Phosphoantigens Overcome Human TCRV\gamma9 + \gamma8 Cell Immunosuppression by TGF-\beta: Relevance for Cancer Immunotherapy

Aude-Hélène Capietto, Ludovic Martinet, Delphine Cendron, Séverine Fruchon, Frédéric Pont and Jean-Jacques Fournié

*J Immunol* 2010; 184:6680-6687; Prepublished online 17 May 2010; doi: 10.4049/jimmunol.1000681

http://www.jimmunol.org/content/184/12/6680

References

This article cites 80 articles, 35 of which you can access for free at:
http://www.jimmunol.org/content/184/12/6680.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Phosphoantigens Overcome Human TCRVγ9+ γδ Cell Immunosuppression by TGF-β: Relevance for Cancer Immunotherapy

Aude-Hélène Capietto,*† Ludovic Martinet,*†,1 Delphine Cendron,*†,2 Séverine Fruchon,*† Frédéric Pont,‡ and Jean-Jacques Fournié*†

Human γδ cells expressing TCRVγ9 are HLA-unrestricted CTLs with high relevance for cancer immunotherapy. Many tumor cell types produce TGF-β, however, a cytokine strongly immunosuppressive for conventional T CD4, CD8, and NK cells. Whether TGF-β also inhibits TCRVγ9+ lymphocytes was unknown. Because phosphoantigens (PAgs), such as bromohydrin pyrophosphate, selectively activate the antitumor functions of TCRVγ9+ T cells, in this study, we investigated whether TGF-β modulates these functions. We report that TGF-β does not block activation of TCRVγ9+ T cells but inhibits their PAg/IL-2–induced proliferation and maturation into effector cells and finally reduces the cytotoxic activity of these γδ T cells when exposed to lymphoma target cells. TGF-β did not bias their differentiation pattern toward γδ Th17 or γδ regulatory T cells. Nevertheless, increasing doses of PAg stimulus countered TGF-β inhibition. So, although TGF-β impairs TCRVγ9+ γδ cells like other cytolytic lymphocytes, PAg alone or combined to therapeutic mAb has the ability to bypass its immunosuppressive activity. The Journal of Immunology, 2010, 184: 6680–6687.

*Département d’Oncogénèse et Signalisation, Institut National de la Santé et de la Recherche Médicale, Unité 563, and Université Toulouse III Paul-Sabatier, Centre de Physiopathologie de Toulouse Purpan, and Institut Fédératif de Recherches 150, Hôpital Purpan, Toulouse, France.
†Current address: Institut de Pharmacologie et de Biologie Structurale, Unité Mixte de Recherches 5089, Toulouse France.
‡Current address: Department of Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN.

Received for publication March 1, 2010. Accepted for publication April 11, 2010.

This work was supported in part by institutional grants from the Institut National de la Santé et de la Recherche Médicale, by the Innate Pharma, Marseille, and by the Institut National du Cancer Lymphoma Program (RITUXOP) and Programme Libre de Physiopathologie de Toulouse Purpan; and ‡Institut Fe´de´ratif de Recherches 5089, Toulouse France.

Address correspondence and reprint requests to Dr. Jean-Jacques Fournié, Institut National de la Santé et de la Recherche Médicale, Centre Hospitalier Universitaire de Purpan, Place Baylac, F-31300 Toulouse, France. E-mail address: jean-jacques.fournie@inserm.fr.

Abbreviations used in this paper: ADCC, Ab-dependent cell cytotoxicity; BrHPP, bromohydrin pyrophosphate; CM, central memory; GzB, granzyme B; fic, freshly isolated cell; KIR, killer Ig-related receptor; MFI, mean fluorescence intensity; N, naive; PAg, phosphoantigens; rh, recombinant human; pcl, primary cell line; TEMh1, T effector memory helper 1; TEMRA, T effector memory RA; Treg, regulatory T.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10/$16.00 by guest on September 16, 2017http://www.jimmunol.org/Downloaded from
thus have related therapeutic applications (17–19). These drugs have led several groups to demonstrate antitumor functions of human γδ T lymphocytes in different in vivo contexts. Studies in nonhuman primates (20, 21) and in cancer patients (7, 22–27) have illustrated the potential of activated TCRVγ9δ T lymphocytes against leukemia, lymphoma, and carcinoma (reviewed in Refs. 28–31). In addition, clinical trials involving PAgS or aminobisphosphonates alone and in combinations are currently assessed by different groups around the world.

