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ANALYSIS OF A "NOVEL" SUBSET OF CD34^{+} HAEMATOPOIETIC PRECURSORS IN PERIPHERAL BLOOD OF PATIENTS WITH CHRONIC INFLAMMATORY DISEASES

Coordinator: Prof. Lucia De Franceschi

Tutor: Prof. Claudio Lunardi

PhD candidate: Dr. Francesca Moretta

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SUMMARY

Background: in chronic inflammatory disorders, both infectious and non infectious, a novel population of $CD34^+$ hemopoietic precursors is detectable in high proportions in peripheral blood (PB). These precursors display peculiar phenotypic characteristics, namely the expression of DNAM-1 (bright), an activating receptor expressed by natural killer (NK) cells and CXCR4, a chemokine receptor that suggests their recent exit from bone marrow (BM). In healthy donors (HD), these precursors are virtually undetectable in PB while they represent 10-15% of BM $CD34^+$ cells. It is conceivable that, in chronic inflammatory processes, pro-inflammatory cytokines may favor the exit of this $CD34^+$ subset. While their presence in PB during chronic inflammation may represent a homeostatic event to compensate an accelerated cell death, a major interest in these cells is related to their differentiating capacity, which has been analyzed in the present study.
Methods: we evaluated by cytofluorimetric analysis the presence of $CD34^+$ $DNAM1^{bright}$ $CXCR4^+$ cells in different pathological conditions characterized by chronic inflammation including selected viral infections and autoimmune diseases and defined the phenotypic and functional characteristics of both NK and T cells generated in vitro from purified $CD34^+$ $DNAM1^{bright}$ $CXCR4^+$ progenitors.
Results: first, we confirmed in our cohorts that patients with autoimmune diseases or with chronic viral infections display high proportions of $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ precursors, which could even outnumber the conventional $CD34^+DNAM\text{-}1^-CXCR4^-$ precursors. Analysis of the progenies of these cells cultured in the presence of appropriate cytokines, showed that both $CD56^+CD3^-$ (NK) and $CD56^-CD3^+$ (T) cells can be generated. Comparison of the lymphoid progenies derived from precursors isolated from patients with viral infections versus patients with autoimmunity revealed a high prevalence of NK cells in the former, while, in autoimmunity, T cell progenies largely prevailed. In the first set of experiments, we focused on NK cell progenies, mostly from HIV and HCV infected patients. $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ cells gave rise, in approximately 20 days of colture, to sizeable populations of fully mature NK cells expressing KIRs, CD57, DNAM-1, NKG2D and NKG2C (a HLA-E specific activating receptor correlated with infection/sieropositivity for cytomegalovirus, CMV). This rapid differentiation towards mature NK cells was never occurring in conventional $CD34^+$ precursors. Notably, the $NKG2C^+$ progenies could undergo selective expansion upon NK cell colture with HLA-E-transfected 221 cell line. Further analysis of T cell progenies derived from highly purified (>99%) $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ precursors isolated from patients with autoimmune diseases, revealed the presence of both $CD4^+$ and $CD8^+$ T cells. Importantly, the large majority of cells expressed CXCR4 and the activating receptors DNAM-1 and NKG2D and 2B4 co-receptor. Analysis of $CD4^+$ T cell progenies showed the presence of both Th1 star (Th1*) and Th17 subsets.
Conclusions: the generation of T cells in the absence of thymic selection together with the expression of activating NK receptors capable of inducing a HLA-independent T-cell activation **(**or to amplify low affinity TCR responses) and the presence of Th1* and Th17 may suggest the possible involvement of these cells in the pathogenesis of self-damage in autoimmunity.

INTRODUCTION

General physiological aspects of T and NK cells

T lymphocytes ($CD3^+$) play a major role in adaptive immunity and display both effector and regulatory function. Thus, while $CD3^+CD8^+$ cells are predominantly cytolytic and kill virus infected and tumor cells upon activation and clonal expansion, $CD3^+CD4^+$ (helper) cells produce different cytokines which regulate the overall adaptive responses, including antibody production from B cells and $CD8^+$ T cells proliferation and acquisition of cytolytic activity. T cell maturation from $CD34^+$ hematopoietic precursors, in most instances, requires the thymus microenvironment that is also responsible of the "thymic selection" resulting in the nearly complete elimination of self-reactive T cells or "unfit" T cells, whose T cell receptor (TCR) does not recognize major histocompatibility complex (MHC). In addition to thymic selection, T cell mediated reactivity against self tissues is downregulated by a number of mechanisms ensuring peripheral tolerance, including the requirement of co-stimulatory receptors for optimal T-cell activation, the activity of checkpoint inhibitors, the presence of T regulatory cells (Treg). While most T cells express an alpha/beta TCR, a small subset expresses gamma/delta TCR, that recognizes CD1 molecules or phosphoantigens (expressed for example on tumor cells). Alpha/beta TCR expressed on $CD8^+$ cells recognize 9 aminoacid-long antigen peptides in the context of MHC-class I (HLA-I in humans), while those expressed by $CD4^+$ cells recognize longer peptides in the context of MHC-class II. A small subset of alpha/beta TCR T lymphocytes is characterized by the expression of an invariant va24-J18 joined with Vbeta11 that recognizes lipid antigens presented by the CD1d molecules.

Natural Killer cells ($CD56^+CD3^-$) are an important component of the innate immunity. They belong to the recently identified family of innate lymphoid cells (ILC) (1) that play an important role in innate defenses against pathogens and in tissue homeostasis, particularly in mucosa and skin. NK cells are involved in the first line immunity against viruses and in responses against tumor growth and dissemination (2).

While NK-cell activity against tumors and virus-infected cells is primarily related to their cytolytic function, the production of chemokines and cytokines promotes

early inflammatory responses, complementing the activity of other innate cell types. In addition, they contribute to regulate downstream adaptive immune responses by modulating both the strength and the quality of T cell responses (3). In turn, NK-cell activity is regulated by cytokines, including IL-2, IL-15 and IL-18 and cellular interactions primarily with dendritic cells (DC), macrophages and mesenchymal stromal cells, but also with neutrophils and eosinophils (4-8).

NK cells are recruited from PB to inflamed tissues or secondary lymphoid organs. Their selective migration is dictated by the expression of chemokine receptors that are differentially expressed in NK subsets (see below).

Definition of the molecular mechanisms by which NK cells discriminate between normal and tumor or virus-infected cells was inspired by the "missing self hypothesis" initially proposed by Ljunggren and Kärre (9), based on the observation that murine NK cells could kill a lymphoma cell line that had lost MHC-class I expression, but not the parental MHC-class I+ cells.

It appeared that NK cells could sense the presence of MHC- class I molecules on potential target cells. A clue that NK cells may detect even MHC class I allelic determinants was provided by the observation that they rejected parental bone marrow (BM) grafts in F1 hybrid mice (hybrid resistance) (10).

In addition, IL-2-cultured human NK cells were shown to kill PHA-induced T lymphoblasts from some allogeneic donors (11).

The expression of inhibitory NK receptors specific for MHC-class I antigens was compatible with these data. The first identified human inhibitory receptors were the killer Ig-like receptors (KIRs), specific for allelic determinants of HLA-class I molecules (12, 13).

Other HLA-class I-specific receptors are CD94/NKG2A (specific for HLA-E) (14) and LIR-1/ILT2 (15).

The existence of an "off" signal preventing NK-cell activation implied the existence of an "on" signal generated upon NK cell interaction with target cells. The first activating NK-cell receptors in humans are NKp46 (16), NKp44 (17) and NKp30 (18), (frequently referred to as natural cytotoxicity receptors, NCR) (19). Other important activating receptors are NKG2D (20), 2B4 (21) and DNAM-1 (22). In general, the ligands of such receptors are either upregulated or expressed de novo

in “stressed” cells, particularly in tumors and virally-infected cells (4). Notably, the interactions of both inhibitory and activating receptors with their ligands represent important checkpoints finely tuning NK-cell activation and function. Thus, the loss of HLA-class I expression results in target cell lysis, provided that ligands of activating receptors are expressed in sufficient amounts. In an autologous setting, this may occur only on interaction with abnormal cells, thus avoiding unwanted NK-cell activation. In addition, downregulation or masking (e.g. by antibodies) of inhibitory receptors may lead to uncontrasted NK-cell activation (23).

