Activation-Induced Expression of CD56 by T Cells Is Associated With a Reprogramming of Cytolytic Activity and Cytokine Secretion Profile *In Vitro*

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ABSTRACT A subset of human T lymphocytes expresses the natural killer (NK) cell-associated receptor CD56 and is capable of major histocompatibility complex (MHC)–unrestricted cytotoxicity against a variety of autologous and allogeneic tumor cells. $CD56^+$ T cells have shown potential for immunotherapy as antitumor cytotoxic effectors, but their capacity to control adaptive immune responses via cytokine secretion is unclear. We have examined the inducibility of $CD56^+$ T cells from human blood *in vitro* and compared the kinetics of Th1, Th2, and regulatory cytokine secretion by $CD56^+$ T cells with those of conventional $CD56^-$ T cells. CD56 was induced on $CD8^+$ and $CD4^-CD8^-$ T cells by CD3/T-cell receptor (TCR)-mediated activation, particularly when grown in the presence of interleukin (IL)-2. Activation-induced $CD56^+$ T cells proliferated less vigorously but displayed enhanced natural cytotoxicity compared with $CD56^-$ T cells. $CD56^+$ T cells released interferon-γ (IFN-γ) and interleukin-13 (IL-13), but not IL-10, upon TCR stimulation. Flow cytometric analysis demonstrated that, compared with $CD56^-$ T cells, elevated proportions of $CD56^+$ T cells expressed IFN-γ, IL-4, and IL-13 within hours of activation. These acquired cytolytic and cytokine secretion activities of $CD56^+$ T cells make them potential targets for immunotherapy for infectious and immune-mediated disease. *Human Immunology 67, 863–873 (2006).* © American Society for Histocompatibility and Immunogenetics, 2006. Published by Elsevier Inc.

ABBREVIATIONS

IFN	interferon
IL	interleukin
mAb	monoclonal antibody
MHC	major histocompatibility complex
NK	natural killer
NKR	natural killer cell receptor
NKT	natural killer T
PBMC	peripheral blood mononuclear cell
PHA	phytohemagglutinin
PMA	phorbol myristate acetate
TCR	T-cell receptor

INTRODUCTION

A subset of human T lymphocytes expresses the cell-surface, immunoglobulin superfamily molecule, CD56, which is typically expressed by natural killer (NK) cells, neurons, and some tumor cells [1, 2]. $CD56^+$ T cells are heterogenous in nature and include $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ cells expressing αβ or γδ major histocompatibility complex (MHC) or CD1d-restricted T-cell receptors (TCRs), and various combinations of NK cell receptors (NKRs), including CD16, CD56, CD94/NKG2, CD161, NKG2D, and killer immunoglobulinlike receptors [3–5]. They generally account for less that 5% of peripheral blood T cells and up to 50% of T cells in the liver [6, 7] and in the intestine [8–10]. Functionally, $CD56^+$ T cells display properties of both NK cells and T cells. They are

capable of potent perforin/granzyme-mediated killing of a variety of tumor target cells *in vitro* and *in vivo* [3, 11, 12]. This MHC-unrestricted cytotoxicity does not require prior antigenic exposure but appears to involve the ligation of stimulatory NKRs and/or the TCR, and exposure to cytokines of diverse cellular origins, including interferon-γ (IFN-γ), interleukin-1 (IL-1) and IL-2 [11], IFN-α, IL-2, IL-12 and/or IL-18 [13], IL-2 and IL-12 [13–17], and IL-15 [4]. The cytotoxic activities of CD56^{+} T cells against autologous and allogeneic leukemic cells are reported to be superior to those of NK cells and lymphokine activated killer cells [11, 18, 19], and phase I trials for leukemia by using autologous CD56^{+} T cells generated *ex vivo* by CD3 stimulation of peripheral blood mononuclear cells (PBMCs) in the presence of cytokines (cytokine-induced killer cells) are under way [19–22].

In addition to NK-like cytotoxicity, CD56^{+} T cells are capable of TCR-mediated and MHC- or CD1d-restricted cytotoxicity and secretion of cytokines, including IFN-γ, TNF-α, and IL-4, which promote Th1 or Th2 adaptive immune responses [3–5, 13, 15, 23–25]. The dual innate and adaptive immune functions place CD56^{+} T cells alongside natural killer T (NKT) cells and subsets of γδ T cells as frontline innate immune effectors and potential regulators of adaptive immune responses against microorganisms and tumors [26, 27]. These properties make CD56^{+} T cells attractive potential targets for therapy for infectious and immune-mediated diseases as well as cancer. In support of this notion, numerical and functional deficiencies and phenotypic alterations of CD56^{+} T cells have been reported in patients with various infectious and autoimmune diseases and cancer [23, 28–32].

In the present study, we have investigated the potential of human CD56^{+} T cells as initiators and regulators of adaptive immune responses by examining the kinetics of their inducibility from human blood *in vitro* and comparing their functional properties with those of conventional CD56^{−} T cells. We demonstrate that CD56 expression can be induced on CD56^{−} T cells by CD3/TCR-mediated activation and that activation-induced CD56^{+} T cells display MHC-unrestricted killing of target cells and IFN-γ and IL-13, but not IL-10 secretion. Flow cytometric analysis demonstrated that CD56^{+} T cells more frequently expressed IFN-γ, IL-4, and IL-13 than CD56^{−} T cells in the first 24 hours following stimulation. Thus, in addition to acquiring innate cytolytic activities, the induction of CD56 on T cells is frequently associated with enhanced capacity to stimulate and polarize adaptive immune responses.

MATERIALS AND METHODS

Subjects

Blood samples were obtained from healthy donors. PBMCs were prepared by standard Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation. Ethical approval for this study was obtained from the Ethics Committee of the National University of Ireland, Maynooth.