Limits to γδ T cell-based cancer immunotherapies are now appearing, however (32). Tumor progression in cancer patients is frequently associated with emergence of malignant cells evading immune surveillance (33). Of the various immunoescape mechanisms used by cancer cells, production of TGF-β is one of the most potent and frequent (reviewed in Refs. 34 and 35). This cytokine has a distinct effect on the main subsets of conventional γδ T lymphocytes (36, 37). It inhibits proliferation and functional differentiation of cytolytic CD8 T cells and promotes differentiation of Th17 cells and regulatory T (Treg) cells to reduce anticancer immunity (38, 39).

Nevertheless, whether this cytokine also targets the unconventional human γδ T cells remains unclear. TGF-β inhibits γδ T cell proliferation induced by Mycobacterium tuberculosis-pulsed monocytes (40) and helps induce FOXP3+ γδ Treg cells in the presence of IL-15 (41), but little is known about its direct bioactivity on the anticancer functions of human γδ T cells. In this study, we investigated whether TGF-β affects the proliferation, maturation, and cytokytic functions of TCRVγ9δ γδ T lymphocytes, either as freshly isolated cells (fics) or as primary cell lines (pcl).

Materials and Methods

**Abs and flow cytometry**

FITC-conjugated Abs to TCRVγ9, PE-Cy5–conjugated anti-CD27 and anti-CD69 or with PE-conjugated Abs to markers TCRVγ9 or with PE-Cy7–conjugated anti-CD16 and anti-NKG2D were from Beckman Coulter (Marsselle, France), Pacific Blue-conjugated Abs to CD45RA and CD3 were from Ozyme (St. Quentin en Yvelines, France), the PE-conjugated Ab to TGF-βRII was from R&D Systems Europe (Lille, France), and allophycocyanin-conjugated anti-killer Ig-related receptor (KIR) mAb [clone 1-7F9] (42, 43) was from Innate Pharma (Marseille, France). The respective isotype-matched conjugated controls were from Beckman Coulter, Ozyme, and R&D Systems Europe, respectively. γδ T lymphocytes intracellular cytokine levels and cytotoxic granule production were measured on cells stained at the cell surface and treated with brefeldin A (Sigma-Aldrich, Lyon, France) for 5 h before intracellular staining. Cell surface Ag stainings involved FITC-conjugated anti-TCRVγ9 (Beckman Coulter) and Pacific Blue-conjugated anti-CD3 (Ozyme). After staining, cells were washed twice, fixed, and permeabilized with 4% paraformaldehyde and 0.1% saponin plus 0.5% BSA (Sigma-Aldrich). Cells were then stained with allophycocyanin-conjugated anti-IFN-γ, A647-conjugated anti-GrB, or FITC-conjugated anti-perforin Abs (BD Biosciences, Le Pont de Claix, France). Flow cytometry was done with LSR-II and the dedicated software FACSDiva (BD Biosciences) and FlowJo 7.5.5 (Tree Star, Ashland, OR). Quantification of TGF-βRII expression on γδ T cell surface was done by using Quantum beads (Bang Laboratories, Fishers, IN) as described previously (12).

**Reagents**

The synthetic PAg BrHPP (Innate Pharma, Marseille, France) has been described previously (13). Recombinant human (rh) IL-2 was provided by Sanofi-Aventis (Toulouse, France), and rituximab was provided by Roche (Neuilly-sur-Seine, France). CFSE was purchased from Molecular Probes (Eugene, OR). rhTGF-β1 was purchased from R&D Systems Europe. Cells were cultured in complete medium containing RPMI 1640 (Invitrogen, Cergy Pontoise, France) supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin, 100 IU/ml penicillin, and 1 mM sodium pyruvate (Cambrex Biosciences, Rockland, ME).

**Vγ9 T cell samples and cultures**

TCRJVγ9δ γδ T lymphocytes were isolated from PBMCs obtained from human healthy individuals (Etablissement Français du Sang, Toulouse, France) after Ficoll-Hypaque density centrifugation. Distinct TCRVγ9δ γδ T cell samples were prepared for this study. On the one hand, freshly isolated γδ T cells (referred below to as γδ fics) were directly purified by immunomagnetic separation using anti-TCRγδ–conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). On the other hand, primary TCRVγ9δ γδ T cell lines (referred below to as γδ pcl) were obtained from PBMCs by in vitro culture for 14 d in complete medium supplemented with 10% Fetal Clone I (HyClone-Thermo Fisher Scientific, Bresbiere, France), BrHPP (1 µM), and rhIL-2 (300 IU/ml) at day 0, with additional IL-2 (300 IU/ml) renewal every 3 d. After checking for TCR phenotype of the cell samples (>90% TCRVγ9δ γδ T cells for fics or γδ pcl), the cells were washed and treated or not with TGF-β for 48 h in IL-2–free complete medium supplemented with 10% FBS (Invitrogen). BrHPP was added or not for the last 24 h of γδ fics and pcl culture (these cells referred below to as activated γδ fics or activated γδ pcl).

