Research Article

Differential Contribution of BLT₁ and BLT₂ to Leukotriene B₄-Induced Human NK Cell Cytotoxicity and Migration

Meng Wang, Nermine Mostafa El-Maghraby, Sylvie Turcotte, Marek Rola-Pleszczynski, and Jana Stankova

Division of Immunology, Department of Paediatrics, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, QC, Canada J1H 5N4

Correspondence should be addressed to Jana Stankova; jana.stankova@usherbrooke.ca

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Accumulating evidence indicates that leukotriene B₄ (LTB₄) via its receptors BLT₁ and/or BLT₂ (BLTRs) could have an important role in regulating infection, tumour progression, inflammation, and autoimmune diseases. In the present study, we showed that LTB₄ not only augments cytotoxicity by NK cells but also induces their migration. We found that approximately 30% of fresh NK cells express BLT₁, 36% express BLT₂, and 15% coexpress both receptors. The use of selective BLTR antagonists indicated that BLT₁ was involved in both LTB₄-induced migration and cytotoxicity, whereas BLT₂ was involved exclusively in NK cell migration, but only in response to higher concentrations of LTB₄. BLT₁ and BLT₂ expression increased after activation of NK cells with IL-2 and IL-15. These changes of BLTR expression by cytokines were reflected in enhanced NK cell responses to LTB₄. Our findings suggest that BLT₁ and BLT₂ play differential roles in LTB₄-induced modulation of NK cell activity.

1. Introduction

Human natural killer (NK) cells with the CD3⁻ CD56⁺ phenotype comprise 10–15% of peripheral blood lymphocytes. They constitute a major component of the innate immune system especially in response to transformed and infected cells [1–3]. Even though priming is not necessary for NK cells to perform their cytolytic function, proinflammatory cytokines, such as IL-2 [4, 5] and IL-15 [6], can induce NK cell proliferation, cytotoxicity, or cytokine production. Chemokine-induced NK cell migration may explain the redistribution of NK cells from the bone marrow and lymph nodes to blood and other organs [7]. In addition to chemokines, NK cells respond to other chemoattractants such as N-formyl-methionyl-leucyl-phenylalanine (f-MLP), casein, and C5a [8].

Leukotriene B₄ (LTB₄) is a potent lipid mediator of allergic and inflammatory reactions, in addition to modulating immune responses [9, 10]. LTB₄ is a major chemoattractant of granulocytes [11, 12] and can be responsible for T cell recruitment in asthma [13–15]. Two human LTB₄ cell-surface receptors, BLTRs, high-affinity BLT₁ and low-affinity BLT₂,

were cloned and identified in 1997 and 2000, respectively [16, 17]. It has been demonstrated that BLT₁ expression is high in peripheral blood leukocytes and lower in other tissues, whereas BLT₂ expression is ubiquitous in most human tissues with lower expression in peripheral blood leukocytes [18]. Studies using BLT₁^{-/-} mice and specific BLT₁ antagonists have demonstrated that BLT₁ plays critical roles in both host defence and many inflammatory diseases by mediating multiple activities of LTB₄, including inflammatory cell recruitment [19, 20], prolongation of inflammatory cell survival [21, 22], and activation of inflammatory cell functions [23, 24]. Recent studies with BLT₂^{-/-} mice showed that BLT₂ is involved in autoantibody-induced severe inflammatory arthritis [25] but is protective in DSS-induced colitis by enhancing epithelial cell barrier functions [26]. However, the functions and biological activity of BLT₂ in lymphocytes are not completely known at this time.

It has been shown that LTB₄ could augment the cytolytic function of human NK cells [27–29] and induce T lymphocyte recruitment to inflammatory sites [13–15]. These observations led us to examine whether LTB₄ was chemotactic

for NK cells and to define the contribution of BLT₁ and/or BLT₂ to NK cell migration and cytolysis in response to LTB₄. We first determined BLT₁ and BLT₂ expression in NK cells, at both the mRNA and protein levels, and then studied the differential contribution of these receptors in LTB₄-induced NK cell migration and cytotoxicity. We also evaluated the modulation of BLT₁ and BLT₂ expression after cytokine stimulation and the subsequent effect on NK cell responses to LTB₄.

