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List of abbreviations: BTN3, butyrophilin 3 ; MFI, mean fluorescence intensity ; aAPC, artificial APC

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Summary

The human butyrophilin 3 (BTN3 or CD277) molecules belong to the B7 family members and are expressed in various immune cells such as T cells and NK cells. Here, we show that CD277 triggering considerably enhances TCR-induced cytokine production and cell proliferation, even when another costimulatory molecule, CD28 is engaged. These CD277-induced additive functional effects are in accordance with the detection of early T-cell activation events such as TCR-induced cell signaling increased upon CD277 engagement. However, we found that CD277 triggering is not involved in CD16 or NKp46-induced NK cell activation. BTN3/CD277 comprises three structurally related members, BTN3A1, BTN3A2 and BTN3A3. CD277 antibodies recognize all isoforms and we describe a differential expression of BTN3 isoforms between T cells and NK cells that could explain differential CD277 functions between T and NK cells. Our results show that while T cells express all BTN3 / CD277 transcripts, NK cells express mostly BTN3A2, which lacks the B30.2 intracellular domain. Furthermore, NKp30-induced cytokine production is decreased by the specific engagement of BTN3A2, but not by BTN3A1 triggering. Thus, we provide new insights into CD277 costimulatory pathway that may differentially participate in the regulation of various cell-mediated immune responses.

Introduction

The human butyrophilin (BTN) 3 (also known as CD277) molecules belong to the B7 family members and are expressed in various immune cells such as T cells and NK cells [1]. The molecules comprise three structurally related members, BTN3A1, BTN3A2 and BTN3A3 [2, 3]. Structurally, the butyrophilins are composed of an extracellular IgV-like domain, followed by an IgC-like domain and a heptad repeated sequence [2-7]. Some BTNs harbor an intracellular domain of 166 amino acids, named B30.2, presumably involved in intracellular signal transduction, notably the BTN implied in the regulation of superoxide concentrations [8, 9]. BTN3A1, 3A2 and 3A3 exhibit 95% identity and form a mono-phylogenetic group along with the B7/BTN-related members [1]. However, only BTN3A1 and BTN3A3 display the B30.2 signal transducing domain, whereas the BTN3A2 is a decoy receptor that does not contain any intracellular domain.

High expression levels of BTN3 transcripts could be found in human lymphoid tissues, mainly spleen, lymph node (LN) and peripheral blood lymphocytes (PBLs) [1]. Using anti-CD277 monoclonal antibody, it was also demonstrated that BTN3A was expressed on most of immune cells, including T and B lymphocytes, but also NK cells, monocytes, dendritic cells, as well as hematopoietic precursors and some tumor cell lines [1]. Research on the counter-receptor of BTN3A showed that neither CD28, CTLA-4, ICOS, PD-1 nor BTLA were involved, and, except from some (but not all) acute T leukemia cell lines, was absent from both resting and activated T cells.

Similar experiments were performed with BTN2A and showed that BTN2A mRNA was expressed in most human tissues, but protein expression was significantly lower in leukocytes. These experiments also revealed that a particular glycosylated-form of BTN2A1 binds a lectin molecule, DC-SIGN, found on dendritic cells (DCs), confirming the

involvement of the butyrophilin family as co-regulators of the immune system [10]. Furthermore, the binding of human BTN2A1 to DC-SIGN was also dependent on heavy glycosylation of the receptor when expressed by tumor cells. In two recent studies, the recombinant murine BTNL2 protein bound an unidentified receptor on B cells and T cells [11], distinct from the known receptors of the B7 molecules [12]. Both groups demonstrated that the activation of mouse T cells, through TCR engagement, was inhibited by the ligation of BTNL2 with its putative receptor on T cells. Recently, a report proposed that BTN3A1 is an additional coinhibitor receptor of T-cell activation [13]. However, the expression of BTN3A1 on lymphocytes as well as on NK cells prompted us to investigate whether BTN3A1 was involved in the regulation of innate effectors (NK) as well as T lymphocytes ant to explore the potential role of BTN3 (CD277) on the regulation of T lymphocyte and NK activation. Our results show that CD277-triggering in $CD4^+$ T cells considerably enhances TCR-induced signaling, cytokine production and $CD4^+$ T-cell proliferation. In contrast, CD277 triggering is not involved in CD16 or NKp46-induced NK-cell activation. The differential behaviour of CD277 in these two immune cell types prompt us to investigate the relative expression of the different BTN3 isoforms in both T cells and NK cells.

Results

The CD277 co-engagement up-regulates TCR-induced cytokine production, but not NK activating receptor-induced NK cell functions.

To identify possible differences at the protein level, the detection of CD277 surface expression was performed on several T and NK differentiation subsets from healthy donors (n = 4). Using multi-parametric flow cytometry, $CD3^+$ $CD4^+$, $CD3^+$ $CD8^+$ and NK cell populations were analyzed (see online supplemental informations, Fig. S1 & S2). The CD277 mAb staining with the CD277 mAb reveals a strong expression of CD277 in all cell types $CD4^+$ helper T cells, cytolytic $CD8^+$ T cells and NK cells (Fig. S1*B* & S2*B*). Taken together, CD277 is expressed on all subtypes of T cells in the peripheral blood as well as in lymph nodes and also in NK cells, and its expression is not modulated after cell activation (Fig. S1 & S2). This relative stability of the CD277 surface expression prompted us to further investigate the potential action of the CD277 engagement in immune cells.

The role of CD277 engagement was investigated on TCR-induced cytokine production. Purified $CD4^+$ T cells from healthy donors were cultured during 24 to 72 h with CD3 + CD28 mAbs or CD3 + CD277 mAbs or CD3 mAb + IgG1 (control condition). After 24 h of culture, IL-2 and IFN-γ production by $CD4^+$ T cells were measured by ELISA. As expected, these two cytokines were secreted in large amounts after CD3 + CD28 stimulation by comparison with the control condition (Fig. 1*A*: IL-2, 120 pg/ml, $p = 0.0079$, Fig. 1*B*: IFN-γ, 7000 pg/ml, $p = 0.0317$). Although the IL-2 levels produced by the CD3 + CD277 co-activated $CD4^+$ T cells were lower than the IL-2 levels obtained with CD3 + CD28 co-stimulation, the quantity of IL-2 induced by CD3 + CD277 co-activation was significantly higher than that induced with the IgG1 control (Fig. 1*A*: IL-2, 40 pg/ml, $p = 0.0159$). Moreover, IFN-γ secretion was strongly

enhanced by CD3 + CD277 co-activation (Fig. 1*B*: IFN-γ, 9000 pg/ml, *p = 0.0159*) compared to the control situation, and surprisingly, the production was even greater than that obtained after CD3 + CD28 co-activation. A similar effect was obtained regarding the expression profile of the activation marker CD25 under CD3 + CD277 co-stimulation (Fig. 1*C*). Altogether, these results suggest that the CD277 molecule acts as a T cell costimulatory molecule for cytokine production.

To investigate whether similar costimulatory effects are obtained in NK cells, CD107 expression under P815 redirected cytotoxicity (Fig. 1*D*) and IFN-γ assays (Fig. 1*E*) were performed. The NK cells are stimulated via two different activation receptors, CD16 or NKp46 using specific mAbs, in presence of isotypic control, CD277 mAb, anti-DNAM (positive control for a co-stimulation of the activation receptors) or anti-NKG2A (positive control for a co-inhibition of the activation receptors). The CD277 triggering alone did not induce any effect on NK cell stimulation. Moreover, at the contrary of DNAM (co-stimulation) or NKG2A (co-inhibition), the CD277 engagement fails to modulate CD16 or NKp46-induced NK cell activation, both for degranulation as evaluated by CD107 a&b staining and IFN-γ secretion. These results show that CD277 is not involved in the regulation of NK cell activation, contrary to what was observed with T cells.

The CD277 co-engagement up-regulates TCR-induced early signaling events in CD4$^+$ T cells.

These BTN3/CD277-mediated positive signals shown in T cell cytokine production (Fig. 1*A&B*) are not in accordance with a previous work where another CD277 mAb clone has been used [13]. To further test the robustness of our results, we investigate the capacity of the CD277 triggering to regulate TCR-induced early T cell events such as signaling pathways. The TCR engagement encodes intracellular signals implicated in the initiation of general

signaling pathways such as PI3K/Akt and MAP kinases (ERK-1/2) involved in T cell functions including cytokine production, proliferation and regulation of apoptosis [14]. To investigate the co-stimulatory role of CD277 in T cell signaling, CD3 mAb versus CD3 + CD277 mAbs coated beads were used to stimulate $CD4^+$ T cells and phosphoflow analysis was performed. $CD4^+$ T cells were stimulated with mAbs coated beads for various periods of time (Fig. 2). An induction of Akt and ERK-1/2 phosphorylation using CD3 mAb coated beads was detected specially at late time points such as 30 min (Fig. 2*A*&*B*). These TCR-induced phosphorylation events were enhanced when a combination of CD3 plus CD277 mAbs were used. Moreover, this CD277 costimulation revealed phosphorylation events as early as 2 min after stimulation (Fig. 2*B*). These results show that CD277 stimulation is involved in the regulation of T cell signaling induced by the TCR-CD3 complex.

As the CD28 molecule is known to be a potent costimulator of TCR-induced signaling events in primary T cells [15], the role of CD277 was analyzed in the modulation of an optimal (CD3 + CD28)-induced T cell stimulation. Purified $CD4^+$ T cells were stimulated with various concentrations of mAbs against CD277 (from 5 to 17 μg/ml) or isotype control IgG1, together with anti-CD3 plus anti-CD28 for different periods of time (2, 5, 10 and 30 min) (Fig. 2). The CD277 cross-linking strongly increases the phosphorylation of Akt and ERK-1/2 induced by CD3 + CD28 stimulation (Fig. 3*A*&*B*). This effect was dose and time dependent (Fig. 3*B*).

Hence, we thus demonstrated that CD277 triggering potentialize the TCR signal as expected for a costimulatory signal and that it further enhanced the cosignals provided by CD28. We next decided to investigate the functional consequences of the activation of these signaling pathways.