NK cells originate from HSC primarily in the BM. However, NK-cell precursors, at different stages of differentiation, are present in thymus (24), tonsils (25), lymph nodes (26), liver and decidua (27).

Tonsil and decidua precursors could give rise to NK cells and ILC3, but not to myeloid cells (28).

The presence of such committed precursors at peripheral sites may ensure their prompt proliferation and differentiation towards mature NK and ILC upon appropriate signaling. Interestingly, a different balance of NK/ILC3 is determined by the source of HSC and may be infected by the use of G-CSF (29).

These notions are important for haplo-HSCT due to the central role of NK cells in clearing leukemia cells escaping the preparative regimen (30).

Peripheral NK cells are generally subdivided into two major subsets, based on the surface density of CD56 antigen (31). While $CD56^{dim}$ cells predominate in PB, $CD56^{bright}$ are prevalent in secondary lymphoid tissues. The two subsets differ in their proliferative potential, cytolytic activity and cytokine production. Notably, $CD56^{dim}$, in addition to cytotoxicity, can rapidly produce IFN-γ and TNF-α upon receptor-induced cell triggering (but not in response to cytokines). $CD56^{bright}$ cells derive from HSC via phenotypically-identified stages (32). It is now clear that $CD56^{bright}$ give rise to $CD56^{dim}$ NK cells. Their progression to $CD56^{dim}$ and, further, to more differentiated NK cells, is traced by the gradual downregulation of CD94/NKG2A and the acquisition of KIRs, CD16 and, later, CD57 that marks terminally differentiated cells characterized by the highest cytolytic activity (33).

Importantly, during NK-cell maturation, a process known as NK cell “licensing” or “education” promotes the selection of NK cells expressing inhibitory receptors

specific for self HLA-class I alleles. These cells acquire full functional capability, while the remaining KIR^+ cells, potentially autoreactive, become anergic and may be deleted (34). Thus, the licensing process has a relevant effect on shaping the KIR repertoire.

NK cells in pathologic conditions

The function of NK cells may be severely impaired in some diseases. Frequently, functional inhibition of NK cells represent true mechanisms of escape developed by viruses or tumors to evade the NK cell-mediated surveillance. Thus, NK-cell activation may be inhibited by the downregulation of different activating receptors. This phenomenon has been well documented in viremic HIV-1 infections in which both NKp46 and NKp30 are sharply downregulated (35).

Similar downregulation of NKp46, NKp30 and DNAM-1 was detected in NK cells of chronic viremic HCV-infected patients. Receptor expression was rescued by standard antiviral treatment (36).

In tumors, activating receptor downregulation is induced by hypoxia and/or by soluble inhibitory factors/cytokines such as PGE2, ID0-induced Kinurenin, TGFβ, IL1β (in AML). Notably, in solid tumors, at least some of these inhibitory factors may be released not only by tumor cells, but also by cells present in the tumor microenvironment, including fibroblasts, M2 macrophages, Tregs, myeloid suppressor cells (37). Also soluble ligands recognized by activating receptors may induce receptor modulation or blocking. Another important inhibitory mechanism is related to the activation of PD1-PDL1 axis. While PD1 was originally discovered in T lymphocytes (38), it has recently been shown that it may be expressed in mature NK cells, particularly in cancer patients and inhibit NK-cell function upon interaction with $PDL1^+$ targets (39). Type 2 cytokines may act on the promoters of genes encoding for IFN-γ and TNF and inhibit cytolytic function (40). CMV-induced expression of UL40 protein, that mimics the HLA-class I molecules may protect infected cells from NK-mediated cytotoxicity. Virus- or tumor-induced secretion of IFN-γ by T or NK cells themselves may lead to upregulation of HLA –class I molecules in target cells, thus inducing them to be resistant to NK-mediated attack.

$CD34^{+}DNAM\text{-}1^{bright}CXCR4^{+}$ precursors

As revealed by recent studies, a peculiar subset of lymphoid precursors is present in peripheral blood of patients affected by different diseases characterized by chronic inflammation(41) including HCV infection, tuberculosis, chronic obstructive pulmonary disease and PAPA (Pyogenic sterile Arthritis, Pyoderma gangrenosum, Acne) Syndrome (42).

These cells are lineage negative (lin-), i.e. are not belonging to the T/B/monocyte/NK cell lineages, are positive for CD34, an hematologic stem cell marker, express DNAM-1, a receptor involved in killing of tumor cell targets (43) and in transendothelial cell passage, normally expressed by NK cells(44), and CXCR4, a chemokine receptor that suggests their recent egression from BM.

Such population of peculiar cell precursors resides in the bone marrow, hardly circulates in healthy donors and is absent in cord blood. Their proportion was reported to correlate with the degree of inflammation, likely reflecting lymphoid cell turnover/reconstitution occurring during chronic inflammation.

These cells also express various molecules important for the release and trafficking of precursors from the bone marrow, such as metallopeptidases and metalloproteinases (41).

Remarkably, these precursors could generate *in vitro* two different lymphoid cell population: NK cells with a mature phenotype able to produce Interferon-γ upon stimulation and displaying cytotoxic activity against different target cells and mature T cells expressing TCR α/β and high levels of DNAM-1 and NKG2D, two activating receptors normally expressed by NK cells (45).

Therefore, according to their phenotypic and trancriptional characteristics, in addition to their ability to generate NK and T cells in culture, the authors propose that $CD34^{+}$ $DNAM1^{bright}$ $CXCR4^{+}$ cells may represent committed lymphoid precursors released from the BM during chronic inflammation.

Notably, their proportion in peripheral blood correlated with NK and T cell numbers, suggesting a fundamental contribution for their homeostasis. In addition, also a correlation was found between the size of $CD34^{+}$ $DNAM1^{bright}$ $CXCR4^{+}$ cells and the plasma fibrinogen concentration in peripheral blood (PB) of patients, indicating a direct correlation also with the degree of chronic inflammation (41).

AIM OF THE PROJECT

The aim of this PhD project was to evaluate the presence of $CD34^+$ $DNAM1^{bright}$ $CXCR4^+$ cells in different pathological conditions characterized by chronic inflammation including selected viral infections and autoimmune diseases and to define the phenotypic and functional characteristics of both NK and T cells generated in vitro from $CD34^+$ $DNAM1^{bright}$ $CXCR4^+$ progenitors.

Moreover, we evaluated whether the size of the subset of $CD34^+$ $DNAM1^{bright}$ $CXCR4^+$ precursors present in peripheral blood (PB) could correlate with selected autoimmune diseases and/or their duration, severity and patient age.

PATIENTS AND METHODS

Patients

Blood samples from patients affected by different diseases characterized by chronic inflammation both of infectious and non-infectious origin were obtained as follows: 68 Combination Antiretroviral Therapy (cART) treated, virologically suppressed (HIV-RNA, viral load<50cp/ml), HIV infected patients; 12 HCV infected patients; 3 cases of rheumatoid arthritis (RA), 1 case of acute graft versus host disease (aGVHD), 16 cases of chronic graft versus host disease (cGVHD), 3 cases of B-cell chronic lymphocytic leukemia (B-CLL); 11 cases of systemic lupus erythematosus (SLE), 3 cases of sarcoidosis, 67 cases of systemic sclerosis (SSc), 1 case of sjogren syndrome (SS), 1 case of diffuse idiopathic skeletal hyperostosis (DISH). Peripheral blood of 18 healthy donors (HD) were obtained from the blood bank. Written informed consent was obtained by all the patients enrolled in this study.

Isolation of PBMCs

Peripheral blood mononucleate cells (PBMCs) were isolated from heparinized blood through density gradient centrifugation using Lymphoprep Ficoll-Isopaque (Axis-Shield, Oslo, NO) according to manufacturer's instructions. Ten mL peripheral blood (PB) were diluted with 10 mL PBS and then layered over 10 mL Lymphoprep in a 50 mL centrifuge tube. Samples were centrifuged at 800 x g for 20 minutes and cells collected using a Pasteur pipette. Obtained cells were washed twice with PBS at 1200 rpm for 10 minutes and then counted using acridin orange staining in a Burker chamber.

