IL-21-Mediated Potentiation of Antitumor Cytolytic and Proinflammatory Responses of Human Vγ9Vδ2 T Cells for Adoptive Immunotherapy[1]

Aurélie Thedrez,* Christelle Harly,* Alexis Morice,* Samuel Salot,† Marc Bonneville,[2]* and Emmanuel Scotet[2]*

Vγ9Vδ2 T lymphocytes are a major human γδ T cell subset that react against a wide array of tumor cells, through recognition of phosphorylated isoprenoid pathway metabolites called phosphoantigens. Immunotherapeutic protocols targeting Vγ9Vδ2 T cells have yielded promising, yet limited, signs of antitumor efficacy. To improve these approaches, we analyzed the effects on γδ T cells of IL-21, a cytokine known to enhance proliferation and effector functions of $CD8^+$ T cells and NK cells. IL-21 induced limited division of phosphoantigen-stimulated Vγ9Vδ2 T cells, but did not modulate their sustained expansion induced by exogenous IL-2. Vγ9Vδ2 T cells expanded in the presence of IL-21 and IL-2 showed enhanced antitumor cytolytic responses, associated with increased expression of CD56 and several lytic molecules, and increased tumor-induced degranulation capacity. IL-21 plus IL-2-expanded Vγ9Vδ2 T cells expressed higher levels of inhibitory receptors (e.g., ILT2 and NKG2A) and lower levels of the costimulatory molecule NKG2D. Importantly, these changes were rapidly and reversibly induced after short-term culture with IL-21. Finally, IL-21 irreversibly enhanced the proinflammatory Th1 polarization of expanded Vγ9Vδ2 T cells when added at the beginning of the culture. These data suggest a new role played by IL-21 in the cytotoxic and Th1 programming of precommitted Ag-stimulated γδ T cells. On a more applied standpoint, IL-21 could be combined to IL-2 to enhance γδ T cell-mediated antitumor responses, and thus represents a promising way to optimize immunotherapies targeting this cell subset. ***The Journal of Immunology,*** **2009, 182: 3423–3431.**

Most peripheral blood γδ T cells in human adults express a particular combination of TCR variable regions, Vγ9 and Vδ2. Vγ9Vδ2 T cells, which make up 0.5–5% of the peripheral lymphoid pool, recognize in vitro a wide array of transformed and infected cells and are activated in vivo in a variety of infectious and tumor contexts. This broad reactivity pattern is due to Vγ9Vδ2 TCR-dependent recognition of a particular set of phosphorylated compounds, referred to as phosphoantigens. Phosphoantigens are produced through the isoprenoid pathway shared by either mammalian cells (mevalonate pathway) or microorganisms (desoxyxylulose-phosphate pathway) (1–3). Owing to their ability to directly kill tumor cells and produce inflammatory cytokines boosting antitumor properties of other immune effectors (such as IFN-γ), Vγ9Vδ2 T cells probably contribute to protective immunity against cancers. These observations, and the recent availability of synthetic clinical grade phosphoantigens or pharmacological inhibitors of the mevalonate pathway (e.g., aminobisphosphonates) able to trigger Vγ9Vδ2 T cell proliferation and antitumor responses (4–7), have fostered development of immunotherapeutic approaches targeting this γδ subset (see for recent reviews Refs. 7–9). Phosphoantigens or aminobisphosphonates together with IL-2 can trigger the selective outgrowth of Vγ9Vδ2 T cells in vitro and in vivo in both preclinical (nonhuman primate) models and in cancer patients (10, 11). Passive immunotherapy, resting on adoptive transfer of autologous in vitro expanded Vγ9Vδ2 T cells (12–15), has been so far evaluated in phase I trials in metastatic renal carcinoma patients (16, 17). Regarding active immunotherapy, both preclinical studies and phase I/II trials performed in myeloma, lymphoma, and metastatic renal carcinoma and prostate cancer patients have demonstrated efficient but transient in vivo Vγ9Vδ2 T cell systemic expansions after treatment with γδ agonists and IL-2 (10, 18, 19). These treatments are generally well tolerated with limited side effects and may lead to disease stabilization or partial tumor regression in some treated patients (17–19).

Although yielding encouraging results in terms of feasibility, tolerance and preliminary efficacy, Vγ9Vδ2-based immunotherapies might not yet be optimal owing in particular to the limited, yet significant, killing of primary tumors by these lymphocytes. A way to improve clinical efficacy of Vγ9Vδ2 T cell-based immunotherapies could be through enhancement of the overall antitumor effector response, possibly with potentiators of cytolytic and proinflammatory properties. In this respect, IL-21 represents a good candidate for such a purpose. IL-21R is broadly expressed by lymphoid cells, including activated γδ T cells (20). This cytokine acts synergistically with IL-2, IL-7, or IL-15 to induce proliferation of conventional naive and memory

*Institut National de la Santé et de la Recherche Médicale, Unité 892, Centre de Recherche en Cancérologie Nantes-Angers, Nantes, France, and †Innate Pharma, Marseille, France

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[2] Address correspondence and reprint requests to Dr. Emmanuel Scotet or Dr. Marc Bonneville, Institut National de la Santé et de la Recherche Médicale, Unité 892, Centre de Recherche en Cancérologie Nantes-Angers, Institut de Biologie, Centre Hospitalier de l'Université, 9 quai moncousu, 44035 Nantes Cedex, France. E-mail addresses: Emmanuel.Scotet@inserm.fr or bonnevil@inserm.fr

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$CD8^+$ T cells (21) as well as CD1d-restricted NKT cells (22, 23). Similarly IL-21 has been shown to drive short-term proliferation of human γδ T cells (20, 24). On a functional standpoint, IL-21 has been involved in the functional polarization of T cells, more particularly toward production of proinflammatory Th17 cytokines like IL-21, IL-22, and IL-17, and it may affect Th1/Th2 polarization as well, reviewed in (25). It also enhances antitumor immunity in vitro and in vivo through mechanisms involving up-regulation of perforin and granzyme production by NK or $CD8^+$ T cells (26–28). Although IL-21 leads to acquisition of a follicular B helper-like phenotype by human Vγ9Vδ2 T cells (20, 24), its effects on cytokine and cytolytic responses elicited by tumor cells have not yet been assessed.

In this study, we show that human recombinant IL-21: 1) does not sustain, by itself, long-term Ag-induced Vγ9Vδ2 T cell proliferation in vitro, 2) neither inhibits nor enhances IL-2-induced proliferation of Ag-stimulated γδ T cells, 3) dramatically increases, in a rapid but reversible manner, the expression of several lytic effector molecules and antitumor lytic activity of γδ T cells and, 4) drives Vγ9Vδ2 T cell cytokine polarization during in vitro expansion toward an effector proinflammatory phenotype. Altogether our results indicate that IL-21 can be combined with IL-2 for in vitro or in vivo generation of Vγ9Vδ2 T cells showing enhanced antitumor functions. Moreover, our data provide new evidence that IL-21 can affect the differentiation/polarization of human γδ T cells, by driving them toward a proinflammatory Th1 cytokine profile.

