle (EtOH) at a concentration of 100nM, using the Boyden chamber technique. Cells were then collected and analyzed by flow cytometry. The bar chart shows the migration index of NK cells to LTB_4.

This bar chart shows the mean and the standard error of the migration index for 7 individual donors.

2.3. Migration of NK cell subsets to LTB_4

Taking in consideration that the NK subsets show differences in the level of expression of BLT1, we sought to determine whether NK cells migrate as a homogenous population or whether the different subsets have different potentials in migrating towards LTB_4.

Chemotaxis assay was performed on freshly isolated PBL as described earlier in the Results. In order to determine if there was a difference in the migration of the two subsets, we gated on $CD56^+$ lymphocytes (figure 13A) then the quadrant was placed in order to divide the $CD56^+$ population into the two subsets using dot plots of FL2-height for the CD56 versus FSC-height as seen in figures 13B and C, and then we could quantify the percentage of each subset in the migrated NK cells. If both subsets had the same potential of migration in response to LTB_4, one would expect the same relative percentages of the subsets to be found in the initial population as in the migrated one.

Figures 13B and C show representative dot plots from one donor. These dot plots confirm results shown in figure 12 that more NK cells migrate to LTB_4 compared to EtOH as shown by the total number of migrated NK cells to LTB_4 (2241) and EtOH (1106) at the upper left corner of the dot plots. In addition, they also show that a higher percentage of $CD56^{bright}$ cells migrate to LTB_4 than $CD56^{dim}$ cells as shown by the numbers at the upper right corner of the plots.

Although the difference is small, it was consistent for the five different donors used. Figure 13D shows a modest, but statistically significant difference in the migration index of fresh $CD56^{bright}$ cells to LTB_4 compared to migration to EtOH, which indicates that relatively more fresh $CD56^{bright}$ cells than $CD56^{dim}$ cells migrate to LTB_4.

Incubation with IL-15 resulted in another modest but significant increase of $CD56^{bright}$ cells migrating to LTB_4 comparing that to cells incubated in medium. This indicates that $CD56^{bright}$ cells which express more BLT1 than $CD56^{dim}$ cells showed better migration to LTB_4 and this migration was further enhanced following up-regulation of the BLT1 with IL-15 (figure 13E).