**Vγ9 T cell division**

Freshly isolated PBMCs (5 × 10^6 cells/ml) were labeled with CFSE (1 µM) for 10 min at 37°C and washed with fresh culture media, according to the manufacturer’s instructions. Labeled cells were then stimulated with BrHPP (100 nM), rhIL-2 (0–200 IU/ml as indicated), and TGF-β (0–100 ng/ml as indicated). Cells were harvested, stained for TCRVγ9, and analyzed for CFSE dilution and cell surface expression of TCRVγ9 by flow cytometry after 7 d of culture. The progressive dynamics of CFSE dilution by dividing TCRVγ9δ γδ T cells was measured after specified durations following PAg stimulation of PBMCs as above and compared with the theoretical cell growth Cyton model (CPSM software, kindly provided by P.D. Hodgkin (44)). The maturation stages of CFSE-labeled TCRVγ9δ γδ T cells (1 × 10^5 cells/ml) activated with BrHPP (100 nM) and rhIL-2 (10 IU/ml) with or without TGF-β (2 ng/ml) were determined by simultaneous immunostaining of TCRVγ9δ, CD27, and CD45RA surface markers and flow cytometry.

**Cytotoxicity assays**

The Daudi and Raji human Burkitt’s lymphoma cell lines used as target cells were cultured at 37°C in complete medium with 10% FBS and 25 mM HEPES (Invitrogen). Specific lysis by Vγ9 T cells was measured by standard 4-h [^{3}^{1}^{H}]{TdR} release assays. The lysis rates were obtained by (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. Maximum and spontaneous releases were determined, respectively, by adding 0.1% Triton X-100 or complete medium to [^{3}^{1}^{H}]-labeled tumor target cells in the absence of γδ T cells. Data present as the mean of triplicate samples. Specific lysis assays by ADCC involved the same settings as above, except the presence of rituximab (10 µg/ml) added to the 4-h cell coinoculation and to the spontaneous release and maximum release controls.

**TCRVγ9δ T cell transcriptomes**

PBMCs were obtained from four human healthy individuals (Etablissement Français du Sang, Toulouse, France) as depicted above. For each donor, TCRVγ9δ γδ T cells were purified (>99% for each sample) by immunomagnesturing and flow cytometry cell sorting either before activation (γδ fics resting control) or 6 h after PAg plus IL-2 stimulation (γδ fics activated 6 h) or 7 h after PAg stimulation and culture with IL-2 (γδ pcl 7 d). Total RNA was isolated from each of these cell samples using TRIzol reagent (Invitrogen Life Technologies, Paisley, U.K.), according to the manufacturer’s instructions. The quality and integrity of the RNA obtained were assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) after denaturation at 70°C for 2 min. cRNAs were then prepared according to one-Cycle Target Labeling protocol (Affymetrix, Santa Clara, CA) starting from 1 µg total RNA. The cRNAs were then fragmented and hybridized to Affymetrix HG-U133 plus 2.0 arrays. The chips were washed and scanned, according to the manufacturer’s instructions. GeneChip Operating Software (version 1.1; Affymetrix) was used for the primary image analysis of the array; for the normalization (global scale normalization, target value of 500) and standard comparisons. Expressions of the selected genes are summarized as means and SDs of the raw expression data from the four donors. Asterisks indicate significant (p < 0.05) changes to the γδ fics resting control, and a full line indicates the absent/present threshold. Lists of significantly changed (up- and downregulated) gene expression levels were built using as criteria p < 0.01 and p > 10-fold changes in expression level for genes identified at baseline expression in resting controls was above the cutoff threshold. The resulting sublists of genes were compared with 193 biological signatures defined in Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg) by using an in-house developed program based...
on our search engine nwCompare (45) and hypergeometrical distribution for ρ values.

Statistics

Data shown for transcriptome analysis are means and SDs and means and SEs of the mean (SEM) otherwise. The normality and equal variance of each sample series were evaluated prior to statistical analysis by the specified tests. A one-tailed, paired Student t test was used whenever appropriate or one-way Mann-Whitney rank-sum test was used otherwise using α = 5% for significant differences. All statistical analyses were performed using the SigmaStat 3.0 (SPSS, Chicago, IL) and XL Stat 2008 (AddinSoft, Paris, France) software.