Materials and Methods

Abs and flow cytometry

The following mAbs (with clone number) were used for extracellular stainings: Vδ2-FITC (IMMU389), CD3-PC5 (UCHT1), CD56-PE (N901), CD45RA-PE (ALB11), CD27-PC5 (1A4), CD244-PE (C1.7), NKG2D-PE (ON72), NKG2A-PE (Z199), ILT2-PE (HP-F1) from Immunotech/Beckman Coulter; CD107a-FITC (H4A3) and CD107b-FITC (H4B4) from BD Biosciences; and Vγ9-allophycocyanin (7B6) provided by Innate Pharma. For intracellular stainings, IFN-γ-allophycocyanin (B27), IL-4-PE (MP4-25D2), granzyme A-FITC (CB9), granzyme B-FITC (GB11), and perforin-PE (γG9) were obtained from BD Biosciences. Isotype-matched mAbs were obtained from BD Biosciences, Beckman Coulter, or R&D Systems and used as staining controls. Flow cytometry acquisition and analysis were performed by using LSR/FACSCalibur (BD Biosciences) and CellQuest Pro (BD Biosciences) systems with FlowJo (Tree Star) software.

Reagents

Synthetic C-HDMAPP (IPH1201/Picostim) was provided by Innate Pharma (29). Recombinant human IL-2 (Proleukin) and IL-21 were obtained, respectively, from Chiron Therapeutics and Clinisciences. CFDA-SE, (CFSE) was obtained from Molecular Probes/Invitrogen. Brefeldin A, L-glutamine, streptomycin, penicillin, monensin, PMA, ionomycin, and RPMI 1640 were purchased from Sigma-Aldrich.

Tumor cell lines

The 786-0 (renal adenocarcinoma) and Raji (Burkitt's lymphoma) human tumor cell lines were obtained from the American Type Culture Collection and cultured at 37°C in RPMI-FCS medium (RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin).

Expansion of human Vγ9Vδ2 PBL

PBMC of human healthy donors (identified in this study as Donor A–H) were isolated from blood samples and obtained from the Etablissement Français du Sang (Nantes, France) after Ficoll-Hypaque (Eurobio) density centrifugation. For Vγ9Vδ2 PBL expansions, fresh or frozen PBMC (1×10^6 cells/ml) were specifically activated by C-HDMAPP (80 nM) in RPMI-FCS medium supplemented with recombinant human IL-2 (20 ng/ml) or IL-21 (10 ng/ml). After 4 days, cultures were supplemented with IL-2 (60 ng/ml) or IL-21 (30 ng/ml). Specific expansion of $V\delta2^+$ T cells within PBL was measured by calculating frequency (Vδ2/CD3 stainings) and absolute (Vδ2 T cell number) at days 5, 7, 10, 14, and 20 following activation. The fold amplification rates of $V\delta2^+$ T cells were calculated according to the following formula: (absolute number of $V\delta2^+$ T cells)/(absolute number of $V\delta2^+$ T cells at day 0). Resting Vγ9Vδ2 PBL lines expanded under these conditions were checked for purity by flow cytometry (>80% of $V\delta2^+$ T cells) and subsequently used in phenotype/functions experiments. For gene expression analysis, γδ T cells were purified following in vitro expansion by positive magnetic selection by using MACS technology (Miltenyi Biotec), according to the manufacturer's instructions.

Cell division analysis

Freshly isolated PBMC were labeled with CFSE (2 μM) for 15 min at 37°C, washed and maintained for 15 min at 37°C in RPMI-FCS medium to allow the release of dye excess, according to the manufacturer's instructions. Labeled cells (1×10^6 cells/ml) were activated by C-HDMAPP (80 nM) in the presence of IL-2 (60 ng/ml) or IL-21 (30 ng/ml). After 3 days of culture, cells were harvested and stained for TCR Vγ9 chain, CD27, and CD45RA surface expression. Cell division profile and surface markers expression were measured by flow cytometry. Peaks of cell division and frequency were calculated by using the *Proliferation Platform* of the FlowJo analysis software.

^{51}Cr release assays

Cytolytic activity of Vγ9Vδ2 PBL was measured by a standard 4-h ^{51}Cr release assay (30). Percentage of tumor target cell lysis was calculated according to the following formula: (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. Maximum and spontaneous release were determined, respectively, by adding 0.1% Triton X-100 or medium to ^{51}Cr-labeled tumor target cells in the absence of γδ T cells. Data are presented as the mean of triplicate samples. Lytic units were calculated by using the formula described by Friberg and colleagues (31). Lytic units were used to represent lytic activity that increases with potency per lytic batch, where a lytic batch equals 10^6 effector cells. An estimate of lysis at 20% was calculated from a regression line plotting percentage of lysis by E:T ratio of 20 by using the following formula: $10^6/(20)(3 \times 10^3)$, where 10^6 was the standard number of effector cells and 3×10^3 was the standard number of targets.

CD107 mobilization assays

Vγ9Vδ2 T cells (2×10^5 cells/well) were activated by either C-HDMAPP (80 nM) or tumor cells at a γδ T cell to target cell ratio of 1:1 at 37°C in RPMI-FCS containing monensin (10 μM) and a combination of FITC-conjugated anti-CD107a and anti-CD107b mAbs. After 4 h, cells were harvested, stained with a TCR Vγ9-specific mAb and fixed with 0.5% paraformaldehyde. Double-stained cells were analyzed by flow cytometry.

Comparative gene expression analysis

Vγ9Vδ2 PBL were amplified within human PBMC of three different healthy donors under IL-2 or IL-2+IL-21 conditions and purified 3 wk after the initial C-HDMAPP activation by using magnetic positive γδ selection (purity >99%) and stored as frozen pellets. RNA was extracted (NucleoSpin RNA II; Macherey-Nagel) and quality checked (RNA integrity number between 9.6 and 10) using an Agilent 2100 Bioanalyzer platform (Agilent Technologies). The total of 1 μg of total RNA was used for a linear T7-based amplification step and checked as described. Fluorescently labeled (Cy3/Cy5) cDNAs were hybridized to topic-defined glass slides (1076 genes spotted in quadruplicates) PIQOR Immunology Microarrays (Miltenyi Biotec) and scanning was performed by the Miltenyi Microarray Service. Hybridization, scanning, and data analysis were performed according to the PIQOR protocol and comply with the MIAME standards (minimum information about a microarray experiment standards). In this assay, the PIQOR Analyzer calculates all normalized mean Cy5/Cy3 ratio (IL-2+IL-21 to IL-2 ratio) of the four replicates per gene (including the respective coefficient of variation). A fold change ≥1.7 was considered to be a good discriminatory criteria to select genes of interest. Genes that did not pass the quality filtering for very low signal intensity were not evaluated (<2-fold above average signal intensity of the background in both Cy3 and Cy5 channels). Data have been deposited in the NCBI GEO database (http://geo@ncbi.nlm.nih.gov) as accession number GSE13912.

