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NK cells from tuberculous pleurisy express high ICAM-1 levels and exert stimulatory effect on local T cells

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Tuberculous pleurisy, one of the most common manifestations of extrapulmonary tuberculosis, is characterized by a T-cell-mediated hypersensitivity reaction along with a Th1 immune profile. In this study, we investigated functional cross-talk among T and NK cells in human tuberculous pleurisy. We found that endogenously activated pleural fluid-derived NK cells express high ICAM-1 levels and induce T-cell activation *ex vivo* through ICAM-1. Besides, upon *in vitro* stimulation with monokines and PAMP, resting peripheral blood NK cells increased ICAM-1 expression leading to cellular activation and Th1 polarization of autologous T cells. Furthermore, these effects were abolished by anti-ICAM-1 Ab. Hence, NK cells may contribute to the adaptive immune response by a direct cell-contact-dependent mechanism in the context of *Mycobacterium tuberculosis* infection.

Key words: ICAM-1 · NK cells · Pleurisy · Th1 · Tuberculosis

Supporting Information available online

Introduction

Pleuritis is the most frequent clinical manifestation of extrapulmonary tuberculosis (TB) among young adults, and is normally considered a relatively benign form of disease since it may resolve without chemotherapy [1]. Tuberculous pleurisy is caused by a severe delayed-type hypersensitivity reaction in response to the rupture of a subpleural focus of *Mycobacterium tuberculosis* (*Mtb*) infection, but it may also be developed as a complication of primary pulmonary TB [2, 3]. This pleural effusion is characterized by a strong granulomatous inflammatory response to *Mtb* together with high levels of IFN-γ [4].

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NK cells are $CD56^{+}CD16^{+/-}/CD3^{-}CD19^{-}$ lymphocytes of the innate immune system whose function is controlled by an array of germline-encoded activator/inhibitory receptors [5, 6]. Two subsets of NK cells have been identified in humans according to CD56 expression differing in phenotype and function, and also in terms of chemokine receptors and adhesion molecules expression [7, 8, 9]. Functionally, $CD56^{bright}$ cells are effective cytokine producers, whereas $CD56^{dim}$ cells are efficient effectors of natural and Ab-dependent target cell lysis. Recently, we have described that an endogenously activated pleural fluid NK (PF-NK) population is a major source of IFN-γ in response to *Mtb* [10, 11]. Consistently, PF-NK are enriched in $CD56^{bright}$ cells, as has been observed in other chronic inflammatory sites [12, 13] or in tumor-affected tissues [14]. Together with the classical NK functions (*i.e.* cytotoxicity and cytokine production), novel skills have recently been described in niche-specific and *in vitro*-activated human NK cells. These unconventional capabilities include

angiogenesis and tissue remodeling [15], functional cross-talk with T cells [16, 17, 18] and direct pathogen recognition [19, 20]. In this regard, a novel innate immune cell population called Interferon-producing killer dendritic cells has been recently described in mice [21, 22].

ICAM-1/CD54 is the major ligand of costimulatory $\alpha_L\beta_2$ integrin (CD11a/CD18), commonly known as lymphocyte function-associated antigen (LFA-1) [23, 24]. The surface enhancement of ICAM-1 on endothelial, APC and B cells have pleiotropic effects in LFA-1-mediated T lymphocyte responses including T-cell homing [25], formation and stabilization of immunosynapse [26, 27], T-cell memory differentiation [28], and Th1 polarization [29, 30], among others. However, little is known about ICAM-1/LFA-1 interaction in NK/T-cell cross-talk in physiological settings.

Herein, we have investigated whether the proinflammatory microenvironment found in tuberculous pleurisy could selectively up-regulate T-cell-activating molecules on NK cells. The findings of our study demonstrate that through ICAM-1, PF-NK cells co-activate PF-T cells, information the notion that NK cells link innate with adaptative immune response in mycobacterial infections.

Results

NK cells from tuberculous pleural fluid express high ICAM-1 levels

The role of ICAM-1 has been widely studied in APC and endothelial cells, however, little is known about its expression and function on human lymphocytes in an inflammatory context [31–34]. To address this, we evaluated ICAM-1 expression on lymphocyte subpopulations in PBMC and PF mononuclear cells (PFMC) from patients with tuberculous pleurisy (TB-PF). As is shown in Fig. 1A, two pleural lymphocyte populations significantly increased ICAM-1 expression, $CD4^+$ T and $CD3^-CD56^+$ cells. The former doubled its MFI and the latter increased ~3,5-fold with respect to its peripheral blood (PB) counterpart and, furthermore, PF-NK expressed more surface ICAM-1 than $CD4^+$T cells ($p<0.01$). ICAM-1 expression on $CD8^+$ T and NK-T-like ($CD3^+/CD56^+$) populations gave variable results among samples but they tended to be increased in pleural fluid (PF). On the contrary, PB- and PF-B cells maintained high ICAM-1 levels which are consistent with their classical function as accessory cells.

ICAM-1 and CD11a are differentially expressed on $CD56^{dim}$ and $CD56^{bright}$ subsets

To discern whether the enhanced ICAM-1 expression could be due to differential expression between $CD56^{bright}$ and $CD56^{dim}$ or to activation mediated by the proinflammatory microenvironment found in TB-PF [1, 4], we analyzed ICAM-1 expression on NK subsets from PB and PF. As it is shown in Fig. 1B, higher ICAM-1 levels were detected on $CD56^{bright}$ than on $CD56^{dim}$ from PB and PF, but up-regulation of ICAM-1 was observed on both PF subsets compared with PB. Hence, differential expression and cellular activation may explain ICAM-1 enhancement on PF-NK. Besides, CD11a, the major ICAM-1 receptor on lymphocytes, was also differentially expressed on both NK subsets from PB and PF. PB-$CD56^{dim}$ NK cells expressed higher CD11a levels than $CD56^{bright}$ but this molecule was again up-regulated in both PF-NK subsets (Fig. 1B).

PF-NK that respond to Mtb stimulation are ICAM-1^{high}

We have previously described that upon *in vitro* stimulation with *Mtb*, a large percentage of PF-NK produce IFN-γ [10, 11]. To evaluate whether a relationship between ICAM-1 expression and *Mtb*-induced IFN-γ response by PF-NK cells could be ascribed, PFMC were incubated for 24 h with or without *Mtb* and, thereafter, ICAM-1 and IFN-γ co-expression was assessed on NK cells. As it can be seen in Fig. 2, almost all IFN-γ^+ cells were also ICAM-1^{high} NK cells. Hence, within PF-NK, $CD56^{bright}$ ICAM-1^{high} cells are also IFN-γ^+ cells.