2. Materials and Methods

2.1. Antibodies and Reagents. Mouse anti-human CD56 and CD3 antibodies and 7AAD were purchased from BD Biosciences (Mississauga, ON, Canada). FITC-conjugated goat anti-rabbit IgG (GAR-FITC) and DTAF-conjugated streptavidin (SA-FITC) were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Polyclonal rabbit anti-human BLT₁R and BLT₂R antibodies, LTB₄, CAY10583, U75302, and LY255283 were from Cayman Chemical (Ann Arbor, MI, USA). Isotype control rabbit IgG was from InterSciences (Markham, ON, Canada). Biotinylated mouse anti-human BLTR antibody and isotype control were from AbD SeroTec (Raleigh, NC, USA). Human IL-2 and IL-15 were purchased from PeproTech (Dollard des Ormeaux, QC, Canada). MIP-1 α was from Abcam (Cambridge, MA, USA). All other chemical agents were obtained from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise mentioned.

2.2. Cell Culture. Peripheral blood mononuclear cells (PBMCs) and lymphocytes (PBLs) were isolated as described previously [30]. Briefly PBMCs were isolated from healthy volunteers' peripheral blood using density gradient centrifugation with Ficoll-Paque PLUS (GE healthcare) and PBLs were collected after monocyte depletion of PBMCs by adherence. Human NK cells were purified from fresh PBLs using Macs magnetic system (Miltenyi Biotec, Cambridge, MA, USA) with human NK cell enrichment kits (StemSep, Vancouver, BC, Canada), according to the manufacturer's directions. Enrichment routinely resulted in greater than 95% purity as determined by cytometric analysis with anti-CD56 antibodies. PBLs or NK cells (2×10^6 cells/mL) were cultured in RPMI 1640 (Invitrogen, Burlington, ON, Canada) with 80 IU/mL penicillin G (Novopharm, Toronto, ON, Canada), and 100 μ g/mL streptomycin and 5% FBS (PAA, Etobicoke, ON, Canada) in the absence or presence of IL-2 or IL-15, 10 ng/mL, in a humidified atmosphere with 5% carbon dioxide at 37°C. The antagonists, U75302 10 μ M and LY255283 50 μ M, were added 30 minutes prior to stimulation with LTB₄.

2.3. Semiquantitative End Point or Real-Time PCR Analysis. After appropriate treatment, total cellular RNA was isolated using TRIzol reagent (Invitrogen). After treatment with RNasin (Promega, Madison, WI, USA) and DNase kit (Fermentas, Burlington, ON, Canada) to exclude genomic DNA contamination, 1 μ g of RNA was converted to cDNA with oligo(dT) (Fermentas) and M-MLV reverse transcriptase (Promega) in a volume of 20 μ L.

End point RT-PCR was performed in a final volume of 50 μ L containing 2 μ L cDNA, 1 μ M primer, and the reaction buffer of Taq DNA polymerase kit (Feldan, Quebec, QC, Canada), using a Biometra thermocycler (Montreal Biotech, Montreal, QC, Canada) using an initial denaturation step at 95°C for 2 min, 24 cycles (for GAPDH) or 32 cycles (for BLT₁/BLT₂) of 30 s denaturation at 95°C/30 s annealing at 58°C/30 s extension at 72°C, and a final 8 min extension at 72°C. Positive controls of cloned human BLT₁ or BLT₂ cDNA were described previously [31]. Negative controls, in which the reverse transcription step was omitted, confirmed that the PCR products reflected mRNA levels rather than contaminating genomic DNA. PCR products (10 μ L) were electrophoresed in a 1.2% (w/v) agarose gel and visualized with ethidium bromide. Densitometric quantification was done with NIH ImageJ software.

Real-time PCR was performed with Rotor-Gene 3000 system (Corbett Research, Concorde, NSW, Australia) using the SYBR Green I detection method. Each sample for the real-time PCR consisted of 1 μ L of cDNA, 1 μ M primer, 2.5 mM MgCl₂, the reaction buffer of Taq DNA polymerase kit (Feldan), and 0.8 μ L of SYBR Green I (1/1000 stock dilution; Molecular Probes, Invitrogen) in a reaction volume of 25 μ L. The cycling program consisted of an initial denaturation at 95°C for 5 minutes, 45 cycles of amplification conditions as follows: 95°C (30 s), 58°C (30 s), and 72°C (30 s) and a final extension at 72°C for 6 min. Comparison of the expression of each gene between its control and stimulated states was determined with the delta-delta ($\Delta\Delta$)Ct, according to the following formula:

$$\Delta\Delta Ct = [(Ct \text{ G.O.I.}_{\text{Ctl}} - Ct \text{ H.K.G.}_{\text{Ctl}}) - (Ct \text{ G.O.I.}_{\text{STIM.}} - Ct \text{ H.K.G.}_{\text{STIM.}})] \quad (1)$$

Results were then transformed into fold variation measurements: fold increase = $2^{\Delta\Delta Ct}$. Each experiment was performed in duplicate.