The CD277 engagement further enhances CD3+CD28 costimulation to enhance T cell proliferation and cytokine production.

To investigate the CD277 cosignaling effects on T cell proliferation and cytokine production induced by CD3 + CD28 costimulation, $CD4^+$ T cells were stimulated with various concentrations of CD277 mAb (from 5 to 17 μg/ml), together with CD3 plus CD28 mAbs (Fig. 4). The amount of mAbs able to bind on the beads stays equal along the stimulation conditions by adding IgG1 isotype control and anti-MHC class I (MHC I). The proliferation was evaluated by measuring the dilution of CFSE cytosolic dye in stimulated $CD4^+$ T cells (Fig. 4*A*). The CD277 cross-linking on $CD4^+$ T cells strongly activates $CD4^+$ T cell proliferation mediated by CD3 plus CD28 mAbs in a dose-dependent manner. Among the CD3+CD28 stimulated, only 60% of these cells are divided at day 5 (Fig. 4*C*). The CD277 mAb cross-linking strongly enhances $CD4^+$ T cell division already induced by CD3 plus CD28 mAbs in a dose-dependent manner, such as 90% of cells are divided (Fig. 4*C*). In parallel, our results also showed that the engagement of CD277 increased the proliferation (Fig. 4*B*) and the secretion of cytokines induced by CD3 + CD28 stimulation in a dose-dependent manner (Fig. 4*D*). Altogether, these data support a role of costimulatory molecule for CD277 even after optimal costimulation provided by CD28.

CD277 transcripts are differentially expressed in T and NK cells.

The CD277 molecule is expressed in both T and NK cells [1, 13] (Fig. S1&S2). CD277 has three isoforms *btn3a1, btn3a2 and btn3a3,* with (*btn3a1 and btn3a3),* or without (*btn3a2)* the B30.2 domain in their cytoplasmic part [5] (Fig. 5*A*). The used mAb (clone 20.1) does not discriminate between the Ig domains of the three *btn3a* isoforms, which share a very high level of identity (>95%). Moreover, the CD277 mAb recognizes in a similar manner all the

different isoforms expressed in an ectopic cellular model (Fig. 5*B*). Quantitative PCRs were performed to determine the different relative levels of mRNA expression for each isoform in T and NK cells isolated from human PBMCs (Fig. 5*C*). Both *btn3a1* and *btn3a2* represented the main forms expressed by $CD4^+$ and $CD8^+$ T-cell subsets whereas the decoy form, *btn3a2* was the unique form strongly expressed on NK cells (Fig. 5*C&D*). *Btn3a3* is poorly expressed in these immune cells. These results are further confirmed using available data from GEO omnibus (data not shown).

The two CD277 isoforms, BTN3A1 and BTN3A2 regulate differentially the NKp30-induced IFN-γ production in a NK cell line.

To identify a role for these two major CD277 isoforms (Fig. 5*D*), the KGHYG-1 NK cell line has been nucleofected with constructs encoding for FLAG epitope tagged-BTN3A1 or BTN3A2. This cell line is expressing the Natural Cytotixicity Receptor, NKp30 and a stimulation of this receptor by specific antibodies is able to induce an IFN-γ production in this NK cell line (data not shown). The overexpression of the BTN3 isoforms is monitored by an anti-FLAG mAb cell surface staining (Fig. 6*A*). The transiently transfected NK cells were stimulated by anti-NKp30 and/or anti-FLAG mAbs, and the IFN-γ production is assessed by FACS analysis (Fig. 6*B*). The NKp30 stimulation, but not BTN3A1 or BTN3A2 triggering alone, induces IFN-γ production. However, a co-engagement of NKp30 with a CD277 isoform, modulates the NKp30-induced IFN-γ production. BTN3A1 stimulation seems to increase this cytokine production, whereas BTN3A2 stimulation decreases the NKp30-induced IFN-γ production. These results suggest a differential functional role of these two CD277 isoforms in NK cells.

Discussion

In this study, we describe differential effects of the CD277 molecule as a co-regulator of the immune signal in T cells but not in NK cells (Fig. 1). There is no effect noted on NK cells consistent with the selective expression of the BTN3A2 isoform that lacks much of the cytosolic domain (Fig. 5). However, in the context where only the BTN3A2 isoform is co-engaged, this molecule could induce some negative signals in NK cells (Fig. 6).

CD277 cross-linking elicits a robust costimulation of T cell proliferation, cytokine production and CD25 expression. We showed that the stimulation of BTN3/CD277 proteins with a home-made mAb (clone 20.1, [1]) increases in a dose-dependent manner, the rates of early and late T-cell activation events induced by a combination of CD3 plus CD28 mAbs (Fig. 3 & 4). The experiments were performed using both mAbs coated on microplates or on epoxy beads together with CD3 +/- CD28 mAbs. This protocol has been calibrated in our hands to be very efficient for analyzing costimulation and coinhibition properties. For instance, we reported a strong coinhibition function of PD-1/CD279 and BTLA/CD272 molecules in $CD4^+$ human T cells via similar experiments [16]. To exclude the possible artifact that the CD277 mAbs are acting as adhesion molecules, facilitating T cell -aAPCs (mAb-coated beads) interactions, anti-MHC class I mAbs have been used as control (Fig. 4*C*) showing that CD277-mediated T cell division enhancement is not due to a simple adhesion process.

A negative regulation of T-cell activation using another mAb against BTN3 proteins (clone 232-5), has been reported [13]. Both mAbs (20.1 and 232-5) recognize overlapping but not identical epitope of BTN3 molecule and belong to the different murine IgG classes [13]. While 20.1 exhibits an equal binding to the three BTN3 isoforms (Fig. 5*B*), recognition of BTN3A1 and BTN3A2 by 232-5 mAb is not known. An additonal difference might stand at the level of cross-linking of the receptors. Here, most of our experiments were performed

using CD277 mAbs coated on beads together with CD3 +/-CD28 mAbs. These bead-based aAPCs enable the most efficient reported growth of human $CD4^+$ T cells and permit to develop a useful tool to monitor the receptor signaling pathways for T cell activation [17]. Slightly different conditions used by Yamashiro et al. [13] might be less optimal to provide costimulation. Moreover, CD277 has been recently reported to be a cosignaling molecule in another immune cell type, the dendritic cells, by using the CD277 mAb (clone 20.1) [18].

Recently, CD277 expression at the surface of aAPCs (K32 cell line) has been reported to induce an impaired TCR-induced cell proliferation, suggesting that a counter-receptor at the T cell surface will act as an inhibitory receptor [19]. Altogether, the identification of the putative BTN3 ligand(s) will help to further investigate the biology of the CD277 molecule in the immune system. Using BTN3A1-Fc fusion proteins, we found that a BTN3 ligand is expressed on various tumor cell lines and endothelial cells [1]. However, we do not know if the BTN3A1-Fc protein binds one or multiple ligands that might upon BTN3 binding elicit distinct signals.

In order to understand the differences observed in our study between T cells and NK cells, we compared the mRNA isoforms of *btn3* expressed by T cells and NK cells. We found that *btn3a1* is the main form expressed by T cells whereas the decoy form, btn3a2 is mostly expressed on NK cells (Fig. 5). This result can explain the absence of co-stimulation in response to CD277 stimulation of NK cells. The three genes are expressed in most tissues including cancer cells (http://ist.genesapiens.org) indicating that numerous subsets of cells that might be regulated by CD277. Surprisingly, when we compared the expression on immune cells, most expressed the three isoforms except NK cells in which the btn3a2 isoform was almost exclusively detected (Fig. 5 and data not shown). The three CD277 isoforms share more than 95% identity in their extra-cellular domain but differ dramatically in the sequence of their cytoplasmic domain. The intracytoplasmic domains of BTN3A1, BTN3A2

and BTN3A3 correspond to 242, 65 and 315 amino acid respectively. BTN3A1 and BTN3A3 possess a B30.2 (or PRY/SPRY) domain, a module that mediates diverse functions in at least 11 categories of human molecules/receptors by binding to targets through an interface resembling that of an antibody [9]. The presence of a B30.2 domain on the tripartite motif (TRIM) proteins, including TRIM5α, is important for the antiviral activity of these proteins [20]. By contrast, the B30.2 domain is not present in the BTN3A2 isoform. Based on our data obtained in NK cells (Fig. 1), BTN3A2 could be a putative decoy receptor, devoid of cosignaling function in NK cells, when compared to two well know costimulatory (DNAM-1) and coinhibitory (NKG2A) molecules. However, when NKp30 is co-engaged with BTN3A2 (but not the other isoforms), BTN3A2 is able to induce some negative signals in NK cells (Fig. 6). The cytoplasmic part of BTN3A2 contains 65 amino acids, but no identified signaling motif is found in this peptide sequence. For BTN3A1, it is possible to investigate intracellular signalling as the cytoplasmic part of BTN3A1 contains a B30.2 domain. Some intracellular proteins have been described to interact with the B30.2 domain of a BTN family member, such as the xanthine oxidoreductase that binds to the B30.2 domain of BTN1A1. These interactions are involved in the BTN1A1 functions in the mammary gland and it has been speculated that these interactions could occur in immune cells [21]. Actually, the potential partners of the B30.2 domain of BTN3A1 and/or BTN3A3 are still unknown. The identification of these B30.2 interactors will be necessary to dissect the immunoregulatory mechanims associated with the engagement of BTN3/CD277 molecule at the surface of T cells versus NK cells.

Smith et al. demonstrated that BTN1A1 and BTN2A2 –Fc fusion proteins bound to activated T cells [22]. Immobilized BTN1A1 and BTN2A2 –Fc fusion proteins inhibit the proliferation of murine $CD4^+$ and $CD8^+$ T cells activated by CD3 mAbs. Hence, they bind to ligands that are involved in the regulation of the threshold of T cell activation. Consequently these

molecules should act as ligand for receptors(s) present on activated T cells that will regulate their function. In addition to our results, there is a growing body of literature on these BTN family members that suggests that when the BTN counter-receptors are discovered, they may constitute a huge immunoregulatory network such as the CD28/B7 family. These pathways are likely to be major receptors in immune responses and also the inflammatory reaction.