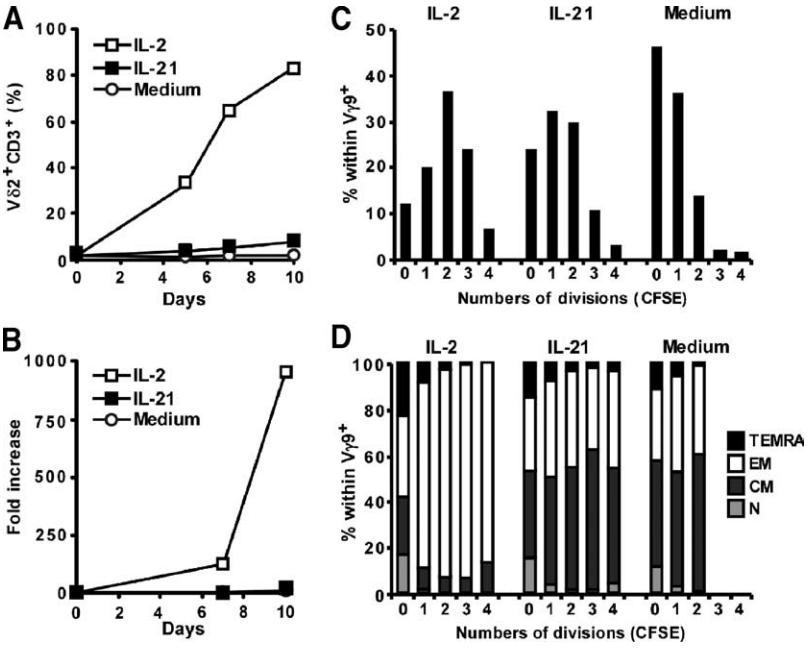

FIGURE 1. Effects of IL-21 on the expansion of Vγ9Vδ2 PBL following activation by phosphoantigens. *A* and *B*, kinetics of $V\delta2^+CD3^+$ cells frequency within PBMC of a healthy human donor (Do#A) (*A*) and corresponding fold amplifications relative to day 0 (*B*) were measured following a specific activation (C-HDMAPP, 80 nM) performed in the absence of any cytokine (Medium) or in IL-2- or IL-21-supplemented medium. *C*, distribution of Vγ9Vδ2 PBL according to their CFSE fluorescence is shown at day 3 in culture performed under conditions described in *A* and *B*. *D*, analysis of the memory status of Vγ9Vδ2 PBL generated in the same experiment. The percentage of naive (N) ($CD27^+/CD45RA^+$), central memory (CM) ($CD27^+/CD45RA^-$), effector memory (EM) ($CD27^-/CD45RA^-$), and terminally differentiated effector memory (TEMRA) ($CD27^-/CD45RA^+$) cells are indicated within each dividing γδ subset. One representative result of at least three experiments performed by using PBMC samples from different donors is shown.

Measurement of IFN-γ and IL-4 responses

γδ T cells (3×10^4 cells/well) were activated with C-HDMAPP (0.8 and 80 nM) or PMA/ionomycin at 37°C in RPMI-FCS medium (200 μl/well). After 24 h, supernatants were harvested and analyzed for IFN-γ and IL-4 secretion by ELISA following standard procedures. Data are presented as the mean of triplicate samples. For intracellular cytokine detection, γδ T cells (2×10^6 cells/well) were activated for 5 h at 37°C in RPMI-FCS by adding grading doses of C-HDMAPP (up to 100 nM) or PMA/ionomycin. After 2 h, intracellular accumulation of cytokines was induced by adding brefeldin A (10 μg/ml). After 3 h, cells were collected, stained for TCR Vδ2 chain expression and fixed at 4°C with 0.5% paraformaldehyde. Fixation was followed by a cell permeabilization with BD PhosFlow Perm/Wash buffer (BD Biosciences) for 20 min and by incubation with IFN-γ- and IL-4-specific mAbs for 30 min. Stained cells were analyzed by flow cytometry.

Results

IL-21 fails to induce long-term proliferation of ex vivo Vγ9Vδ2 PBL following phosphoantigen activation

We studied the effects of recombinant human IL-21 on human ex vivo Vγ9Vδ2 PBL, by measuring its ability to trigger either cell division or differentiation, following exposure to C-HDMAPP, a potent synthetic Vγ9Vδ2 T cell agonist. Grading doses of IL-21 induced limited and transient increase of the frequency of $V\delta2^+$ T cells within PBMC (from 10 to 20%) but did not promote efficient γδ T cell expansion when compared with IL-2 (>80%) (Fig. 1, *A* and *B*) (also see supplemental Fig. S1).[3] Accordingly γδ T cells could not be maintained more than 10 days in IL-21-supplemented cultures. These observations suggest that IL-21 induces cell division but is not sufficient, by itself, to sustain full proliferation of activated Vγ9Vδ2 T cells. To further address this issue, we combined CFSE dilution assays and phenotypic analysis of the memory status of Vγ9Vδ2 T cells, 3 days after initial antigenic activation. As shown in Fig. 1*C*, IL-21 induced a significant entry into cell division (more than 75% of dividing cells in which more than 50% achieved 1–2 divisions). However, the average number of cell divisions was much higher in IL-2-supplemented cultures, in which the majority of activated γδ T cells achieved 2–3 divisions at the same time point. In line with a previous study (24), γδ T cells with a central memory ($CD27^+CD45RA^-$) phenotype were more frequent in IL-21- than IL-2-supplemented cultures,

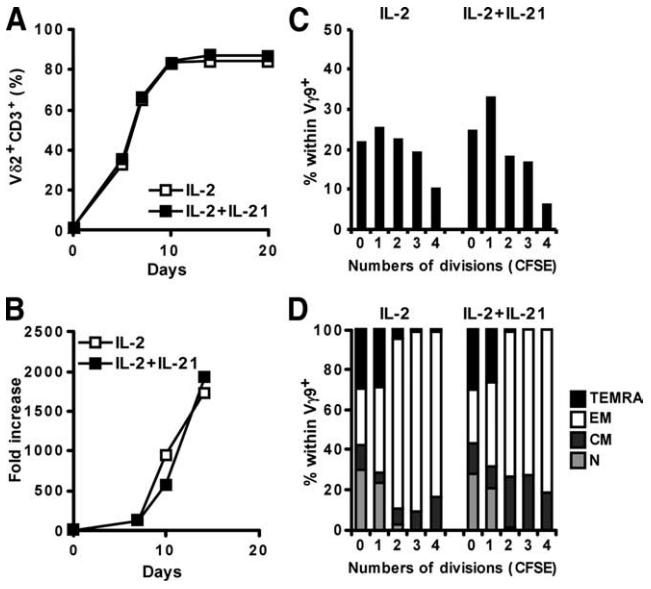

FIGURE 2. Effects of IL-21 in combination with IL-2 on the expansion of Vγ9Vδ2 PBL following activation by phosphoantigens. *A* and *B*, Kinetics of $V\delta2^+CD3^+$ cell frequency within PBMC of a healthy human donor (Do#B) (*A*) and corresponding fold amplification relative to day 0 (*B*) were measured following a specific activation (C-HDMAPP, 80 nM) performed in the absence of any cytokine (Medium) or in IL-2- or IL-2+IL-21-supplemented medium. *C*, distribution of Vγ9Vδ2 PBL according to their CFSE fluorescence is shown at day 3 in culture performed under the conditions described in *A* and *B*. *D*, analysis of the memory status of Vγ9Vδ2 PBL generated in the same experiment. Percentages of naive (N) ($CD27^+/CD45RA^+$), central memory (CM) ($CD27^+/CD45RA^-$), effector memory (EM) ($CD27^-/CD45RA^-$), and terminally differentiated effector memory (TEMRA) ($CD27^-/CD45RA^+$) cells are indicated within each dividing γδ subset. One representative result of at least three experiments performed using PBMC samples from different donors is shown.