Results

PAg-induced TCRVγ9+ T cell proliferation reversibly inhibited by TGF-β

We first monitored the kinetics of γδ cell proliferation by the CFSE dilution assay at several time points after PAg (BrHPP) stimulation to optimize this readout for the further experiments. Cell cultures were supplemented with IL-2, because the PAg-driven expansion of TCRVγ9+ γδ cells requires an exogenous supply of this cytokine (46). We observed the CFSE was progressively diluted from days 5 to 7, in good match with a stochastic cell division model (44) involving 70% of responding cells, 80 h for first division and 13 h for subsequent divisions, as reported for CD8αβ T cells (47) (Fig. 1A). In addition, the transcriptome of γδ cells obtained 7 d after stimulation confirmed gene expression signatures (45) of cell cycle (47 genes; p < 10-53), purine and pyrimidine metabolisms (37 genes; p < 10-18), and p53 signaling pathway (19 genes; p < 10-16) (data not shown). These experiments indicated that day 7 of culture in IL-2-containing medium was optimal to analyze γδ cells dividing in response to PAg stimulation.

We then asked whether TGF-β inhibits the proliferation of PAg-activated (100 nM BrHPP) γδ T cells in cultures supplemented with IL-2, and we observed a TGF-β dose-dependent inhibition of TCRVγ9+ cell proliferation. The above modelization of cytometry results from cultures at the highest TGF-β/IL-2 ratio suggested ~20% of responding cells (data not shown). The rate of proliferating γδ cells is known to increase with doses of IL-2, as in CD8 T cells (48), and increasing concentrations of IL-2 rescued this inhibition accordingly (Fig. 1B, 1C). Furthermore, increasing dose of PAg also augments the rate of proliferating TCRVγ9+ γδ cells (13, 20, 21, 49) and increasing concentrations of BrHPP rescued the proliferating γδ cells from TGF-β inhibition (Fig. 1D, 1E). Thus, TGF-β inhibits proliferation of TCRVγ9+ γδ T lymphocytes but higher doses of either IL-2 or PAg bypass this inhibition.

TGF-β delays PAg-induced maturation of TCRVγ9+ T lymphocytes

In most adults, the circulating TCRVγ9+ T lymphocytes essentially are made up of CM cells plus a few N and effector memory cells (Fig. 2, left) (4, 5). We thus asked whether TGF-β modulated the γδ T cell maturation in the above proliferation assay by analyzing cell surface expression of the CD27 and CD45RA markers on CFSE-labeled γδ cells stimulated with PAg and cultured with IL-2 and with or without TGF-β. The phenotype of undivided parental cells comprised N and CM lymphocytes in both conditions. By contrast, the maturation of dividing cells diverged in the presence of TGF-β. In control conditions without TGF-β, the divided cells mostly made up CM (CD27+CD45RA-) and TEMh1 lymphocytes (CD27-CD45RA-) (4, 5). By contrast, the few cells that divided in presence of TGF-β not only made up the CM and TEMh1 cells as above but also encompassed a sizeable proportion of N cells (CD27+CD45RA+) (Fig. 2, right). Recent reports showed IL-17+ γδ T cells from individuals with active pulmonary tuberculosis (50) or with HIV infection (51). Although the TCR repertoire of those cells did not contain TCRVγ9+ cells, we asked whether TCRVγ9+ fics and pcls derived from healthy individuals contained IL-17+ γδ T cells. We found no IL-17+ TCRVγ9+ lymphocytes among these cells with or without TGF-β. In addition, we did not detect any FoxP3+CD25hiTREG+ TCRVγ9+ cells in these experiments (data not shown).

So, TGF-β delays the maturation of PAg-activated TCRVγ9+ lymphocytes without inducing Th17 or Treg cells.