Antibodies

Mouse anti-human mAbs used: PeCy7-conjugated anti-CD56 and anti-CD34 (Immunotech-Coulter, Marseille, France; BD Pharmigen, San Jose, CA, USA); PerCp/Cy5.5 anti-CD38 (BioLegend, San Diego, CA, USA), -CD117 (BD Pharmigen, San Jose, CA, USA); BV510-conjugated anti-CD3, -CD14, -CD19, (BD Biosciences, San Jose, CA, USA respectively), FITC-conjugated anti-CD33, -CD34, -CD3, -CD7, -Lin (containing mixture of anti-CD3, -CD14, -CD19, -CD20,

-CD56 mAbs) and PE-conjugated anti-CD107a, (BioLegend, San Diego, CA, USA; BD Pharmigen, San Jose, CA, USA respectively), anti-CD69 (BioLegend, San Diego, CA, USA); APC-CY7-conjugated anti-CD16 (BD Pharmigen, San Jose, CA, USA), APC-conjugated anti-DNAM-1 (BioLegend, San Diego, CA, USA), HorizonV450-conjugated anti-NKp46, Alexa Fuor® 647 anti-NKp30 (BD Pharmigen, San Jose, CA, USA), PerCp/Cy5.5 conjugated anti-NKG2D, -HLADR (BioLegend, San Diego, CA, BD Pharmigen, San Jose, CA, USA). Purified anti-CD4 (IgG1), -CD8 (IgG1), -TCR (IgG1) -CXCR4 (IgG2a), (BD Pharmigen, San Jose, CA, USA), -NKG2C (IgG2b; R&DSystems, Inc. Minneapolis, MN 55413). APC conjugated Anti TCR V alpha 24 J alpha 18, Anti-NKp44 (Z231, IgG1), (BAT221, IgG1), anti-DNAM-1 (F22, IgG1), anti-KIR2DL2/L3/S2 (CD158b1/b2,j), anti-KIR3DL1/S1 (CD158e1/e2), anti-KIR2DL1/S1 (CD158a/h) anti-NKG2A (Z270, IgG1; Z199, IgG2a), anti-CD94 (XA185, IgG1), anti-CD85j (F278, IgG1 kindly provided by Dr. D. Pende), anti-2B4 (ST39, IgG2a), anti-CDw328 (QA79, IgG1), anti-CD161 (MA311, IgG1) all produced in the laboratory (A. Moretta, Genova). Anti HLA-DR (D1-12, IgG2a) was kindly provided by Dr R.S. Accolla (University of Insubria, Varese, Italy). For intracellular staining APC conjugated anti-IFN-gamma (BD PharMingen, San Jose, CA, USA), PE conjugated anti-Perforin (Ancell Corporation, USA).

Immunofluorescence analysis

Cells were analyzed by two-, three, four and five -color cytofluorometry. Briefly, cells were incubated with primary mAbs at 0.1μg/mL, followed by PE- or FITC-conjugated anti-isotype-specific goat anti-mouse secondary reagents at final concentration indicated by datasheets. Direct staining was performed by fluorochrome-conjugated mAbs as a third step at final concentration indicated by datasheets. For cytofluorimetric analysis, cells were gated using forward and side light scatter parameters (FACSFortessa, BD, Mountain View, CA, USA) and at least 10,000 events were always acquired. Mean fluorescence Intensity ratios are calculated as follows: MFI sample/MFI negative contro. Data were analyzed using FlowJo (Tree Star, Inc.). **Figure 1** shows the gating strategy used.

Statistical analysis

Statistical analysis was performed using the Mann Whitney U tests for unpaired datasets for comparisons. Chi-square and Fisher's exact test were used for comparison analysis. Tests were two-sided. Analysis was performed using JMP 10.0 (SAS) if not otherwise stated in the text.

RESULTS

Identification of increased proportion of $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ in patients with chronic inflammation.

Flow cytometric analysis of PBMC in patients with chronic infection shows the presence of high proportions of $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ cells in comparison with healthy donors (0,55 ± 0,78 vs. 5,57 ± 6,07%, mean ± S.D.; P<0.01) (**Figure 2A**). Such increased level is present also when we consider the total pool of $CD34^+$ circulating precursors, (prevailing over conventional $CD34^+DNAM\text{-}1^-CXCR4^-$ cells; p<0.05) (**Figure 2B**).

We then evaluated the presence of $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ cells in different diseases characterized by chronic inflammation of non-infectious origin: we analyzed 106 patients, the majority of whom affected by autoimmune/autoinflammatory diseases characterized by chronic inflammation (**Figure 3A**). Also in these patients we found the presence of $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ cells in significant proportions in most patients (84%) (**Figure 3B**).

We then assessed the presence of possible associations between clinical variables and percentages of $CD34^+DNAM\text{-}1^{bright}CXCR4^+$precursors (1% and 10% cut-off) within the group of patients with systemic sclerosis, since this was the largest available in our study, and almost all cases showed a sizeable population of $CD34^+DNAM1^{bright}$ precursors.

Specifically, 55 patients with systemic sclerosis were evaluated: 21 (38%) had diffuse disease, 46 (90%) had organ involvement, 17(33%) had a disease duration ≥ 10 years, 23 (47%) were immunosuppressed, 22 (42%) had associated Sjogren syndrome, 28 (54%) had $CD34^+DNAM\text{-}1^{bright}CXCR4^+$≥ 10% and 27 (46%) had $CD34^+DNAM\text{-}1^{bright}CXCR4^+$≥ 1% (**Table 1**). No significant associations were found between the proportions of $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ cells and the clinical features of the patients.

Analysis of NK cell progenies of CD34^{+}DNAM-1^{bright}CXCR4 T cells

We then analyzed the progenies deriving in vitro from highly purified (>99% purity) CD34^{+}DNAM-1^{bright}CXCR4^{+} cells. These precursors were cultured in an appropriate cytokine mix as described by Bozzano et al (41). After 20 days of culture, flow cytometric screening of growing cultures using anti CD3, anti CD56 and anti CD33 antibodies revealed the presence of CD56^{+}CD3^{-} cells as well as CD56^{-}CD3^{+} T cells and CD56^{+}CD3^{+} NKT cells (**Figure 4A**). No cells of the mielomonocytic (CD33^{+}) lineages were obtained. Further analysis of NK cell progenies showed that sizeable fractions (around 20%) of NK cells recovered from cultures were represented by CD16^{+}CD56^{dim} cells (**Figure 4B**) expressing KIRs and activating receptors including NKG2D, DNAM1, NKp30, NKp46, CD57 and LIR-1 (**Figure 4C**). Importantly, a proportion of NK cells expressed NKG2C, a HLA-E specific receptor representing the activating counterpart of NKG2A that has been shown to be associated with HCMV infection and with adaptive NK cell responses (46). Of note, coltures of CD34^{+} cells purified from umbilical cord blood did not generate NKG2C^{+} NK cells in vitro (47). In addition, cultures from purified CD34^{+}DNAM-1^{-} sorted cells in the present setting gave rise to delayed or absent mature NK cells progenies with no expression of NKG2C. Thus, altogether, these results indicate that CD34+DNAM-1brightCXCR4^{+} precursors can give rise, within a short time culture "in vitro", to CD56^{+}CD16^{+}KIR^{+}NKG2C^{+}CD57^{+} mature NK cells, while this does not occur in the case of conventional CD34^{+}DNAM-1^{-} precursors.

Functional analysis of NKG2C^{+} NK cell progenies derived from CD34^{+}DNAM-1^{bright}CXCR4^{+} precursors.