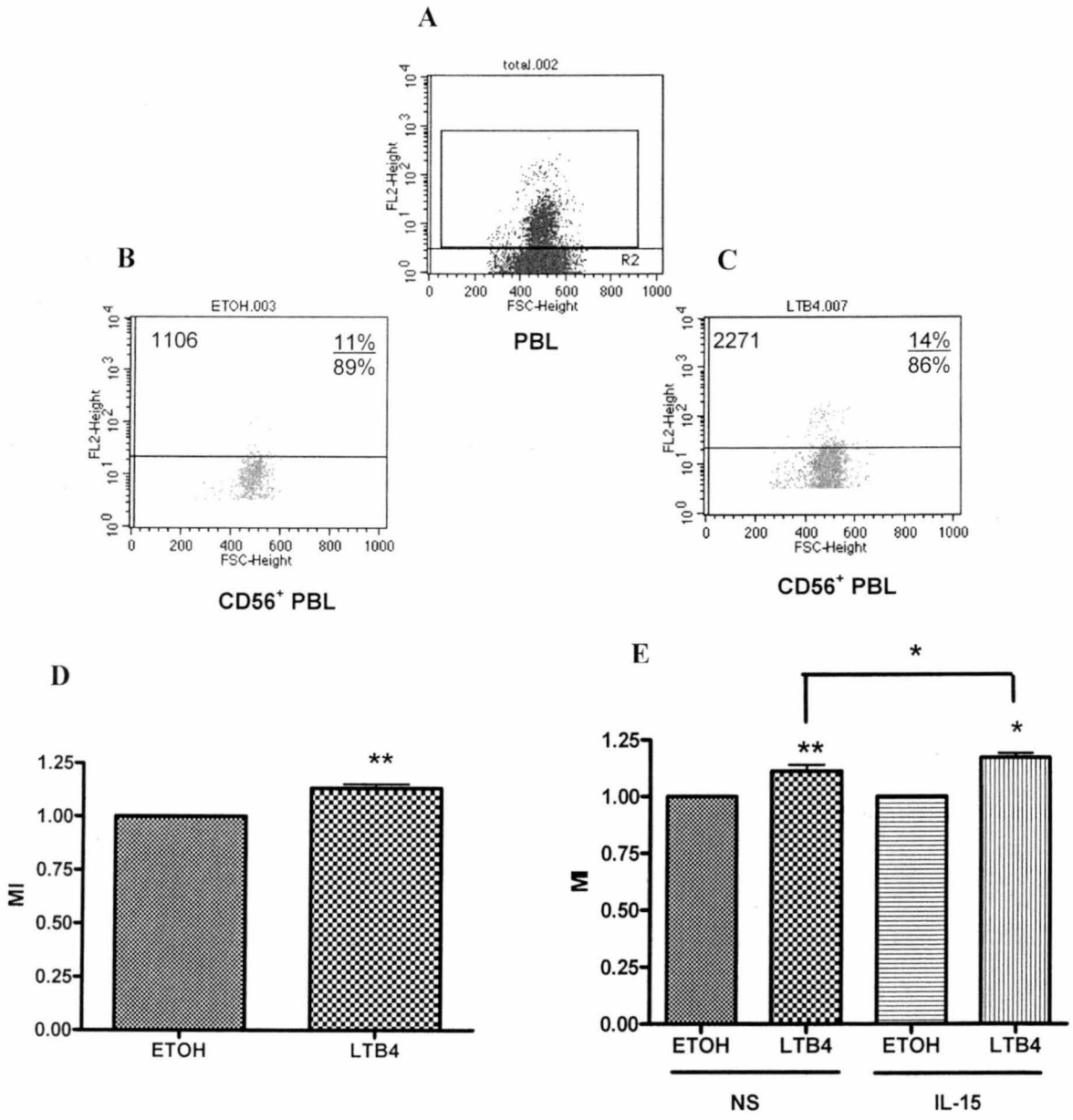

Figure 13. Migration of CD56 subsets in response to LTB_4. (A) Dot plot of PBL with FL2-Height for CD56-PE versus FSC-Height gated on the $CD56^+$ population. Migrated $CD56^+$ lymphocytes in response to (B) EtOH and (C) LTB_4 with the quadrant dividing the $CD56^+$ population into $CD56^{bright}$ (above) and $CD56^{dim}$ (below) with the total number of migrated NK cells at the upper left corner and the percentages of each subset at the upper right corner. (D) Bar chart of the migration

index of the $CD56^{bright}$ subset to LTB_4 and EtOH. (E) Bar chart of the migration index of the $CD56^{bright}$ subset to LTB_4 after incubation in the presence or absence of 10ng/ml IL-15 for 18 hours.

Part 3 Perforin expression by NK cells and their subsets

3.1. Perforin expression by NK cells and their subsets

Perforin is constitutively expressed by NK cells; yet, the relative levels of expression by the two NK subsets are still controversial. A study published in 1995,indicates that both NK subsets express perforin at high levels (97-99%; Konjevic et al, 1995)but a recent study reported that the $CD56^{dim}$ subset expresses significantly more perforin protein and transcripts than the $CD56^{bright}$ subset (Wendt et al, 2006).

Our results show that, for a representative donor, around 80% of NK cells express perforin protein (figure 14B) after gating on the $CD56^{+}$ population (figure 14A). After gating on each subset separately (figure 14C), we also found that 80% of the $CD56^{dim}$ subset expresses perforin protein (figure 14D) compared to approximately 40% of the $CD56^{bright}$ subset (figure 14E) for the same donor.