[3] The online version of this article contains supplemental material.

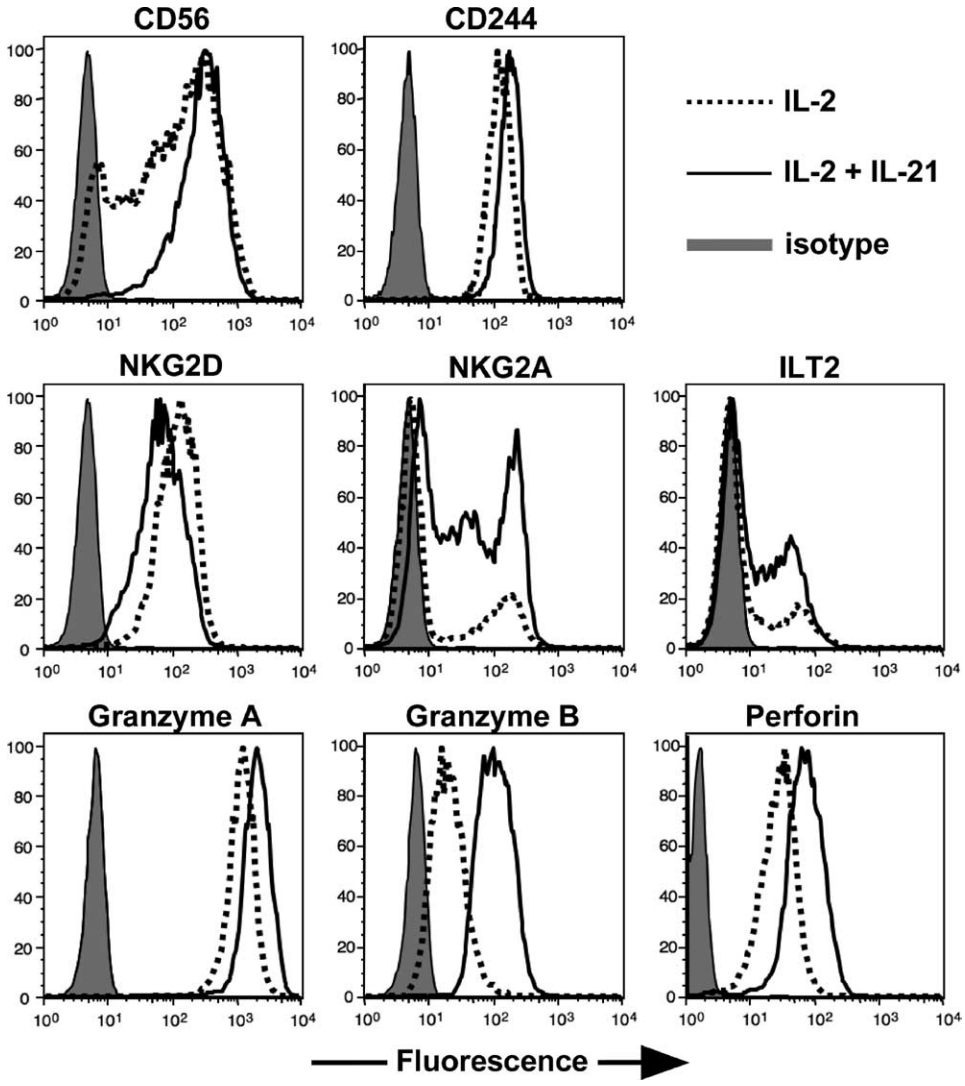

FIGURE 3. IL-21 modulates the expression NK receptors and lytic molecules in Vγ9Vδ2 PBL expanded following activation by phosphoantigens. Expression of cell surface markers (CD56, CD244, NKG2D, NKG2A, and ILT2) and intracellular lytic molecules (granzyme A, granzyme B, and perforin) was measured at day 25 postactivation by flow cytometry in Vγ9Vδ2 PBL (Do#D) generated in IL-2- or IL-2+IL-21-supplemented medium. Background stainings obtained using isotype control Abs are also shown (gray-filled histogram).

even among cells having achieved several divisions (Fig. 1*D*). Altogether these results indicate that IL-21, though triggering γδ T cell division with maintenance of the central memory subset, is unable to promote long-term ex vivo expansion of activated human PBMC-Vγ9Vδ2 T cells.

IL-21 neither synergizes nor interferes with IL-2

We further analyzed the pro-proliferative activity of IL-21 in combination with IL-2 on the ex vivo expansion of PBMC-Vγ9Vδ2 T cells. IL-21 did not synergize or interfere with IL-2-induced proliferation because similar enrichment for Vγ9Vδ2 T cells (>80% of $V\delta2^+$ T cells) and expansion (>2.000-fold) were observed in IL-2- vs IL-2+IL-21-supplemented cultures (Fig. 2, *A* and *B*). Moreover, IL-21 did not synergize nor inhibited entry into cell division of activated γδ T cells, as indicated by the similar CFSE profiles of PBMC cultured in both conditions (Fig. 2*C*). Finally, like IL-2-supplemented cultures, most Vγ9Vδ2 T cells stimulated by both IL-21 and IL-2 displayed an effector memory phenotype (Fig. 2*D*). This response suggests that the increased frequency of central memory cells within IL-21-supplemented cultures reflects the limited proliferative activity of these cells rather than a specific effect targeting a particular memory subset.