Antibodies and Flow Cytometry

Monoclonal antibodies (mAb) specific for human CD3, CD4, CD8αβ, CD56, γδTCR, αβTCR, CD161, CD69, HLA-DR, CD25, CD45RO, the invariant Vα24Jα18 TCR α-chain found on CD1d-restricted NKT cells [24, 27] (6B11), IFN-γ, IL-4, IL-5, IL-10, and IL-13 were obtained from BD-Pharmingen (Oxford, UK). The expression of cell surface or intracellular antigens by fresh or cultured PBMCs or purified PBMC subsets was detected by mAb staining and four-color flow cytometry (FACSCalibur, Becton Dickinson, Oxford, UK) with CellQuest software (Becton Dickinson).

In Vitro Stimulation and Expansion of T Cells

PBMCs or enriched PBMC subsets were suspended in complete RPMI medium (RPMI 1640 containing 25 mM HEPES, 2 mM L-glutamine, 50 μg/ml streptomycin, 50 U/ml penicillin, and 10% fetal calf serum) at a density of 0.5×10^6 cells/ml and stimulated for up to 120 hours with either 10 ng/ml phorbol myristate acetate (PMA) plus 1 μg/ml ionomycin, or with anti-CD3 mAb (0–3 μg/ml HIT3a; BD-Pharmingen Erembadege, Belgium) that was bound to microwell plates by incubation at 37°C for 4 hours in 0.1 M Na$_2$HPO$_4$, pH 9. For longer-term stimulation and expansion of T cells, PBMCs were suspended in complete RPMI medium containing 5 μg/ml phytohemagglutinin (PHA, Sigma-Aldrich, Dublin, Ireland), and 25 ng/ml recombinant human IL-2 (National Cancer Institute, Frederick, Maryland) was added 2 days later and at 4–5 day intervals thereafter. After 12–14 days, the cells were restimulated with PHA and an equal number of irradiated (5000 rad) allogeneic PBMCs and fed with IL-2 as above. The cultures were analyzed by flow cytometry or separated into CD56^{+} and CD56^{−} subsets on day 28.

Purification of CD56^{+} and CD56^{−} T Cells

CD56^{+} and CD56^{−} T cells were purified from fresh PBMCs or PBMCs that were stimulated twice with PHA and cultured with IL-2, as described above, which resulted in the death of all non-T (CD3^{−}) cells and selective expansion of CD56^{+} T cells (see results). CD56^{+} and CD56^{−} cells were then purified using CD56 microbeads (Miltenyi Biotec, Gladbach Bergische, Germany). The purities of enriched fractions were deter-

mined by flow cytometry, and only preparations that yielded purities of >98% were used in functional studies.

Cytotoxicity Assays

MHC-unrestricted cytotoxicity by CD56$^+$ and CD56$^-$ T cells, purified as above, was assayed in 4-hour ^{51}chromium-release assays [25] using K562 cells as targets.

Analysis of Cytokine Production

The release of IFN-γ, IL-13, and IL-10 into supernatants of stimulated cells (0.5 × 10^6/ml) over 3 days was quantified by enzyme linked immunosorbent assay (ELISA) by using antibody pairs and recombinant cytokine standards purchased from R&D Systems (Abingdon, UK).

Intracellular expression of IFN-γ, IL-4, IL-5, IL-10, and IL-13 by PBMC subsets was detected by flow cytometry after stimulating the cells with PMA and ionomycin or anti-CD3 mAb in the presence of brefeldin A (10 μg/ml, Sigma-Aldrich), an inhibitor of protein translocation from the endoplasmic reticulum to the Golgi apparatus. Cells were then stained with mAbs specific for surface CD3 and CD56, fixed with 4% paraformaldehyde, and permeabilized with 0.2% saponin (Sigma-Aldrich) before adding the anti-cytokine mAbs [25]. As controls, unstimulated cells were treated similarly.

Statistical Analysis

Mean frequencies of lymphocyte subsets and mean cytokine levels were compared using the paired Student's t-test and p values of <0.05 were considered significant.

RESULTS

CD56 Expression by T Cells Is Induced by Activation

Flow cytometric analysis of freshly isolated PBMCs (Figure 1A) from 33 healthy donors indicated that CD56 was expressed by 0.7%–14.2% (mean 5%) of CD3$^+$ cells. Up to 6.9% (mean 1.9%) of CD56$^+$ T cells, compared with 9.5 % (range 1.7%–24.7%) of NK cells, expressed high levels of CD56 (CD56^{bright} cells). About 50% (range 17.6%–80%) of CD56$^+$ T cells expressed the CD8 coreceptor, whereas the remainder expressed CD4 or double-negative CD4$^-$CD8$^-$ phenotypes in approximately equal proportions. Of the CD56$^+$ T cells, 14.4% (range 9.3%–27.9%) had γδ TCRs, compared with 2.6% of CD56$^-$ T cells. A higher proportion (1.6%) of CD56$^+$ T cells (range 0.6%–2.5%) had invariant Vα24Jα18 TCR chains compared with 0.02% of CD56$^-$ T cells (0.007%–0.03%, Table 1 and data not shown).
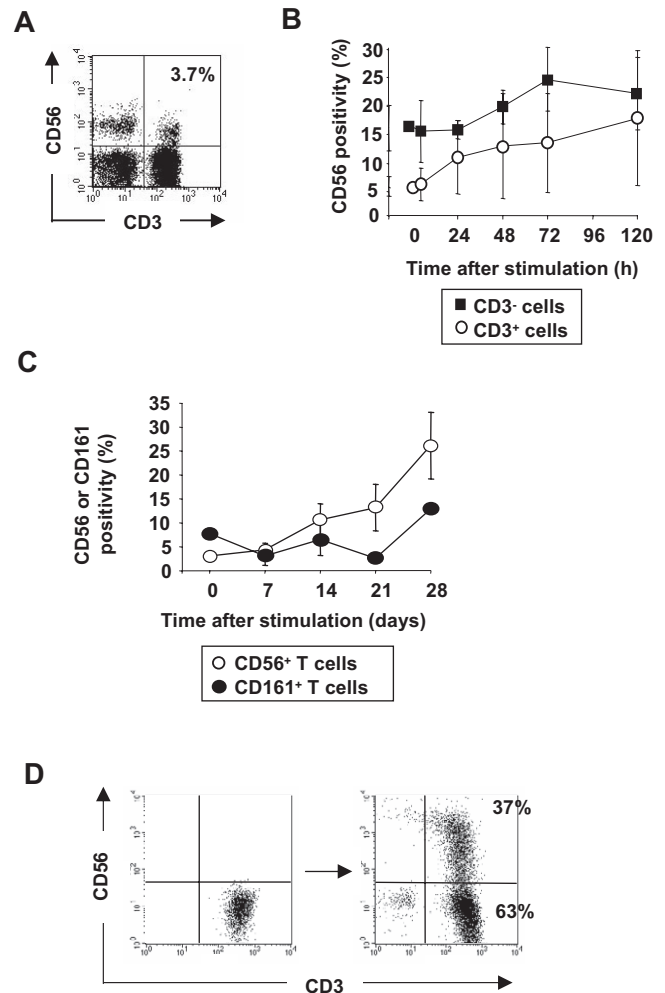
FIGURE 1 CD56 is induced on peripheral blood T cells by activation *in vitro*. (A) Representative flow cytometric dot plot demonstrating the expression of CD56 by CD3$^+$ and CD3$^-$ lymphocytes in fresh peripheral blood mononuclear cells (PBMCs). (B) Kinetics of CD56 positivity of CD3$^+$ and CD3$^-$ PBMCs after stimulation with 0.3 μg/ml plate-bound anti-CD3 monoclonal antibody (means of 9 samples). (C) Kinetics of CD56 and CD161 expression by peripheral T cells after stimulation with phytohemagglutinin and culture with interleukin (IL)-2 (means of three samples). (D) Representative flow cytometric dot plots illustrating expression of CD56 by T cells before (left) and after (right) expansion for 28 days with phytohemagglutinin and IL-2 when PBMCs that were depleted of CD56$^+$ T cells were used as starting material. Error bars illustrate standard errors of the means.