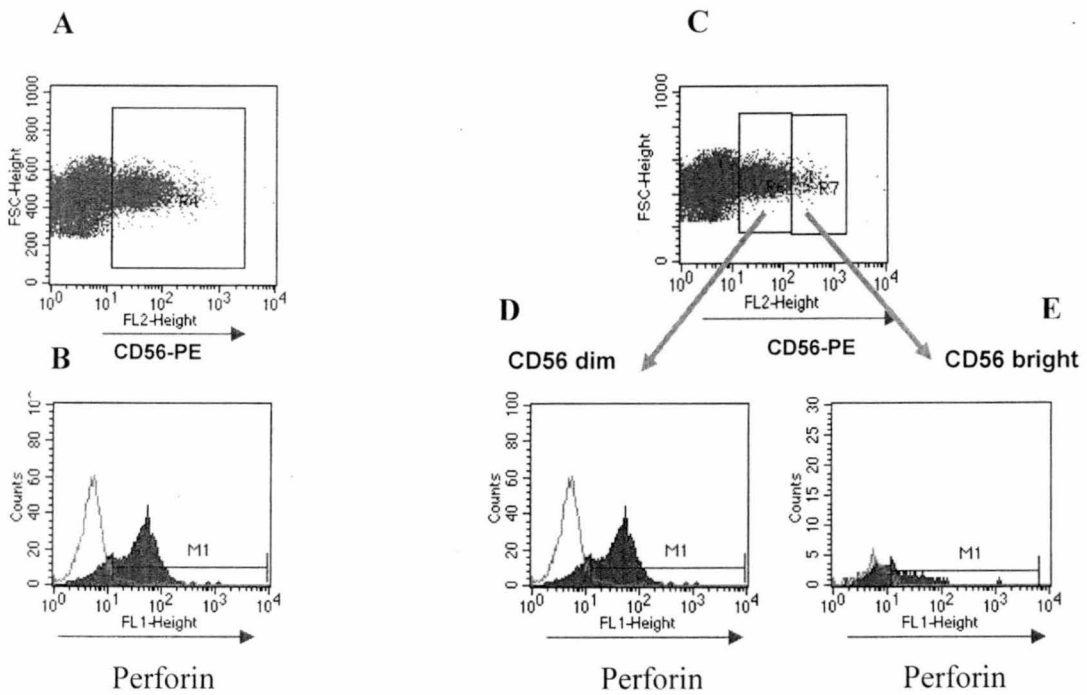

Figure 14. Perforin expression by NK cells and their subsets. Freshly isolated lymphocytes were fixed, permeabilized and labeled with anti-CD56 and anti-PFP antibodies as described in the Materials and Methods. (A) Dot plot of CD56-PE versus Forward scatter showing PBL gated on the $CD56^{+}$ population. (B) Histogram of the total $CD56^{+}$ population showing perforin expression. (C) Dot plot of CD56-PE versus Forward scatter showing PBL gated on each of the $CD56^{dim}$ and $CD56^{bright}$ subsets separately. Histograms of (D) the $CD56^{dim}$ subset and (E) the $CD56^{bright}$ subset showing perforin expression. Perforin expression is represented by the filled histogram, while the control is represented by the light histogram. These data are representive of one donor out of five donors examined.

3.2. Effect of LTB4 on perforin expression

In order to examine the effect of LTB_4 on perforin expression by NK cells, LTB_4 concentration response and kinetics were first performed. Freshly isolated lymphocytes were incubated with various concentrations of LTB_4 or its vehicle EtOH (10^{-7}-10^{-11} M) for 24 hours. They were then labeled with anti-CD56 and anti-PFP antibodies and analyzed for expression of perforin by flow cytometry.

At this time point, the effect of LTB_4 concentration response on perforin expression by NK cells was very modest and not statistically significant. However, a slight modulation of PFP was seen with LTB_4 at a concentration of 10^{-8} M (figure 16A). Kinetic experiments with LTB_4 (10^{-8} M) were performed with freshly isolated lymphocytes over 6 – 96 hours, labeled for CD56 and PFP expression and analyzed by flow cytometry.

Down-regulation of the perforin protein was seen in NK cells at 72 hours. There was 20% reduction in perforin expression for a representative donor shown in figure 16B. Although this effect of LTB_4 on perforin expression in NK cells is modest, it was consistent and statistically significant (*p= 0.0233). NK cells preincubated with LTB_4 (10^{-8} M) were found to express 87.4% ± 3.97 SEM of the perforin expressed by NK cells preincubated in EtOH.