PCR primers (IDT, Coralville, IA, USA) were designed with Primer3, and their sequences are as follows: GAPDH (housekeeping gene, 246 bps) 5'-GAT GAC ATC AAG AAG GTG GTG AA-3' (forward), 5'-GTC TTA CTC CTT GGA GGC CAT GT-3' (reverse); hBLT₁ (216 bps) 5'-GTT TTG GAC TGG CTG GTT GC-3' (forward), 5'-GGT ACG CGA GGA CGG GTG TG-3' (reverse); hBLT₂ (183 bps) 5'-GAG ACT CTG ACC GCT TTC GT-3' (forward), 5'-AAG GTT GAC TGA GTG GTA GG-3' (reverse).

2.4. Flow Cytometry

2.4.1. Cell-Surface Staining. Freshly isolated cells (1×10^6) were suspended in 5 μ L PBS-2% BSA and labelled with 5 μ L anti-BLTR-Biotin or isotype antibodies for 30 minutes on ice. After washing with PBS, cells were incubated with SA-FITC, anti-CD3-APC, and anti-CD56-PE antibodies for 30 minutes, then washed, and resuspended in 200 μ L PBS.

2.4.2. Intracellular Staining. Freshly isolated cells (1×10^6) were fixed with 2% paraformaldehyde and permeabilized

with 0.1% saponin at room temperature. Cells were then incubated for 15 minutes with human IgG to block binding to Fc receptors, resuspended in 100 μ L PBS-2% BSA, and labelled with polyclonal anti-BLT₁ Ab (1:2000 dilution), polyclonal anti-BLT₂ Ab (1:1000 dilution), or isotype control for 30 minutes at room temperature. After washing with PBS, cells were incubated with GAR-FITC, anti-CD3-APC, and anti-CD56-PE antibodies for 20 minutes at room temperature, then washed, and resuspended in 200 μ L PBS.

2.4.3. Flow Cytometry Analysis. 100,000 events/sample were recorded and analyzed with FACSCalibur (BD Biosciences) and Flowjo Software (Treestar, Ashland, OR, USA). Each experiment was performed in duplicate.

2.5. Chemokinesis and Chemotaxis Assays. NK cell chemotactic activity was evaluated using a modified Boyden chamber assay. A volume of 200 μ L RPMI 1640-2% BSA with MIP-1 α (1 ng/mL) and graded concentrations of LTB₄ or control (medium or EtOH) alone was added to the lower chamber. A volume of cells (6×10^5), which were prestained with anti-CD3-FITC/CD56-PE antibodies and preincubated with or without antagonists, in 200 μ L RPMI 1640-2% BSA only or LTB₄ (for the chemokinesis assay), was added to the upper chamber. The two chambers were separated by a 5 μ m pore size polycarbonate filter (Neuroprobe, Gaithersburg, MD). After a 3-hour incubation, migrating cells were collected from the lower chamber and on the lower side of the filter for counting by flow cytometry with fixed time acquisition. Each experiment was performed in triplicate. The number of migrating NK cells was quantitated by counting CD3⁺ CD56⁺ cells in the migrating population. The results were then converted to a migration index (MI): MI = mean number of cells migrating to chemoattractant/mean number of cells migrating to control (EtOH or medium).

2.6. Cytotoxicity Assay. Target cells, K562 (an erythroleukemia cell line, ATCC), were labelled with 0.1 μ M CFSE for 5 minutes at room temperature, washed twice with PBS-2% FBS, and suspended at 5×10^5 cells/mL in RPMI 1640-5% FBS. Effector cells, PBLs or enriched NK cells, were preincubated with or without antagonists. Effector and target cells were then cocultured at indicated effector : target ratios in a final volume of 200 μ L. Graded concentrations of LTB₄ or vehicle control were used during the 2-hour cytotoxicity assay. Target cells alone were incubated in medium to measure spontaneous cell death. After a 2 h incubation, 2 μ L 7AAD was added to every sample and kept on ice for 15 min. Samples were immediately acquired by flow cytometry (BD FACSCalibur). The analysis was performed on gated cells that fell within the CFSE positive population (K562). Within this population of cells, we quantified the 7AAD labeling for each sample. Cytotoxicity was determined as

$$\text{cytotoxicity (\%)} = \frac{\text{CFSE}^+ 7\text{AAD}^+}{\text{CFSE}^+} \times 100\% \quad (2)$$

– spontaneous death.

Each experiment was performed at least in duplicate.