In conclusion, CD277/BTN3 proteins should be also considered as positive immunomodulators in T-cell responses. An elegant mechanism to directly modulate these effects for an immune cell would be to differentially regulate the expression of the BTN3 isoforms. Altogether, these results are the basis of new investigations on defining the nature of extracellular and intracellular ligands of the CD277 molecule.

Materials and Methods

Human T cell isolation

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteer donors provided by the "Etablissement Français du Sang" (EFS, Marseilles, France) and isolated by fractionation over a density gradient of Lymphoprep© (Abcys). Human $CD4^+$ T cells were negatively selected from isolated PBMCs by depletion of non-$CD4^+$ T cells with magnetic beads using the T cell isolation kit II from Miltenyi Biotec®. Isolated CD4 cells were used for further experiments when purity was superior to 90%.

Expression profile of CD277 on T cells subpopulations

PBMCs from healthy donors were stained with 5 μl of the following mouse anti-human mAbs per million of cells: ECD-conjugated anti-CD3, PC5-conjugated anti-CD14, PC5-conjugated anti-CD19 (to select $CD3^+$ $CD14^-$ $CD19^-$ cells) (all from Beckman Coulter, Marseilles, France), Pacific Blue-conjugated anti-CD4, Alexa700-conjugated anti-CD8 (all from BD

Pharmingen, San Diego, CA, USA), APC-Alexa750-conjugated anti-CD27 (Caltag, Invitrogen, USA), PC7-conjugated anti-CD45RA (BD Biosciences), Alexa647-conjugated anti-CD277 (clone 20.1, IgG1) [1]. The CD277 mAb (clone 20.1) was labeled with Alexa Fluor 647 using a commercial kit (Invitrogen, Paisley, UK). APC-conjugated IgG1 (Beckman Coulter) was used as negative control and LIVE/DEAD Fixable Dead Cell Stain Kit was used for viability. Cells were incubated 20 min at 4°C, then washed twice in PBS fixed with 2% paraformaldehyde, and analyzed by a FACSAria flow cytometer (BD Biosciences). Data were analyzed using FlowJo Software (TreeStar, Ashland, OR).

Kinetics of CD277's expression profile on naive CD4$^+$ T cells

Purified CD4$^+$ T cells ($2 \cdot 10^5$ cells/well) from thawed human PBMCs were cultured during 96 h in RPMI 1640 10% FBS in flat bottom 96-well plates (Microtest^{TM} 96, Becton Dickinson) which have been previously incubated with CD3 mAb (clone OKT3) plus CD28 mAb (clone CD28.2) [23] or isotypic control (IgG1). Anti-CD3 (and anti-CD28 mAbs were used at 0.3 μg/ml and 10 μg/ml respectively. Cells were placed into an atmosphere of 5% CO_2 at 37°C in a humidified incubator. Every 24 h, cells were transferred in a conic bottom 96-well plate (Nunc^{TM}, Denmark) and stained for 30 min at 4°C with 3 μl of purified anti-PD-1 (clone PD-1.3.1) [24] , washed 3 times in PBS- FBS 0.2%- NaN3 0.02%, then stained with PE-conjugated goat anti-mouse (1/80, Beckman Coulter), washed and stained with 3 μl of each of PC7-conjugated anti-CD4, FITC-conjugated anti-CD3 (all from BD Biosciences) Alexa647-conjugated anti-CD277 and 6 μl of 7-AAD (BD Biosciences) for 30 min at 4°C. Purified IgG1 and APC-conjugated IgG1 were used as controls. Immunostained cell samples fixed with 2% paraformaldehyde were analyzed on a BD FACS Canto (BD Biosciences). Data were analyzed using FlowJo Software (TreeStar, Ashland, OR).

Expression of CD277 in lymph nodes

Mononuclear cells were obtained from lymph nodes by crushing fresh tissue samples in RPMI 1640 10% FBS. Detection of Follicular T Helper (TF_H) cells was performed by incubation for 20 min at 4°C with PE-conjugated anti–ICOS, biotinylated anti–CXCR5 (all from BD Biosciences), PC5-conjugated anti-CD14, Pacific blue-conjugated anti–CD4 (all from Beckman Coulter) and LIVE/DEAD Fixable Dead Cell Stain Kit©. Cells were then washed in PBS and incubated with anti–biotin–allophycocyanin–Alexa Fluor 750 (Invitrogen, Carlsbad, CA) for 20 min at 4°C. After staining, each cell preparation was washed twice in PBS, fixed with 2% paraformaldehyde, BD FACS Canto (BD Biosciences). Data were analyzed using FlowJo Software (TreeStar, Ashland, OR).

Functional assays on CD4+ T cells using plate-immobilized mAbs

Purified $CD4^+$ T cells ($2 \cdot 10^5$ cells/well) from thawed human PBMCs were cultured in RPMI 1640 10% FBS in flat bottom 96-well plates (Microtest™ 96, BD Biosciences), which had been previously incubated with mouse anti-human CD3 (clone OKT3)/CD28 (clone CD28.2) [23] or anti-CD3/anti-CD277 (clone 20.1) or anti-CD3/isotypic control (IgG1). Purified anti-CD3 was used at 0.3 μg/ml. Anti-CD28, anti-CD277 and isotypic control were used at 10 μg/ml. Cells were placed into an atmosphere of 5% CO_2 at 37°C in a humidified incubator. After 2 days of culture, cytokine production (IL-2 and IFN-γ) was measured by ELISA assay according to the manufacturer's protocol (OptEIA, human IFN-γ or IL-2 Set, BD Pharmingen). After 5 days, cells were stained with 3 μl of PE-conjugated anti-CD25 (BD Biosciences), and 5 μl of 7-AAD for 30 min at 4°C then washed twice in PBS, fixed with 2% paraformaldehyde and analyzed on a BD FACS Canto (BD Biosciences). Data were analyzed using FlowJo Software (TreeStar, Ashland, OR).

Functional assay on CD4+ T cells with artificial APCs (aAPCs) and *carboxyfluorescein diacetate succinimidyl diester* (CFSE) labeling

Human CD4+ T cells were purified by negative selection from PBMCs using magnetic beads (Miltenyi Biotec) according to the manufacturer's protocol. CD4 T cells were routinely more than 97% pureCD4+ T cells were labeled with 0.5 μM CFSE (Invitrogen) for 10 min at 37°C, washed and stimulated ($1.5 \cdot 10^5$ cells/well) with aAPCs at a ratio of 1:1 (cells to beads) in triplicate in 96-well round-bottom plates (Falcon; BD Biosciences). As described previously [16], magnetic beads (Dynabeads M-450 Epoxy, Dynal Biotech) were coated with the following mAbs : anti-CD3 (clone OKT3), anti-CD28 (clone CD28.2), and/or various concentrations of anti-CD277 (clone 20.1) or anti-MHC class I (MHC I) (clone YJ4) or IgG1 control. These aAPCs were coated with suboptimal CD3 mAb (5%), suboptimal levels of CD28 mAb (10%), and either IgG1 Ab (CD3/CD28/IgG1), CD277 mAb (CD3/CD28/CD277 + IgG1) or anti-MHC class I (CD3/28/MHC I + IgG1), constituting the remaining 85% of protein added to the bead. The amount of protein was kept constant at 20 μg/ml by the addition of control IgG1. Cultures were incubated at 37°C, 5% CO_2 for 5 days and then proliferation of CFSE labeled $CD4^+$ T cells were measured by flow cytometry (FACS Canto, Beckman Coulter).

Functional assay on NK cells, cytolytic activity

Fresh NK cells were sorted with Easy Sep® negative selection kit and incubated over night in medium completed with sub-optimal concentrations of IL-2 (100 U/ml) and IL-15 (10 ng/ml). NK cell receptors functions were tested in re-directed cytolytic experiments against the FcRγ-positive P815 mastocytoma murine cell line. Briefly, effector cells were incubated with P815 cells pre-coated for 30 minutes with the mAb of interest (irrelevant mouse IgG1: 11 μg/ml, anti-NKp46 (clone BAB281, Beckman Coulter): 1 μg/ml, anti-DNAM (clone DX11, BD

Biosciences): 5 μg/ml, anti-NKG2A (clone 131411, R&D Systems): 5 μg/ml, anti-CD277 20.1: 10 μg/ml) according to a 1:1 effector:target (E/T) ratio. Similar stimulation conditions have been used with the CD16 mAb (clone 3G8, BD Biosciences). Cytotoxic tests were performed in 4-hours assays in the presence of GolgiStop® and soluble FITC-labeled CD107 (a&b) mAbs (both from BD Biosciences), then the cells were stained for surface markers (PeCy7-CD56 (Beckman Coulter, Immunotech), fixed and permeabilized (Cytofix/Cytoperm®) then stained with anti- IFN-γ mAb (Beckman Coulter, Immunotech). Cells were finally re-suspended in PBS 2% para-formaldehyde and extemporaneously analyzed on a BD FACS Canto® (BD Biosciences, San Jose, CA). The degree of activation of NK cells was measured based on the percentage of cells positive for CD107 a&b (degranulation) and/or the production of inflammatory cytokine (IFN-γ).

Measurements of cytokine production

To determine the production of cytokines, cell-free supernatants were collected at 48 h and assayed for IL-2 and IFN-γ by ELISA using OptEIA kits (BD Pharmingen) according to the manufacturer's instructions. After 8 h of transfection, KGHYG-1 cells were incubated with plate-bound mAbs in a 96-well plate. For NKp30 and/or CD277 isoform stimulation, anti-FLAG mAb was preadsorbed at 4 μg/100 μl/well and anti-NKp30 mAb at 1 μg/100 μl/well. Upon 4 h of stimulation, intracellular IFN-γ stainings are performed with a PE-labelled specific Ab (Beckman Coulter).