The inducibility and stability of CD56 expression by T cells was examined by stimulating PBMCs with plate-bound anti-CD3 mAb and analyzing CD56 expression by CD3$^+$ cells at intervals up to 120 hours. Figure 1B illustrates that short-term (5-day) T-cell activation with anti-CD3 mAb alone resulted in a gradual increase in the percentages of T cells that expressed CD56, from 6%–17.9% (threefold, means of nine samples). No expansion

TABLE 1 Phenotypic properties of freshly isolated human peripheral blood CD56$^+$ T cells and CD56$^+$ T cells induced by two rounds of stimulation with PHA and culture with IL-2[a]

Cell-surface phenotype	Percent of fresh CD56$^+$T cells (mean and range)	Percent of activation-induced CD56$^+$ T cells (mean and range)
CD4$^+$CD8$^-$	23 (7-41)	0.6 (0-1.5)
CD8$^+$CD4$^-$	52 (18-80)	74 (61-88)
CD4$^-$CD8$^-$	15 (10-34)	25.9 (12-40)
CD4$^+$CD8$^+$	0.6 (0-1.0)	0.3 (0-0.9)
αβTCR	86 (72-91)	96 (93-98)
γδTCR	14 (9.3-28)	4 (1.7-7.4)
Vα24Jα18 TCR	1.6 (0.6-2.5)	2 (1.3-2.7)

Abbreviations PHA = phytohemagglutinin; IL = interleukin.

[a] Data are means and ranges of 4-15 samples.

of overall cell numbers was observed. After repeated stimulation with PHA and culturing with IL-2, total cell numbers increased by a mean factor of 4.2, and the mean frequencies of CD56$^+$ T cells rose from 3%–26.2% (ninefold), whereas the frequencies of T cells expressing another NKR, CD161, remained constant (means of three samples, Figure 1C). This amounts to a mean 37-fold expansion of CD56$^+$ T cells. To determine whether these expansions of CD56$^+$ T cells are a result of the induction of CD56 expression by activated CD56$^-$ T cells or the selective expansion of CD56$^+$ T cells, PBMCs were depleted of CD56$^+$ T cells by using mAb-coated magnetic beads and were expanded with PHA and IL-2. After 28 days, CD56 was expressed by a mean of 37% of T cells, where purified CD56$^-$ T cells were used as starting material (Figure 1D). These results provide evidence that CD56 can be induced on T cells by activation.

Phenotypes of Activation-Induced CD56$^+$ T Cells

CD56$^+$ T cells were expanded from PBMCs by subjecting them to two rounds of stimulation with PHA and culturing them with IL-2. Flow cytometry was used to compare the frequencies of CD4, CD8 and αβ, γδ and Vα24Jα18 TCR expression by activation-induced CD56$^+$ T cells with those of CD56$^+$ T cells in fresh blood. Table 1 demonstrates that, whereas a mean of 23% of CD56$^+$ T cells in fresh blood express CD4, this coreceptor was not present on activation-induced CD56$^+$ T cells, all of which expressed CD8$^+$ (mean 74%) or double-negative CD4$^-$CD8$^-$ (26%) phenotypes. Activation-induced expansion of CD56$^+$ T cells was not associated with expansions of γδ T cells, which accounted for 14% of CD56$^+$ T cells in fresh blood compared with 4% of induced CD56$^+$ T cells. In contrast, CD1d-restricted NKT cells expressing invariant Vα24Jα18 TCRs and CD56 were expanded by activation, these TCR chains being present on 2% of induced