2.7. Statistical Analysis. Data are presented as mean $\pm$ SEM. Statistical tests (Student's *t*-test, one-way ANOVA, or 2-way ANOVA, as appropriate) were performed using GraphPad Prism 5.0 (GraphPad Prism Software, San Diego, CA). *P* < 0.05 was considered significant.

3. Results

3.1. BLT₁ and BLT₂ Expression on NK Cells. Initially it was believed that BLT₁ was expressed only in phagocytes (granulocytes, eosinophils, and macrophages) [17, 32–34]. However, BLT₁ mRNA was found in differentiated CD4⁺ T_H cells and CD8⁺ T_{EFF} cells and the LTB₄-BLT₁ pathway was found to be involved in inflammation-induced T_H and T_{EFF} cell recruitment [35, 36]. We, and others, have reported that LTB₄ could augment NK cell cytotoxicity [27, 37–39]. Thus, we sought to determine the pattern of BLTR expression in NK cells. We assessed BLT₁ and BLT₂ mRNA expression by RT-PCR in each population of cells, PBLs, enriched NK cells, and monocytes (Figure 1(a)). Densitometric analysis of six donors' data, shown in Figure 1(b), indicated that BLT₁ and BLT₂ mRNA expression was similar in PBLs, NK cells, and monocytes, with a tendency for higher BLT₂ mRNA expression in these cell populations.

BLTR expression on fresh PBMCs was then evaluated by flow cytometry. Polyclonal anti-BLT₁ and anti-BLT₂ antibodies, which are directed toward an intracellular (C-terminus) domain of the receptor, were used to evaluate total (intracellular and extracellular) expression in permeabilized cells. In addition, a monoclonal anti-BLT₁ antibody, which recognizes the N-terminal receptor epitope (extracellular), was used to evaluate cell-surface expression. Histogram graphs in Figure 1(c) illustrate the BLTR expression from a representative donor on lymphocytes, CD56⁺ NK cells, and monocytes, respectively. Figure 1(d) illustrates a compilation of individual donors and indicates that BLT₁ receptors are expressed both intracellularly and on the cell-surface of all three cell populations. Around $25.19\% \pm 3.69\%$ PBLs expressed BLT₁ on the cell-surface, whereas almost 50% PBLs expressed BLT₁ when intracellular receptors were taken into account ($47.76\% \pm 3.23\%$). The discrepancy was smaller in NK cells and monocytes, as the expression of BLT₁ on cell-surface ($25.09\% \pm 4.75\%$ and $61.76\% \pm 5.27\%$, resp.) was around 80% of total expression ($31.64\% \pm 3.39\%$ and $76.23\% \pm 2.56\%$). Although the antibodies used for cytometry were different, it appeared that the expression of BLT₂ protein was lower than that of BLT₁ in PBLs and monocytes, but it was similar in NK cells. Due to a lack of an antibody directed at the extracellular region of BLT₂, we could not evaluate differences between its cell-surface and its total cellular expression. Interestingly, more NK cells ($15.69\% \pm 2.49\%$) expressed both BLTRs than PBLs ($8.53\% \pm 0.79\%$) (Figure 1(e)). Our findings of the heterogeneous expression of BLTR in NK cells led us to study the involvement of BLT₁ and/or BLT₂ in LTB₄-mediated effects in these cells.

3.2. NK Cell Migration Response to LTB₄. LTB₄ is a potent neutrophil chemoattractant and recruits neutrophils to inflammatory sites in skin or lung by directing cell migration

FIGURE 1: Continued.

FIGURE 1: Expression of BLT₁ and BLT₂ in NK cells. RT-PCR of BLT₁, BLT₂, and GAPDH from PBLs, isolated NK cells, and monocytes were analyzed by agarose gel electrophoresis (a): lanes 1 and 2, human BLT₁ and BLT₂ cDNAs as positive controls; lane 3, polymerase-only, RT-negative samples for each oligonucleotide pair; lane 4, PBL mRNA; lane 5, NK cell mRNA; and lane 6, monocyte mRNA. (b) Expression levels of BLT₁ and BLT₂ mRNA were determined by densitometry and the ratio to GAPDH (the mean $\pm$ the SEM) from six donors is shown. (c) Whole cell and cell-surface expression of BLT₁ (top and middle rows, resp.) and whole cell expression of BLT₂ (bottom row) on PBLs, NK cells, and monocytes were measured by flow cytometry as described in Section 2. In the overlaid histogram graphs, grey background indicates control histograms, where cells were incubated with Ig isotype, and solid line indicates cells incubated with anti-BLTR antibodies. (d) Results represent the percentage of BLTR positive cells as population comparison analysis. Bars represent means $\pm$ SEM of ten to seventeen independent experiments. (e) Coexpression of BLT₁ and BLT₂ was measured as the percentage of events that were BLT₁ and BLT₂ double positive, gating on PBLs and NK cells (CD56⁺), respectively. Bar graphs represent means $\pm$ SEM of four independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, paired Student's *t*-test.