TGF-β reduces cytotoxic functions of TCRVγ9+ T lymphocytes

The above experiments indicated that TGF-β inhibits proliferation and functional maturation of γδ T cells, which normally generate cytolytic effector lymphocytes. Because TGF-β reduces the lytic activity of CD8+ T lymphocytes (52) and NK cells (53), we assessed its activity on cytolytic functions of TCRVγ9+ cells. Thus, TCRVγ9+ fics and pcls were cultured with or without PAg activation for 48 h in complete medium with or without TGF-β, and their intracellular GzB, perforin, and IFN-γ were assessed by immunostaining and flow cytometry. The percentage of cells producing both GzB and perforin among ex vivo TCRVγ9+ fics was quite low as previously described (5) (data not shown) and strongly increased upon PAg activation with or without TGF-β. By contrast, resting TCRVγ9+ pcls made up a vast majority (~80%) of GzB and perforin-producing cells, in line with their maturation as cytolytic effector cells. These phenotypes were strongly decreased in resting pcls treated with TGF-β as compared with untreated pcls controls: 55% of GzB+ perforin+ and 39% of GzB+perforin+ cells with TGF-β versus 14% of GzB+perforin+ and 83% of GzB+perforin+ cells in control cultures without TGF-β. With secondary PAg activation, however, the intracellular content of TCRVγ9+ pcls comprised both GzB+ cells and GzB+perforin+ cell subsets within frequencies that were not reduced by TGF-β (Fig. 3A). TGF-β reduced the rates of IFN-γ+ cells among activated fics and resting pcls but conversely enhanced this rate among activated pcls. These results were confirmed by dosages of IFN-γ secreted in culture supernatants (Fig. 3B, 3C). The cell surface expression of NKG2D was reduced on resting pcls treated with TGF-β relative to control cells, but TGF-β did not cause this reduction on γδ pcls and γδ fics stimulated with PAg. In the same experiments, the cell surface expression of KIRs and CD16 were unchanged (Fig. 3D). This set of data indicated that the lytic machinery of γδ cells was reduced by TGF-β but rescued by secondary PAg activation of pcls.

We then challenged these conclusions by using functional assays of cytolytic activity, the standard [51Cr] release assays from Daudi Burkitt’s lymphoma target cells, and specific ADCC of the CD20+ Raji target cells with anti-CD20 rituximab. These distinct assays reflect activation of the γδ T cell’s lytic activity for allogenic targets, either driven by TCRVγ9+ plus NKG2D (54, 55) or by TCRVγ9+ plus FcγRIIIA (12). Both spontaneous lysis and ADCC were higher with γδ pcls than with activated γδ fics; nevertheless, they were both inhibited by TGF-β. By contrast, ADCC mediated by PAg-activated γδ cells, either pcls or fics, was almost insensitive to TGF-β (Fig. 4). In addition, other functions of PAg-activated γδ cells, such as secretion of chemokines RANTES, MCP-1, MIP-1α, and MIP-1β (56), were not inhibited by TGF-β (data not shown).

Thus, TGF-β inhibits the spontaneous cytolytic activity of γδ T lymphocytes but not ADCC induced by rituximab in presence of PAg.

Mechanism of PAg rescue from TGF-β

Because the previous results indicated that TCRVγ9 activation can rescue γδ cells from inhibition by TGF-β, we investigated the
mechanism of this recovery. We checked whether PAg activation reduced the expression of high-affinity TGF-βR at the cell surface of TCRVγ9+ lymphocytes. Immunolabeling and quantitative flow cytometry of the TGF-βRII protein on γδ T lymphocytes revealed similar expression levels in all culture conditions, however, excluded this possibility (Fig. 5A). Furthermore, comparing the transcriptomes of PAg-activated and resting TCRVγ9+ T cells ruled out reduction of the TGF-β response pathway with activation. PAg-activated cells had less TGF-β1 mRNA, as many transcripts for TGF-βRII and TGF-βRIII (in line with the protein expression), and more TGF-βRI mRNA. In addition, activated cells had more of the TGF-β transducer SMAD2 and less inhibitors SKI and SMAD7, corresponding to a fully functional TGF-β pathway (Fig. 5B). Thus, TCRVγ9 activation does not reduce the TGF-β response pathway.

We then attempted to address this point by analyzing TCRVγ9 downmodulation and CD69 upregulation as readouts of PAg signaling in culture conditions with and without TGF-β. PAg induced
a weak TCRγ9 downmodulation and a strong CD69 upregulation on γδ fics (57, 58). Reciprocally with γδ pcls, PAg induced a strong TCRγ9 downmodulation and a weak CD69 upregulation. Furthermore, in the presence of TGF-β, the activation of γδ fics was unchanged whereas that of γδ pcls was modified. The TCRγ9 downmodulation by γδ pcls was reduced by TGF-β, and their CD69 upregulation was therefore increased (Fig. 5C, 5D), in line with their above-depicted IFN-γ response (Fig. 3B, 3C).

These findings suggest that TCR can rescue the γδ cells from TGF-β inhibition by triggering additional activation signaling rather than by impairing their TGF-β response pathway.

Discussion
This study aimed at characterizing the bioactivity of the TGF-β cytokine on the PAg-responsive TCRγ9+ T lymphocytes. We showed in this study that TGF-β inhibits proliferation, maturation, and cytolytic functions of these lymphocytes like other T and NK cells (36, 37, 39). In contrast with these latter, however, the TGF-β-mediated immunosuppression of γδ cells can be rescued not only by IL-2 but also by PAg-driven TCR signaling.