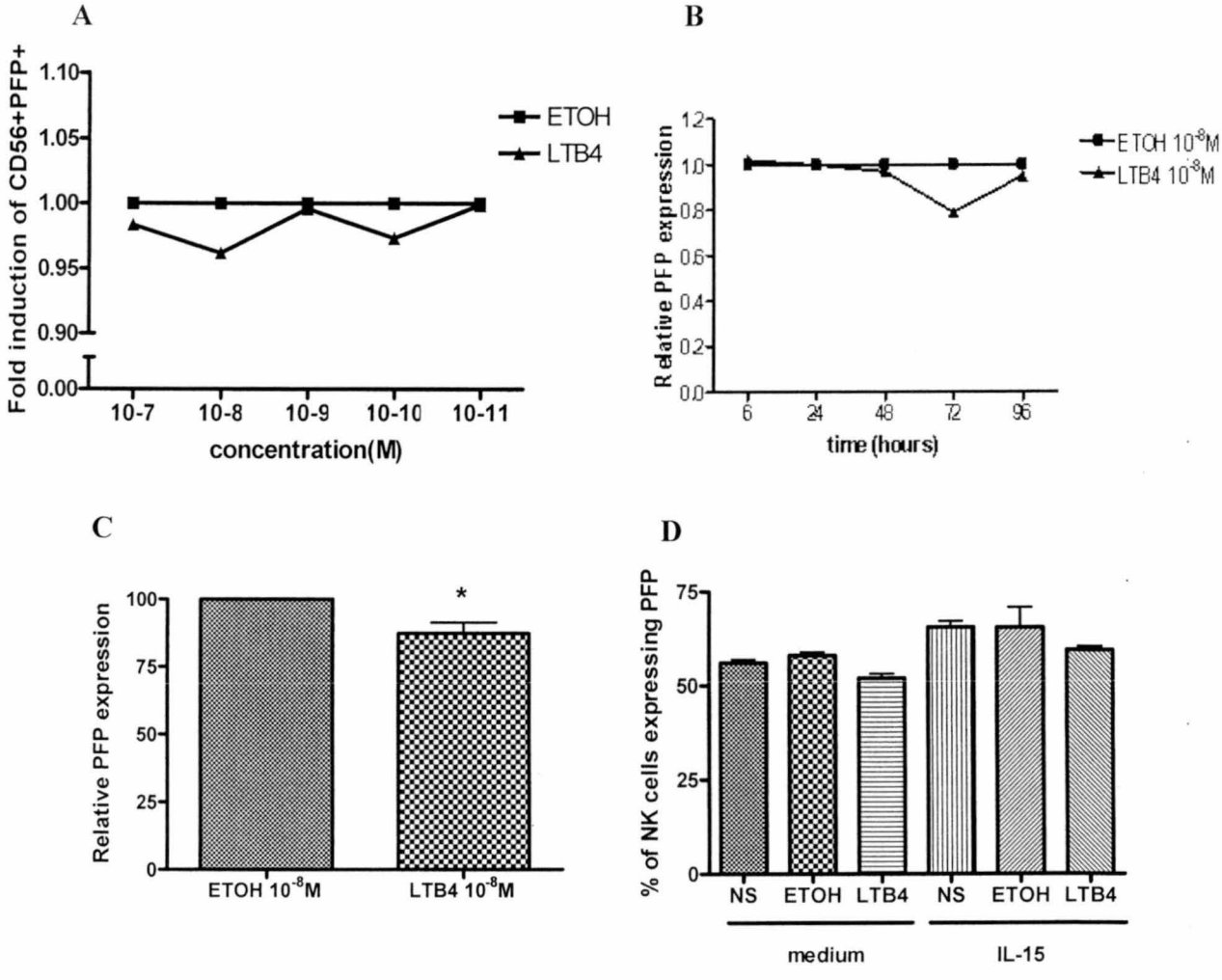

Figure 15. Effect of LTB_4 on perforin expression in NK cells. (A) Effect of LTB_4 concentration response (10^{-7}-10^{-11} M) on perforin expression by NK cells after 24 hours, in terms of fold expression after standardizing EtOH to 1, n=1. (B) Effect of LTB_4 (10^{-8} M) on perforin expression by NK cells over a time range of 6 to 96 hours. (C) Regulation of perforin expression in NK cells by LTB_4 (10^{-8} M) after 72 hours, mean and SEM of five different experiments. (D) Effect of LTB_4 on perforin protein expression in NK cells following preincubation with IL-15 or medium from a representative donor out of five donors examined. *$p<0.05$

3.3. Impact of modulation of BLT1 on perforin protein expression by NK cells stimulated with LTB_4

We have found that LTB_4 resulted in a down-regulation of perforin in NK cells (figure 15C), we then sought to determine if modulating BLT1 expression in NK cells can further impact the effect of LTB_4 on perforin expression. PBL were preincubated with IL-15 at a concentration of 10 ng/ml or medium for 18 hours and then incubated with LTB_4 or EtOH (10^{-8} M) or unstimulated (NS) for 72 hours. PBL were then fixed, permeabilized and labeled for CD56 and PFP and analyzed by flow cytometry.