Plasmid contructs and cell line tranfections

The construct p3XFlagBTN3A1 (BTN3A1) corresponding to the wild-type full-length human BTN3A1 cDNA deleted from its signal peptide sequence and tagged with 3x Flag epitope in the 5' end, was generated by subcloning of pCR-BluntII-TOPO vector containing BTN3A1

(cDNA clone IRCMp5012H1242D, Source BioScience LifeSciences, Nottingham, UK) into the p3xFLAG-myc-CMV-25™ vector (SIGMA Life Science), using the restriction sites EcoRI/XbaI. The construct p3XFlagBTN3A2 (BTN3A2) corresponding to the wild-type full-length human BTN3A2 cDNA deleted from its signal peptide sequence and tagged with 3x FLAG epitope in the 5' end, was generated by subcloning of pOBT7 vector containing BTN3A2 (cDNA clone IRAUp969E0222D, Source BioScience LifeSciences, Nottingham, UK) into the p3xFLAG-myc-CMV-25™ vector, using the restriction sites EcoRI/XbaI. The construct p3XFlagBTN3A3 (BTN3A3) corresponding to the wild-type full-length human BTN3A3 cDNA deleted from its signal peptide sequence and tagged with 3x FLAG epitope in the 5' end, was generated by subcloning of pOTB7 vector containing BTN3A1 (cDNA clone IRAUp969E1250D, Source BioScience LifeSciences, Nottingham, UK) into the p3xFLAG-myc-CMV-25™ vector, using the restriction sites EcoRI/XbaI. To exclude the Myc tag expression at the carboxy-terminal part of BTN3 isoforms, a stop codon has been introduced at the 3' end of BTN3Ax sequences.

2 x 10^6 COS-7 cells seeded in 100-mm plates were transfected with 5 μg p3xFlagBTN3Ax contructs using 15 μl of FuGENE 6 Transfection Reagent (Roche). The human NK cell line, KHYG-1 is growing in RPMI 1640 medium supplemented with 20% FCS and 450 UI/ml rIL-2 [25]. 5 x 10^6 KHYG-1 cells were transfected with 2 μg p3xFlagBTN3Ax contructs using the Amaxa™ Nucleofector™ Technology (Solution T, program Y-001) (Lonza Cologne AG).

Screening of the different *btn3a* isoforms transcripts in PBMC from healthy subjects. Public and home-made Affymetrix U133+2 data sets of purified CD4, CD8 and, NK cells were collected. CD8 and CD4 data were retrieved from the public GEO datasets [26] (http://www.ncbi.nlm.nih.gov/gds), while NK sets were personal. We used Robust Multichip Average (RMA) with the non-parametric quantile algorithm as normalization parameter.

RMA was applied to the raw data collected from the various series. Quantile normalization and Loess' correction were done in R using Bioconductor and associated packages. The probe set corresponding to the three isoforms of BTN3A was retrieved from the normalized data sets and the corresponding log values were linearized for graphical representation. We used the respective Affymetrix probesets corresponding to BTN3A1, BTN3A2 and BTN3A3 isoforms : STP201623_s_at, 213282_at, 204171_at.

Phosphoflow by FACS analysis

Human $CD4^+$ T cells were purified by negative selection from PBMCs using magnetic beads (Miltenyi Biotec) according to the manufacturer's protocol. $CD4^+$ T cells were routinely more than 97% pure. Cells were incubated 24 h in RPMI 1640 10% FBS at 37°C. $CD4^+$ T cells were washed with PBS 1% FCS and stimulated with aAPC at a ratio of 1:3 (cells to beads) comprised of magnetic beads (Dynabeads M-450 Epoxy, Dynal Biotech) coated with anti-CD3, anti-CD28 and/or anti-CD277 mAbs as described above. The contacts between cells (10^6 in 50 µl) and beads (3 x 10^6 in 30 µl) are performed at 37°C in water bath for different times (2, 5, 10 and 30 min) in PBS 1% FCS.

Phosphoflow analysis was performed by cytometry as previously described [27]. Briefly, cells were fixed and permeabilized, incubated with anti-phospho-Akt S473 (# 4058, Cell Signaling Technology) or anti-phospho-ERK-1/2 T202/Y204 (# 4377, Cell Signaling Technology) antibodies and appropriate biotinylated secondary antibodies. Finally, revelation was performed using Streptavidin-phycoerythrin solution (#IM3325, Beckman Coulter). FACS data were acquired on a FACSCanto flow cytometer (BD Biosciences) using Diva software. FACS data were analyzed using Flowjo software (TreeStar, Ashland, OR).

Statistical analysis

All data were analyzed using GraphPad Prism version 5.00 for (GraphPad, San Diego, CA) and Microsoft Excel (Microsoft Office). The Mann-Whitney test matched nonparametric test was used to examine: the variations of CD277 and PD-1 expression from lymphoid tissue on living T lymphocyte subsets (in Fig. 1, Fig. S1 & S2), the variation of Akt and ERK phosphorylation levels on CD277 stimulated T lymphocyte cells (in Fig. 2-3), and the difference of secretion of cytokines on T cells (in Fig. 1 & 4). The comparisons were made between different conditions of stimulation. The Wilcoxon paired test was used to compare between different conditions of stimulation on NK cells (in Fig. 1). Differences were considered as statistically significant when $p < 0.05$.

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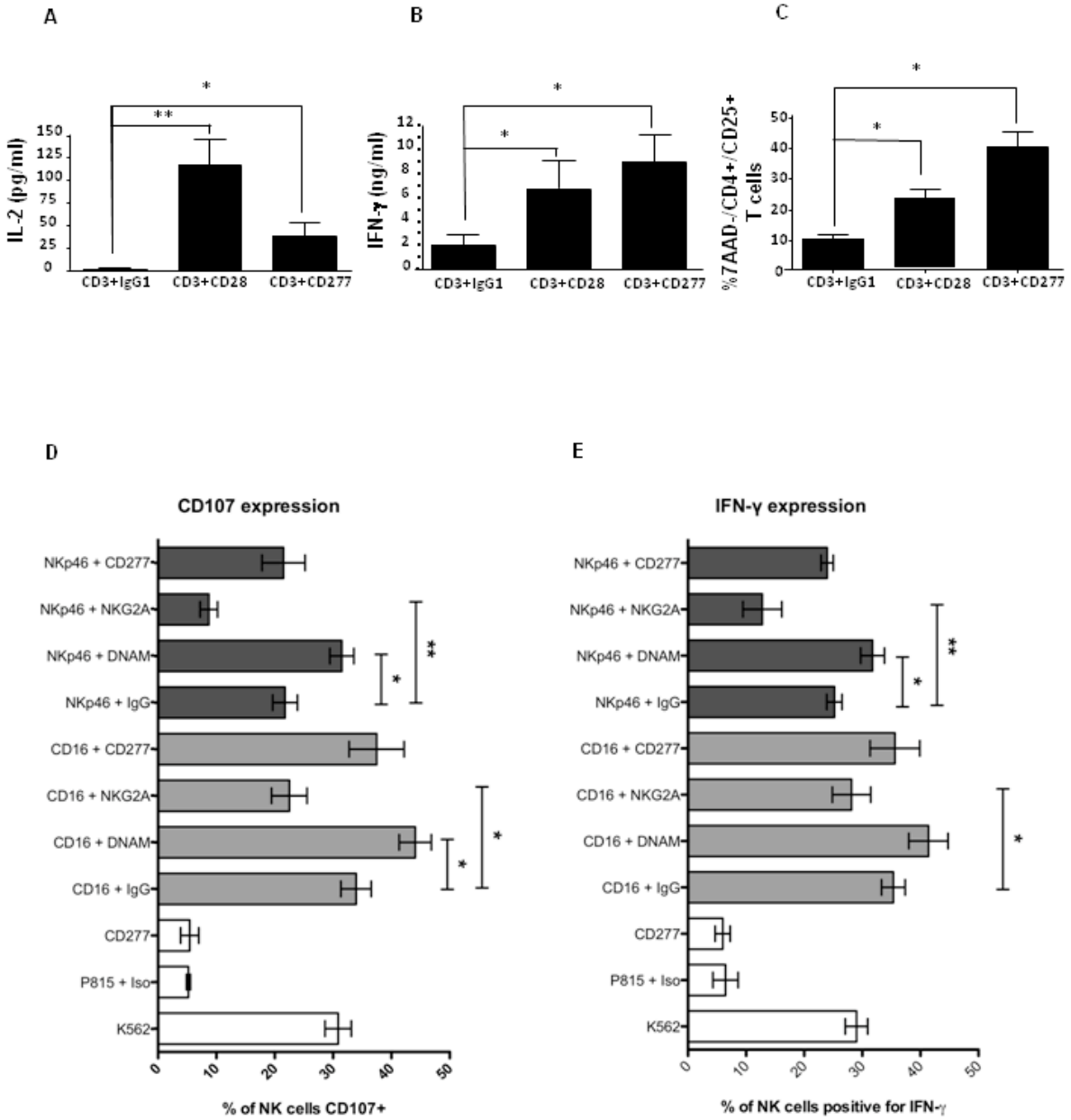
Figure legends

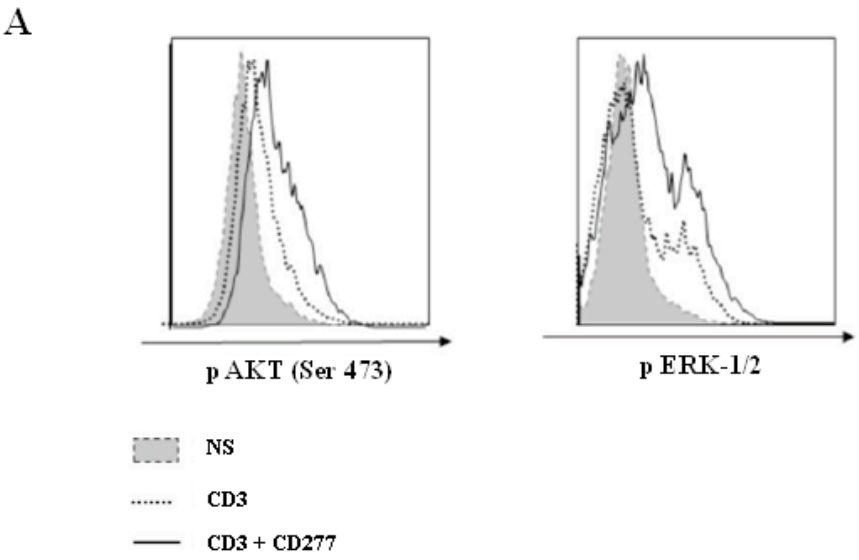
Figure 1. CD277 triggering upregulates cytokine production in T cells but in NK cells.