[40, 41]. Recent studies demonstrated that LTB₄ can also induce migration of mast cells [42], dendritic cells [43], and T cells [35, 36, 44]. Thus, we investigated whether LTB₄ could induce migration of human NK cells. As shown in Figure 2(a), LTB₄ induced a 1.5-fold migration of NK cells at 10^{-8} M, and the magnitude of migration was similar to that induced by MIP-1 α , a potent chemoattractant of NK cells [7]. This increase in chemotaxis was due to directional migration as LTB₄ did not modulate NK cell chemokinesis (Figure 2(b)). NK cells migrated in response to a wide range of LTB₄ concentrations, from 10^{-9} to 10^{-5} M, with a maximum at 10^{-9} M LTB₄ (Figure 2(c)). In order to study the contribution of the two receptors to chemotaxis, we used selective antagonists. We found that LTB₄-induced chemotaxis was abolished following preincubation with the BLT₁ antagonist, U75302 at 10 μ M, for 30 minutes. However, the BLT₂ antagonist, LY255283, blocked LTB₄-induced chemotaxis only at the highest concentrations of the ligand. Moreover, the selective BLT₂ agonist CAY10583 was capable of inducing significant NK cell migration (Figure 2(d)) and this migration was only blocked by LY255283 (Figure 2(e)).

Our data suggest that the high-affinity BLT₁ receptor mediates most of the LTB₄-induced chemotactic activity in NK cells, with the lower affinity BLT₂ receptor participating preferentially when LTB₄ concentrations are very high.

3.3. BLT₁ but Not BLT₂ Mediates LTB₄-Induced NK Cell Cytotoxicity. To determine which of the two receptors was

necessary for the LTB₄-induced effect on NK cell cytotoxic function, we again used the selective antagonists. LTB₄ significantly enhanced NK cell cytolytic activity at 10^{-10} M to 10^{-7} M, with a maximal effect between 10^{-10} M and 10^{-8} M of LTB₄ (Figure 3(a)). Preincubation with U75302 at 10 μ M for 30 minutes abrogated the enhanced cytotoxicity. In contrast, LY255283 did not significantly block LTB₄-induced cytotoxicity. Moreover, the selective BLT₂ agonist Cay10583 failed to affect the level of NK cell activity (Figure 3(b)), suggesting that BLT₂ was not involved in LTB₄-induced cytotoxicity.

3.4. Modulation of LTB₄ Receptor Expression by IL-2 and IL-15. Pro- or anti-inflammatory cytokines can modulate BLT₁ expression in human monocytes, with decreased expression induced by IFN- γ and TNF α , and increased expression stimulated by IL-10 and dexamethasone [45]. TNF α and dexamethasone also regulate BLT₁ expression in neutrophils [21, 46]. Therefore, we sought to investigate whether IL-2 and IL-15, two NK-activating cytokines, could regulate BLTR expression. Real-time quantitative PCR was used to examine BLT₁ and BLT₂ mRNA expression in purified NK cells, incubated with IL-2 or IL-15 for 2 or 6 hours. IL-2 was found to induce a 2.4-fold increase of BLT₁ expression after a 2 h stimulation but did not significantly increase BLT₂ expression. IL-15 induced a 2.1-fold increase of BLT₁ and a 1.9-fold increase of BLT₂ expression after a 6 h incubation (Figure 4(a)). BLTR protein expression was then examined in NK cells incubated with IL-2 or IL-15 for 18 h. IL-2

FIGURE 2: NK cell migration in response to LTB_4 . (a) Bar graphs represent NK cell migration in response to MIP α , 1 ng/mL, and LTB_4 , 10^{-8} M. (MI: migration index, number of cells migrating in response to stimulus divided by number of cells migrating in response to control medium. Graph represents mean $\pm$ SEM of four independent experiments.) (b) Results illustrate the comparison of chemokinesis and chemotaxis to 10^{-8} M LTB_4 . Spontaneous migration in the presence of vehicle alone (ethanol 0.0033%) was normalized to 1. Bar graphs represent means $\pm$ SEM of four independent experiments, * P < 0.05 paired Student's t -test. (c) PBLs were preincubated without antagonist ($\blacksquare$), with U75302 10 μ M ($\triangle$) or LY255283 50 μ M ($\square$) for 30 minutes at 37°C, before a chemotaxis assay with graded concentrations of LTB_4 or vehicle control. The number of migrating cells was measured by FACS, gating on the NK cell population (CD3 $^-$ CD56 $^+$). Each curve represents mean $\pm$ SEM of five independent experiments. # P < 0.05, and ### P < 0.001 by one-way ANOVA with Dunnett posttest to vehicle control. * P < 0.05, ** P < 0.01, and *** P < 0.001 by two-way ANOVA with Bonferroni posttests to no-antagonist data. (d) NK cell migration in response to graded concentrations of CAY10583. Data are expressed as means $\pm$ SEM of ratios of migrating cells in response to CAY10583 versus vehicle (n = 8), * P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001. (e) NK cell migration in response to 10^{-9} M CAY10583 in the absence or presence of LY255283 (LY) or U75302 (U). **** P < 0.0001, n = 5.