LTB_4 resulted in down-regulation of perforin in NK cells, as compared to its vehicle EtOH. However, preincubating PBL with IL-15 did not have any further impact on LTB_4-stimulated decrease of perforin compared to PBL preincubated with medium as shown in figure 15D.

DISCUSSION

LTB_4 is a potent leukocyte activator and chemoattractant, which mediates its biological action through LTB_4 receptors, BLT1 and BLT2. BLT1 has been reported to be primarily expressed by granulocytes and monocytes (Pettersson et al, 2000; Dasari et al, 2000), to a lesser extent in antigen primed $CD4^+$ and $CD8^+$ T cells (Islam et al, 2006) and not to be expressed by NK cells as shown by Pettersson et al in 2003. In this study, we present evidence that BLT1 is expressed on human NK cells and this expression can be modulated by selected cytokines such as IL-15, IL-2 and IFN-γ. Moreover, LTB_4 induces human NK cell chemotaxis which is further augmented by the up regulation of the BLT1. Interestingly, we also found differential expression of BLT1 by NK cell subsets with subsequent variations in their functional activities.

The expression of BLT1 by the various subsets of immune cells has been the focus of many studies since the discovery of the receptor. Despite that, little is known about the expression of this receptor on NK cells, as it was either indicated that they do not express BLT1 in one study, or it was not studied in other reports, although it had been shown that LTB_4 augments NK cell cytotoxicity (Jondal, 1985; Stankova et al, 1992).

In this study, we have presented the first evidence for BLT1 expression on NK cells. Flow cytometric analysis of fresh peripheral blood lymphocytes, using specific anti-BLT1 antibodies, revealed that 37.89% ± 11.36% SD of the classical ($CD56^+$) NK cells express BLT1.

It is now well established that pro- and anti-inflammatory cytokines as well as microbial products modulate the expression of BLT1. For example, proinflammatory mediators such as interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and lipopolysaccharide (LPS) down-regulate BLT1 expression, while the anti-inflammatory cytokine, interleukin-10, and dexamethasone up-regulate BLT1 expression on monocytes (Pettersson et al., 2005). Conversely, LPS down-regulates BLT1 expression on human endothelial cells (Qiu et al., 2006). In addition, BLT1 expression was up-regulated by dexamethasone (Stankova et al., 2002), and down-regulated by TNF-α on polymorphonuclear neutrophils (O'Flaherty et al., 1991).

Several cytokines were found to have diverse effects on NK cells, including IL-2, IL-15 (Carson et al, 1994; Carson et al, 1997), IFN-γ (Zhang et al, 2004), IL-12 (Smyth et al, 2000), type I interferons (Hunter, 1997), TNF-α (Tripp et al, 1993), IL-1 (Hunter, 1997), IL-10, IL-18 (Cai et al, 1999) and others. However, the cytokines with the most profound effects on NK cells were found to be IL-2, IL-15 and IFN-γ. We hypothesized that these cytokines may have an effect on the expression of BLT1 on NK cells. Consistent with this hypothesis, IL-2 has been reported to up-regulate several chemokine receptors including CCR2, CCR4, CCR5, CCR7, CCR8, and CX3CR1 on human NK cells (Barlic et al, 2003; Robertson, 2002). While, IL-15 up-regulates expression of CX3CR1 on NK cells (Barlic et al, 2003) and CC chemokine receptors on human T cells (Perera et al, 1999), but their effects on BLT1 expression on NK cells or on any other cell type have not been reported.

In the present study, we have analyzed the regulation of BLT1 surface expression on human NK cells by IL-2, IL-15 and IFN-γ. Freshly isolated peripheral blood lymphocytes were incubated for 18 hours with each of these cytokines, and the effect on BLT1 expression on NK cells was investigated by flow cytometric analysis. Expression was addressed by two parameters. The first is the percentage of cells expressing the receptor and the second is the average receptor expression per NK cell. We found that IL-2 and IL-15 resulted in the up-regulation of the receptor on these cells while IFN-γ resulted in down-regulation, when both parameters were studied.