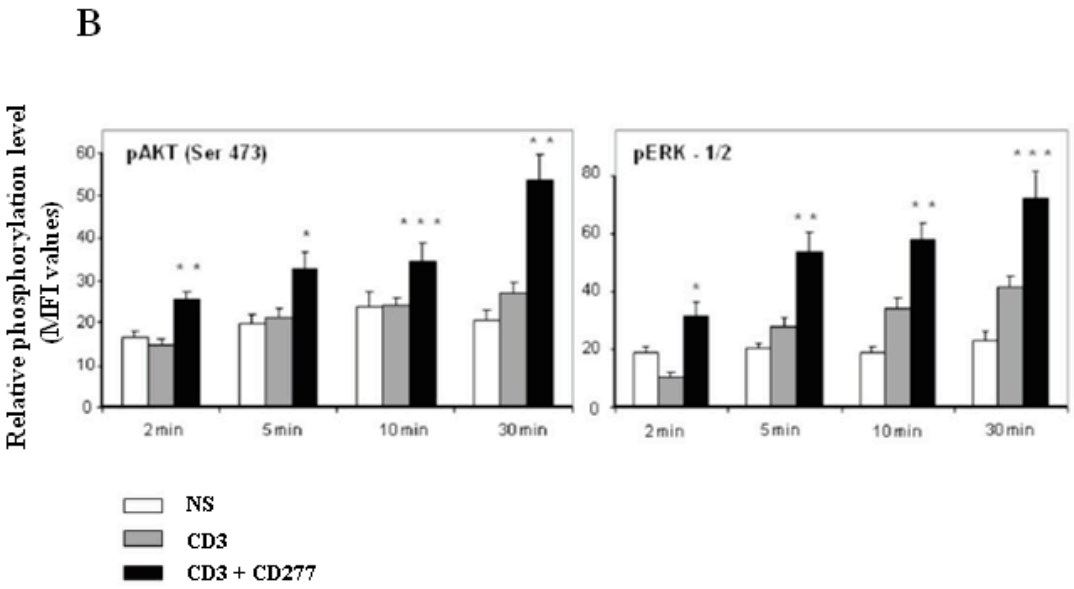


(A and B) $CD4^+$ T cells were stimulated by plate-immobilized anti-CD3 (0.3 μg/ml), together with 10 μg/ml of anti-CD28 or anti-CD277 mAbs, or isotypic control (IgG1). After 48 h of culture, supernatants were collected and (A) IL-2 or (B) IFN-γ levels were analyzed by ELISA. (C) After 72 h, cells were collected and expression of CD25 was analyzed by flow cytometry**.** Data are shown as mean + SEM of 4 donors. * $p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$, Mann-Whitney test. The comparisons were made between CD3+CD28 and CD3+CD277 stimulation conditions or between CD3+CD277 and CD3+isotype control stimulation conditions.

(D and E) NK cells were isolated from the peripheral blood of healthy donors, and maintained in IL-2 + IL-15 overnight. The NK cells were then used in a direct cytotoxic assay against the K562 leukemia cell line (positive control for NK-cell activation). NK cells were also used in a redirected cytotoxic experiment after coculture with the P815 mastocytoma cell line coated with anti-CD277, anti-NKp46 or anti-CD16 mAbs. Co-stimulatory effects were also tested with P815 coated with anti-NKp46 or anti-CD16 mAb plus mouse isotypic controls (IgG1), anti-DNAM (activating co-receptor), anti-NKG2A (inhibitory receptor) or anti-CD277 mAb. Histograms represent (D) the percentage of NK cells positive for CD107 a&b and (E) the percentage of NK cells positive for IFN-γ**.** Data are shown as mean + SEM of 4 donors. The p values were calculated between both stimulation conditions using the the Wilcoxon paired test. * $p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$.

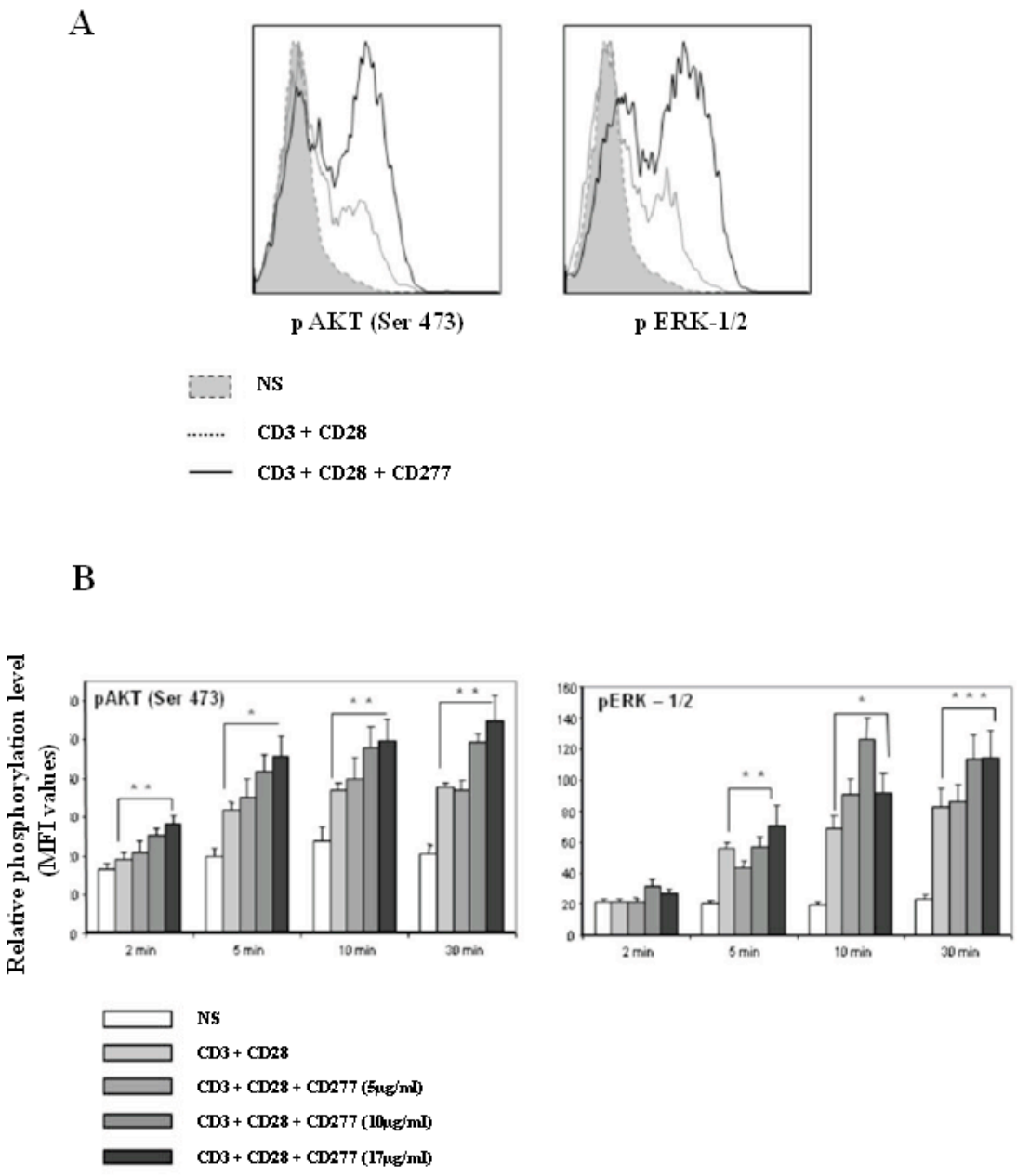
Figure 2. Regulation of AKT and ERK phosphorylation via CD277 in TCR-activated T cells.