The presence of NKG2C^{+} cells in NK cell progenies derived from CD34^{+}DNAM-1^{bright}CXCR4^{+} precursors raised the question of their specificity and function. Indeed, the CD94/NKG2C receptor complex is known to recognize the non-classical HLA-E molecule. Thus, we assessed whether the NKG2C progenies generated in our cultures could be HLA-E specific. In order to address this question, we investigated whether a preferential expansion of NKG2C positive NK cells could be obtained by colturing NK cells derived from CD34^{+}DNAM-1^{bright}CXCR4^{+}

precursors in the presence of 721.221AEH cell lines (221E) transfectants, stably expressing surface HLA-E molecules. Flow cytometric analysis of cell progenies after a 6 day culture day showed a sharp increase of $NKG2C^+$ cells (78.2% versus 19.7%, U test, $P<0.005$), (**Figure 5**). Such increase of $NKG2C^+$ cells was selective as it was not paralleled by increased expression of other surface markers, thus suggesting a selective expansion of $NKG2C^+$ NK cells.

Analysis of T-cell progenies arising from $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ precursors from patients with chronic infections or patients with autoimmune disease.

As shown in **Figure 4A**, the lymphoid progenies obtained from cultured $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ precursors contained high proportions of $CD3^+$ T cells in addition to NK cells. We further analysed wheter differences existed in the proportions of T cells present in chronic viral infection versus autoimmune diseases. As shown in **Figure 6A**, the progeny of precursors from patients with chronic HIV infection contained very few T cells while the highly prevalent population was represented by $CD56^+CD3^-$ NK cells. In contrast, in patients with autoimmune diseases, the majority of cells generated in culture were represented by $CD3^+$ T cells. Although not shown, similar results were obtained in limiting dilution experiments, thus excluding a possible contamination of $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ precursors with mature peripheral T cells. Cells co-expressing CD3 and CD56 could be identified as NKT cells expressing V-alpha24J18 (data not shown). No substantial differences existed between the two groups of patients in terms of NKT cell proportions. **Figure 6B** shows the T cell progenies stained with anti-CD4 or anti-CD8 antibodies; it is evident that both CD4 and CD8-positive cells could be generated and that no substantial differences existed in the two groups of patients. Next, we focused on T cells arising from $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ precursors isolated from patients with autoimmunity. As shown in **figure 6C**, it was analysed the expression of a large number of informative receptors/markers expressed by such T cell progenies.

Remarkably, a sizable fraction of cells expressed NK cell receptors, both activating (NKp46 and NKG2D) and inhibitory (KIR and NKG2A). In addition, the large

majority of cells expressed the chemokine receptor CXCR4 already present on their precursors. Importantly, most T cells expressed 2B4, a surface molecule functioning as co-receptor in NK cells (48), and two major activating NK receptors, namely DNAM-1 and NKG2D. These findings suggest that these T cell progenies are potentially capable of undergoing activation upon cross-linking of such receptors with their ligands expressed on stressed cells present in inflammatory conditions. In addition, they may potentiate T-cell triggering induced via TCR, also in case of low affinity TCR. These peculiar phenotypic characteristics provide further evidence that T cells present in culture are not derived from contaminating mature peripheral T cells which do not express significant amounts of NK cell receptors.

Analysis of $CD4^+$ T-cell progenies of $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ precursors from patients with autoimmunity.

The role of specific T cell subsets in the pathogenesis of autoimmune disease is now well established. In particular, it appears that, in addition to $CD8^+$ cytolytic cells, also $CD4^+$ T cell subsets, identified more recently, correlate with autoimmunity, namely the Th17 and the so-called Th1-star (Th1*), both originated from a $CD161^+$ precursor (49).

Importantly, a study by Rivino and co-workers revealed that the use of a limited number of markers allows the identification of different T-helper ($CD4^+$) subsets (50); these markers are represented by 3 chemokine receptors, namely CCR6, CXCR3 and CCR4. As illustrated in **Figure 7A**, $CD4^+$ T cells lacking CCR6 and expressing CXCR3 correspond to classical Th1 cells; those lacking CCR6 and expressing CCR4 represent the Th2 subsets. Among $CD4^+$ T cells expressing CCR6, those co-expressing CXCR3 correspond to Th1* while those co-expressing CCR4 correspond to Th17. We applied this gating strategy to analyze the composition of $CD4^+$ T cell progenies. **Figure 7B** shows gated $CD4^+$ cells from a representative patient were stained with antibodies to these different chemokine receptors. It can be seen that a substantial fraction of cells co-expressed CCR6 and CXCR3 and could thus be identified as Th1* cells (bottom left panel). In addition, also cells co-expressing CCR6 and CCR4 could be identified (bottom right panel),

corresponding to Th17 cells. Thus, two different T helper subsets both implied in autoimmunity are highly represented in the progeny of $CD34^{+}$DNAM-$1^{bright}CXCR4^{+}$ precursors.

DISCUSSION

In the present study, we show that the recently identified $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ lymphoid precursors isolated from PB of patients with chronic viral infection or autoimmune diseases give rise to rapidly maturing, functional NK cells and T cells. In vitro maturation of NK cells from $CD34^+$ precursors isolated from PB or other tissues has been well documented. However, so far, it has not been described a rapid growth of $CD34^+$ cells towards mature and functional $KIR^+CD57^+NKG2C^+$ NK cells. Since the colture conditions and the cytokines used in this study do not differ from those of previous investigations, it appears that the development potential of $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ precursors is unique and different from that of "conventional" $CD34^+$ cells isolated from PB, BM and umbilical cord blood. Notably, $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ and $CD34^+DNAM\text{-}1^-CXCR4^-$ cells represent concurrently circulating precursors with the potential to undergo development towards NK cells with different phenotipic and functional characteristics and timing of generation. This finding is in line with the concept of heterogeneity in human $CD34^+$ precursors.

During HIV infection, increased circulating $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ precursors has been shown to correlate with the intensity of inflammation. Since most HIV patients are HCMV seropositive, the presence of two different circulating precursors both capable of giving rise to mature $NKG2C^+$ NK cell progenies (although within different time intervals) supports the idea of a major effort of the immune system to control HCMV replication in peripheral tissues. In this context, in patient with persistent HCMV infection, HCMV-specific T cells are detectable at sites of viral persistence in peripheral tissues (51). Interestingly, mature $NKG2C^+$ NK cell progenies obtained in colture also express tissue-tropic chemokine receptors (CCR1 and CXCR3), a finding in line with an attempt of the immune system to rapidly recruit HCMV-specific innate effector cells, possibly preceding or accompanying HCMV-specific T cell responses.

Our study supports the hypothesis that some tissue resident NK cells (with so far unknown progenitors) may derive through peripheral seeding of $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ precursors expressing tissue-directing chemokine receptors such as CXCR1 and CXCR3 (41).

The generation of adaptive NK cells with a mature NKG2C^{+} phenotype is associated with CMV infection both in humans and in mice. These cells are able to expand upon virus infection (52). The new pathway allowing a rapid generation of these cells directly from specialized circulating lymphoid precursors is likely to represent a new, relevant mechanism by which the innate immune system faces CMV reactivation/replication. These innate mechanisms may be of great value in the control of HCMV replication, particularly in patients with defects of T cell-mediated HCMV specific responses including HIV patients and HSC-transplanted patients. This novel pathway of NK cell differentiation may be complementary, or even preferential, to the generation of memory-like NK cells from mature peripheral CD56^{dim} NK cells. Indeed, it could represent a redundant mechanism by which the immune system controls CMV replication in peripheral tissues.

It should be noted that conventional CD34^{+} cells isolated from different sources can give rise in vitro and in vivo to different innate lymphoid cell (ILC) populations (53). Whether CD34^{+}DNAM-1^{bright}CXCR4^{+} precursors may give rise to ILC subsets other than NK cells it is still undefined and will be evaluated in the future.

As far as the generation of T cells from CD34^{+}DNAM-1^{bright}CXCR4^{+} precursors concerns, a first important consideration is that they can undergo maturation and TCR re-arrangement in the absence of a thymic microenviroment, thus escaping thymic selection. That T cells isolated from our cultures are indeed the progenies of CD34^{+}DNAM-1^{bright}CXCR4^{+} precursors and are not due to contamination with peripheral blood T cells has been ruled out by the following considerations: 1) the high degree of purity of cultured precursors (>99%); 2) experiment performed under limiting dilution condition in which T cell clones were obtained in coltures containing 1-10 precursor cells per each well; 3) the peculiar phenotypic charcteristics of T cell progenies expressing particular surface markers/receptors virtually absent in the peripheral T cell populations of healthy subjects.