A remarkable feature of the immune system is the directed migration of leukocytes during inflammatory events. This immune cell trafficking involves the interaction between chemotactic signals and their corresponding receptors.

NK cells are one of the major players in immunosurveillance and antitumor responses. NK cells circulate in peripheral blood, and rapidly migrate to sites of immune reactions in peripheral tissues, in response to gradients of chemokines. The mechanisms by which NK cells home into areas of inflammation and tumors are of great relevance to the human health and disease (Campbell, 2001), but the molecular basis responsible for NK cell recruitment into tissues is not completely known.

NK cells were found to express many chemokine receptors including, CXCR1, CCR2, CCR5, CCR7 and CX3CR1 (Qin et al, 1996; Nieto et al, 1998; Kim et al, 1999; and Imai et al, 1997). NK cells were also found to migrate in response to many factors including, classical chemotactic factors as casein, C5a (Bottazzi et al, 1985), cytokines such as TNF-α, IL-2, and IL-12 (Maghazachi, 1991; Allavena et al, 1994)

and chemokines including MCP-1, -2, and -3, RANTES, MIP-α and IL-8 (Allavena et al, 1994; Sebok et al 1993).

LTB_4, through its high-affinity receptor BLT1, was found to be able to direct the migration of many leukocytes, including granulocytes (Ford-Hutchinson et al, 1980), eosinophils (Wardlaw et al, 1986), monocytes, macrophages (Migliorisi et al, 1987); (Crooks et al, 1998) and effector T cells (Tager et al, 2003; Goodarzi et al, 2003; Ott et al, 2003). As for the ability of LTB_4 to direct NK cell migration, there was no information at this point, we therefore sought to investigate this aspect. A migration assay using the Boyden chamber technique for fresh peripheral blood NK cells was performed. We showed that NK cells migrate significantly more towards LTB_4 compared to EtOH (vehicle). The migration index reached up to 2 fold in some cases.

The high-affinity receptor for LTB_4, BLT1, is expressed by subsets of immune cells and is needed for an efficient immune response to invading pathogens (Talvani et al, 2002). Furthermore LTB_4/BLT1 interactions are emphasized in the pathogenesis of many inflammatory conditions such as rheumatoid arthritis (RA), asthma, cystic fibrosis, psoriasis, inflammatory bowel disease, and atherosclerosis (Ahmadzadeh et al, 1991; Montuschi and Barnes, 2002; Konstan et al, 1993). We have shown that BLT1 is expressed in human NK cells and that LTB_4 is capable of inducing the migration of these cells. We may speculate that the elevated levels of LTB_4 at inflammatory sites which lead to the local recruitment of BLT1-expressing leukocyte subsets from the bloodstream, also includes NK cells. However, further studies have

to be done on this aspect in order to confirm the presence of NK cells at these sites containing high levels of LTB_4 and examine their role in the pathogenesis of these diseases. Given that no specific markers for NK cells were used in former studies, there is no precise information as yet about the presence of NK cells at these sites.

NK cells constitutively express IL-2/15Rβγc, but the lack of abundant IL-2 during the early innate immune response, as IL-2 is produced primarily by antigen-activated T cells located in the periphery, positioned IL-15 as the most important physiological ligand for NK cell proliferation, cytotoxicity, and cytokine production (Carson et al, 1994). Therefore, in our studies, we focused on IL-15 for the further exploration of BLT1 modulation and the consequence of this modulation on the different functions of human NK cells.

Fresh peripheral blood lymphocytes were incubated with IL-15 at a concentration of 10ng/ml for 18 hours, same conditions that induced BLT1 up-regulation by IL-15 on NK cells. Significant migration was detected by NK cells in response to LTB_4 in comparison with the control cells incubated with medium, in absence of IL-15. This indicates that the prior incubation with IL-15 resulted in up regulation of BLT1, resulting in enhanced migration of the NK cells to the ligand LTB_4. Therefore, increased expression of BLT1 on NK cells by IL-15 may have a potential role in the diverse immune responses.