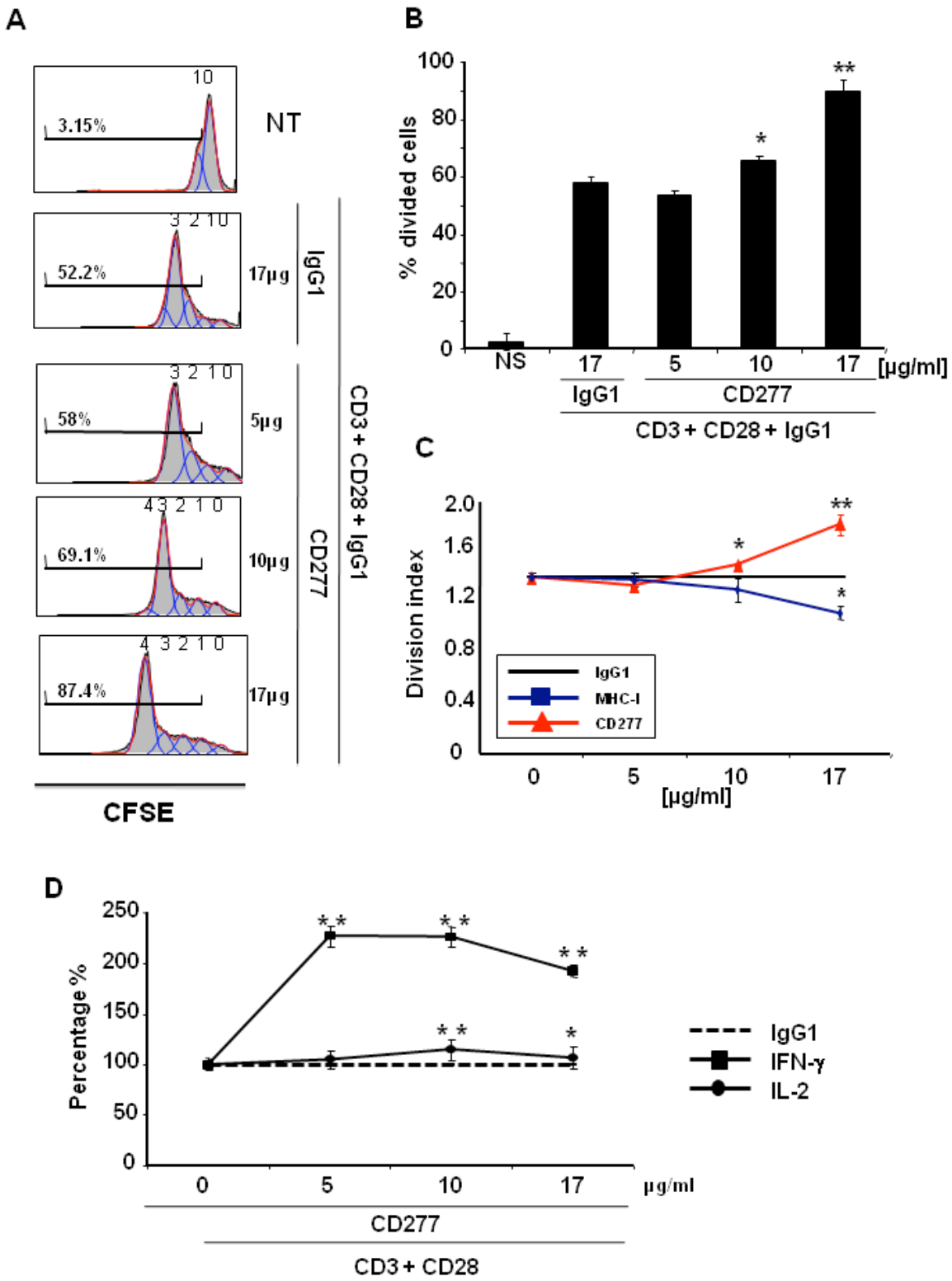
(A) Histograms depict representative AKT and ERK-1/2 phosphorylation in non-stimulated (NS) $CD4^+$ T cells (dotted gray histogram) and $CD4^+$ T cells stimulated for 30 min with antibody-coated Epoxy dynabeads (1μg/ml of anti-CD3 plus 17μg/ml of IgG1 control mAbs, dotted open histogram ; 1μg/ml of anti-CD3 plus 17μg/ml of anti-CD277 mAbs, solid line histogram). (B) Purified $CD4^+$ T cells from healthy donors were stimulated or not with antibody-coated Epoxy dynabeads (1 μg/ml of anti-CD3 plus anti-CD277 mAb or IgG1 (isotype control). Every 2, 5, 10 and 30 min, the intracellular phosphorylation of AKT and ERK-1/2 on $CD4^+$ T cells were detected by flow cytometry and MFI values (mean + SEM) are shown. Four independent studies were performed. The p values were calculated between non-stimulated (NS) T cells and CD3+CD277 stimulated T cells using the Mann-Whitney test. * $p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$.

Figure 3. Regulation of AKT and ERK phosphorylation via CD277 in (TCR+CD28)-activated T cells.



(A) Histograms depict representative AKT and ERK-1/2 phosphorylation in non-stimulated (NS) $CD4^+$ T cells (dotted gray histogram) and $CD4^+$ T cells stimulated for 30 min with antibody-coated Epoxy dynabeads (1 μg/ml of anti-CD3 and 2 μg/ml of anti-CD28 plus 17 μg/ml of IgG1 (isotype control), solid gray line ; 1 μg/ml of anti-CD3 and 2 μg/ml of anti-CD28 plus 17 μg/ml of anti-CD277 mAb, solid black line. (B) Purified $CD4^+$ T cells from thawed PBMCs of 4 healthy donors stimulated or not with antibody-coated Epoxy dynabeads (1 μg/ml of anti-CD3 and 2 μg/ml of anti-CD28 plus various concentrations of anti-CD277 mAb or IgG1 (isotype control). Every 2, 5, 10 and 30 min, the intracellular phosphorylation of AKT and ERK-1/2 on $CD4^+$ T cells was detected by flow cytometry. Results are presented as MFI (mean fluorescence intensity expression) on $CD4^+$ T cells at different time points after treatment from 2 to 30 min and are mean + SEM of N=4 samples. The p values were calculated between both stimulation conditions using the Mann-Whitney test. * $p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$.

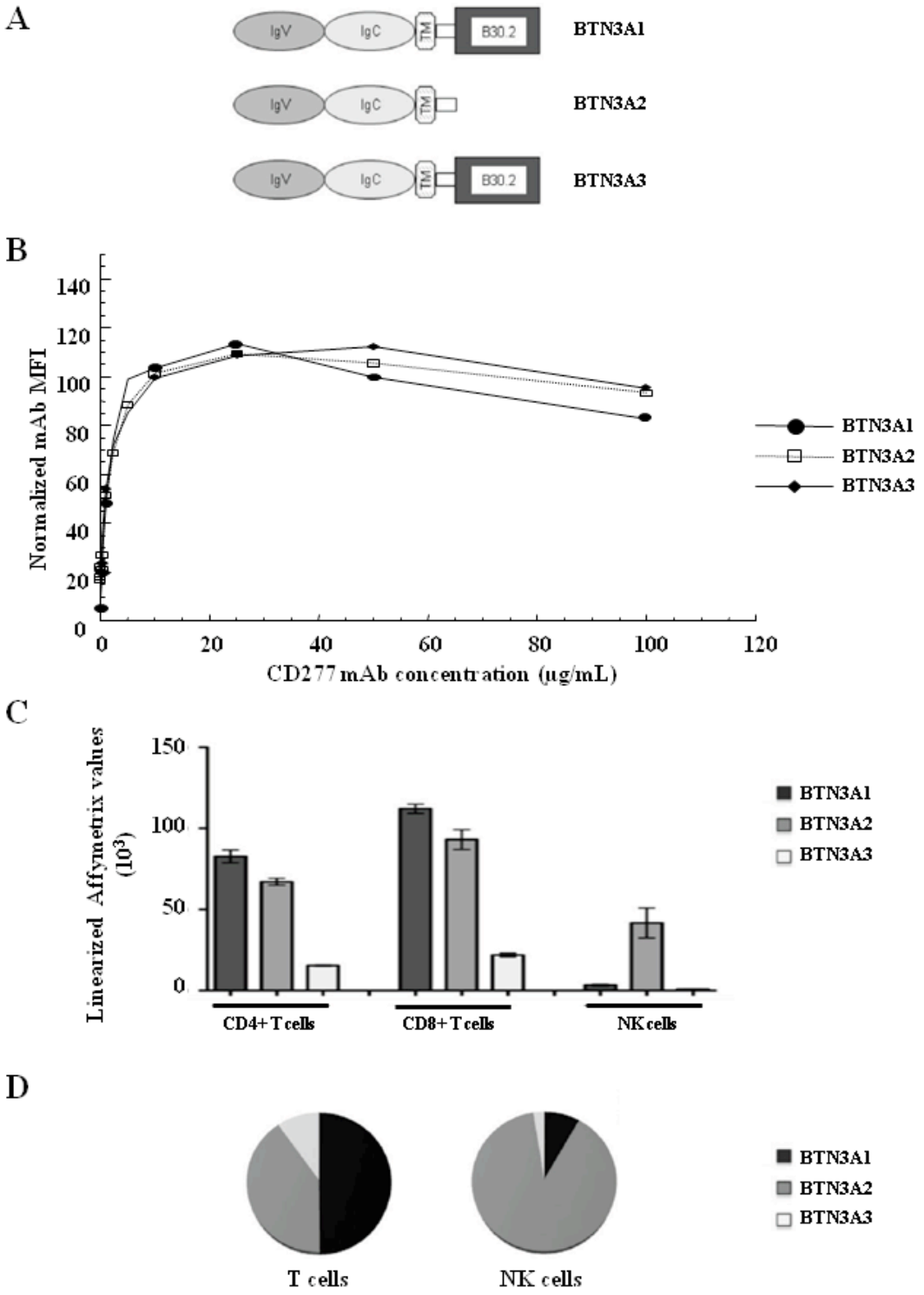
Figure 4. CD277 is a costimulatory molecule for CD4$^+$ T cells.



(A) CD4$^+$ T cells were purified from PBMCs from healthy donors (n=4). CFSE-stained purified CD4$^+$ T cells were stimulated with antibody-coated Epoxy dynabeads (1 μg/ml of