TGF-β suppresses proliferation of conventional T cells by blocking expression of their endogenous IL-2 growth factor (59, 60) and of their high-affinity IL-2R, transferring receptor, c-myc transcription factor, and cell cycle regulators (61–63). We showed above that TGF-β also suppressed the proliferation of γδ cells. We propose that TGF-β targets, on the one hand, the endogenous IL-2 production of TCRγ9+ T lymphocytes presumably through blockade of its gene promoter as in αβ T lymphocytes (64, 65) because exogenous IL-2 partially reversed this blockade. On the other hand, TGF-β inhibited the functions induced by TCR signaling, but this was reversed by increasing doses of PAg stimulus. TCRγ9+ γδ lymphocytes usually represent ~1% of the circulating mononuclear cells, so their expansion is critically required to bring a significant contribution to cancer immunotherapies. By underlining the negative incidence of TGF-β may have on therapeutic protocols involving in vivo γδ cell expansions, this study also provides a rationale for γδ cell-based protocols composed of either in vitro expansions (49) or in vivo activation by high-dose PAg supplemented with exogenous IL-2 (12, 20, 21, 25, 66, 67). In addition, the proliferative response of PAg/IL-2–stimulated γδ lymphocytes is essentially—if not exclusively—mediated by cells from the N and CM compartments of TCRγ9+ lymphocytes (4, 8), suggesting that TGF-β might target more selectively these maturation stages.

TGF-β induces substantial in vitro differentiation of regulatory FOXP3+CD25highTCRγ9+ cells when combined with IL-2, IL-15, and the weak PAg isopentenyl pyrophosphate (41). Nevertheless, TGF-β alone or combined with the potent agonist BrHPP and IL-2 induced neither γδ Treg cells nor γδ T cells with Th17 phenotype. Both lack of the Treg-promoting IL-15 cytokine (68, 69) and relative strength of the PAg agonist used in our and in most in vivo studies (70) and clinical trials might account for this discrepancy.

We found in this study that TGF-β slows the PAg-induced maturation of TCRγ9+ cells into effector memory cells. TGF-β induces apoptosis of effector memory T cells during infection-induced clonal expansions (71). Low rates of TCRγ9+ cell death because of TGF-β were noticed in our study, arguing against shorter life spans of the most mature γδ cell subsets in this paper. Rather, we hypothesize that in cultures supplemented...
with TGF-β, IL-2, and PAg, the γδ cells, which have maintained a CD27+/CD45RA+ phenotype after several divisions, had preserved the N phenotype of their progenitors. This suggests that by analogy with its action on melanocyte or neural stem cells (72, 73), TGF-β might favor the maintenance by self-renewal of an N γδ cell pool. This peculiar bioactivity resembles that of Wnt3a protein (and glycogen synthase kinase-3β inhibitors) on the maturation of CD8+ CTLs (74), but its lymphoproliferative suppression makes it a distinct one. Nevertheless, the maintenance of N T lymphocytes among cells proliferating to Ag in the presence of TGF-β had not been characterized previously and extends the range of the bioactivities of this cytokine.

As discussed above, the differential sensitivity of γδ cell cytolytic functions to TGF-β was inherent to the presence of PAg stimulus; resting γδ pcls were sensitive, whereas PAg-activated γδ pcls were more resistant to TGF-β. In addition to presence of PAg, however, the maturation of γδ cells was also important for response to TGF-β, with higher sensitivity to this mediator in PAg-activated pcls than in PAg-activated fics, as depicted in conventional T lymphocytes (75). In addition, the most mature γδ cells upregulate various surface coreceptors that confer better responsiveness to PAg (4, 5, 8) by improving the efficiency of stimulus transduction and therefore lowers their sensitivity to TGF-β (76, 77). Along this line, the direct cell–cell binding facilitated by therapeutic rituximab not only provides γδ cells with a physical contact to the Raji cell target to eradicate but also strengthens their stimulatory signaling (12). From the current study, we propose that stimulation with PAg strengthens intracellular signaling for ADCC by TCRVγ9+ cells and thus resistance to TGF-β.