FIGURE 3: (a) LTB₄ enhances NK cell cytolytic function via the BLT₁ receptor. PBLs (effector cells) were preincubated without antagonist (■) or with U75302 10 μ M (Δ) or LY255283 50 μ M ($\square$) for 30 minutes at 37°C and then were cocubated with CFSC-labelled K562 cells at $E:T = 50:1$ ratio in the presence of graded concentrations of LTB₄. Cytotoxicity was measured using the percentage of 7AAD positive events gating on K562 cells. Each curve represents mean $\pm$ SEM of seven independent experiments. # $P < 0.05$, and ## $P < 0.01$ by one-way ANOVA with Dunnett posttest to controls. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by two-way ANOVA with Bonferroni posttests to no-antagonist data. (b) Effects of LTB₄ or CAY10583 on NK cell cytotoxicity in the absence or presence of LY255283 or U75302. Data are expressed as means $\pm$ SEM of cytotoxicity ratios. ** $P < 0.01$ using Student's paired t -test; $n = 9$.

only increased expression of BLT₁, whereas IL-15-activated NK cells showed approximately 1.4- and 1.3-fold higher expression of both BLT₁ and BLT₂, respectively (Figure 4(b)).

3.5. Regulation of LTB₄-Induced NK Cell Migration by IL-2 and IL-15. Dexamethasone-treated human neutrophils show a higher response to LTB₄ in chemotaxis, as a result of enhanced BLT₁ expression [21]. For this reason, we hypothesized that IL-2 and IL-15 may increase the chemotactic response of NK cells to LTB₄. We treated PBLs with IL-2,

IL-15, or medium for 18 h and NK cell migration in response to 10⁻⁹ M and 10⁻⁶ M LTB₄ was examined (Figure 5(a)). IL-15 induced a significant augmentation of NK cell migration to both concentrations of LTB₄. However, IL-2 did not significantly increase NK cell chemotactic response to LTB₄ at either concentration. As illustrated in Figure 5(b), U75302 abolished the migration of IL-15-activated NK cells in response to both concentrations of LTB₄; meanwhile, LY255283 totally abrogated the migration of IL-15-activated NK cells to higher concentration LTB₄ (10⁻⁶ M) but only partially blocked

FIGURE 4: Modulation of BLTR expression on human NK cells by IL-2 and IL-15. (a) NK cells were incubated for 2 or 6 h in the absence or presence of IL-2 (50 ng/mL) or IL-15 (10 ng/mL). Total RNA was then extracted and analyzed by q-PCR. Bar graphs represent mean $\pm$ SEM of five independent experiments. * $P < 0.05$, by one-way ANOVA with Dunnett posttest to untreated cells (control). (b) PBLs were incubated for 18 h in the absence or presence of IL-2 or IL-15. Total expression of BLT₁ (light grey) and BLT₂ (dark grey) on NK cells was measured by FACS and expressed as fold induction relative to untreated cells. Bar graphs represent mean $\pm$ SEM of seven independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by paired Student's t -test, treated versus untreated cells.

FIGURE 5: Effect of IL-2 or IL-15 pretreatment on LTB₄-induced NK cell migration. (a) PBLs were incubated for 18 h in the absence or presence of IL-2 (50 ng/mL) or IL-15 (10 ng/mL). Cells were stained with anti-CD3 and anti-CD56 antibodies and then placed in a chemotaxis assay in response to LTB₄ 10⁻⁹ M or 10⁻⁶ M. Migrating CD3⁺ CD56⁺ cells were counted by FACS, and results were expressed as migration index (LTB₄/vehicle). Bar graphs represent mean $\pm$ SEM of seven independent experiments. * $P < 0.05$ by paired Student's t -test, treated versus untreated cells. (b) IL-15-activated PBLs were preincubated with or without U75302 or LY255283 before chemotaxis assays. Migrating CD3⁺ CD56⁺ cells were counted by FACS, and results were expressed as migration index (LTB₄/vehicle). Bar graphs represent mean $\pm$ SEM of seven independent experiments. * $P < 0.05$ by paired Student's t -test.

the migration to lower concentration (10⁻⁹ M). Our data suggest that IL-15-enhanced chemotactic response of NK cells to LTB₄ is also predominantly dependent on BLT₁ signaling.