As discussed above, T cell progenies represented the large majority of lymphoid cells recovered from CD34^{+}DNAM-1^{bright}CXCR4^{+} precursors isolated from patients with autoimmunity. Interestingly, both CD4^{+} and CD8^{+} cells could be detected. Perhaps more importantly, T cell progenies expressed a series of non HLA-specific activating NK receptors capable of mediating cell triggering in a

TCR-independent fashion. In particular, while only few cells expressed NCR, most T cells expressed the 2B4 co-receptor as well as DNAM-1 and NKG2D receptors. Since the ligands of these receptors are *de novo* expressed or upregulated upon cell stress (occurring in inflammatory conditions)(54), it is possible that T cells expressing the corresponding receptors may be directly triggered. Alternatively, weak TCR signaling, due to low affinity recognition of self-antigen, could be amplified by the concomitant engagement of these receptors and result in T cell activation and autoreactivity. In the case of $CD8^+$ T cells, this could lead to cytotoxicity and tissue damage. Regarding $CD4^+$ T cells, different subsets may be generated, in particular, subsets that are thought to be involved in autoimmune phenomena, such as Th17 and Th1*. In order to detect these subsets, it has been applied a flow cytometry assay based on the combined use of three chemokine receptors such as CCR6, CXCR3 and CCR4 on gated $CD4^+$ T cells. According to Rivino and coworkers (F. Sallusto's group), Th1* are identified by the co-expression of CCR6 and CXCR3 while Th17 by the co-expression of CCR6 and CCR4. Thus, T cell progenies originated from $CD34^+$DNAM-$1^{bright}CXCR4^+$ precursors maturing in the absence of thymus microenvironment contain a number of T cell subsets potentially involved in autoimmune phenomena and offer an important clue for the identification of new mechanisms involved in the pathogenesis of at least some autoimmune diseases. Therefore, it is possible to speculate that T lymphocytes generated from $CD34^+$DNAM-$1^{bright}CXCR4^+$ precursors may be involved in autoimmunity by two mechanisms: 1) T cell triggering occurring in a totally TCR-independent fashion by the concomitant engagement of triggering receptors (DNAM-1 and NKG2D) and co-receptor (2B4) (48), typical of NK cells; 2) T cell triggering involving a low affinity recognition of autoantigen by TCR, amplified by the engagement of the above NK receptors. The occurrence of these activation pathways in determining tissue damages may possibly explain why autoantigen(s) involved in autoimmunity have remained, at least in part, elusive.

It should be stressed that our patient cohort was mostly limited to certain autoimmune diseases, primarily SSc and SLE, while for other diseases, only few or single patients could be analyzed. However, the presence of $CD34^+$DNAM-

$1^{bright}CXCR4^{+}$ precursors appears to be common to different inflammatory conditions (both infectious and non infectious), as well as their ability to give rapidly rise to NK and T cell progenies.

The analysis of the frequency of $CD34^{+}DNAM\text{-}1^{bright}CXCR4^{+}$ precursors in patients with autoimmune diseases did not reveal substantial correlations with severity, duration of disease and age. This data may not be surprising: indeed, these cells represent circulating precursors recently emerged from BM, most likely on their way to homing into different tissues, as suggested by the expression of specific chemokine receptors and by preliminary data in which these precursors have been detected in healthy tissue. Thus, it is conceivable that their development and the generation of NK or T cell progenies would occur in peripheral sites in which they may exert their defensive or pathogenetic activity (A. De Maria, personal communication).

Although our studies provide a number of interesting, novel clues to interpret not only lymphoid cell differentiation but perhaps also the pathogenesis of certain autoimmune diseases, it is evident that further studies extended to the gene expression of these novel $CD34^{+}DNAM\text{-}1^{bright}CXCR4^{+}$ precursors in comparison with conventional $CD34^{+}$ cells as well as a careful molecular dissection of cells in the transition from precursors to mature NK cells are clearly required. In addition, it would be important to identify such developing cells in vivo, either in peripheral blood or in tissues, in order to gain further insight particularly in relation to the pathogenesis of autoimmune diseases.

CONCLUSIONS

In this work we demonstrate that:

1) patients with autoimmune diseases or with chronic viral infections display high proportions of $CD34^{+}DNAM\text{-}1^{bright}CXCR4^{+}$ precursors;

2) the analysis of the progenies of these cells cultured in the presence of appropriate cytokines, show that both $CD56^{+}CD3^{-}$ (NK) and $CD56^{-}CD3^{+}$ (T) cells can be generated;

3) comparison of the lymphoid progenies derived from precursors isolated from patients with viral infections versus patients with autoimmunity revealed a high prevalence of NK cells in the former, and of T cell progenies in autoimmunity;

4) NK cell progenies from $CD34^{+}DNAM\text{-}1^{bright}CXCR4^{+}$ precursors from patients with chronic infection give rise, to sizeable populations of fully mature NK cells expressing KIRs, CD57, DNAM-1, NKG2D and NKG2C, a HLA-E specific activating receptor correlated with infection/sieropositivity for HCMV; moreover the NKG2C+ progenies could undergo selective expansion upon NK cell culture with HLA-E-transfected 221 cell line;

5) $CD34^{+}DNAM\text{-}1^{bright}CXCR4^{+}$ precursors isolated from patients with autoimmune diseases revealed the presence of both $CD4^{+}$ and $CD8^{+}$ T cells, both in bulk cultures and in clonal populations obtained under limiting dilution conditions.

6) T cell progenies expressed low but significant proportions of both activating (NCR) and inhibitory (KIR and CD94/NKG2A) NK receptors, CXCR4 and the activating receptors 2B4, DNAM-1 and NKG2D; the expression of these receptors may induce T cell activation independent on TCR-mediated signals. In the case of both $CD4^{+}$ and $CD8^{+}$ T cells T cells, they may release cytokines (e.g. IFN-γ) involved in inflammatory responses.

7) analysis of CD4+ T cell progenies demonstrated the presence of both Th1* and Th17 subsets, which may play a role in the pathogenesis of self-damage in autoimmunity.