This study indicates that signaling pathways for TCR-mediated activation and TGF-β-mediated inhibition coexist in γδ T cells. So, the intracellular dominance of signaling from γδ TCR versus TGF-βR determines the outcome of functional γδ cell responses to their microenvironment, like the γδ TCR versus KIR relationship (78–80). Because increasing doses of stimulating PAg/IL-2 can overcome the inhibition of cytolytic TCRVγ9+ T cells by TGF-β, strategies bypassing its bioactivity could be envisaged. Therapeutic protocols based on TCRVγ9+ T lymphocytes stimulated by BrHPP and IL-2 proved more easily tunable than those based on conventional CTL or NK cells, because these γδ cells do not require tumor-derived peptide Ags presented by HLA molecules. In this aim, however, this work suggest the need to adapt PAg doses delivered to patients in function of their tumor’s ability to produce TGF-β. Furthermore, synergizing this stimulus with rituximab, trasmutumab, or other therapeutic mAbs might also provide effector γδ cells with more robust cytolytic activity (12). Future studies will determine whether such combinations will defeat particularly immunosuppressive tumors of advanced cancers of the breast, prostate and pancreas.
Acknowledgments

We thank Richard A. Flavell and members of our laboratory for critical suggestions on this work. Inna Pharma for providing BrHP, laboratoires Sanofi-Aventis for rHuIL-2, and laboratoires Roche for gifts of rituximab.

Disclosures

The authors have no financial conflicts of interest.

References

The authors have no financial conflicts of interest.

Sanofi-Aventis for rhIL-2, and laboratoires Roche for gifts of rituximab.

6686 PHOSPHOANTIGENS BYPASS TGF-β SUPPRESSION OF HUMAN γ T CELLS

1. Poupot, M., and J. J. Fournié. 2004. Non-peptide antigens activating human γ-γ T lymphocytes. Immunol. Lett. 95: 129–135.

2. Belmant, C., D. Decise, and J. J. Fournié. 2006. Phosphoantigens and amino-biphosphonates: new leads targeting γ γ T lymphocytes for cancer immunotherapy. Drug Discov. Today Ther. Strateg. 5: 17–23.

3. Hamann, D., M. Sirenelli, H. Regnault, S. R. Kerckhof-Garde, M. R. Klein, and R. A. van Liere. 1997. Phenotypic and functional separation of memory and effector human CD8+ T cells. J. Exp. Med. 186: 1407–1418.

4. Dieli, F., P. Foccia, M. Lipp, G. Stefani, N. Caccamo, C. Di Sano, and A. Salerno. 2005. Identification of effector/memory Vα2 T cells and migratory routes in lymph node or inflammatory sites. J. Exp. Med. 198: 391–397.

5. Angelini, D. F., G. Borsellino, R. Poupot, A. Diamantini, R. Poupot, G. Borsellino, R. A. Kroczt, C. La Mendola, E. Scetoc, et al. 2006. CCR5 identifies a subset of VγVδ2 T cells which secrete IL-4 and IL-10 and help B cells for antibody production. J. Immunol. 177: 2590–2595.

6. Dieli, F., N. Gebbia, F. Poccia, N. Caccamo, C. Montesano, F. Fulfaro, C. Arcara, M. R. Valerio, S. Meraviglia, C. Di Sano, et al. 2003. Induction of γγ T-lymphocyte effector functions by biphosphonate zolendronic acid in cancer patients in vivo. Blood 102: 2310–2311.

7. Dieli, F., C. Belmant, F. Poccia, N. Caccamo, C. Montesano, F. Fulfaro, C. Arcara, M. R. Valerio, S. Meraviglia, C. Di Sano, et al. 2003. Induction of γγ T-lymphocyte effector functions by biphosphonate zoledronic acid in cancer patients in vivo. Blood 102: 2310–2311.

8. Caccamo, N., S. Meraviglia, V. Ferlauzo, D. Angelini, G. Borsellino, F. Poccia, L. Bastiatti, F. Dieli, and A. Salerno. 2005. Differential requirements for antigen or homeostatic cytokines for proliferation and differentiation of human γγ T cells. Eur. J. Immunol. 35: 1764–1772.

9. Focca, P., L. Bastiatti, B. Cipriani, G. Mancino, F. Martini, M. L. Gougeon, and V. Colilli. 1999. Phosphoantigen-reactive VγVδ2 T lymphocytes suppress in vitro human immunodeficiency virus type 1 replication by cell-release anti-viral factors in CC chemokines. J. Infect. 30: 1858–867.

10. Roncin-Rouroco, B., V. Kunzmann, P. Wrobel, D. Kabelitz, A. Steinle, and T. Herrmann. 2005. Activation of VγVδ2 T Cells by NGK2D. J. Immunol. 175: 2144–2151.