3.6. Higher Response of IL-2- and IL-15-Activated NK Cells to LTB₄ in Cytotoxicity. We next tested whether IL-2- and IL-15-activated NK cells could also increase their cytotoxic responses to LTB₄. We treated purified NK cells with IL-2,

FIGURE 6: Effect of IL-2 and IL-15 pretreatment on LTB₄-induced NK cell cytotoxicity. Resting NK cells (a), IL-2-activated NK cells (b), and IL-15-activated NK cells (c) were coincubated with CFSE-labelled K562 cells at different *E*:*T* ratios for a 2 h cytotoxicity assay in the presence of LTB₄ 100 pM (solid lines) or vehicle control (dotted lines). The killing ability was measured using the percentage of 7AAD positive K562 cells. Each curve represents the means $\pm$ the SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by two-way ANOVA with Bonferroni posttests to vehicle control.

IL-15, or medium for 18 h and then coincubated NK cells and K562 target cells at a graded effector:target cell ratio from 0.1:1 to 10:1. As shown in Figure 6, LTB₄ induced a significant augmentation of cytotoxicity in resting NK cells at *E*:*T* ratios of 2.5 to 5:1. IL-2- and IL-15-activated NK cells showed an enhanced cytotoxicity in response to LTB₄ at lower *E*:*T* ratios of 1:1 and 2.5:1 and 0.5:1 to 1:1, respectively. The BLT₁ antagonist U75302, but not the BLT₂ antagonist LY255283, blocked NK cell responsiveness to LTB₄ (data not shown), suggesting that IL-2- and IL-15-induced upregulation of BLT₁ expression may be involved in the augmented cytotoxic response to LTB₄ by NK cells.

4. Discussion

In the present study we investigated the differential involvement of BLT₁ and BLT₂ receptors in LTB₄-induced chemotaxis and cytotoxic activity of NK cells.

Previously, we and others have shown that LTB₄ increases NK cell cytotoxicity in several species [37, 39, 47]. However, BLTR expression in NK cells was still unclear. We found that the BLT₁ receptor was expressed on human NK cells, which is in contrast to the findings of Pettersson et al. [48] who reported that no CD56⁺ cells were BLT₁-positive, although they found low expression on some CD16⁺ cells. Interestingly,

we found that the expression of BLT₁ was quite variable from individual to individual. The highest BLT₁ cell-surface expression on PBL and NK cells was 10 times higher than the lowest one. Moreover, we found expression of both mRNA and protein, which confirmed BLT₁ and BLT₂ expression in human NK cells. Meanwhile, we found that the expression of both receptors in monocytes and lymphocytes was similar to the findings of Islam et al. [13] and Yokomizo et al. [33]. The fact that protein expression of BLTR was different, especially that of BLT₁, between PBLs, NK cells, and monocytes whereas mRNA expression was similar, suggests that there might be not only transcriptional regulation of BLT₁ expression [49], but also posttranscriptional regulation of expression of these proteins. The higher expression of BLT₂ mRNA in comparison to BLT₁ mRNA might be due to the special gene structure, as the open reading frame of BLT₂ is found in the promoter of BLT₁ [49].

NK cells are rapidly distributed throughout the body in order to exercise their effector functions. However, the chemotactic signals responsible for their migration are not well known. We show here that LTB₄ could induce a similar level of NK cell migration as MIP-1 α , which has been shown as one of the most potent NK cell chemoattractants [7]. This suggests that LTB₄ may induce early and rapid NK cell recruitment and trafficking to inflammatory sites, given that LTB₄ is synthesized very rapidly [10] compared to chemokines. The peak of NK cell migration was in response to a low (1 nM) concentration of LTB₄, which was lower than the optimal concentration inducing CD8⁺ T cell migration (10 nM) [36] or mast cell migration (100 nM) [42].

Similar to T cells [13–15] and neutrophils [11, 12], the BLT₁ receptor was the principal signal transducer of LTB₄-induced chemotaxis of human NK cells. However, unlike in the other cell types, BLT₂ plays a partial role in the chemotactic response of NK cells to LTB₄. This agrees with the observations of Yokomizo et al., who found that both BLT₁ and BLT₂ could mediate LTB₄-induced cell migration in BLT₁-BLT₂ cotransfected CHO cells [33]. Since 12-HHT, a fatty acid derived from the COX pathway, may be the more effective agonist for BLT₂ [50], it may also potentially be involved in NK cell chemotaxis.