ICAM-1 is enhanced in PB-NK cells upon stimulation with monokines and PAMP

Early studies have shown that IL-2-stimulated human NK cells up-regulate ICAM-1 and other cell adhesion molecules [35]. In addition, *Mtb*-derived PAMP and cytokines such as IL-12, IL-15 and IL-18 that activate NK cells have been found in TB-PF [1, 4]. Thus, we evaluated whether ICAM-1 could be up-regulated on NK cells from PBMC upon stimulation with Pam_3CyS or LPS, which signal through TLR2 and TLR4, or different combinations of cytokines. NK activation (measured as % of $CD69^+$) did indeed correlate with surface ICAM-1 up-regulation (Fig. 3A), suggesting that this molecule is also an NK activation marker. Interestingly, Pam_3CyS increased the percentage of $CD69^+$ on NK cells but it did not induce a strong ICAM-1 up-regulation. For comparison, cytokine mediated-ICAM-1 up-regulation was concomitantly measured among T cells during these experiments. As it is shown in Fig. 3B, cytokine stimulation did induce a bigger fold increase in MFI ICAM-1 on NK than on T-cell (Mean ± SEM, 3.0 ± 0.6 *versus* 1.2 ± 0.2, $p<0.05$), a result that closely resemble that observed on endogenously primed PF lymphocyte populations (Fig. 1A).

PB-NK mediate T-cell functional activities by ICAM-1-dependent mechanisms

Having observed that activated PB-NK cells up-regulate ICAM-1, we investigated whether this increase could affect functional activities from T cells. Thus, NK cells were purified from PBMC and stimulated as indicated above, and co-cultured with autologous T cells. After that, ICAM-1-dependent development of Th profile and cellular activations were analyzed.

First we investigated whether NK cells could favor Th1 development through ICAM-1, as has been previously observed for APC [30]. To test this, T cells were co-cultured with autologous resting

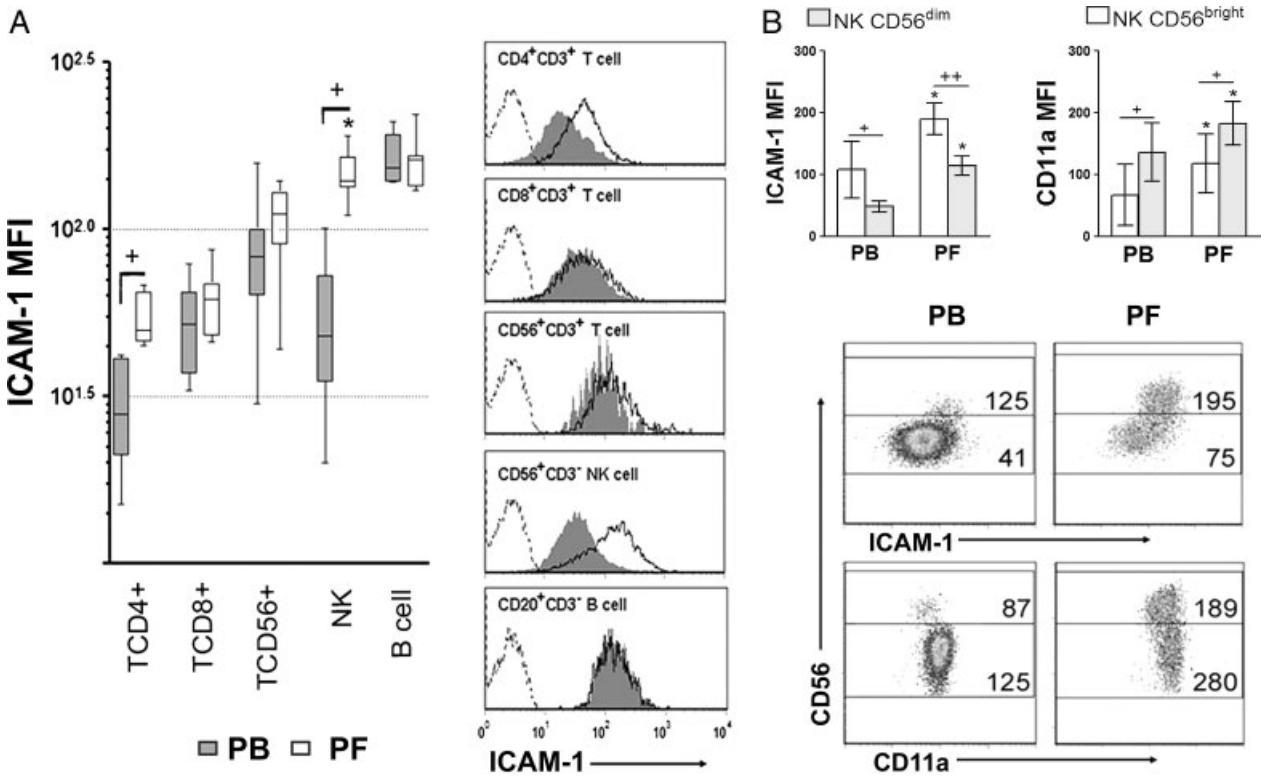

Figure 1. NK cells from TB-PF show increased ICAM-1 levels and their expression is differential between CD56^{dim} and CD56^{bright} subsets. Freshly isolated PB or pleural fluid (PF) lymphocytes were surface stained for ICAM-1, CD11a and surface phenotypic markers. (A) Gated lymphocyte populations were analyzed for surface ICAM-1 expression (median fluorescence intensity, MFI ± CI$_{95\%}$). Box plots represent 95% confidence interval and error bars indicate maximum and minimum values. PF *versus* PB (Wilcoxon paired test): $^{+}p<0.01$. PF-NK *versus* T CD4^{+} or T CD8^{+}: $^{*}p<0.01$ ($n = 12$ patients). FACS histogram plots of a representative patient (PB, filled gray histogram; PF, black line histogram; isotype, dotted line). (B) Differential expression of ICAM-1 ($n = 12$ patients) and CD11a ($n = 8$ patients) in PF- and PB-NK subsets (mean MFI ± SEM). CD56^{bright} *versus* CD56^{dim} (Wilcoxon paired test): $^{+}p<0.05$, $^{++}p<0.005$. PF *versus* PB: $^{*}p<0.05$. FACS dot plots are representative of eight patients.

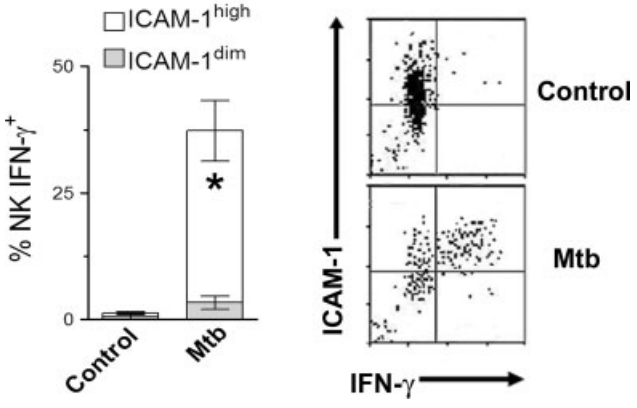

Figure 2. PF-NK ICAM-1^{high} cells express IFN-γ upon *Mtb* stimulation. PFMC, cultured alone (control) or stimulated with *Mtb* for 24 h in complete medium, were stained and analyzed for intracellular IFN-γ and surface ICAM-1 coexpression in gated PF-NK. Bar graph indicates mean ± SEM ($n = 4$ PF form TB). Percentage ICAM-1^{high}/IFN-γ^{+} *versus* ICAM-1^{dim}/IFN-γ^{+} (Paired Student's *t*-test): $^{*}p<0.005$. Dot plots are representative of four patients. Horizontal quadrant marker is placed in MFI of control cells to arbitrarily separate ICAM-1^{high} and ICAM-1^{dim} NK cells.