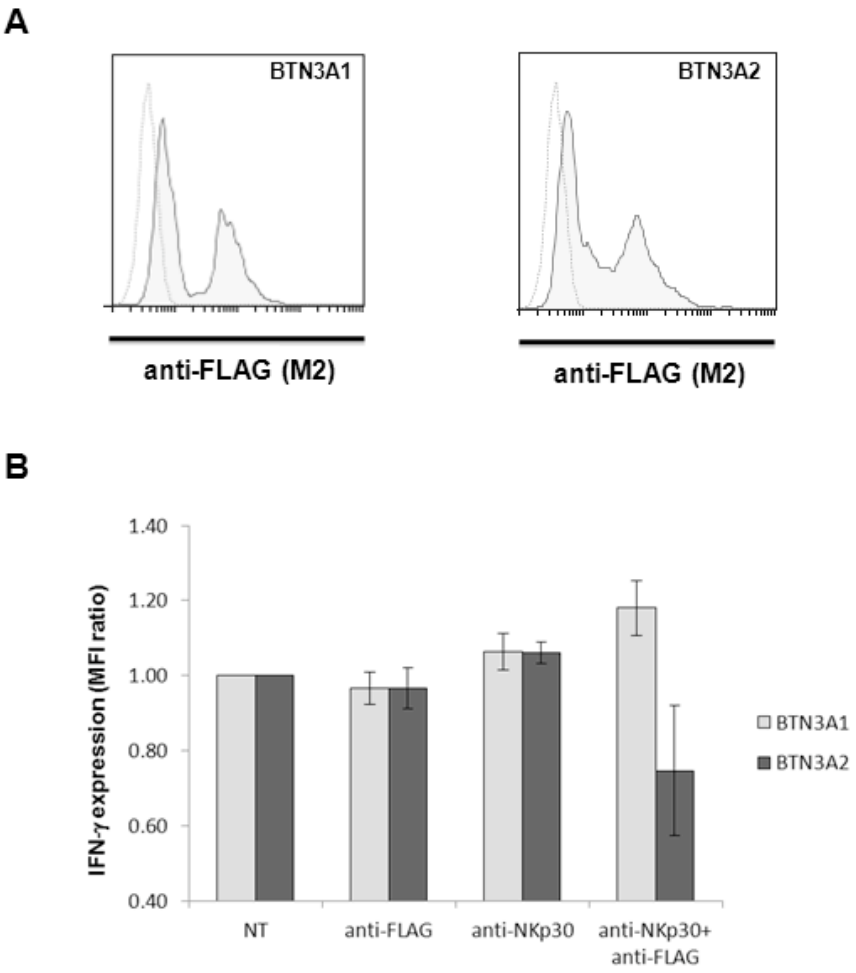
anti-CD3 plus 2 μg/ml of anti-CD28 mAbs and with various concentrations of CD277 mAb or IgG1 (isotype control) for 5 days, and proliferation was analyzed by CFSE dilution. Anti-MHC I (clone YJ4) mAbs were added in a dose-dependent manner in parallel with CD277 mAbs. CFSE-stained T-cell proliferation is shown as (A) the percentage of divided cells as determined on a representative histogram and (B) the percentage of divided cells**.** Data are representative of three independent experiments. (C) The division index is calculated as the ratio between the total number of divisions and the number of cells included into the tissue culture well. Data were analyzed using Flowjo software (TreeStar, Ashland, OR). (D) $CD4^+$ T cells were purified from PBMCs from healthy donors and stimulated with antibody-coated Epoxy dynabeads (1 μg/ml of anti-CD3 plus 2 μg/ml of anti-CD28 mAb plus various concentrations of anti-CD277 mAb or IgG1 (isotype control). Supernatants were collected on day 2 of culture for IFN-γ and IL-2 detection by ELISA. Data shown are mean + SEM of n=4 donors. The p values were calculated between CD3+CD28+IgG1 stimulated T cells and CD3+CD28+CD277 stimulated T cells using the Mann-Whitney test. * $p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$.

Figure 5. The three CD277 isoforms are differentially expressed in NK cell and T cell populations.



(A) Schematic representation of the three different human butyrophilin 3 (BTN3) / CD277 isoforms containing a stretch of IgV and IgC domain in their extracytoplasmic part and a transmembrane domain (TM). Only two isoforms (BTN3A1 and BTN3A3) contain a large intracytoplasmic part encoding for a B30.2 domain. (B) CD277 antibody titration. COS cells were transfected 24 h with each of the three BTN3 isoforms. 2 x 10^5 transfected cells were incubated with increasing amounts of CD277 mAb (0.01 to 100 μg/ml). The stained samples were analysed by flow cytometry and the mean fluorescence intensity (MFI) was determined and normalized with an internal antibody control of transfection. The normalized mAb fluorescence intensity of the positively stained cell populations is plotted against concentration. The binding activity of the CD277 mAb on COS transfected cells is the same for the three isoforms. ((Please indicate the number experiments the data are representative of.)) (C) Quantitative PCRs were performed from purified human NK cells or $CD4^+$/$CD8^+$ T cell subsets. Linerarized Affymetrix values are representated corresponding to the mean value of 4 donors and micro-arrays + SEM. BTNA3A2 is the main isoform expressed by NK cells, while $CD4^+$ and $CD8^+$ T cells also express high level of BTNA3A1. (D) Representative expression of the three CD277 isoforms in NK cells or in T cells (both $CD4^+$ and $CD8^+$ values are used into this condition).

Figure 6. The two CD277 isoforms, BTN3A1 and BTN3A2, differentially regulate NKp30-induced IFN-γ production.



The NK cell line, KGHYG-1 was nucleofected with constructs encoding for FLAG epitope tagged-BTN3A1 (right) or BTN3A2 (left). (A) The overexpression of the BTN3 isoforms was monitored by anti-FLAG mAb cell surface staining. (B) NK cells were stimulated by anti-NKp30 (1 μg/ml, clone 210847) and/or anti-FLAG (4 μg/ml, clone M2) mAbs for 4 h, and IFN-γ production was assessed by flow cytometric analysis. Upon gating the FLAG-tagged positive cells, the MFI of IFN-γ producing cells was measured. The MFI ratio values were calculated using the MFI of IFN-γ staining on non-treated (NT) cells. Data presented are representative of (A) and mean + SEM (B) of 3 independent experiments.

Supplemental information

Expression profile of CD277 on T and NK peripheral cell subsets

It has been shown that T and NK cells are expressing the CD277 molecule [1, 2]. Here, we deeply screen the different T and NK cell subsets for their CD277 surface expression. The detection of CD277 surface expression was performed on several T and NK differentiation subsets from healthy donors (n = 4). Using multi-parametric flow cytometry and based on the differential expression of CD27 and CD45RA molecules, the CD277 expression level on naïve and memory T cell populations was analyzed (Fig. S1*A*). Any significant difference between the different T-cell subsets was detected (Fig. S1*B)*. In parallel, the CD277 expression on the NK cells was monitored by analyzing $CD56^{+}$ $CD3^{-}$ cells (Fig. S2*A*). Thawed alive NK cells (Live dead© negative cells) were selected based on the expression of $CD56^{+}$ $CD3^{-}$ from healthy human PBMCs after 20 min incubation at 4°C with the anti-CD277-Alexa647 (clone 20.1). Cells were further washed twice in PBS (PBS, Lonza), then fixed with 2% formaldehyde and analyzed on a BD FACS Canto (BD Biosciences). Data were analyzed using FlowJo Software (TreeStar, Ashland, OR). Independently of their $CD56^{Bright}$ (helper) or $CD56^{Dim}$ (cytotoxic) phenotype, all NK cells also expressed high level of CD277 (Fig. S2*B*), showing that CD277 molecules are similarly found on the two major circulating subsets of NK cells.

Expression profile of CD277 on CD4 TF_H cells in lymph nodes

Multiple immune T cell populations are found in lymphoid organs where they play specialized functions. Among them we particularly analyzed T follicular helper (TF_H) cells, which are present in the germinal centers and required for proper B cell responses. TF_H cells express the chemokine receptor CXCR5 and high levels of the ICOS molecule [3]. As

previously described [4], the CXCR5^{+} ICOS^{High} TF_H cells obtained from reactive lymph nodes expressed high levels of the programmed death-1 (PD-1) molecule (Fig. S1*D, left panel*). Based on the differential expression of CXCR5 and ICOS molecules, the CD277 expression level on TF_H cells was analyzed (Fig. S1*D, right panel*). These results show that CD277 is expressed on TF_H cells at similar levels as other T-cell subsets. Hyperplasic lymph nodes were collected from patients presenting with enlarged lymph nodes clinically suspicious for lymphoma. In all cases, routine histology and immunochemistry showed features of non-specific lymphoid hyperplasia and ruled out the diagnosis of lymphoma. The CD277 immunostainings were performed on total frozen sections of reactive lymph nodes as previously described [5]. The final concentration for CD277 mAb (clone 20.1) was 1.5 μg/ml. Negative control samples were prepared by omitting the primary mAb. To visualize CD277 expression in T cells present in the interfollicular T cell zone, total frozen sections of reactive lymph nodes were stained with CD277 mAbs (Fig. S1*E*). The results of immunohistochemical analysis show a strong positivity on both interfollicular T-cell area and mantle B-cell zone, indicating that CD277 is express on T cells but also on B cells in the lymph nodes. Surprisingly, the pattern of staining was totally different in the germinal center (GC). Most of the GC zone is negative suggesting that B cells are loosing the expression of CD277 during the differentiation process. However, few scattered cells are harboring a positive staining that could correspond to TF_H cells, as these cells (CXCR5^{+} ICOS^{+} PD-1^{+}) are expressing CD277 (Fig. S1*D*), confirmed by performing a flow cytometry analysis (data not shown). The TF_H cells (CXCR5^{+} ICOS^{+} PD-1^{+}) were positive for CD277. Thus, CD277 was equally present on the TF_H cells and the CXCR5^{-} conventional T cells, whereas there is no significant staining in GC resident B cells. Taken together, CD277 is expressed on all subtypes of T cells in the peripheral blood as well as in lymph nodes and also in NK cells. The expression of the B7/CD28 family members or many other molecules involved in lymphocyte regulation, is

regulated under conventional stimuli. The regulation of CD277 expression should be also assessed under lymphocyte activation.

Modulation of CD277 on activated T cells and NK cells

As the expression of co-signaling molecules like PD-1 and their ligands could be regulated after TCR stimulation [6], we were wondering whether CD277 could be also modulated under T cell activation. To test this hypothesis, we compared the expression profile of CD277 and PD-1 at the surface of activated $CD4^+$ T cells. Purified $CD4^+$ T cells from healthy donors were thus incubated from 24 to 96 h with CD3 and CD28 mAbs or with the respective isotype controls. As expected, the CD3+CD28 costimulation resulted in a seven-fold increase of PD-1 expression after 72 h of culture, whereas CD277 expression was not modified at any time point (Fig. S1*C*). Similar results were obtained on $CD8^+$ T cells (data not shown).

In parallel, the regulation of CD277 expression was analyzed upon IL-2 and IL-15-induced NK cell activation (Fig. S2*C*). The HVEM molecule expression is down-regulated during lymphoid activation [7]. HVEM is highly expressed on NK cells and decreased upon NK cell stimulation. However, the CD277 expression was not modulated after NK cell activation (Fig. S2*C*). Altogether, these results demonstrated that T and NK cells constitutively express the CD277 molecule, but its expression is not modulated after cell activation.

This set of experiments on CD277 expression profiles (Fig. S1 & S2) using CD277 mAb (clone 20.1) showed that the CD277 molecule appears to be stably expressed in T and NK cells.