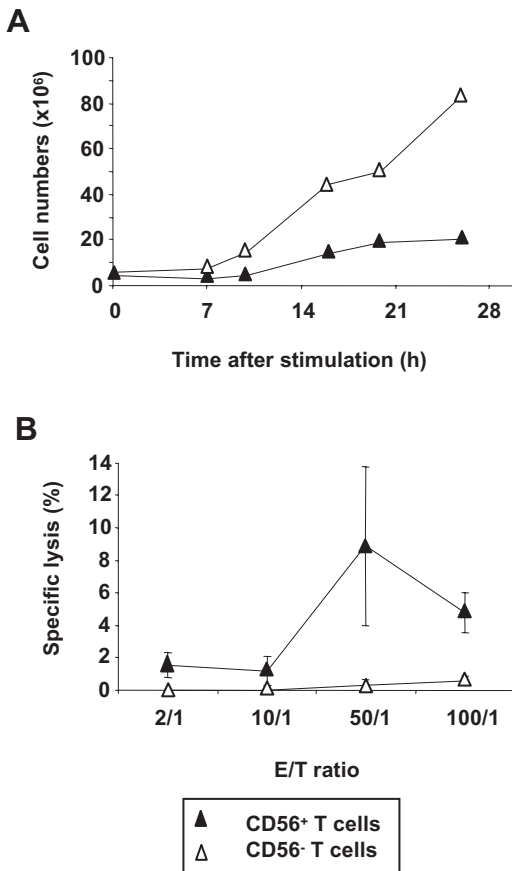CD56$^+$ T cells and 1.6% of CD56$^+$ T cells from fresh blood (Table 1).

CD56$^+$ T Cells Display Reduced Proliferative Capacity and Enhanced MHC-Unrestricted Cytotoxicity Compared With CD56$^-$ T Cells

CD56$^+$ and CD56$^-$ T cells were purified from freshly isolated PBMCs, and 5×10^6 cells of each type were expanded for 28 days with PHA and IL-2, as described above. At various time intervals, the number of viable cells was determined. Figure 2A illustrates that purified CD56$^-$ T cells underwent a mean 17-fold expansion, whereas CD56$^+$ T cells expanded about fourfold in this time, indicating that they have either a reduced proliferative capacity or an increased sensitivity to apoptosis. CD56$^-$ and CD56$^+$ T cells purified from PBMCs that were expanded with PHA and IL-2 were used as effectors in ^{51}chromium-release cytotoxicity assays against the K562 target cell line. Figure 2B illustrates that CD56$^+$ T cells displayed moderate cytotoxicity, giving a mean specific lysis of 9% at effector/target ratios of 50:1, whereas CD56$^-$ T cells displayed negligible (0.23%) cytotoxicity.

Kinetics of Activation Marker Expression by Resting and Activated CD56$^+$ and CD56$^-$ T Cells

Flow cytometric analysis of freshly isolated PBMCs revealed that the early activation marker, CD69, was not expressed by resting CD56$^-$ T cells, but was present on up to 55% (mean 24%) of CD56$^+$ T cells (Figure 3). Within 1 hour of culture with PMA and ionomycin, both cell subsets expressed CD69, and by 24 hours this activation marker was expressed by all lymphocytes (data not shown). Significantly higher frequencies of CD56$^+$ (mean 63.9%) compared with CD56$^-$ (mean 31.1%, $p < 0.001$) T cells upregulated CD69 in response to anti-CD3 mAb stimulation, peaking at 24 hours for both lymphocyte subsets (Figure 3). CD25 was found to be

A

B

- ▲ CD56$^+$ T cells
- △ CD56$^-$ T cells

FIGURE 2 CD56$^+$ T cells display reduced proliferative capacity and enhanced major histocompatibility complex–unrestricted cytotoxicity compared to CD56$^-$ T cells. (A) Viable cell numbers recovered at various times after stimulation of 5 × 10^6 CD56$^+$ or CD56$^-$ T cells purified from PBMCs. (B) Specific lysis of K562 target cells by CD56$^+$ and CD56$^-$ T cells purified from PBMCs expanded with phytohemagglutinin and IL-2. Results are means of three experiments. E/T = effector/target ratio. Error bars illustrate standard errors of the means.

expressed by less than 17% of resting CD56$^+$ and CD56$^-$ T cells, but its expression was induced on 100% of T cells within 72 hours of activation with PMA and ionomycin (data not shown), and 75.1% and 64% of CD56$^+$ and CD56$^-$ T cells, respectively, stimulated with anti-CD3 mAb for 96 hours (Figure 3). HLA-DR was expressed by <5% of freshly isolated PBMCs but was induced on about 50% of CD56$^+$ and CD56$^-$ T cells in response to 48 hours stimulation with PMA and ionomycin (data not shown), and about 10% of both cell populations following anti-CD3 mAb stimulation (Figure 3). CD45RO was expressed by around 29% of resting CD56$^+$ and CD56$^-$ T cells. This antigen was slowly induced on CD56$^+$ and CD56$^-$ T cells by stimulation with PMA and ionomycin over a 5-day period, reaching maximum frequencies of 60% of CD56$^-$ T cells and

40% of CD56$^+$ T cells (not shown). However, CD45RO was not induced on either cell type by anti-CD3 activation (Figure 3).

CD56$^+$ T Cells Release IFN-γ and IL-13, but Not IL-10, Upon CD3 Stimulation

PBMCs were expanded with PHA and IL-2, and CD56$^+$ and CD56$^-$ T cells were purified by selection with mAb-coated magnetic beads. Similar numbers of CD56$^+$ and CD56$^-$ T cells were stimulated with anti-CD3 mAb bound to microwell plates at varying concentrations. After 24, 48, or 72 hours, supernatants were collected and assayed for IFN-γ, IL-13, and IL-10 by ELISA. Figure 4 demonstrates that IFN-γ release by both lymphocyte subsets was detectable 24 hours after stimulation with 0.03–3 μg/ml anti-CD3 mAb. Consistently higher amounts of IFN-γ were released by CD56$^+$ T cells compared with similar numbers of CD56$^-$ T cells after 48 hours ($p = 0.04$) and 72 hours ($p = 0.01$). The kinetics of IFN-γ secretion by both cell types was similar, increasing gradually from 0–72 hours. IL-13 was released by both CD56$^+$ and CD56$^-$ T cells in similar amounts and with similar kinetics after stimulation with 0.03–3 μg/ml anti-CD3 mAb (Figure 4). Purified CD56$^-$ T cells consistently released moderate amounts