(NKrest) or activated (NKact) NK cells with or without anti-ICAM-1, and T-bet as well as Gata-3 expression levels were determined on purified CD4^{+} cells. To avoid polarizing effects of endogenous cytokines produced during the assay, and in accordance with previous data [30], an anti-cytokine cocktail (αCK) was added (Fig. 4A). As is shown in Fig. 4A, NKact cells expressing elevated ICAM-1 levels induced higher T-bet expression in CD4^{+} T cells than NKrest. In addition, ICAM-1 dependence in Th1-transcription factor inducing effect was demonstrated by the reduction of T-bet expression by anti-ICAM-1 mAb.

To further evaluate whether T-bet expression was associated with Th1 cytokine expression, T and NK co-cultures were subsequently restimulated with PMA and ionomycin and IFN-γ/IL-4 were detected by intracellular staining. According to T-bet expression, the percentage of T CD4^{+}IFN-γ^{+} cells increased above the basal levels in the presence of NK (Fig. 4B). In addition, a significant increase in the percentage of CD4^{+}IFN-γ^{+} cells was detected by co-culture with NKact cells with respect to NKrest, which was also associated with the increment in T-bet expression. Furthermore, the presence of anti-ICAM-1 decreased the percentage of CD4^{+}IFN-γ^{+} cells at the levels found with NKrest cells. Hence, the enhanced expression of IFN-γ may be explained by the increase in ICAM-1 on NKact cells. In spite of the undetectable Gata-3 levels, low percentage of CD4^{+}IL-4^{+} cells could be observed after PMA/ionomycin stimulation and it was enhanced by the presence of NK. However, neither the activation

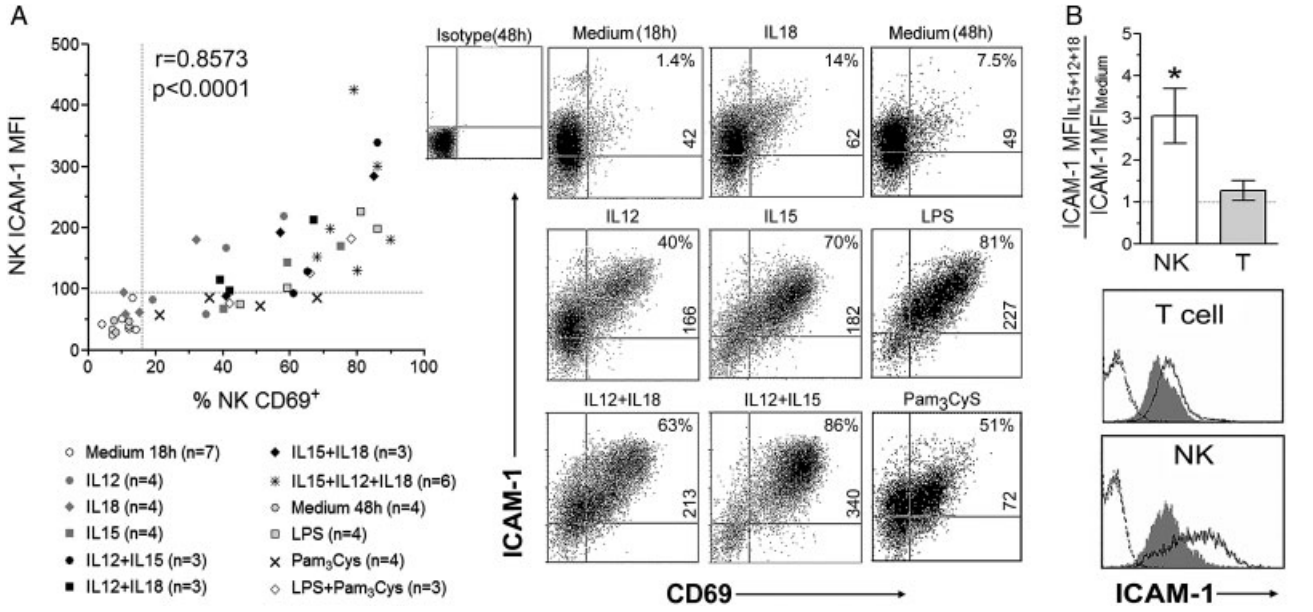

Figure 3. Resting peripheral NK cells up-regulate ICAM-1 after *in vitro* stimulation. PBMC from seven healthy donors, cultured alone (medium) or stimulated with cytokines (18 h) or PAMPs (48 h) were stained and analyzed for CD69 and ICAM-1 coexpression in gated NK (A and B) and T cells (B). (A) Dispersion plot includes data from n independent experiments for each treatment. Dotted ticks were set up above unstimulated cell values. Spearman two-tailed correlation test: $r = 0.8573$; $p<0.0001$. Dot plots are representative of three experiments and percentage of $CD69^+$ and MFI ICAM-1 in NK are indicated (B) Bar graph indicates fold increase in ICAM-1 MFI upon IL-12+IL-15+IL-18 stimulation on NK or on T cells from the same assays (mean ± SEM). NK *versus* T cells (Paired Student's *t*-test): $^*p<0.005$ ($n = 5$ healthy donors). Histogram data are representative of five healthy donors.

status of NK (NKact *versus* NKrest $p>0.05$) nor the presence of anti-ICAM-1 (NKact+αICAM-1 *versus* NKact+IgG1 $p>0.05$) modified the percentage of T $CD4^+IL4^+$ levels.

Finally, we wanted to explore ICAM-1 dependence of NK-mediated-T-cell activation. To address this, we performed autologous co-cultures of T cells with NKrest or NKact with or without anti-ICAM-1, and CD69 expression was determined on $CD4^{+/-}$ T cells. Basal and positive activation levels were obtained by incubating T cells alone or with Con A, respectively. As it can be seen in Fig. 5A, higher relative percentage of $CD69^+$ cells in $CD4^+$ T cells were induced by NKact than by NKrest, and this effect was inhibited by anti-ICAM-1. Besides, among $CD4^{neg}$ T cells, NKact mediated-activation was lesser and more variable between assays, and ICAM-1 inhibition had not significant effect.

PF-NK promote PF-T-cell activation in an ICAM-1-dependent fashion

Having observed that *in vitro*-activated NK cells are able to modulate T-cell activities through ICAM-1-dependent signals, and given that high ICAM-1 levels were detected on PF-NK cells, we wondered whether local NK-dependent T-cell co-activation could also be observed. To do this, freshly purified PF-NK were co-cultured overnight with PF-T cells in the presence or absence of anti-ICAM-1. Consistent with the previously described activated phenotype of PF-T cells [36], a weak level of basal CD69 expression was detected on PF-T cells, which was further increased upon Con A stimulation. Besides, co-culture with PF-NK increased the percentage $CD69^+$ in $CD4^+$ and $CD4^{neg}$ T cells and this effect was reduced to basal levels in the presence of anti-ICAM-1 (Fig. 5B).

Discussion

The ICAM-1/LFA-1 interaction has a pleiotropic effect during Ag presentation, as it not only plays an important role in T-cell recirculation and inflammation but also in T-cell activation [37, 38]. The role of ICAM-1/LFA-1-mediated adhesion has been established as a critical event for strengthening the contact between DC and Th cells leading to optimal Th cells activation [39], Th1-differentiation [30] and cytotoxicity [40], among others. Besides, ICAM-1/LFA-1 interaction also controls NK cell functional responses [41]. However, the role of ICAM-1 has not been elucidated in NK-T-cell activation in a physiological setting.