IL-2 was an early candidate for the immunotherapy of cancer and already approved for use in renal cell cancer (Figlin, 2000). However, a lot of work has been done

recently focusing on the unique in vitro and in vivo functions of IL-15 and the promising applications for its use in cancer immunotherapy. It is a likely candidate for exogenous cytokine therapy as it potentially expands NK cells, memory $CD8^+$ T cells, and some non-classical T cell lineages (Fehniger et al, 2002). In addition, higher amounts of IL-15 used for a limited duration may be effective to activate innate immune cells, activate NK cell killing and ADCC of tumor targets in a similar fashion as intermediate doses of IL-2 (Carson et al, 1994), and stimulates cytokine production (e.g. IFN-γ, TNF-α, GM-CSF) in combination with other cytokines by the $CD56^{bright}$ NK cells (Fehniger and Caligiuri, 2001). These in vitro effects suggest that providing IL-15 in intermediate doses in vivo may activate the cytotoxicity and cytokine-producing function of NK cells.

There is compelling evidence for the role of NK cells in tumor immunosurveillance and their beneficial effects on many experimentally successful immunotherapy strategies. Our research further adds to the previously mentioned advantages of IL-15, its use in the immunotherapy of some cancers where there are elevated levels of LTB_4 such as in colon cancer (Ihara et al, 2007). The up-regulation of BLT1 leading to increased migration of NK cells to these sites where they can play their role in mediating cell contact-dependent cellular cytotoxicity and producing pro-inflammatory cytokines and thus help in their anti-tumor role.

One and most important function of NK cells is cytotoxicity, where NK cells were first identified by their ability to detect and kill certain types of target cells, mainly cancerous and virally infected cells. NK cells can accomplish this by different

mechanisms, but the primary mechanism is through granule exocytosis, where it is capable of secreting many proteins from their cytoplasmic granules, including perforin, granzymes and others leading to the destruction of their target cells. In this study, we focused on perforin, as a key element in the NK cell cytotoxicity machinery.

As has already been shown by others, we found that around 70-90% of human peripheral blood NK cells express perforin protein by flow cytometry, using anti-CD56 and anti-PFP antibodies to detect the $CD56^{+}PFP^{+}$ population. However, we found contradictions in the literature regarding the expression of perforin by the different human peripheral blood NK cell subsets. A study that was published by Konjevic et al in 1995 showed that both $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets were positive for perforin from 97-99%. Other studies reported that the $CD56^{dim}$ NK cell subset expresses significantly more perforin protein and transcripts than in $CD56^{bright}$ subset (Jacobs et al, 2001; Wendt et al, 2006).

Our results show that a higher percentage of $CD56^{dim}$ NK cell subset expresses perforin compared to $CD56^{bright}$ NK cell subset, where 80% of $CD56^{dim}$ expressed perforin compared to 40% of $CD56^{bright}$ subset, which agrees with the more granular nature of the $CD56^{dim}$ NK cell subset and its more cytotoxic nature (Jacobs et al, 2001).

We investigated the effect of LTB_4 on perforin expression by peripheral blood human NK cells. We found that LTB_4 resulted in down-regulation of the perforin protein in these cells in a concentration- and time-dependent manner, reaching a maximum

decrease at a concentration of 10^{-8} M at 72 hours. This down-regulation was very modest, but it was consistent and statistically significant.

Furthermore, we proceeded to investigate the effect of BLT1 modulation on perforin expression and down regulation, but BLT1 modulation did not appear to have any significant effect. However, it is still to be investigated whether the down-regulation induced by LTB_4 on perforin in NK cells is a decrease of protein expression or degranulation. Especially that LTB_4 is known to induce the degranulation of other cells such as neutrophils (Showell et al, 1982), eosinophils (Takafuji et al, 1998) and basophils (Iikura et al, 2005).

In recent years, the heterogeneity of human NK cells has received increasing attention. Two major subsets can be detected based on the differences in the expression of CD56 molecules. In healthy individuals, around 90 % of circulating NK cells express low levels of CD56 ($CD56^{dim}$), whereas around 10 % express high levels of CD56 molecule and are called $CD56^{bright}$ (Cooper et al, 2001). In addition, recent studies have revealed that NK cell receptors, cytolytic molecules, adhesion structures, and chemokine ligands showed differential expression patterns in the two NK subpopulations and suggested that these two subpopulations represent independent populations with functionally diverse capabilities (Wendt et al, 2006).We were interested to further investigate the expression of BLT1 by the two subpopulations. We found a significant difference in the expression of BLT1 by the two subsets. $CD56^{bright}$ subset expressed significantly more BLT1 per cell and also a greater percentage of cells of this subset expressed BLT1 compared to the $CD56^{dim}$ subset.