- ▲ CD56$^+$ T cells
- △ CD56$^-$ T cells
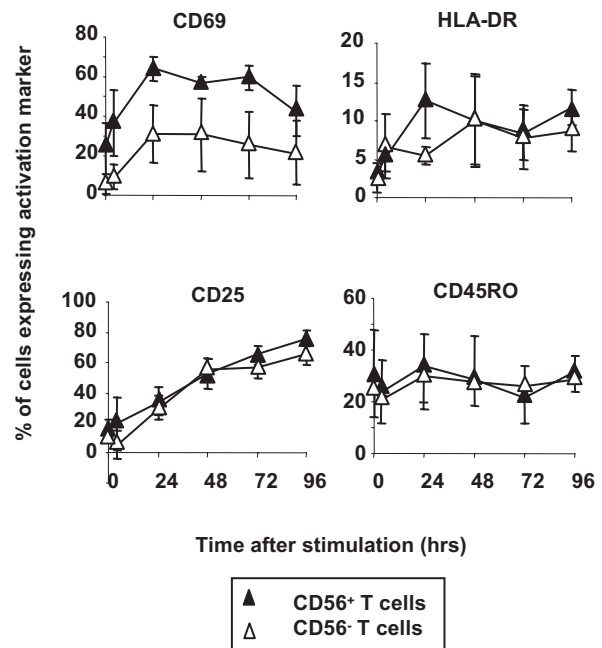
FIGURE 3 Kinetics of activation marker expression by CD56$^+$ and CD56$^-$ T cells after PBMC stimulation with 0.3 μg/ml plate-bound anti-CD3 mAb. Results demonstrate mean frequencies of CD56$^+$ and CD56$^-$ T cells in three samples that express CD69, HLA-DR, CD25, and CD45RO, as determined by flow cytometry. Error bars illustrate standard errors of the means.
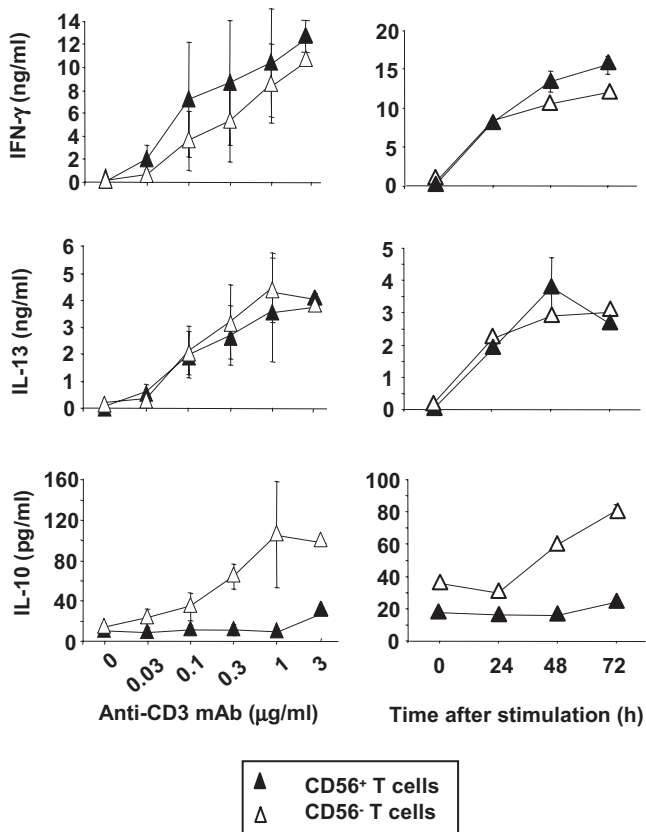
FIGURE 4 Quantification of cytokine secretion by CD56$^+$ and CD56$^-$ T cells after stimulation with plate-bound anti-CD3 monoclonal antibody (mAb). Left, mean levels of interferon-gamma (IFN-γ) and IL-13 released after 48 hours and IL-10 released after 72 hours, after stimulation with 0–3 μg/ml anti-CD3 mAb as measured by enzyme linked immunosorbent assay (means of six experiments). Right, kinetics of IFN-γ, IL-13, and IL-10 release after stimulation with 0.3 μg/ml anti-CD3 mAb (means of three experiments). Error bars illustrate standard errors of the means.

of IL-10 at 48–72 hours after stimulation with 0.03–3 μg/ml anti-CD3 mAb, whereas little or no IL-10 was secreted by resting or stimulated CD56$^+$ T cells (Figure 4). The differences in IL-10 levels released by CD56$^+$ and CD56$^-$ T cells were significant at 48 hours ($p = 0.006$) and 72 hours ($p = 0.01$) after stimulation. These data indicate that CD56$^+$ T cells can release Th1 and Th2 cytokines upon stimulation, but unlike CD56$^-$ T cells, they cannot be induced to release the regulatory cytokine IL-10 by TCR stimulation.