Herein we showed that endogenously activated PF-derived NK cells express increased amounts of ICAM-1 and LFA-1 molecules, as has been observed in peripheral lymphocytes from other inflammatory diseases [32–34]. Furthermore, we observed differential expression of ICAM-1 and LFA-1, which is consistent with the functional divergent activities of $CD56^{bright}$ and $CD56^{dim}$ subsets [7]. According to its cytotoxic effector function [41], $CD56^{dim}$ from PB and PF expressed high LFA-1 levels, whereas NK-mediated helper functions may be related to the high ICAM-1 levels on $CD56^{bright}$ [42]. Accordingly, IFN-γ production was also ascribed to $CD56^{bright}$ subset [10]. These data lead us to explore functional consequences on NK-T cells interactions.

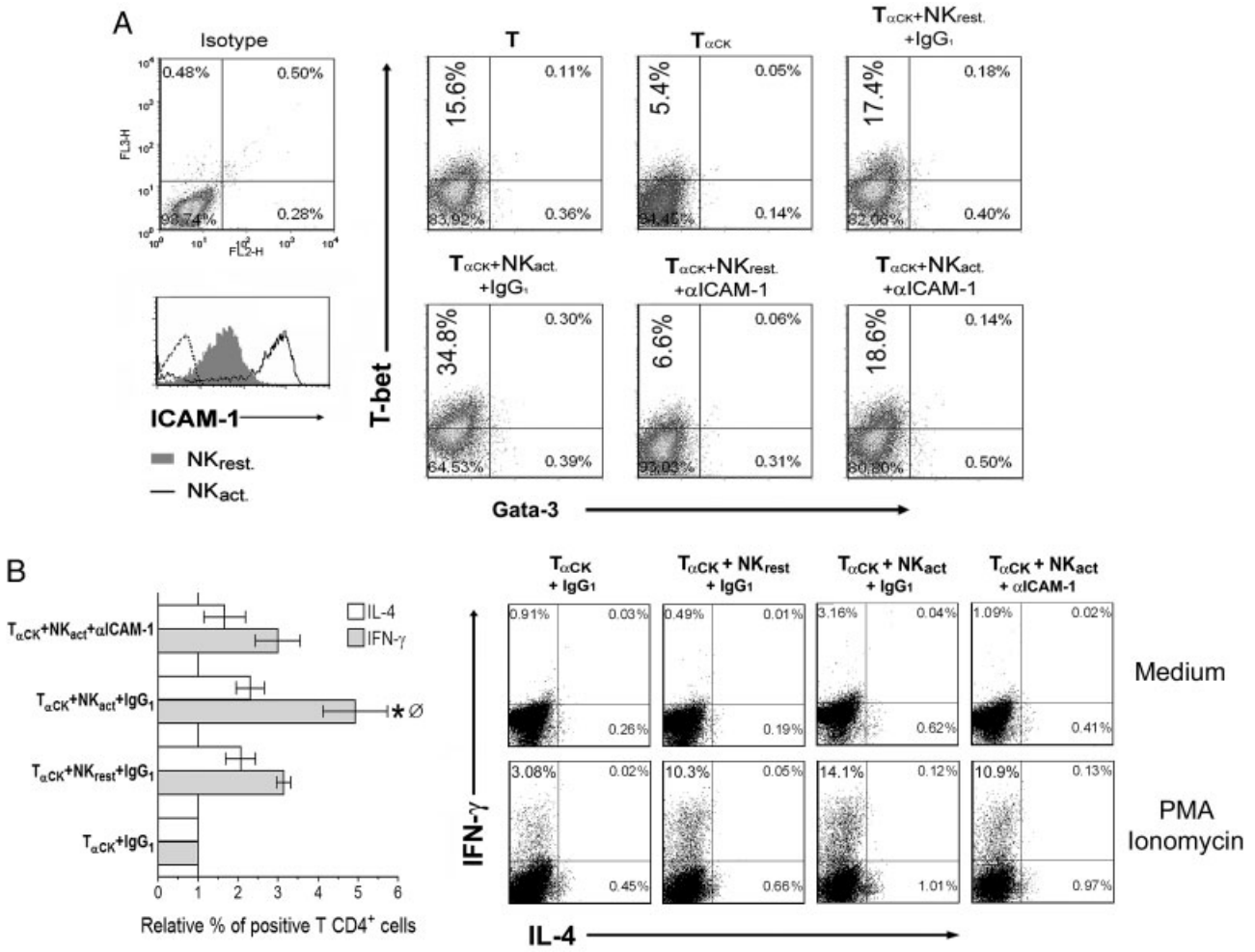

Figure 4. NK-mediated Th1 polarization is dependent on ICAM-1. *In vitro* Th1/2 profile was determined by intracellular T-bet/Gata-3 and IFN-γ/IL-4 staining in purified T $CD4^+$cells cultured in IL-2-supplemented-mediun alone (T) or anti-cytokine cocktail ($T_{\alpha CK}$). Co-cultures of $T_{\alpha CK}$ with autologous NK_{rest} or NK_{act} were carried out in the presence of anti-ICAM-1 (αICAM-1) or the corresponding isotype control Ab (IgG1). (A) Dot plot analysis of T-bet/Gata-3 coexpression is representative of four healthy donors. Histogram plot of ICAM-1 expression on NK_{act} (black line) and NK_{rest} (gray filled) at the start of the co-culture is depicted. (B) IFN-γ^+ (gray bars) and IL-4^+ (white bars) in PMA/ionomycin restimulated $CD4^+$ cells normalized to cytokine expression in $T_{\alpha CK}$ (mean ± SD). NK_{rest}+IgG_1 *versus* NK_{act}+IgG_1: $^{\varnothing}p<0.05$; NK_{act}+IgG_1 *versus* NK_{act}+α-ICAM-1:$^{*}p<0.05$ (Paired one-way ANOVA, $n = 3$ healthy donors). A representative dot plot analysis of three healthy donors.

In our *in vitro* approaches, we were able to induce up-regulation of ICAM-1 by short stimulation of PB-NK with monokines known to be present in tuberculous PF (TB-PF) [4, 10, 43]. These monokines include IL-12, which enhances IFN-γ production [44], IL-15 that promotes survival and differentiation [45], and IL-18, which potentiates the effect of IL-12 by inducing IL-12R on NK cells [46]. However, in our hands, those monokines did not up-regulate ICAM-1 on $CD3^+$ T cells to the same extent, suggesting a possible explanation for the stronger ICAM-1 enhancement from PF-NK cells than from PF-T subpopulations. In addition, the remarkable correlation between CD69 and ICAM-1 expression indicates that the latter molecule may be used as a sensitive NK cell activation marker. However, an exception was observed by the TLR2 agonist, Pam_3CyS, which was able to induce CD69 expression but failed to up-regulate ICAM-1, suggesting an independent mechanism for modulation of these two activation markers [47].