FIGURES AND TABLE

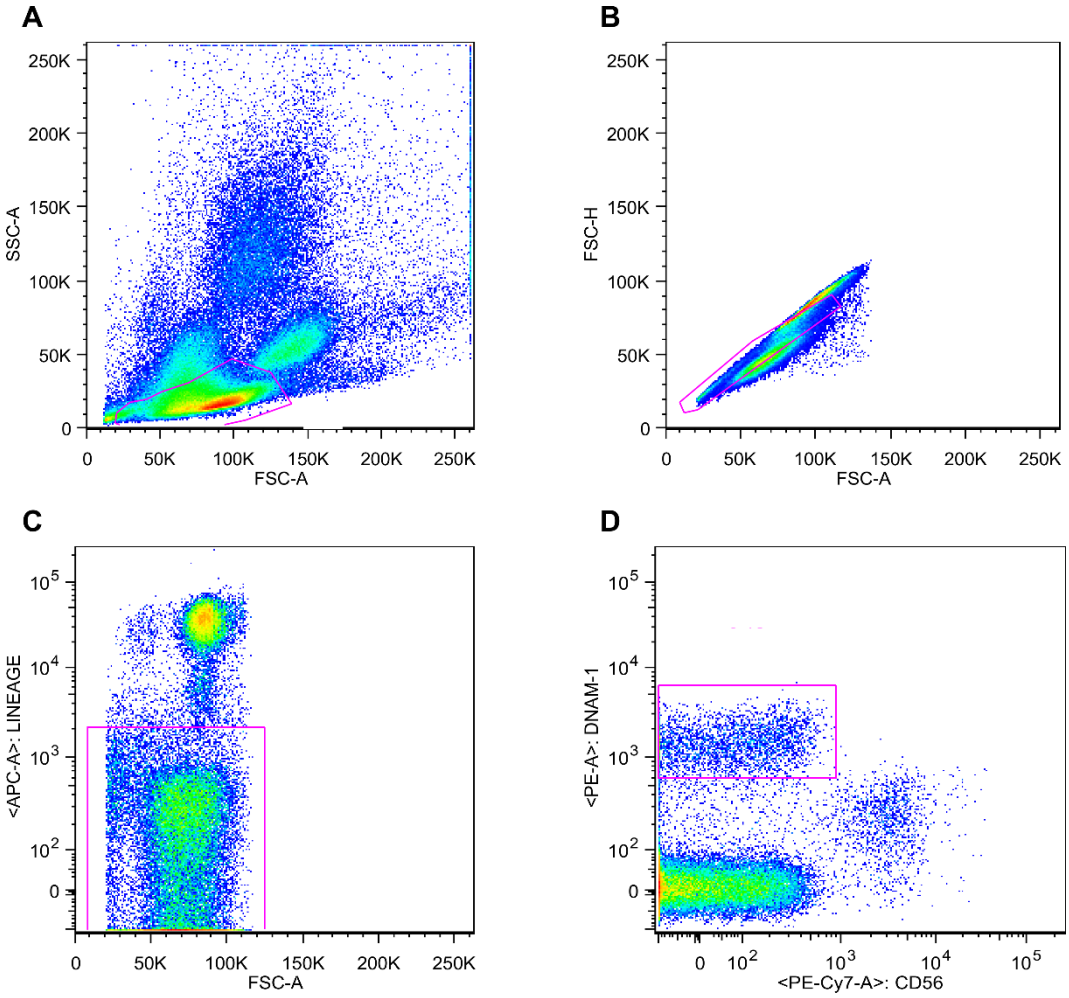

Figure 1: Gating strategy. As a first step, we performed a gate on lymphocytes as shown in **(A)**; after "doublets"elimination **(B)**, lineage negative cells are considered **(C)**. The resulting populations are analyzed for CD56 and DNAM-1:
cells that are brightly positive for DNAM-1 and negative for CD56 are shown **(D)**.

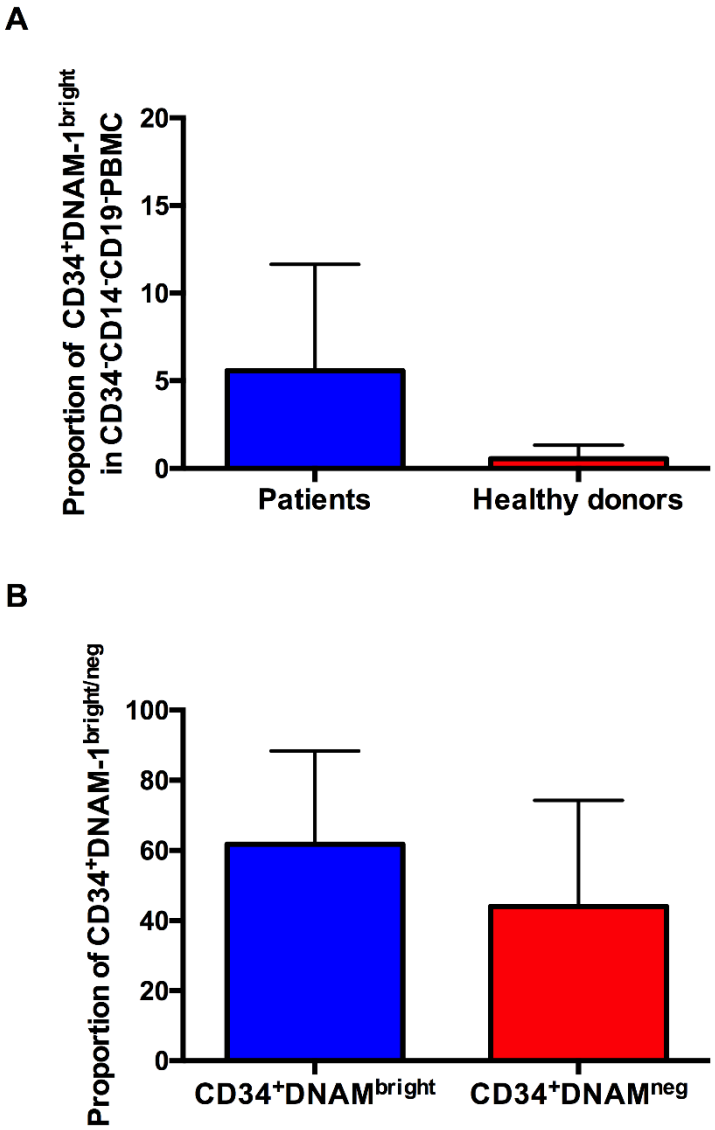

Figure 2: proportion of CD34$^+$DNAM-1^{bright}CXCR4$^+$ cells in patients with chronic inflammation (both infectious and non-infectious). (A): CD34$^+$DNAM-1^{bright}CXCR4$^+$ PBMC are highly increased during chronic inflammation as compared to healthy donors (HD). Histograms show the proportion ± SD of cells identified by flow cytometry of PBMC (15 patients and 18 HD). **(B):** The proportions of CD34$^+$DNAM-1^{bright}CXCR4$^+$ and CD34$^+$DNAM-1$^-$CXCR4$^-$ cells in patients with chronic inflammation.

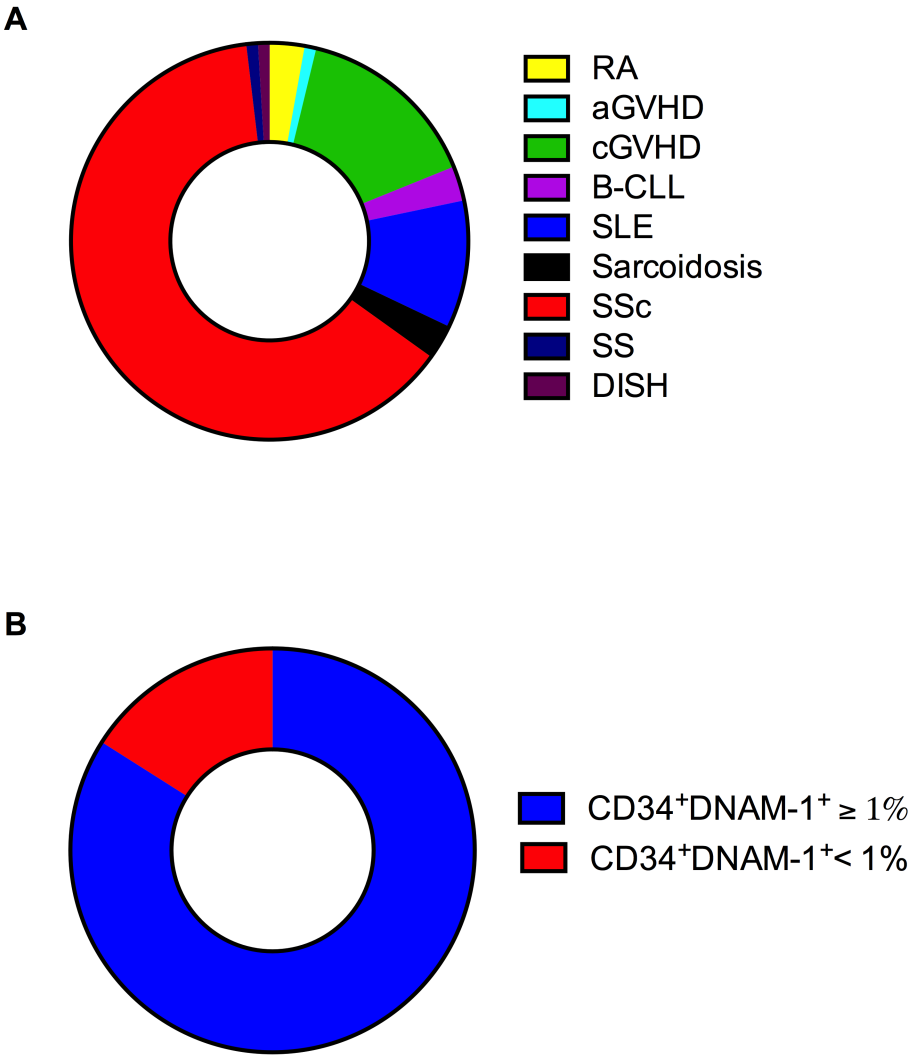

Figure 3: CD34$^+$DNAM-1$^+$ precursors in patients with chronic inflammation of non infectious origin.
(A): diseases evaluated (RA: rheumatoid arthritis; aGVHD: acute graft versus host disease; cGVHD: chronic graft versus host disease; B-CLL: B-cell chronic lymphocytic leukemia; SLE: lupus erythematosus systemicus; SSc: systemic sclerosis; SS: sjogren syndrome; DISH: Diffuse idiopathic skeletal hyperostosis **(B):** proportion of cases with sizeable proportions of CD34$^+$DNAM-1$^+$ cells (≥ 1%).