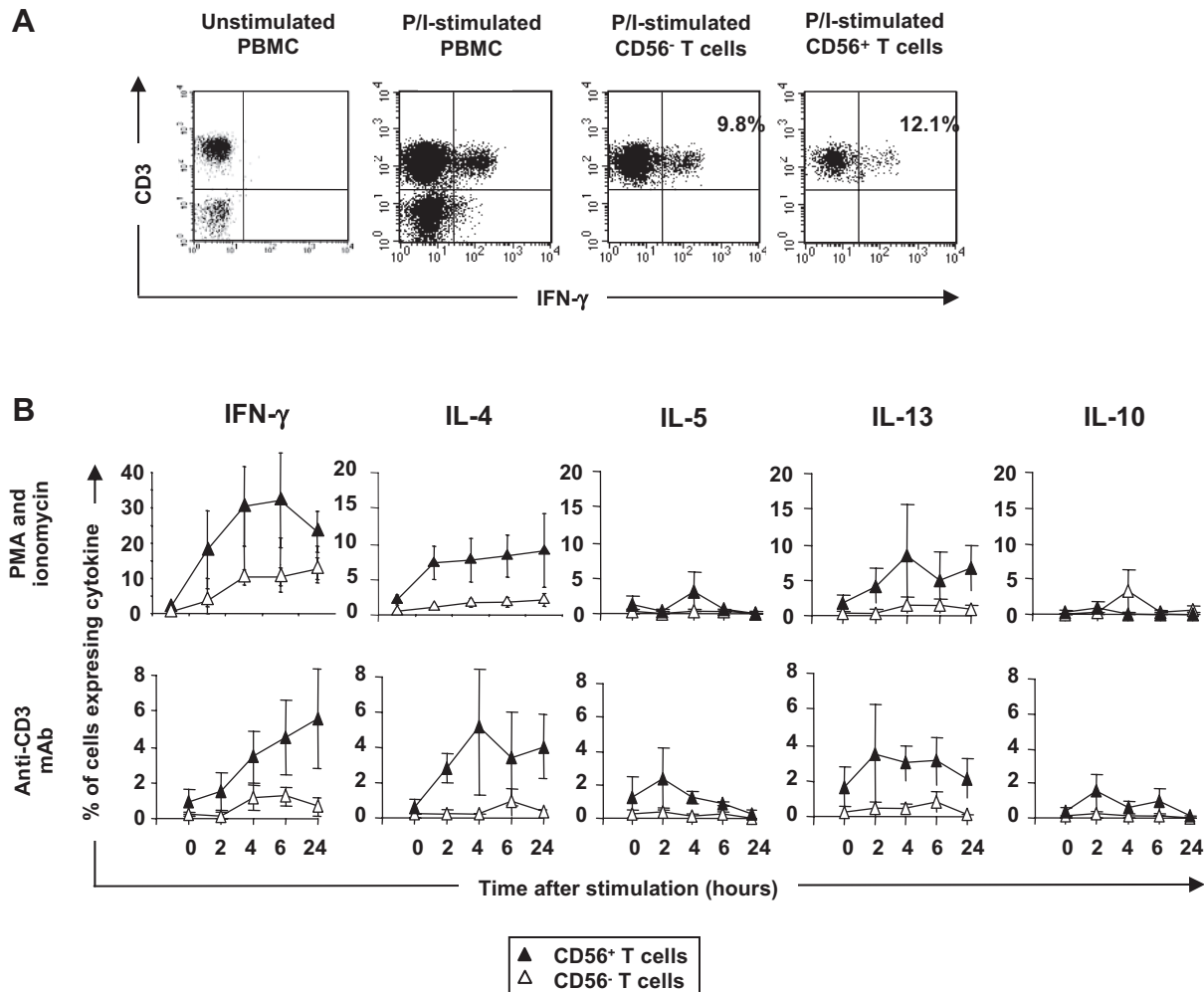### CD56$^+$ T Cells Rapidly Express Intracellular Cytokines Upon Activation

Figure 4 illustrates that CD56$^+$ T cells release IFN-γ and IL-13, whereas CD56$^-$ T cells can release IFN-γ, IL-13, and IL-10 within 24 hours of stimulation with anti-CD3 mAb. Since CD56$^+$ T cells display properties

of innate lymphocytes, it is possible that they become activated to release cytokines in the first few hours after activation. We investigated the early kinetics of intracellular cytokine expression by CD56$^+$ and CD56$^-$ T cells within freshly isolated PBMCs from five donors by using flow cytometry (Figure 5A). No IFN-γ was expressed by unstimulated cells. IFN-γ was expressed by significant proportions of CD56$^+$ T cells (mean 30.3%) and CD56$^-$ T cells (mean 10%) within 2–6 hours of PBMC stimulation with PMA and ionomycin (Figure 5B). Lower proportions of CD56$^+$ T cells (mean 4.5%) and CD56$^-$ T cells (mean 1.2%) expressed IFN-γ after stimulation with anti-CD3 mAb. The proportions of CD56$^+$ T cells that produced IFN-γ, after both types of stimulation, were significantly higher than those of CD56$^-$ T cells ($p = 0.004$ for PMA and ionomycin stimulation and $p = 0.03$ for anti-CD3 mAb stimulation). The mean intensities of fluorescence staining for IFN-γ were similar for CD56$^+$ and CD56$^-$ T cells, suggesting that similar amounts of IFN-γ were produced by both cell types (Figure 5A).

Significant frequencies of CD56$^+$ T cells (mean 8.4%) and CD56$^-$ T cells (mean 1.5%) produced IL-4 after PBMC stimulation for up to 6 hours with PMA and ionomycin, and lower percentages (means 5.2% and 0.9%, respectively) produced IL-4 after anti-CD3 mAb stimulation (Figure 5B). Consistently higher frequencies of CD56$^+$ T cells produced IL-4 compared with CD56$^-$ T cells ($p = 0.03$ for PMA and ionomycin and $p = 0.02$ for anti-CD3 mAb). Intracellular IL-13 was also detected in significantly greater frequencies of CD56$^+$ T cells compared with CD56$^-$ T cells (after 4 hours means 8.4% and 1.5%, respectively, $p = 0.01$ for PBMC stimulation with PMA and ionomycin and means 3.0% and 0.5%, respectively, $p = 0.001$ for stimulation with anti-CD3 mAb, Figure 5B). IL-5 and IL-10 were expressed by very low proportions (<3%) of CD56$^-$ and CD56$^+$ T cells after stimulation of the PBMCs with PMA and ionomycin or anti-CD3 mAb (Figure 5B).

DISCUSSION

CD56$^+$ T cells belong to a heterogenous group of human T cells that express various stimulatory, costimulatory, inhibitory, and adhesion receptors that control NK cell activity [3–5, 33–35]. T cells with NKRs, including CD56$^+$ T cells, are often present in cultures of stimulated T cells [4, 11, 13–17], and they frequently express memory phenotypes [2, 36, 37]. They can be activated by TCR ligation and by antigen-nonspecific signals through NKRs and cytokine receptors [4, 11, 25, 33–35] and display potent MHC-unrestricted cytotoxicity against a range of tumor cells *in vitro* and *in vivo* [3, 11, 12, 18, 19, 25].