Recent studies have demonstrated that human NK cells efficiently enhance $CD4^+$ as well as $CD8^+$ T-cell proliferation. This process is dependent on direct contact-mediated interactions between ligands for TCR co-stimulatory receptors expressed on stimulated human NK cells such as CD80, CD86, CD70, OX40L and 2B4 receptors [16, 17, 48]. These studies have been carried out by employing long-time-IL-2-stimulated NK cells or NK cell clones and up to date, formal evidence of this interaction *in vivo* in humans has not been provided [48, 49]. Herein, by using short-time monokine-stimulated primary NK cells, we found that these cells have the capacity to up-regulate T-bet and IFN-γ expression driving Th1 polarization of $CD4^+$ cells and to induce CD69 expression on $CD4^+$ T cells in an ICAM-1-dependent fashion. In addition, we also found that NK cells form conjugates with T cells in an ICAM-1 dependent way, as previously observed with *Plasmodium falciparum*-infected erythrocytes [50] and with

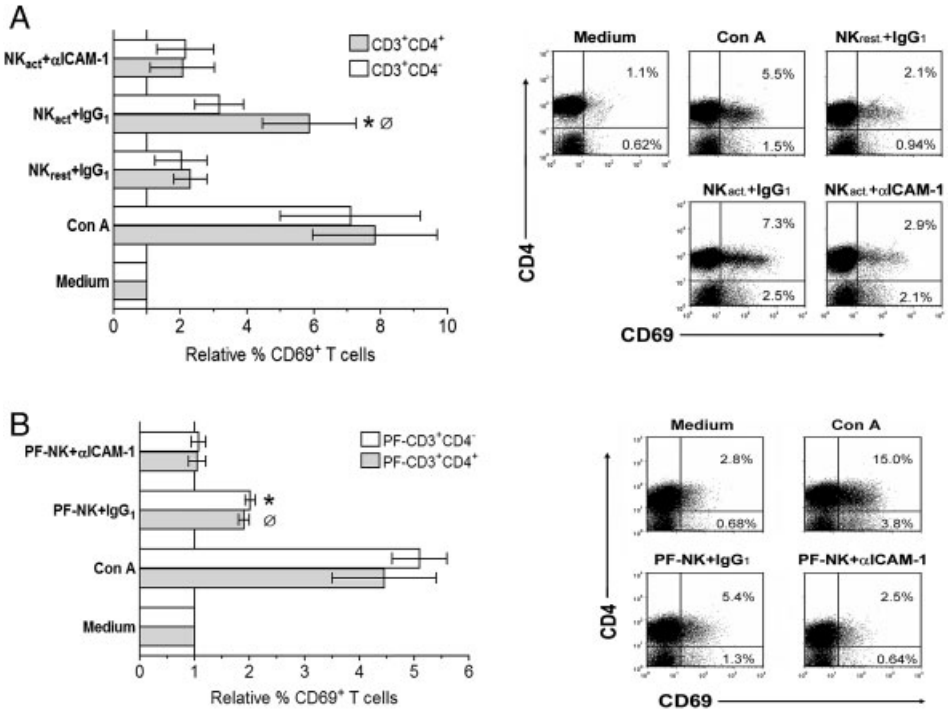

Figure 5. *In vitro*-activated and PF-derived NK cells induce T-cell activation by ICAM-1 engagement. Purified T cell from PB (A) or PF (B) were cultured 12 h with complete medium (Medium), Con A (1 μg/mL) or NK_{act} or NK_{rest} (A) or PF-NK (B). Co-cultures were carried out in the presence (αICAM-1) or not (IgG_1) of anti-ICAM-1 mAb. Data were expressed as mean ± SD of relative percentage $CD69^+$ of T CD4+(gray bars) or T $CD4^{neg}$ (white bars) cells normalized to medium. (A) $NK_{act}+IgG_1$ *versus* $NK_{rest}+IgG_1$: $^{ø}p<0.01$ or $NK_{act}+IgG_1$ *versus* $NK_{act}+\alpha$ICAM-1:* $p<0.01$ on $CD3^+CD4^+$ (Paired one-way ANOVA, $n = 6$). Dot plots are representative of six healthy donors. (B) PF-NK+IgG1 *versus* PF-NK+αICAM-1: $^*p<0.05$ on $CD3^+CD4^{neg}$ or $^{ø}p<0.05$ on $CD3^+CD4^+$ (Paired one-way ANOVA, $n = 3$ PF from TB). Dot plots of a representative pleural fluid from three TB patients.

γδ Tcells [51] (see Supporting Information). Furthermore, taking advantage that the inflammatory focus of TB pleurisy constitute an *in vivo* activating microenviroment [10, 11, 36], we were able to show that endogenous up-regulation of ICAM-1 by pass the requirement of *in vitro* NK pre-activation and was indeed sufficient to enhance CD69 expression in PF-T cells.

In summary, we demonstrate that ICAM-1/LFA-1 are differentially expressed between PB- and PF-NK cells being $CD56^{bright}$ ICAM-1^{high} cells the major NK population within TB-PF, and also the producers of IFN-γ [10]. Moreover, herein we provide evidence of another mechanism mediated by endogenously activated NK cells, specifically, T-cell costimulation through ICAM-1. We conclude that NK cells play a beneficial role through the early production of IFN-γ and cell-mediated costimulation, contributing to direct and sustain an effective adaptative immune response by driving a Th1 profile in this physiologically relevant human model of chronic intracellular infection.

Materials and methods

Patients and healthy blood donors

Patients with newly diagnosed moderate and large pleural effusions were identified at the Servicio de Tisioneumonología, Hospital F.J Muñiz, Buenos Aires, Argentina. Written informed consent was obtained according to Ethics Committee from Hospital F.J. Muñiz. Physical examination, complete blood cell count, electrolyte, chest x-ray and HIV test were performed in this institution. Exclusion criteria included positive HIV test or the presence of concurrent infectious diseases. TB-PF together with PB samples were obtained during therapeutic thoracentesis as described previously [10]. A total of 17 PF were studied (average age = 29 year, range = 20–46 year) and among them, six had pulmonary disease according to chest X-ray findings. PF were classified as exudates according to at least one of Light's criteria [3]. Buffy coats were the source of normal PBMC (Blood Transfusion Service, Hospital Fernandez, Buenos Aires, Argentina).

Mononuclear cells

PF and PB were dispensed into 50 mL polystyrene tubes (Corning, NY, USA) containing heparin. PBMC and PFMC were isolated by Ficoll-Hypaque gradient centrifugation and suspended in RPMI 1640 tissue culture medium (Gibco Lab, NY, USA) containing gentamycin (85 μg/mL) and 10% heat inactivated FBS (Gibco Lab) (Complete medium). Purity and viability were tested using Trypan-Blue exclusion dye.