References

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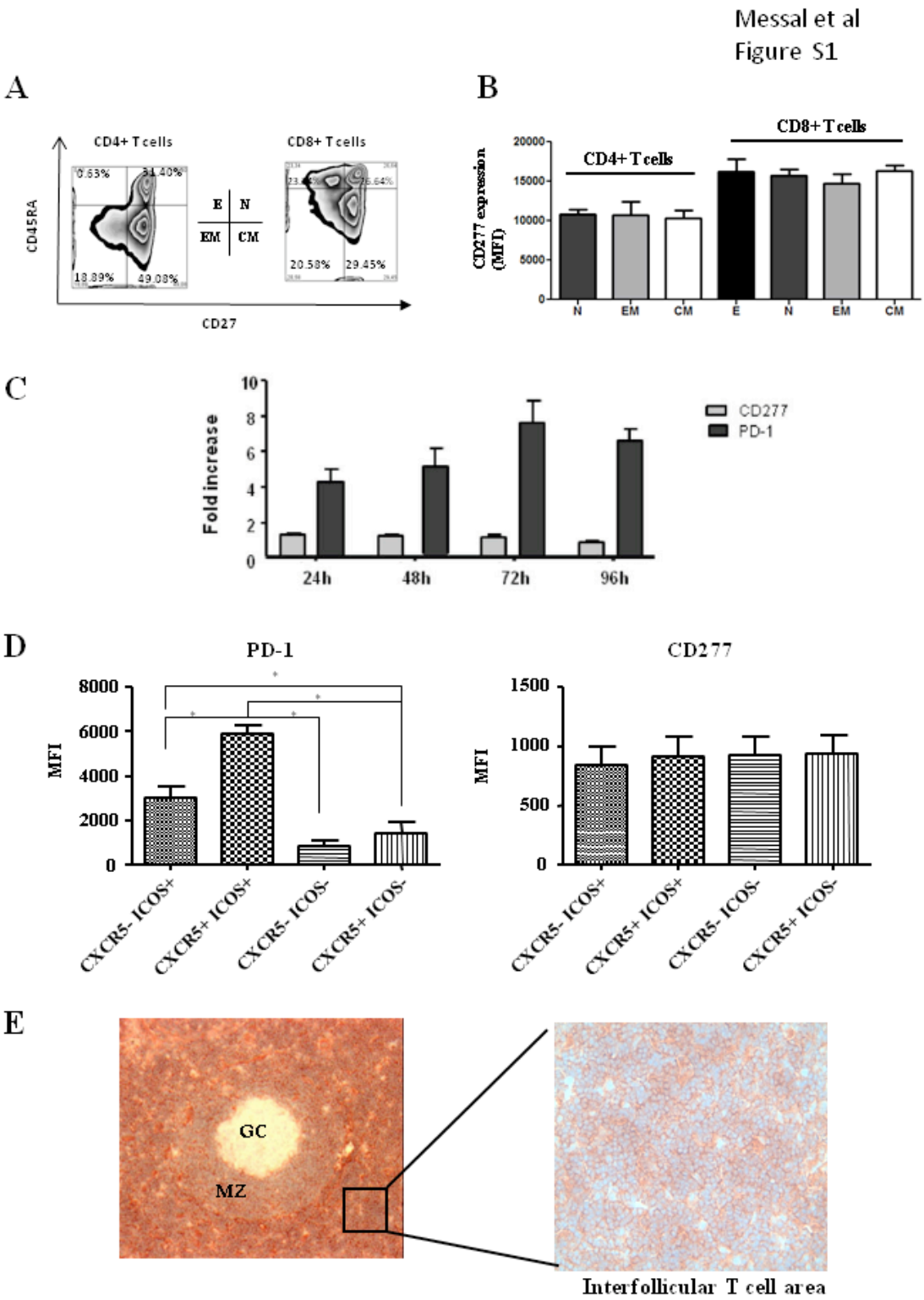
Supplemental Figures

Figure S1. CD277 expression in T cells.



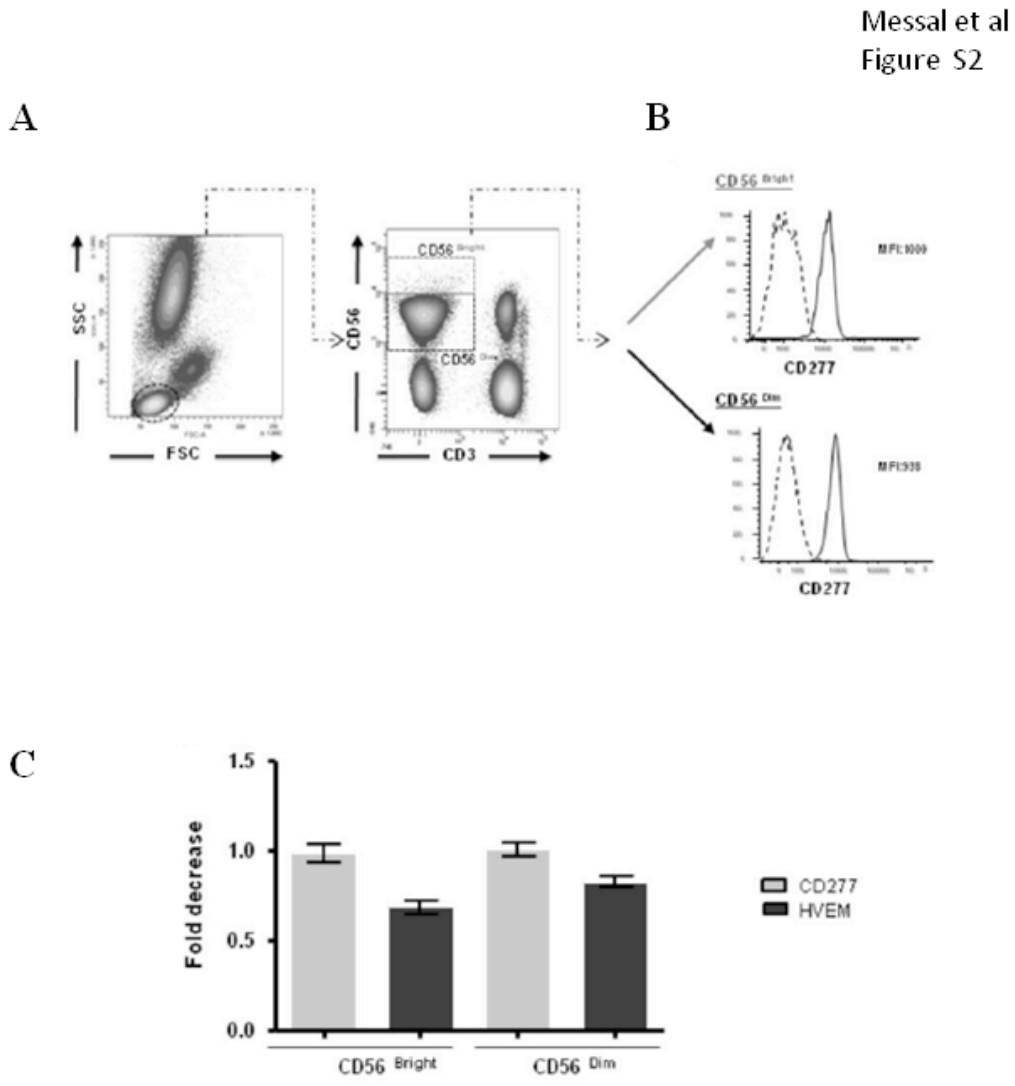
(A) $CD4^+$ and $CD8^+$ T cell subsets were subdivided based on differential CD27 and CD45RA expression into $CD27^+$ $CD45RA^+$, Naive (N) ; $CD27^+$ $CD45RA^-$, Central Memory (CM) ; $CD27^-$ $CD45RA^+$, Effectors (E) ; and $CD27^-$ $CD45RA^-$, Effector Memory (EM). (B) MFI of CD277 among these cell subsets is plotted + SEM.

(C) Expression of the CD277 and PD-1 molecules represented as fold induction over the unstimulated condition. Expression as determined by fold induction ± SEM of CD277 and PD-1 on purified $CD4^+$ T cells from PBMCs stimulated or not with plate-immobilized anti-CD3 (0.3 μg/mL) and anti-CD28 (10 μg/mL) mAbs during 96 h. Every 24 h, $CD4^+$ T cells are stained with anti-PD-1 and anti-CD277 Alexa 647-coupled mAbs as described in the material and methods section. Data are representative of 4 independent experiments.

(D) Expression profile of CD277 and PD-1 in lymphoid organs. Living cells from lymphoid tissue were identified as Vivid negative. T follicular Helper cells were further gated using staining with CD4, ICOS and CXCR5 mAbs. Results are provided as MFI values (n = 7, mean + SEM). Data are representative of 7 independent studies. The p values were calculated using the Mann-Witney paired test to compare differences between CD277 or PD-1 expression on T cells subsets. * $p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$.

(E) CD277 immunohistochemical pattern on reactive lymph nodes. Cells teased from lymph nodes were collected from patients who had given informed consent according with the Institutional Review Board of the Institut Paoli-Calmettes (Marseille, France). Only a few positive small lymphocytes were present scattered within the Germinal Center (GC). The CD277 expression was found on both interfollicular T-cells area and mantle zone (MZ) B-cells whereas there is no significant staining in GC B-cells.

Figure S2. CD277 expression in NK cells.



(A) Gating strategy of $CD3^{-}$ $CD56^{+}$ NK cells. (B) Histogramms are showing the CD277

expression of on $CD56^{Bright}$ and $CD56^{Dim}$ NK cells isolated from a representative donor. 4 donors have been analyzed.

(C) Expression of the CD277 and HVEM molecules on cytokine-activated NK cells. Fresh NK cells were sorted with the Easy Sep® negative selection kit and incubated overnight in medium completed with IL-2 (100 U/ml) and IL-15 (10 ng/ml) for 24 h. Cells were analyzed for CD277 expression as described in the material and methods section, data are representative of 4 independent experiments.