FIGURE 5 Flow cytometric detection of intracellular cytokine expression by CD56⁺ and CD56⁻ T cells after stimulation of fresh PBMCs with phorbol myristate acetate (PMA) and ionomycin (P/I), or 0.3 μg/ml plate-bound anti-CD3 mAb. (A) Representative flow cytometric dot plots of IFN-γ expression by unstimulated and PMA and ionomycin-stimulated total PBMCs, and PMA and ionomycin-stimulated electronically gated CD56⁻ and CD56⁺ T cell subsets. (B) Kinetics of IFN-γ, IL-4, IL-5, IL-13, and IL-10 expression by CD56⁺ and CD56⁻ T cells after stimulation of PBMCs with PMA and ionomycin (top) or anti-CD3 mAb (bottom). Results are means of five experiments for IFN-γ and IL-4, and three experiments for IL-5, IL-13, and IL-10. Error bars illustrate standard errors of the means.

Many NKR⁺ T cells can recognize nonprotein components of pathogens and host cells. Such "innate lymphocytes" include NKT cells, which recognize glycolipids presented by the MHC class I-like molecule CD1d [27, 38], and γδ T cells, which respond to soluble metabolites, heat shock proteins, or glycolipids presented by CD1c [26]. NKT cells and γδ T cells most notably display rapid Th1, Th2, and/or regulatory cytokine secretion and are thought to be important for the initiation, polarization, and regulation of adaptive immune responses [24, 39–42]. Defects in cytokine production by NKT cells and γδ T cells have been associated with failure to eliminate pathogens and tumors and the development of autoimmune diseases in murine models and

in humans [26, 27, 38]. Human CD56⁺ T cells display functional similarities (responsiveness to innate cytokines and MHC-unrestricted cytotoxicity) to these innate lymphocytes [3, 4, 11, 12, 18, 19, 25, 33, 34] and are altered in disease [23, 28–32], but it is not clear whether these cells can similarly initiate and/or regulate adaptive immune responses.

In the present study, we confirm previous reports that human CD56⁺ T cells can be expanded from fresh PBMCs by TCR-mediated activation, particularly when grown in the presence of exogenous IL-2 [4, 11, 13–17]. We found that a single CD3/TCR stimulation of PBMCs in the absence of added growth factors resulted in three-fold expansions of CD56⁺ T cells but no increase in total

cell numbers. Expansion of PBMCs by two rounds of PHA stimulation and culturing with IL-2 resulted in a mean 37-fold increase in CD56$^+$ T cell numbers, expanding from 3%–27% of all T cells in 28 days. Consistent with previous reports that CD56 is present on T cells at a later stage of differentiation [13], purified expanded CD56$^+$ T cells displayed a lower proliferative potential than CD56$^-$ T cells from the same cultures. As previously reported [11, 25], CD56$^+$ T cells, only, were capable of killing the NK-sensitive target cell line, K562, but this cytolytic activity was less potent than that of NK cells. We found that PHA and IL-2–induced expansions of CD56$^+$ T cells are the result both of the induction of CD56 expression by CD56$^-$ T cells and of the proliferation of CD56$^+$ T cells. Expansion of PBMCs that were first depleted of CD56$^+$ T cells with PHA and IL-2 resulted in the expression of CD56 by a mean of 37% of T cells after 28 days. This suggests that CD56 can be induced on T cells by activation. It is possible that the CD56$^+$ T cells in these cultures arose from expansions of small numbers of contaminating CD56$^+$ T cells; however, we feel that this is unlikely since CD56$^+$ T cells were found to proliferate less vigorously than CD56$^-$ T cells.

Phenotypic analysis of CD56$^+$ T cells generated by mitogen activation and culturing with IL-2 *in vitro* indicated that CD56 is induced on CD8$^+$ and double-negative CD4$^-$CD8$^-$ T cells, but not CD4$^+$ T cells, even though CD56$^+$ T cells expressing CD4 were present in fresh blood. CD4$^+$, CD8$^+$, and double-negative CD56$^+$ T cells are also present in the liver [6, 7] and the small intestine [8–10]. CD56 was induced on αβ T cells and NKT cells expressing the invariant Vα24Jα18 TCR but less frequently on γδ T cells, even though CD56$^+$ γδ T cells are present in significant numbers in blood. The phenotypic differences between naturally occurring and *ex vivo* activation-induced CD56$^+$ T cells indicate that different signals are required for the expansion of different populations of CD56$^+$ T cells, and the changes in CD56$^+$ T cell numbers in disease [23, 28–32] may reflect changes in subpopulations of this group of cells.

Examination of activation marker expression by freshly isolated PBMC subsets by flow cytometry revealed that the early activation marker, CD69, is expressed by greater frequencies of CD56$^+$ T cells compared with CD56$^-$ T cells, but CD25, HLA-DR, and CD45RO are expressed by similar proportions of each lymphocyte subset. Each activation marker examined was induced on a proportion of CD56$^+$ and CD56$^-$ T cells with similar kinetics after treatment with PMA and ionomycin, but CD69 and CD25, only, were induced on significant proportions of both T cell subsets following anti-CD3 mAb stimulation. Since both CD56$^+$ and CD56$^-$ T cells in fresh PBMCs include cells that are positive and cells that are negative for activation markers, and since both T cell subsets demonstrate similar kinetics of activation marker expression following activation, it is possible that CD56$^+$ T cells in fresh blood include both naive and effector T cells. Further studies are required to ascertain whether induced CD56$^+$ T cells express memory cell phenotypes. Our observations also suggest that CD56$^+$ T cells are not activated more rapidly than CD56$^-$ T cells.