Antigen, agonists and biological reagents

The γ-irradiated *Mtb* H37Rv strain employed here were kindly provided by J. Belisle (Colorado State University, Fort Collins, CO, USA). Mycobacteria were suspended in free pyrogen PBS, sonicated and adjusted at a concentration of $\approx 1 \times 10^8$ bacteria/mL ($OD_{600nm} = 1$). Synthetic lipoprotein Pam_3CyS-SK4 (Pam_3CyS), *Escherichia coli* 0111:B4 LPS and Con A were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human interleukin (rhIL-)12, rhIL-15 and rhIL-18 were from Peprotech (Rocky Hill, NJ, USA). Hybridoma culture supernatant against human CD3 (Clone 145-2C) and CD16 (3G8) were kindly provided by M. Giordano (IIHema, Buenos Aires, Argentina) and the purified mAbs anti-CD19, anti-CD56, anti-CD4 (Becton Dickinson, BD, Mountain View, CA, USA), anti-CD14 (Immunotech, Marseille, France) and anti-MHC-II (HLA-DP,DR,DQ) (Ancell Bayport, MN, USA) were used for depletion assays. Neutralizing, azide free, purified Abs against human (α-h) CD54/ICAM-1 (clone HA58, BD), α-hIL-12, α-hIL-10, α-hIL-4, α-hTNFα (Peprotech) or isotype-matched controls were employed in blocking experiments. FITC, PE and/or Cy5-PE conjugated mAbs against CD3, CD4, CD8, CD56, CD69, CD54 (e-Bioscience, San Diego, CA, USA), CD56, CD11a (Immunotech), CD19, IL-4 (BD), CD20 (Ancell), IFN-γ (Caltag, Burlingam, CA, USA) and isotype-matched mAb were used in immunofluorescence analysis.

Cell surface and intracellular expression by flow cytometry analysis

Surface markers expression were evaluated on freshly isolated or cultured PB and PFMC by staining 0.5×10^6 cells for 20 min at 4°C with adequate amount and combinations of labeled mAb in ~50 μL volume of PBS 1% SFB. For intracellular IFN-γ and IL-4 assessment, brefeldin A (5 μg/mL, Sigma) was added for the last 4–5 h. Then, cells were washed and surface stained as indicated above, followed by fixation, permeabilization and staining with anti-cytokines mAb, according to the manufacturer's instructions (IntraPrep™, Immunotech). For intracellular T-bet/Gata-3 staining, commercial Foxp3 staining Buffer set was used according manufacturer's proceedings (eBioscience). Analysis was carried out in FACScan cytometer (BD) using FCS Express V3 software (De Novo Software™, Los Angeles, CA, USA) by acquiring $3–8 \times 10^4$ events. Gates were set on lymphocytes according to forward and side-scatter properties. NK cells were defined as lymphocytes $CD3^-CD56^+$ or $CD3^-CD19^-$ (>90% NK cells), NKT-like as $CD3^+CD56^+$ cells, T as $CD3^+CD4^+/CD8^+$ and B as $CD3^-CD20^+$ cells.

In vitro stimulation of PFMC, PBMC and purified NK

PF or PBMC (1×10^6 cells/mL) were incubated in Falcon 2063 tubes (Becton Dickinson, Lincoln, NJ, USA) at 37°C in an humidified 5% CO_2 atmosphere, in complete medium with or without rhIL-12 (10 ng/mL), rhIL-15 (10 ng/mL) and/or rhIL-18 (50 ng/mL) for 18 h, with *Mtb* (10^6 bac/mL) for 24 h or with Pam_3CyS (100 ng/mL) or LPS (1 μg/mL) for 48 h. Thereafter, % $CD69^+$ and ICAM-1 co-expression were determined on PB-NK cells or % IFN-γ^+ and ICAM-1 co-expression in PF-NK.

NK ICAM-1^{high} (NK_{act}) cells used in T-cell functional assays were obtained by pre-incubation of purified PB-NK with a cytokine cocktail (IL-12+IL-15+IL-18) for 18 h. NK control cells (NK_{rest}) were culture in complete medium alone.

NK and T-cell purifications

PB and PF-T $CD3^+$ cells were obtained by negative selection. Briefly, ~10^7 cells were pre-incubated at 4°C for 30 min with anti-CD16, -MHC-II, -CD14, -CD56 and -CD19 followed by magnetic depletion with goat anti-mouse IgG (GAM)-coated Dynabeads® (Dynal, Oslo, Norway) according to manufacture's instructions. In general, one cycle of treatment was sufficient for effective depletion (>94% $CD3^+$ cells, as assessed by FACS) with a minor activation (less than Δ1% $CD69^+$ compared with undepleted cells). For resting PB-T $CD4^+$ isolation used in Th1/2 polarization assays, purified T $CD3^+$ cells were depleted using anti-CD8 and -CD25 mAbs. Purity of T $CD4^+$ cells were above 95%.

PB or PF-NK cells were obtained by two-step purification, briefly, ~10^7 PBMC or PFMC were depleted of $CD3^+$, $CD19^+$ and $CD14^+$ cells using GAM Dynabeads®. Next, the cells were positively selected with anti-CD56 mAb employing GAM-coated MACS® following manufacture's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Typical procedure yield less than 5% of contaminating $CD3^+/CD19^+/CD14^+$ cells even in the scarce NK population from PF [11].

NK co-cultures for T-cell functional assays

ICAM-1-mediated T-helper polarization

NK_{rest} or NK_{act} and autologous T $CD4^+$ cells were co-cultured in order to assess the influence of NK cell ICAM-1 expression on Th1/2 profile commitment [30]. Briefly, purified $CD4^+$ T cells were placed in complete medium supplemented with rhIL-2 (100 U/mL)+anti-CD3mAb (5 μg/mL) alone (T) or with a cocktail containing neutralizing Ab against the human cytokines IL-12 (1 μg/mL), IL-4 (2 μg/mL), IL-10 (0.5 μg/mL) and TNF-α (0.5 μg/mL) ($T_{\alpha CK}$). T or $T_{\alpha CK}$ were left alone or co-cultured with NK_{act} or NK_{rest} at 2:1 T/NK ratio for 72 h at 37°C in the presence or absence of anti-ICAM-1 mAb. Finally, intracellular co-expression of T-bet and Gata-3 was determined in gated $CD4^+$ T cells. For cytokine production by co-cultured T cells, cells were washed three times and restimulated with PMA (10 ng/mL) and ionomycin (1 μg/mL) for 6 h, the last 5 h in the presence of brefeldin A (5 μg/mL). Thereafter, IFN-γ and IL-4 co-expression were determined in gated $CD4^+$ T cells.

ICAM-1-mediated T-cell co-activation

NK_{rest}, NK_{act} or endogenously primed PF-NK cells were co-cultured with autologous T cells in order to determine whether NK ICAM-1^{high} may affect T-cell activation. Briefly, purified PB or PF-T $CD3^+$ cells placed in complete medium, were left alone or co-cultured with NK_{act}, NK_{rest} at 2:1 or with PF-NK at 3:1 T/NK ratio for 12 h at 37°C in the presence or absence of anti-ICAM-1 mAb. Con A (1 μg/mL) was employed as positive activation control. Finally, percentage of $CD69^+$ cells were determined on $CD3^+CD4^+$ or $CD3^+CD4^{neg}$.

Statistical analysis

Wilcoxon (non-parametric) paired test were employed for comparison of paired PB and PF samples. Correlation analysis was performed with Spearman two-tailed (non-parametric) test. Comparison of two different treatments was drawn using paired Student's *t*-test, and multiple comparisons among several treatments were drawn using repeated measures (paired) one-way ANOVA with Bonferroni post test. A $p<0.05$ value was assumed as significant. Some data were normalized to internal control in order to minimize inter-experimental variation.