IL-21 polarizes Vγ9Vδ2 T cells toward a CTL effector phenotype

To further investigate the effects of IL-21 on human γδ T cell differentiation, we performed a comparative phenotypic analysis of ex vivo Ag-stimulated PBMC-Vγ9Vδ2 T cells in IL-2 alone or in combination with IL-21, using mAbs specific for chemokine and NK receptors, adhesion molecules, activation and memory markers, and lytic effector molecules. Vγ9Vδ2 T cells that expanded in the presence of either IL-2 plus IL-21 or IL-2 alone expressed similar levels of γδ TCR, chemokine receptors (e.g., CXCR3), adhesion molecules (e.g., CD11a), and activation/memory markers (e.g., CD16) (see supplemental Table S2).[3] However IL-2+IL-21-supplemented cultures expressed significantly higher levels of lytic granules such as granzyme A, granzyme B, and perforin, when compared with standard IL-2 conditions (Fig. 3) (see also supplemental Table S2).[3] Accordingly semiquantitative PCR analysis showed higher levels of mRNA coding for granzymes and perforin in IL-2+IL-21- than in IL-2-supplemented Vγ9Vδ2 T cells. Therefore, this observation suggests that IL-21 acts primarily at the level of transcription or stability of mRNA encoding these lytic molecules (data not shown). Interestingly, increased expression levels of inhibitory NK receptors (e.g., NKG2A, ILT2, and CD244 (2B4)) and

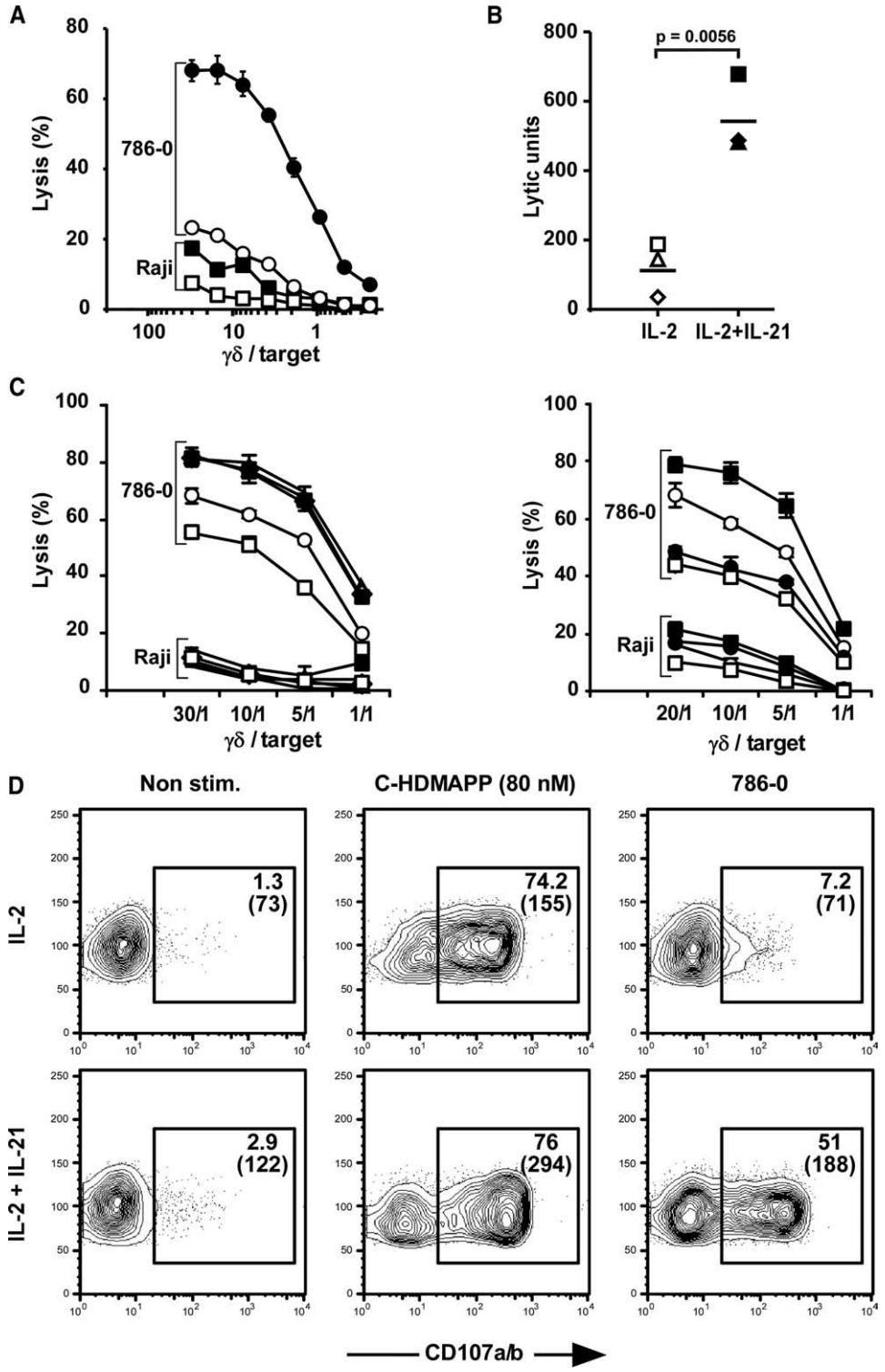

FIGURE 4. IL-21 enhances antitumor cytolytic activity of Vγ9Vδ2 T cells. *A*, cytolytic activity in Vγ9Vδ2 PBL lines from healthy human donor (Do#C), generated following activation by phosphoantigen in IL-2- or IL-2+IL-21-supplemented medium, were measured by ^{51}Cr release assay after coculture together with Vγ9Vδ2-susceptible (786-0) or Vγ9Vδ2-resistant (Raji) tumor cell targets at increasing γδ T cell to target ratios. *B*, Lytic units (per 10^6 effector cells) in Vγ9Vδ2 PBL expanded in IL-2-supplemented (*open symbols*) or IL-2+IL-21-supplemented (*filled symbols*) medium were calculated according to the formula described in *Materials and Methods*. Three independent experiments are shown. Horizontal bar indicates calculated median values. *C*, Vγ9Vδ2 PBL (Do#C) expanded in IL-2-supplemented medium (*left*) were preincubated in IL-2+IL-21 medium for 5 days (△), 2 days (◇), or 16 h (○) before coculture at day 29 with tumor target cells. Cytolytic activity of Vγ9Vδ2 PBL was measured at the indicated γδ T cell to target ratio and compared with the values obtained with Vγ9Vδ2 PBL expanded and maintained for 29 days in IL-2 (□) or IL-2+IL-21 (■) medium. Vγ9Vδ2 PBL were expanded in IL-2- or IL-2+IL-21-supplemented medium and cultured at day 18 (*right*), respectively, in IL-2-supplemented (●) or in IL-2+IL-21-supplemented (○) medium. Cytolytic activity of Vγ9Vδ2 PBL were measured at day 30 and compared with cultures maintained with IL-2 (□) and IL-2+IL-21 (■). Data are presented as the mean ± SEM of triplicate samples. *D*, CD107 mobilization was measured by flow cytometry in Vγ9Vδ2 PBL expanded with IL-2 or IL-2+IL-21 and activated at day 27 by C-HDMAPP or 786-0 tumor cells (γδT cell to target ratio of 1:1). The percentage of CD107a/b$^+$ cells and mean fluorescence intensity (MFI) are indicated. Cytotoxicity and CD107 mobilization experiments were conducted at least three independent times and one representative result of at least three experiments is shown.