On the other hand, our results show that BLT₂ is probably not involved in LTB₄-induced augmentation of NK cell cytotoxicity since the BLT₁, but not the BLT₂, antagonist could block all the effects of LTB₄ and since the BLT₂ agonist CAY10583 had no effect on NK cell cytotoxicity.

BLT₁ and BLT₂ belong to the seven transmembrane domains, G-protein-coupled receptor (GPCR) family. In some GPCRs, dimerization is associated with better receptor activation [51], and it has been shown that the BLT₁:BLT₁ homodimer does show higher affinity for LTB₄ than the monomer [52]. However, BLT₂ monomers have been shown to be more efficient at activating G proteins than the dimers [53]. In the present study, both BLT₁ and BLT₂ antagonists could block NK cell migration to higher concentrations of LTB₄. This might suggest that, in NK cells, which coexpress the two receptors, BLT₁:BLT₂ heterodimers might exist, and although heterodimers of BLT receptors have not been investigated, it would not be surprising

if one of the monomers influenced signal transduction [54].

We also observed that higher concentration of LTB₄ seemed to induce a lower NK cell migration and cytotoxicity. It has been shown that rapid agonist-induced internalization and desensitization are important characteristics of BLT₁ activation [55]. Thus, possibly, desensitization and internalization could reduce receptor responses at higher concentrations of LTB₄.

It is now well established that pro- and anti-inflammatory cytokines as well as microbial products can modulate the expression of BLT₁ in monocytes [45] and neutrophils [21]. In the present study, we found that cytokines which increase NK cell activation and cytotoxicity could also modulate BLT expression. We found that IL-15 upregulated both BLT₁ and BLT₂ expression, but IL-2 only increased BLT₁ protein expression. These cytokines have also been shown to upregulate chemokine receptors in human NK cells [56, 57]. Although IL-2 and IL-15 belong to the same γ c family of cytokines [6], IL-15R α -chain has a much broader tissue distribution than the IL2R α -chain, which is absent in NK cells [58]. IL-15 efficiently engages IL-2/15R $\beta\gamma$ with IL-15R α for signal transduction, leading to the rapid upregulation of receptors for C-C chemokines, whereas IL-2 is unable to do so [57], in parallel with unaffected BLT₂ expression.

Moreover, IL-2 and IL-15 augmented NK cell responses to LTB₄ in terms of chemotaxis and cytolytic function. Preincubation with IL-2 did not increase NK cell migration to the same degree as treatment with IL-15, especially at higher concentration of LTB₄, suggesting that BLT₂ may be involved. The lower effect of IL-2 on BLT expression may be the consequence of the lack of IL-2R α , which results in a receptor with lower affinity (IL-2R $\beta\gamma$) for IL-2, and this in turn could result in a weaker modulation of BLT₂ expression. However, IL-2- and IL-15-activated NK cells showed a similar augmentation in LTB₄-induced cytotoxicity, which was mediated by BLT₁, possibly due to the equivalent upregulation of BLT₁ expression by both cytokines.

We previously showed that LTB₄ augments IL-2R β expression in CD56⁺ NK cells and induces their responsiveness to 100-fold lower concentrations of IL-2 in terms of cytolytic activity [30]. Moreover, IL-15R α mRNA expression was augmented in NK cells after a 30-minute stimulation with 100 nM LTB₄ (data not shown). The evidence that cytokines and leukotrienes can increase each other's receptor expression suggests that IL-2, IL-15, and LTB₄ may synergize to augment NK cell migration to tumour or infection sites and enhance cytotoxic responses.

In conclusion, functional BLT₁ and BLT₂ receptors are expressed in human NK cells and mediate, to a different degree, LTB₄-induced chemotaxis and cytotoxicity. IL-2- and IL-15-dependent modulation of BLTR expression can upregulate the response of NK cells to LTB₄.

Abbreviations

7AAD: 7-Aminoactinomycin D
ADCC: Antibody-dependent cellular cytotoxicity
APC: Allophycocyanin

BLTR: LTB₄ receptor
 CFSE: Carboxyfluorescein succinimidyl ester
 FITC: Fluorescein isothiocyanate
 GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
 LTB₄: Leukotriene B₄
 NK cell: Natural killer cell
 PBLs: Peripheral blood lymphocytes
 PE: R-Phycoerythrin.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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