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References

1 **Antony, V. B. and Mohammed, K. A.,** Pathophysiology of pleural space infections. *Semin. Respir. Infect.* 1999. **14**: 9–17.

2 **Alemán, M., de la Barrera S., Schierloh, P., Alves, L., Yokobori, N., Baldini, E. Abbate, M. and Sasiain, M. C.,** In tuberculous pleural effusions activated neutrophils undergo apoptosis and acquire dendritic cell-like phenotype. *J. Infect. Dis.* 2005. **192**: 399–409.

3 **Light, R., MacGregor, M., Luschsinger, P. and Ball, W.,** Pleural effusions: the diagnostic separations of transudates and exudates. *Ann. Intern. Med.* 1972. **72**: 507–513.

4 **Hiraki, A., Matsuo, K., Murakami, K., Murakami, T., Onoda, T., Sugi, K., Takeyama, R. and Eda, R.** Simultaneous measurement of T-helper 1 cytokines in tuberculous pleural effusion. *Int. J. Tuberc. Lung. Dis.* 2003. **7**: 1172–1177.

5 **Lanier, L. L.,** Up on the tightrope: natural killer cell activation and inhibition. *Nat. Immunol.* 2008. **9**: 495–502.

6 **Stewart, C. A, Vivier, E. and Colonna, M.,** Strategies of natural killer cell recognition and signalling. *Curr. Top. Microbiol. Immunol.* 2006. **298**: 1–21.

7 **Cooper, M. A., Fehniger, T. A. and Caligiuri, M. A.,** The biology of human natural killer-cell subsets. *Trends Immunol.* 2001. **22**: 633–640.

8 **Frey, M., Packianathan, N. B., Fehniger, T. A., Ross, M. E., Wang, W. C., Stewart, C. C., Caligiuri, M. A. and Evans, S. S.,** Differential expression and function of L-selectin on $CD56^{bright}$ and $CD56^{dim}$ natural killer cell subsets. *J. Immunol.* 1998. **161**: 400–408.

9 **Campbell, J. J., Qin, S., Unutmaz, D., Soler, D., Murphy, K. E., Hodge M. R., Wu L. and Butcher, E. C.,** Unique sub-populations of $CD56^+$ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. *J. Immunol.* 2001. **166**: 6477–6482.

10 **Schierloh, P., Yokobori, N., Alemán, M., Landoni, V., Geffner, L., Musella, R. M., Castagnino, J. *et al.*,** *Mycobacterium tuberculosis*-induced IFN-γ production by natural killer cells requires cross-talk with antigen presenting cells involving toll-like receptors 2 and 4 and mannose receptor in tuberculous pleurisy. *Infect. Immun.* 2007. **75**: 5325–5337.

11 **Schierloh, P., Yokobori, N. Alemán, M., Musella, R. M., Beigier-Bompadre, M., Saab, M. A., Alves, L. *et al.*,** Increased susceptibility to apoptosis of $CD56^{dim}CD16^+$ NK cells induces the enrichment of IFN-γ-producing $CD56^{bright}$ cells in tuberculous pleurisy. *J. Immunol.* 2005.**175**: 6852–6860.

12 **Dalbeth, N., Gundle, R., Davies, R. J., Lee, C. G., McMichael, A. J. and Callan, F. C.,** $CD56^{bright}$ NK cells are enriched at inflammatory sites and can engage with monocytes in a reciprocal program of activation. *J. Immunol.* 2004. **173**: 6418–6426.

13 **Pridgeon, C., Lennon, G. P., Pazmany, L., Thompson, R. N., Christmas, S. E. and Moots, R. J.,** Natural killer cells in the synovial fluid of rheumatoid arthritis patients exhibit a $CD56^{bright}$, $CD94^{bright}$, $CD158^{negative}$ phenotype. *Rheumatology (Oxford)* 2003. **42**: 870–878.

14 **Carrega, P., Morandi, B., Costa, R., Frumento, G., Forte, G., Altavilla, G., Ratto, G.B. *et al.*,** Natural killer cells infiltrating human nonsmall-cell lung cancer are enriched in $CD56^{bright}$ $CD16^-$ cells and display an impaired capability to kill tumor cells. *Cancer* 2008. **112**: 863–875.

15 **Hanna, J., Goldman-Wohl, D., Hamani, Y., Avraham, I., Greenfield, C., Natanson-Yaron, S., Prus, D. *et al.*,** Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat. Med.* 2006. **12**: 1065–1074.

16 **Zingoni, A., Sornasse, T., Cocks, B. G., Tanaka, Y., Santoni, A. and Lanier, L. L.,** Cross-talk between activated human NK cells and $CD4^+$T cells via OX40–OX40 ligand interactions. *J. Immunol.* 2004. **173**: 3716–3724.

17 **Hanna, J., Gonen-Gross, J., Fitchett, T., Rowe, T., Daniels, M., Arnon, T. I., Gazit, R. *et al.*,** Novel APC-like properties of human NK cells directly regulate T cell activation. *J. Clin. Invest.* 2004. **114**: 1612–1623.

18 **Fehniger, T. A., Cooper, M. A., Nuovo, G. J., Cella, M., Facchetti, F., Colonna, M. and Caligiuri, M. A.,** $CD56^{bright}$ natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. *Blood* 2003. **101**: 3052–3057.

19 **Athié-Morales, V., O'Connor, G. M. and Gardiner, C. M.,** Activation of human NK cells by the bacterial pathogen-associated molecular pattern muramyl dipeptide. *J. Immunol.* 2008. **180**: 4082–4089.

20 **Marcenaro, E., Ferranti, B., Falco, M., Moretta, L., and Moretta, A.,** Human NK cells directly recognize *Mycobacterium bovis* via TLR2 and acquire the ability to kill monocyte-derived DC. *Int. Immunol.* 2008. **20**: 1155–1167.

21 **Chan, C. W., Crafton, E., Fan, H. N., Flook, J., Yoshimura, K., Skarica, M., Brockstedt, D. *et al.*,** Interferon-producing killer dendritic cells provide a link between innate and adaptive immunity. *Nat. Med.* 2006. **12**: 207–213.

22 **Vosshenrich, C. A., Lesjean-Pottier, S., Hasan, M., Richard-Le Goff, O., Corcuff, E., Mandelboim, O. and Di Santo, J. P.,** $CD11c^{lo}B220^{+}$ interferon-producing killer dendritic cells are activated natural killer cells. *J. Exp Med.* 2007. **204**: 2569–2578.

23 **Bella, J., Kolatkar, P. R., Marlor, C. W., Greve, J. M. and Rossmann, M. G.,** The structure of the two amino-terminal domains of human ICAM-1 suggests how it functions as a rhinovirus receptor and as an LFA-1 integrin ligand. *Proc. Natl. Acad. Sci. USA* 1998. **95**: 4140–4145.

24 **Luo, B. H., Carman, C. V. and Springer, T. A.,** Structural basis of integrin regulation and signaling. *Annu. Rev. Immunol.* 2007. **25**: 619–647.

25 **Ainslie, M. P., McNulty, C. A., Huynh, T., Symon, F. A. and Wardlaw, A. J.,** Characterisation of adhesion receptors mediating lymphocyte adhesion to bronchial endothelium provides evidence for a distinct lung homing pathway. *Thorax* 2002. **57**: 1054–1059.