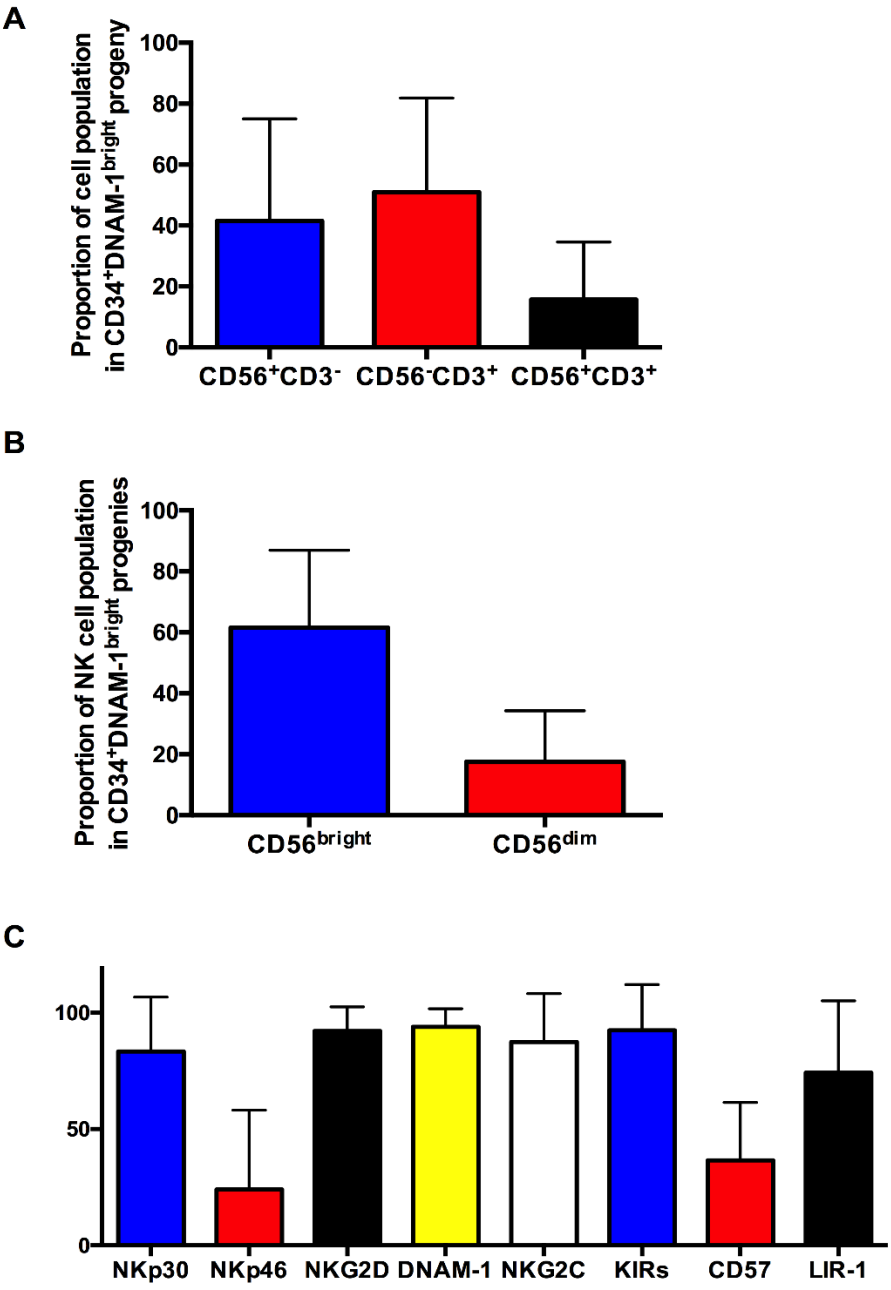

Figure 4: Lymphocyte subsets generated from CD34$^+$DNAM-1^{bright}CXCR4$^+$ precursors. (A): after 20 days of culture with a suitable cytokine mix, both NK (CD56$^+$CD3$^-$) and T cell (CD56$^-$CD3$^+$ and CD56$^+$CD3$^+$) populations are generated. **(B):** the percentages of the NK subsets as defined by the levels of surface expression of CD56 antigen. Note that a reletively high proportions of mature (CD56^{dim}) NK cells can be detected. **(C):** surface expression of the main receptors by gated CD56dim NK cells (mean ± SD of 35 determinations).

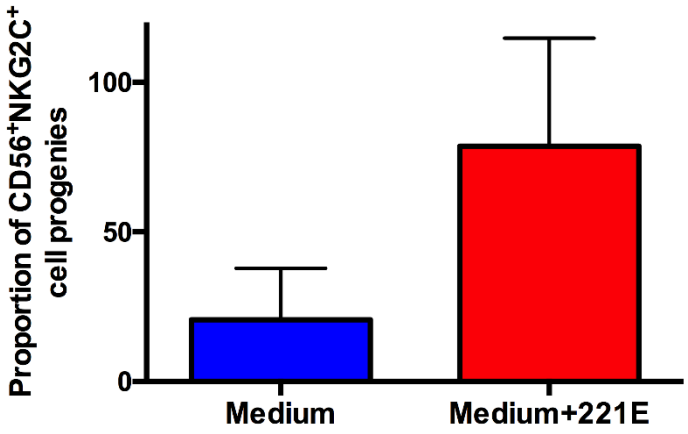

Figure 5: NKG2C$^+$ cells undergo selective expansion upon NK cell colture with HLA-E-transfected 221 cell line. Purified CD3$^-$CD56$^+$ cells cultured for 6 days with irradiated HLA-E bearing cells give rise to high proportions of NKG2C$^+$ NK cell progenies (10 experiments with 10 different patients).