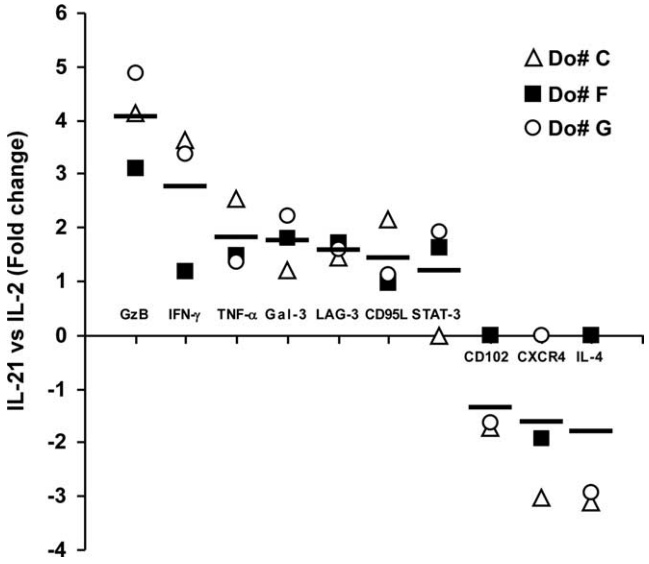

FIGURE 5. Comparative analysis of gene expression in Vγ9Vδ2 PBL expanded with IL-2 or IL-2+IL-21. Values correspond to fold changes in Vγ9Vδ2 PBL generated IL-2+IL-21-supplemented medium and compared with IL-2 cultures, based on quadriplicate microarray experiments. A selection of the products (genes of a known immunological relevance in T cells) of the most strongly upmodulated (positive values) and downmodulated (negative values) transcripts prepared at day 23 from healthy human donors (Do#F and Do#G) or at day 28 (Do#C) postactivation is presented. Granzyme B (GzB), IFN-γ, TNF-α, galectin-3 (Gal-3), CD95 ligand (CD95L), STAT-3, CXCR4, and IL-4 are represented. Microarray data were performed by comparing γδ T cell samples from three different donors (Do#C, Do#F, and Do#G) and generated in both cytokine conditions. Horizontal bar indicates median fold change value.

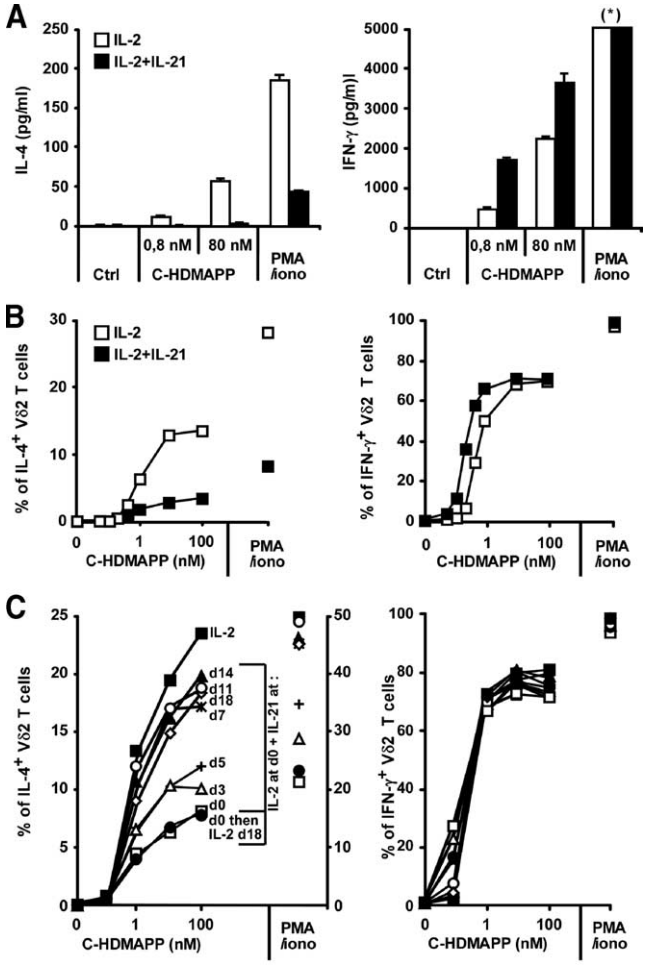

FIGURE 6. IL-21 drives Vγ9Vδ2 PBL functions toward proinflammatory Th1 responses. *A*, ELISA titrations of IL-4 (*left*) and IFN-γ (*right*) produced in culture supernatants by Vγ9Vδ2 PBL of a healthy human donor (Do#C) expanded for 34 days in IL-2- or IL-2+IL-21-supplemented medium and activated by C-HDMAPP (at 0.8 and 80 nM) or PMA/ionomycin (PMA/iono). Data are presented as the mean ± SEM of triplicate samples. Asterisk shows saturating levels of cytokines. *B*, Intracellular accumulation of IL-4 (*left*) and IFN-γ (*right*) in the same Vγ9Vδ2 PBL (IL-2 vs IL-2+IL-21) following activation by grading doses of C-HDMAPP or PMA/ionomycin (PMA/iono). *C*, Kinetics of proinflammatory Th1 programming of Vγ9Vδ2 PBL induced by IL-21. Vγ9Vδ2 PBL (Do#H) were specificallly activated by C-HDMAPP in the presence of IL-2 (day 0). Expanding Vγ9Vδ2 T cells were transferred in IL-2+IL-21-supplemented medium at the indicated time points (days 3, 5, 7, 11, 14, and 18). Intracellular accumulation of IL-4 (*left*) and IFN-γ (*right*) induced following a recall activation at day 34 was measured as described in *B*. Scale (*right side*) indicates percentage of IL-4-producing γδ T cells following activation by PMA/ionomycin.

lower levels of costimulatory NK receptors (e.g., NKG2D) were detected in γδ T cells amplified with IL-21, as compared with standard IL-2 conditions. Also consistent with enhanced differentiation toward an effector phenotype (32), IL-21-supplemented γδ T cells showed increased expression of CD56 (Fig. 3) (and see supplemental Table S2).[3]

IL-21 enhances the specific antitumor cytolytic activity of Vγ9Vδ2 T cells

We next tested whether increased accumulation of lytic molecules within IL-21-treated Vγ9Vδ2 T cells could result in modulation of their specific antitumor cytolytic activity. To this end, we compared cell-mediated lysis of naturally Vγ9Vδ2 T cell-susceptible (786-0) or T cell-resistant (Raji) tumor cell targets. Although IL-21-supplemented Vγ9Vδ2 T cells did not lyse to significant extent Raji cells, their cytolytic activity against susceptible 786-0 cells was approximately five times higher than that of IL-2-supplemented cultures, when estimated as lytic units (see *Material and Methods*) (Fig. 4, *A* and *B*). Similar results were obtained with several other Vγ9Vδ2-susceptible tumor targets, such as SW-1116 (a colorectal adenocarcinoma), 769-P, G401, and G402 (renal carcinomas) (data not shown). Consistent with expression of MHC class I-specific inhibitory NK receptor, Vγ9Vδ2 T cells lyse more efficiently β_2-microglobulin (β_2m)[4] knockdown tumor target than wild-type ones (33). However despite modulation of some activating or inhibitory NK receptor by IL-21 (Fig. 3), IL-21 enhanced to a similar extent lysis of both wild-type and β_2m knockdown target cells (see supplemental Fig. S3).[3]