26 **Qi, S. Y., Groves, J. T. and Chakraborty, A. K.,** Synaptic pattern formation during cellular recognition. *Proc. Natl. Acad. Sci. USA* 2001. **98**: 6548–6553.

27 **Schneider, H., Downey, J., Smith, A., Zinselmeyer, B. H., Rush, C., Brewer, J. M., Wei, B. *et al.*,** Reversal of the TCR stop signal by CTLA-4. *Science* 2006. **313**: 1972–1975.

28 **Perez, O. D., Mitchell, D. and Nolan, G. P.,** Differential role of ICAM ligands in determination of human memory T cell differentiation. *BMC Immunol.* 2007. **8**: 2.

29 **Owaki, T., Asakawa, M., Fukai, F., Mizuguchi, J. and Yoshimoto, T.,** IL-27 induces Th1 differentiation via p38 MAPK/T-bet- and intercellular adhesion molecule-1/LFA-1/ERK1/2-dependent pathways. *J. Immunol.* 2006. **177**: 7579–7587.

30 **Smits, H. H., de Jong, E. C., Schuitemaker, J. H. N., Geijtenbeek, T. B. H., van Kooyk, Y., Kapsenberg, M. L. and Wierenga, E. A.,** Intercellular adhesion molecule-1/LFA-1 ligation favors human Th1 development. *J. Immunol.* 2002. **168**: 1710–1716.

31 **Schierloh, P., Alemán, M., Yokobori, N., Alves, L., Roldan, N., Abbate, E., Sasiain, M. C. and de la Barrera, S.,** NK cell activity in tuberculosis is associated with impaired CD11a and ICAM-1 expression: a regulatory role of monocytes in NK activation. *Immunology* 2005. **116**: 541–552.

32 **Arifhodzic, N. A., Mahmoud, F. F., Abul, H. T., Fiaines, D. D., Al-Dowaisan, A. R., Ammar, I. M., Novotny, L. and Wise, J. A.,** Major lymphocyte populations and T-cell expression of ICAM-1 and L-selectin adhesion molecules in kuwaitis with asthma and rhinitis. *Arch. Environ. Occup. Health.* 2005. **60**: 243–247.

33 **Österlund, P., Smedberg, T., Schröderw, J. and Järvinen, K.-M.,** Expression of intercellular adhesion molecules on circulating lymphocytes in relation to different manifestations of cow's milk allergy. *Clin. Exp. Allergy* 2003. **33**: 1368–1373.

34 **Azeredo, E. L., Zagne, S. M., Alvarenga, A. R., Nogueira, R. M. R., Kubelka, C. F. and de Oliveira-Pinto, L. M.,** Activated peripheral lymphocytes with increased expression of cell adhesion molecules and cytotoxic markers are associated with dengue fever disease. *Mem. Inst. Oswaldo. Cruz.* 2006. **101**: 437–449.

35 **Robertson, M. J., Caligiuri, M. A., Manley, T. J., Levine, H. and Ritz, J.,** Human natural killer cell adhesion molecules. Differential expression after activation and participation in cytolysis. *J. Immunol.* 1990. **145**: 3194–3201.

36 **Mitra, D. K., Sharma, S. K., Dinda, A. K., Bindra, M. S., Madan, B. and Ghosh, B.,** Polarized helper T cells in tubercular pleural effusion: phenotypic identity and selective recruitment. *Eur. J. Immunol.* 2005. **35**: 2367–2375.

37 **Sligh, J. E., jr., Ballantyne, C. M., Rich, S. S., Hawkins, H. K., Smith, C. W., Bradley, A. and Beaudet, A. L.,** Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. USA* 1993. **90**: 8529–8533.

38 **Springer, T. A.,** Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994. **76**: 301–314.

39 **van Kooyk, Y., van de Wiel-van K, P., Weder, P., Kuijpers, T. W. and Figdor, C. G.,** Enhancement of LFA-1-mediated cell adhesion by triggering through CD2 or CD3 on T lymphocytes. *Nature* 1989. **342**: 811–813.

40 **Anikeeva, N., Somersalo, K., Sims, T. N., Thomas, V. K., Dustin, M. L. and Sykulev, Y.,** Distinct role of lymphocyte function-associated antigen-1 in mediating effective cytolytic activity by cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* 2005. **102**: 6437–6442.

41 **Barber, D. F., Faure, M. and Long, E. O.,** LFA-1 contributes an early signal for NK cell cytotoxicity. *J. Immunol.* 2004. **173**: 3653–3659.

42 **Mailliard, R. B., Alber, S. M., Shen, H., Watkins, S. C., Kirkwood, J. M., Herberman, R. B. and Kalinski, P.,** IL-18-induced CD83+CCR7+NK helper cells. *J. Exp. Med.* 2005. **202**: 941–953.

43 **Song, C. H., Lee, J. S., Nam, H. H., Kim, J. M., Suhr, J. W., Jung, S. S., Na, M. J. *et al.*,** IL-18 production in human pulmonary and pleural tuberculosis. *Scand. J. Immunol.* 2002. **56**: 611–618.

44 **Trinchieri, G.,** Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* 2003. **3**: 133–146.

45 **Fehniger, T. A. and Caligiuri, M. A.,** Interleukin 15: biology and relevance to human disease. *Blood* 2001. **97**: 14–32.

46 **Ortaldo, J. R. and Young, H. A.,** IL-18 as critical co-stimulatory molecules in modulating the immune response of ITAM bearing lymphocytes. *Semin. Immunol.* 2006. **18**: 193–196.

47 **Uchihara, J. N., Matsuda, T., Okudaira, T., Ishikawa, C., Masuda, M., Horie, R., Watanabe, T. *et al.*,** Transactivation of the ICAM-1 gene by CD30 in Hodgkin's lymphoma. *Int. J. Cancer.* 2006. **118**: 1098–1107.

48 **Hanna, J. and Mandelboim, O.,** When killers become helpers. *Trends Immunol.* 2007. **28**: 201–206.

49 **Andoniou, C. E., Coudert, J. D. and Degli-Esposti, M. A.,** Killers and beyond: NK-cell-mediated control of immune responses. *Eur. J. Immunol.* 2008. **38**: 2938–2942.

50 **Baratin, M., Roetynck, S., Pouvelle, B., Lemmers, C., Viebig, N. K., Johansson, S., Bierling, P. *et al.*,** Dissection of the role of PfEMP1 and ICAM-1 in the sensing of *Plasmodium falciparum*-infected erythrocytes by natural killer cells. *PLoS ONE* 2007. **2**: e228.

51 **Zhang, R., Zheng, X., Li, B., Wei, H. and Tian, Z.,** Human NK cells positively regulate γδ T cells in response to *Mycobacterium tuberculosis*. *J. Immunol.* 2006. **176**: 2610–2616.

Abbreviations: **αCK:** anti-cytokine cocktail · **LFA:** lymphocyte function-associated antigen · ***Mtb:*** *Mycobacterium tuberculosis* · **NK_{act}:** activated NK cells · **NK_{rest}:** resting NK cells · **PB:** peripheral blood · **PF:** pleural fluid · **PFMC:** PF mononuclear cells · **TB:** tuberculosis · **TB-PF:** tuberculous PF

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