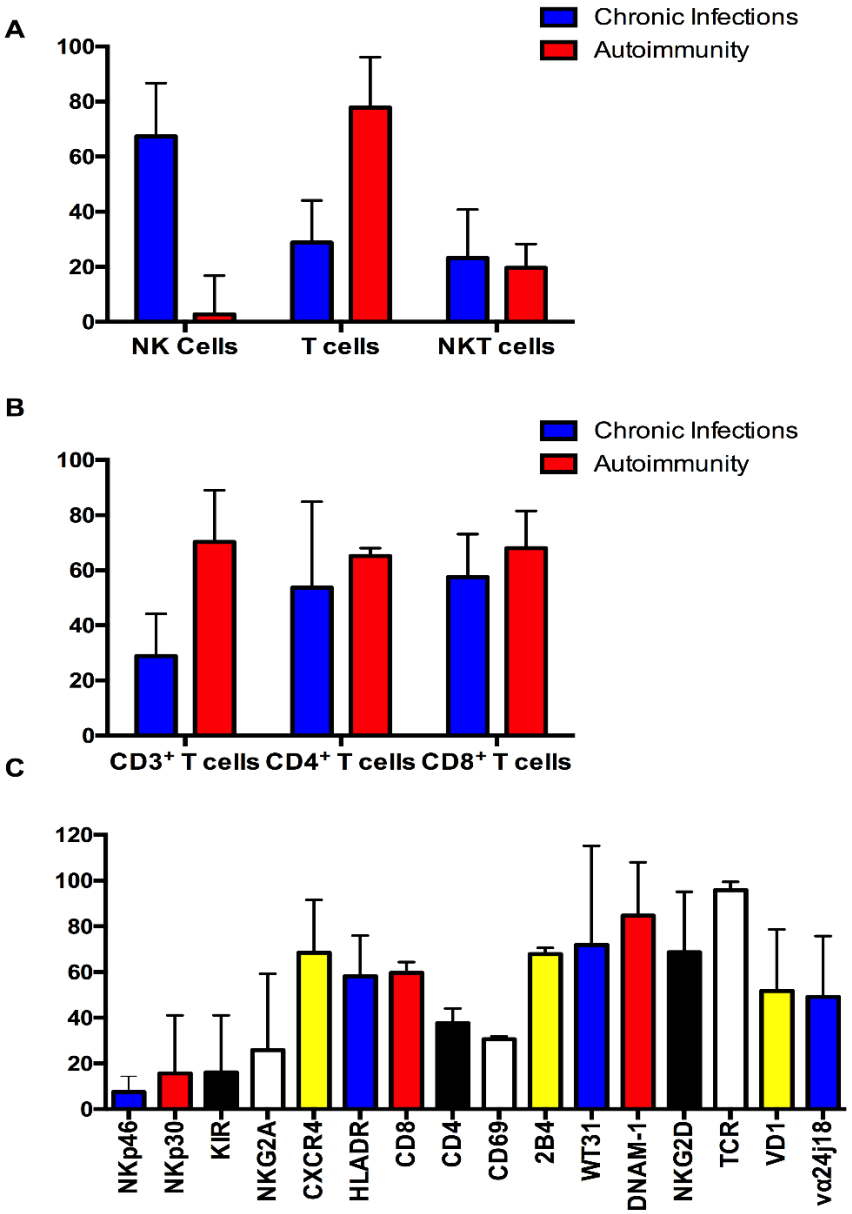

Figure 6: Relative proportions of T, NK or NKT cells and $CD4^+$/$CD8^+$ T cell subsets generated from $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ precursors from patients with chronic infections or patients with autoimmune disease.
(A): the progeny of precursors from patients with chronic HIV infection (6) display high proportions of NK cells and low T cells. Viceversa, those from autoimmunity patients (14) generated high proportions of T cells and very low NK cells. No substantial differences were found for the $CD56^+CD3^+$ NKT cells. **(B):** relative proportions of $CD4^+$ and $CD8^+$ T cell subsets in the same groups of infectious versus autoimmunity patients. **(C):** informative receptors and markers expressed by T cell progenies from autoimmune patients. Note that some T cells in autoimmune patients express low levels of NK receptors (NKp46, NKp30, KIR, NKG2A). Remarkably, most T cells express both DNAM-1 and NKG2D (activating NK receptors); in addition, they largely express the CXCR4 chemokine receptor.

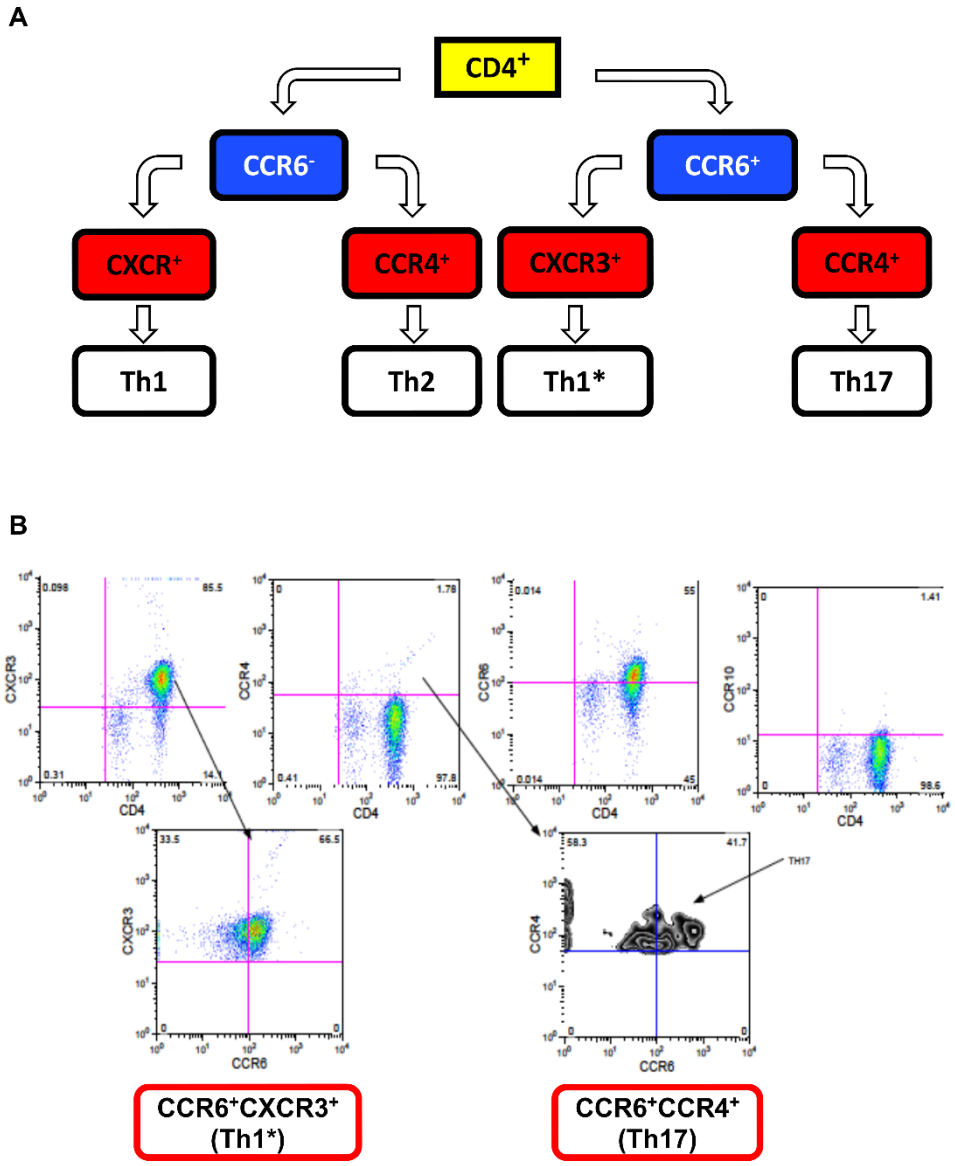

Figure 7: The $CD4^+$ progeny of $CD34^+DNAM\text{-}1^{bright}CXCR4^+$ in a representative patient with autoimmunity contains both Th17 and Th1*.
(A): schematic representation of markers allowing the phenotypic identification of different $CD4^+$ T helper subsets, including Th1 ($CCR6^-CXCR3^+$), Th2 ($CCR6^-CCR4^+$), Th1* ($CCR6^+CXCR3^+$) and Th17 ($CCR6^+CCR4^+$). Notably, both Th1* and Th17 subsets have been implied in autoimmunity. **(B):** in this experiment gated $CD4^+$ T cell progenies were first analyzed for the expression of CXCR3 or CCR6. Analysis of the co-expression of CXCR3 and CCR6 (left bottom panel) reveals the presence of Th1* cells. Analysis of the co-expression of CCR6 and CCR4 (right bottom panel) reveals the presence of Th17 cells.

Table 1. Associations between percentages of $CD34^{+}DNAM\text{-}1^{bright}CXCR4^{+}$ precursors and clinical clinical variables in 55 patients with systemic sclerosis.

	$CD34^{+}DNAM\text{-}1^{bright}CXCR4^{+}$ **(>1%)**		**P**	$CD34^{+}DNAM\text{-}1^{bright}CXCR4^{+}$ **(>10%)**		**P**
SUBTYPE	≥ 1%	<1%	1.00	≥ 10%	<10%	0.77
DIFFUSE	18	3		11	8	
LIMITED	28	6		17	16	
ORGAN INVOLVEMENT			1.00			0.65
YES	38	8		25	21	
NO	4	1		2	3	
DURATION			0.13			0.15
≥ 10	12	5		9	8	
<10	30	4		18	16	
AGE (MEDIAN=65)			0.47			0.03
<65	24	3		17	7	
≥65	22	6		11	17	
IMMUNOSUPPRESSION			0.47			0.77
YES	20	3		12	10	
NO	20	6		12	14	
SECONDARY SJOGREN			0.71			0.09
YES	19	3		15	7	
NO	24	6		13	17	

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