Importantly, IL-21 enhanced within 16 h the specific antitumor cytolytic activity of Ag-stimulated Vγ9Vδ2 PBL expanded in IL-2. Moreover, removal of IL-21 from the culture medium lowered the cytolytic activity of Vγ9Vδ2 T cells expanded in IL-2+IL-21 to the basal levels obtained with IL-2-supplemented cultures. This response indicates that the effect of IL-21 on Vγ9Vδ2 CTL activity is a reversible process (Fig. 4*C*). To extend these observations, we analyzed the polarized exocytosis of secretory lysosomes by measuring cell surface mobilization of CD107a/b (also called LAMP-1/2), which correlates with functional recognition of target cells by T lymphocytes (34). Vγ9Vδ2 T cells amplified under IL-21 conditions underwent much stronger CD107a/b

[4] Abbreviation used in this paper: β_2m, β_2-microglobulin.

degranulation when incubated with 786-0 target cells than γδ T cells cultured in IL-2 alone (50.8% vs 7.3% of positive γδ T cells) (Fig. 4*D*). Altogether, our results indicate that IL-21 can rapidly boost in a reversible fashion the specific antitumor cytolytic functions of cultured Vγ9Vδ2 T cells, presumably through up-regulation of lytic molecules production.

IL-21 drives functional programming of Vγ9Vδ2 PBL toward a proinflammatory Th1 phenotype

To better characterize the effects of IL-21 on the acquisition of particular antitumor effector functions during in vitro expansion of Vγ9Vδ2 PBL, we compared the transcriptomes of magnetically sorted Ag-stimulated Vγ9Vδ2 T cells, from three donors, expanded with either IL-2 or IL-2+IL-21 using microarrays spotted with a selected set of 1076 genes (see *Materials and Methods*). A direct comparison of the two cytokine conditions revealed few changes of the gene expression program (see supplemental Fig. S4).[3] As shown in Fig. 5, IL-21 triggered a significant up-regulation in at least two of three donors of genes coding for molecules associated with a CTL proinflammatory phenotype such as granzyme B, IFN-γ, TNF-α, and CD95 ligand. STAT-3, a major target of the IL-21R signaling cascade, was also up-regulated, though to a lower extent. By contrast CD102 (ICAM-2), CXCR4, and IL-4 were strongly downmodulated by IL-21. Consistent with enhanced Th1 polarization induced by IL-21, we found by semiquantitative PCR decreased levels of mRNA coding for GATA-3, a critical transcription factor implicated in the regulation of IL-4 production by Th2 cells (data not shown).

To confirm these effects at the protein level, we performed a comparative analysis of IFN-γ (Th1) and IL-4 (Th2) production induced after a recall activation (by either C-HDMAPP or PMA/ionomycin) in resting Vγ9Vδ2 T cells that were previously expanded in IL-2 vs IL-2+IL-21. ELISA titrations in culture supernatants (Fig. 6*A*) and intracellular stainings for IFN-γ and IL-4 (Fig. 6*B*) (see also supplemental Fig. S5A)[3] indicated that 10–20% of IL-2 supplemented γδ T cells produced IL-4 following phosphoantigen stimulation. By contrast IL-21-supplemented cultures produced almost exclusively IFN-γ, thus indicating enhanced Th1 polarization. Accordingly IL-2+IL-21-supplemented γδ T cells yielded enhanced tumor-induced IFN-γ responses, when compared with IL-2 cultures (see supplemental Fig. S5B).[3]

We next sought to establish whether, similarly to cytolytic effector function, such a biased Th1 polarization could be induced or reverted by either short-term exposure or removal of IL-21. To this end, we performed kinetics experiments in which IL-21 was added at different time points following the initial C-HDMAPP activation (day 0) in the presence of IL-2. As shown in Fig. 6*C*, IL-21 induced Th1 polarization of day 0, day 3, and day 5 cultures (also see supplemental Fig. S5C).[3] However IL-21 was no longer able to inhibit IL-4 programming of Vγ9Vδ2 T cells cultured at least for 1 wk in IL-2. Moreover, removal of IL-21 from cells expanded in both IL-2 and IL-21 did not restore IL-4 responses, even after 2 wk of culture in IL-2 alone. Importantly, these results could not be accounted for the production of IL-21 by Vγ9Vδ2 T cells (data not shown).

Discussion

The primary aim of this study was to design improved γδ T cell based immunotherapeutic approaches combining synthetic γδ agonists and cytokines known to stimulate effector functions associated with enhanced tumor clearance. To this end, IL-21 represented an interesting candidate as it was shown not only to trigger proliferation of NK cells and Ag-stimulated T cells but also to boost their cytolytic properties and particular cytokine responses. Although our results extend to the γδ system several properties of IL-21 described for other lymphoid subsets, they bring new insights into the role played by this cytokine in the acquisition or potentiation of unexpected functions by in vitro expanded γδ T cells. In particular we show that IL-21 up-regulated in a rapid and reversible way antitumor properties and cytolytic effector functions of Vγ9Vδ2 T cells. Moreover IL-21 enhanced in a slower and irreversible way Th1 differentiation of Vγ9Vδ2 cells stimulated by Ag and IL-2. On a more applied standpoint, these results allowed us to propose new protocols leading to large scale expansion of γδ T cells with enhanced antitumor properties, that could be easily set up under clinical grade conditions.

Because IL-21 was previously shown to trigger proliferation of Ag-stimulated γδ T cells, we initially tested whether this cytokine could be used for large scale expansion of these lymphocytes. In line with previous reports, IL-21 induced short-term proliferation of Ag-stimulated γδ T cells. However, unlike IL-2, IL-21 failed to induce efficient and long-term amplification of stimulated γδ T cells. This result lead us to further assess the combined effect of IL-21 and IL-2 on the proliferation of Ag-stimulated γδ T cells and to compare the functional properties of γδ T cells cultured with either IL-2, IL-21, or both cytokines.

In agreement with previously published data, short-term γδ T cell cultures stimulated in the presence of IL-21 alone were enriched for lymphocytes with a central memory ($CD27^+CD45RA^-$) phenotype, which could reflect polarization toward a follicular helper phenotype, as suggested by enhanced CXCL13 production (20). By contrast, whereas the combination of IL-2 and IL-21 allowed strong expansion of Ag-stimulated γδ T cells, most actively dividing cells displayed an effector memory ($CD27^-CD45RA^-$) phenotype. Because these features were very similar to those of IL-2-supplemented cultures, we tested whether the combination of IL-21 and IL-2 could induce acquisition of unique properties by stimulated γδ cells. In line with previous studies performed on NK, NKT, or $CD8^+$ T cells (23, 26, 35), IL-21+IL-2-supplemented γδ T cells showed up-regulation of 2B4 (CD244), NKG2A, and ILT2 and downmodulation of NKG2D, when compared with IL-2-supplemented cultures. The functional significance of these phenotypic alterations are still unclear; in particular, although several of these NK receptors are known to either enhance or inhibit cytotoxicity upon engagement by β_2m-associated molecules (33), differential susceptibility of β_2m knockdown vs wild-type targets to Vγ9Vδ2 lysis was not affected by IL-21+IL-2 treatment, when compared with IL-2 alone.