We examined the cytokine secretion profiles of human CD56$^+$ T cells to determine whether they display the rapid Th1/Th2/regulatory cytokine secretion properties that are characteristic of innate lymphocytes, such as NKT cells and γδ T cells [24, 39–42]. CD56$^+$ and CD56$^-$ T cells were purified from PHA and IL-2–expanded PBMC cultures by mAb-coated magnetic bead selection. Similar numbers of purified CD56$^+$ and CD56$^-$ T cells were given a single stimulation with varying amounts of crosslinking anti-CD3 mAb, and samples of supernatant were removed at various times poststimulation for analysis of IFN-γ, IL-13, and IL-10 levels by ELISA. As previously reported for sorted CD8$^+$ CD56$^+$ T cells [3, 5], CD56$^+$ T cells were found to release higher levels of the Th1 cytokine, IFN-γ than did similar numbers of CD56$^-$ T cells. We found that the kinetics of IFN-γ release were similar for both lymphocyte subsets, with maximal levels detectable after 48 hours. Furthermore, the strengths of the stimuli, based on the concentrations of anti-CD3 mAb used, required to induce IFN-γ release were the same for CD56$^+$ and CD56$^-$ T cells. Similar amounts of the Th2 cytokine, IL-13, were released by CD56$^+$ and CD56$^-$ T cells following the same stimulus strengths and similar kinetics. However, CD56$^+$ T cells could not be induced to release IL-10, whereas CD56$^-$ T cells consistently released significant amounts of this regulatory cytokine within 48 hours of stimulation with anti-CD3 mAb. The differences in IL-10 secretion by CD56$^+$ and CD56$^-$ T cells were significant. Our data indicate that CD56$^+$ T cells can promote Th1 or Th2 responses but are unlikely to regulate them via IL-10 secretion.

We investigated whether CD56$^+$ T cells are more likely to produce cytokines than CD56$^-$ T cells at the early stages (2–24 hours) after stimulation, by activating fresh PBMCs in the presence of brefeldin A and determining the percentages of CD56$^+$ and CD56$^-$ T cells that express intracellular IFN-γ, IL-4, IL-5, IL-13, or IL-10, by flow cytometry. We found that IFN-γ, IL-4 and IL-13, but not IL-5 or IL-10, were expressed by significant proportions of CD56$^+$ T cells within 2 hours of stimulation with either PMA and ionomycin or anti-CD3 mAb. Significantly lower proportions of CD56$^-$ T cells expressed these cytokines. IFN-γ and IL-4 have

previously been shown to be expressed by higher frequencies of CD56$^+$ compared with CD56$^-$ T cells upon 4 hours of stimulation in fresh peripheral blood [4] and liver [15, 25] mononuclear cells. In contrast, Loza *et al.*, used flow cytometry to demonstrate that IFN-γ expression is higher, but IL-4 and IL-13 expression is lower, in CD56$^+$ T cells compared with CD56$^-$ T cells in cultures that were matured with IL-2, IL-12, IL-18, and/or IFN-α, indicating that the culture conditions used to generate CD56$^+$ T cells are likely to influence the cytokine profiles [13]. For this reason, we avoided using Th cell polarizing cytokines, such as IL-12 or IFN-γ, to expand CD56$^+$ T cells, as was done for the generation of antitumor cytokine-induced killer cells [11, 13–17]. Anfossi and coworkers have demonstrated that murine and human CD8$^+$ T cells that express other NKRs (Ly49 or killer immunoglobulinlike receptors), display reduced frequencies of IFN-γ and TNF-α expression compared with NKR$^-$ CD8$^+$ T cells [43, 44], whereas Kambayashi *et al.* reported that murine influenza-specific NKR$^+$ T cells produce IFN-γ in response to influenza antigens [45].

Our combined ELISA and flow cytometry results indicate that human CD56$^+$ T cells are more likely to produce Th1 (IFN-γ) and Th2 (IL-4 and IL-13) cytokines than CD56$^-$ T cells at early times poststimulation, that CD56$^+$ T cells release higher amounts of IFN-γ but similar amounts of IL-13 compared with CD56$^-$ T cells after 48 hours, and that CD56$^+$ T cells do not produce IL-5 or IL-10. The apparent differences in cytokine production as detected by flow cytometry and ELISA may reflect different cytokine-mediated functions at different stages of an immune response. Cytokines released at an early stage after activation are likely to be directed into the immunologic synapse between the T cell and antigen-presenting cell, and very small quantities of cytokine may be sufficient to saturate this microenvironment. In contrast, the large amounts of cytokine that accumulate in supernatants over 48 hours, and that are detectable by ELISA, are more likely to target multiple cells in the region of the T cell. These two scenarios of cytokine secretion are mediated by two directionally distinct export pathways [46]. The enhanced ability of some CD56$^+$ T cells to produce cytokines at an early stage suggests that they can function as innate lymphocytes like CD1-restricted NKT cells and $\gamma\delta$ T cells, which can stimulate maturation of dendritic cells [47, 48] into antigen-presenting cells as well as releasing Th1/Th2 cytokines.

Our data indicate that CD56 expression can be induced on CD56$^-$ T cells by TCR-mediated activation, and that the expression of this marker is associated with altered cytokine secretion profiles in addition to the acquisition of MHC-unrestricted cytotoxic activity. The acquisition of MHC-unrestricted cytotoxic activity by CD56$^+$ T cells would allow them to kill target cells in an antigen-nonspecific manner, perhaps as a means to combat pathogens and tumors that undergo antigenic drift and can evade conventional MHC-restricted T cells. Their ability to release IFN-γ and IL-13 in response to TCR stimulation indicates that they can also promote Th1 and Th2 responses. In contrast to CD56$^-$ T cells, activation-induced CD56$^+$ T cells do not secrete IL-10. Thus, CD56$^+$ T cells may emerge in parallel with inducible regulatory T cells [49] and may provide a counter-regulatory cell population that can activate adaptive immune responses in certain tissues. This could occur in the liver and intestine, where tolerance is favored over active immunity and where CD56$^+$ T cells are particularly abundant [6–10]. The ease by which CD56$^+$ T cells can be expanded from PBMCs supports their use as possible therapeutic modulators of immune responses in infectious and immune-mediated diseases, in addition to their potential as antitumor cytotoxic effector cells.

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