Showing that the two subsets express different levels of the BLT1, we examined the effect of cytokines on the different NK subsets. Following incubation of NK cells with either of IL-15 or IL-2 for 18 hours, both subsets responded by an increase in the percentage of cells expressing BLT1 and also in terms of increase in the level of BLT1 expressed per cell. Interestingly, the up regulation of BLT1 was significantly higher in the $CD56^{dim}$ subset than in the $CD56^{bright}$ subset. However, the response to IFN-γ was different, the $CD56^{bright}$ subset responded with a higher down-regulation of BLT1 compared to the $CD56^{dim}$ subset.

Recent studies have revealed that the NK cell subsets have different migratory properties and chemokine receptor expression patterns. For example, a very recent study has shown that peripheral blood NK cells are heterogeneous in their expression of certain chemokine receptors and that they have the capacity for differential trafficking within the body. It was found that human NK cells express CXCR1, CXCR3, CXCR4, CCR1, CCR4, CCR5, CCR6, CCR7, CCR9, CXCR5, and CXCR6. These chemokine receptors were expressed at higher percentages by $CD56^{bright}$ NK cells compared to $CD56^{dim}$ NK cells, except for CCR4 (Berahovich et al, 2006). Likewise, we have found that BLT1 is expressed more by $CD56^{bright}$ than by $CD56^{dim}$ NK cells. Because BLT1 is expressed at a lower level in the majority of NK cells or the so called "Classical" or $CD56^{dim}$ NK cell population and is expressed at a higher level in the rare $CD56^{bright}$ NK cell population, we were unable to detect very high migration of these cells in our chemotaxis assays, which are not sufficiently sensitive to detect the migration of very rare cell types within a heterogeneous population. So

we believe that the migration we detected towards LTB_4 is still underestimated and it would have been higher using purified $CD56^{bright}$ subset.

However, we tried to investigate the migratory capacity of the $CD56^{bright}$ NK subset, by gating on this subset during our analysis by flow cytometry. We were able to detect a greater capacity of migration in response to LTB_4 by the $CD56^{bright}$ NK cell subset at the expense of $CD56^{dim}$ subset. As expected, the difference was very modest, especially that it is a rare subset, but it was statistically significant.

The $CD56^{bright}$ NK subset also showed significant migration to LTB_4 following incubation with IL-15 compared to control $CD56^{bright}$ NK cells incubated in absence of IL-15, thus, confirming previous results that IL-15-induced up-regulation of BLT1 results in enhanced migration to LTB_4.

In conclusion, we have shown for the first time that BLT1 is expressed by NK cells. Moreover, BLT1 on NK cells can be modulated by selected cytokines as IL-15, IL-2 and IFN-γ. Finally, LTB_4 induces the chemotaxis of NK cells, which can further be enhanced by up regulation of the BLT1 receptor.

CONCLUSIONS

In the present work, we studied the expression of BLT1 by NK cells and their subsets and the regulation of this receptor by selected cytokines, with the subsequent modulation of the different NK effector functions in response to LTB_4.

We have shown, for the first time that NK cells express BLT1. This expression can be modulated by selected cytokines, namely, IL-2, IL-15 and IFN-γ. Stimulation of lymphocytes with IL-2 and IL-15 resulted in a significant up-regulation of BLT1 on NK cells, while, stimulation with IFN-γ resulted in the down-regulation of the receptor on these cells. $CD56^{bright}$ subset showed significantly higher expression of BLT1 compared to $CD56^{dim}$ subset.

We have also shown that NK cells migrate in response to LTB_4, compared to vehicle, with enhanced chemotaxis following IL-15 incubation, correlating with the up-regulation of BLT1 with IL-15 stimulation. The $CD56^{bright}$ subset also showed enhanced chemotaxis in response to LTB_4 compared to the $CD56^{dim}$ subset, in correlation with the higher expression of BLT1 on this subset.

Lastly, we have demonstrated that LTB_4 stimulation results in the down-regulation of the perforin protein in NK cells. Stimulation with IL-15 resulted in increased expression of perforin in NK cells, without affecting LTB_4 regulation of perforin.

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