One of the most striking effects induced by IL-21+IL-2 on stimulated γδ T cells was a dramatic increase of cytotoxic activity associated with enhanced tumor cell killing. This activity could be due to several nonexclusive mechanisms. IL-21 may increase the overall target avidity of Vγ9Vδ2 effectors. This enhanced activity could be explained by increased expression of either CD95 ligand or TRAIL whose transcript levels were enhanced in γδ T cells by IL-21 treatment. However, we failed to detect increased expression of either these molecules at the protein level. Moreover, blocking Abs directed against CD95 ligand and TRAIL did not affect renal carcinoma cell lysis by IL-21-treated γδ T cells, suggesting a minor role played by either of these two candidates. CD56, which is up-regulated by IL-21, is known to establish homotypic interactions that could enhance binding of Vγ9Vδ2 T cells to $CD56^+$ tumors. Increased CD56 expression may also reflect differentiation into effectors with enhanced cytolytic potential, as suggested by several studies (32, 36). Accordingly, IL-2+IL-21-expanded Vγ9Vδ2 T cells showed strong up-regulation of several cytotoxic effector molecules, such as granzyme A, granzyme B, and perforin. Although a similar up-regulation of these molecules was previously reported in other IL-21-treated cell subsets, strong

downmodulation of granzyme B by IL-21 was recently described by Hinrichs et al. (37). These different behaviors might be explained by the cell differentiation status or the cytokinetic context: whereas IL-21 may inhibit acquisition of cytolytic effector functions during priming of naive precursors, it may have an opposite affect on memory T cells, as it typically the case here. Moreover, IL-21 might repress effector functions when used alone but enhance them when used in conjunction with IL-2. The mechanisms underlying coinduction or corepression of these effectors properties by IL-21 remain unclear but could involve modulation of several transcription factors known to control expression of perforin and granzyme and to be regulated by IL-21, such as Eomesodermin (37–39) or STAT factors (40–42). In any case, the fact that IL-21 up-regulated within a few hours of granzyme and perforin expression on established γδ T cell clones and purified polyclonal lines indicates that this process is a direct consequence of IL-21R signaling on γδ T cells, even though we failed to detect any surface expression of this receptor on these lymphocytes. However, the results of RT-PCR assays indicate that Vγ9Vδ2 T cells express substantial levels of the mRNA transcripts encoding for the specific IL-21R chain (data not shown).

Quite unexpectedly, granzyme and perforin expression in IL-21+IL-2-supplemented cultures returned to baseline levels found in IL-2-treated cells upon removal of IL-21. It will be interesting to determine whether this reversible phenomenon, which to our knowledge has not been reported before, parallels downmodulation or upmodulation of the previously mentioned transcription factors. In line with their enhanced cytolytic potential, IL-21+IL-2-treated γδ T cells showed dramatically enhanced tumor-induced degranulation and cytotoxicity against most Vγ9Vδ2-susceptible tumor targets. Again in this study, this phenomenon was observed within a few hours after exposure to IL-21 and was reversible. Quite interestingly, IL-21 not only increased the percentage of γδ T cells able to translocate CD107a/b to their surface upon tumor recognition, but also increased the surface expression levels of CD107a/b among positive cells, thus suggesting an overall increase in cytotoxic lysosomal granule production (34).

Vγ9Vδ2 T cells are classically considered as proinflammatory Th1 memory cells, able to readily produce TNF-α, IFN-γ but no IL-4 or IL-13 upon ex vivo Ag stimulation (43). For yet unknown reasons, these cells rapidly and stably acquire the capacity to produce IL-4 upon in vitro culture in IL-2 (44). This induced IL-4 production was strongly and irreversibly inhibited by IL-21 when the latter was added early after Ag stimulation of Vγ9Vδ2 T cells in IL-2-supplemented medium. By contrast IFN-γ production was either unaffected or enhanced by addition of IL-21 early after in vitro culture. This observation is reminiscent of previous studies describing enhancement of either Th1 or Th2 polarization by IL-21 (39, 45–47). Altogether, these data suggest that IL-21 is not a primary modulator of Th1/Th2 programming but may strengthen precommitment of memory cells toward either profile. By contrast, IL-21 might inhibit both Th1 and Th2 polarization of naive T cells, and favor instead their programming toward IL-17 production, as suggested by several studies (25). It remains to be determined whether IL-21 acts directly or indirectly on γδ polarization. The fact that IL-21-induced inhibition of IL-4 occurred only when this cytokine was added from the beginning of the culture supports an indirect effect, possibly mediated by APC present in this system. Such an assumption will need to be confirmed through analysis of purified ex vivo γδ T cells.

On an applied standpoint, IL-21 did not allow long-term expansion of Ag-stimulated γδ T cells at least in vitro. This departs from previous studies describing efficient proliferation of NK and $CD8^+$ T cells (21, 48, 49). This apparent discrepancy could reflect involvement of cofactors not provided in the present study. Irrespective of this issue, IL-21 could nevertheless be used in conjunction with IL-2 because it did not inhibit long-term expansion of Vγ9Vδ2 T cells in the presence of the latter cytokine. In functional terms, IL-21 could rapidly up-regulate cytolytic function and antitumor killing mediated by Vγ9Vδ2 T cells; this effect was extremely rapid and could be obtained even on cultured/preactivated cells that had not been exposed before to this cytokine. Therefore, in vitro IL-21 pretreatment of expanded γδ T cells could be exploited in passive immunotherapeutic settings to enhance the antitumor efficacy of adoptively transferred γδ T cells, although this effect might be rapidly lost after in vivo transfer. IL-21 could be also used in conjunction with Vγ9Vδ2 agonists to potentiate in vivo cytotoxic programming of Vγ9Vδ2 T cells. In addition, based on our in vitro findings, IL-21 might enhance IFN-γ response of Vγ9Vδ2 T cells when coadministered with Vγ9Vδ2 agonists and IL-2. This response could be readily tested in nonhuman primates owing to the availability of GMP-grade cytokines and γδ T cell agonists. Nevertheless one should be aware that IL-21 can boost proliferation of some Vγ9Vδ2-susceptible tumors such as multiple myeloma (50), thus precluding its use in vivo in this particular indications.

In conclusion our results indicate that IL-21 could be combined with IL-2 during specific in vitro expansion strategies to boost direct and indirect antitumor properties of human Vγ9Vδ2 T cells. This cytokine might therefore represent a candidate of choice that should be tested for optimization of γδ T cell-based immunotherapeutic protocols.

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Disclosures

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