11. Lapadat, V., J. Li, Liard, J. P. Liardat, and J. Favero. 2001. Production of TFF-α by human VγVδ2 T cells via engagement of FcRRIIA, the low affinity α γ T cell receptor for the Fc portion of IgG, expressed upon TCR activation by nonpeptidic antigen. J. Immunol. 166: 7190–7199.

12. Gennari-Darder, M. R., D. Vermijlen, F. Fulfaro, N. Caccamo, S. Meraviglia, A. H. Capietto, V. Scaglione, M. R. Klein, and R. A. van Liere. 1997. Phenotypic and functional separation of memory and effector T cell subsets. Eur. J. Immunol. 35: 1764–1772.

13. Gennari-Darder, M. R., D. Vermijlen, F. Fulfaro, N. Caccamo, S. Meraviglia, A. H. Capietto, V. Scaglione, M. R. Klein, and R. A. van Liere. 1997. Phenotypic and functional separation of memory and effector T cell subsets. Eur. J. Immunol. 35: 1764–1772.

14. Roncin-Rouroco, B., V. Kunzmann, P. Wrobel, D. Kabelitz, A. Steinle, and T. Herrmann. 2005. Activation of VγVδ2 T Cells by NGK2D. J. Immunol. 175: 2144–2151.

15. Lapadat, V., J. Liard, J. P. Liardat, and J. Favero. 2001. Production of TFF-α by human VγVδ2 T cells via engagement of FcRRIIA, the low affinity α γ T cell receptor for the Fc portion of IgG, expressed upon TCR activation by nonpeptidic antigen. J. Immunol. 166: 7190–7199.

16. Gennari-Darder, M. R., D. Vermijlen, F. Fulfaro, N. Caccamo, S. Meraviglia, A. H. Capietto, V. Scaglione, M. R. Klein, and R. A. van Liere. 1997. Phenotypic and functional separation of memory and effector T cell subsets. Eur. J. Immunol. 35: 1764–1772.

17. Kunzmann, V., E. Bauer, and M. Wilhelm. 1999. Anti-lymphoma effect of TGF-β. Leuk. Lymphoma 46: 671–680.

18. Gober, H. J., M. Kistowska, L. Angman, P. Jeno, L. Moris, and G. De Libero. 1999. Anti-lymphoma effect of TGF-β. Leuk. Lymphoma 46: 671–680.

19. Wojtowicz-Praga, S. 2003. Reversal of tumor-induced immunosuppression by TGF-β1 inhibitors in mice. Immune. Lett. 91: 21–32.

20. Siegel, P. M., and J. Massague. 2003. Cytostatic and apoptotic actions of TGF-β. Annu. Rev. Immunol. 21: 329–360.

21. Kovacs, M., and R. A. Flavell. 2008. TGF-β: a master of all T cell trades. Cell 134: 392–404.

22. Gorelik, L., and R. A. Flavell. 2001. Immune-mediated eradication of tumors through the blockade of transforming growth factor-β signaling in T cells. Nat. Med. 7: 1118–1122.

23. Li, M. O., Y. Y. Wang, S. Sanjaji, A. K. Robertson, and R. A. Flavell. 2006. Transforming growth factor-β regulation of immune responses. Annu. Rev. Immunol. 24: 99–146.

24. Rojas, R. E., K. N. Balaji, A. Subramanian, and W. H. Boom. 1999. Regulation of human CD4+ T-Cell-receptor-positive (TCR+) and γγ TCR regulatory T cells. Immunol. Lett. 57: 1599–1609.

25. Johansson, S. E., B. Hejdelman, J. Hinkula, M. H. Johansson, F. Romagne, B. Wahren, N. R. Wagnman, K. Kalle, and L. Bergström. 2010. NK cell activation by KIR-binding antibody 1-7F9 and response to HIV-infected autologous cells in viremic and controller HIV-infected patients Clin. Immunol. 134: 158–168.

26. Romagne, F., P. André, P. Spee, S. Zahn, N. Anfossi, L. Gauthier, M. Capanna, L. Ruggeri, D. M. Benson, Jr., W. B. Blaser, et al. 2009. Preclinical characterization of 1-7F9, a novel human anti-KIR receptor therapeutic antibody that augments natural killer cell killing of tumor cells. J. Immunol. 182: 6461–6472.

27. Hawkins, E. D., M. L. Turner, M. R. Dowling, C. van Gend, and P. D. Hodgkin. 2003. Stochastic model of T cell activation during TCR engagement and fast algorithm for n-way comparison of proteomic data files. Proteomics 3: 1091–1094.

28. Pechhold, K., D. Wesch, S. Schondelmaier, and D. Kabelitz. 1994. Primary activation of Vγ9-expressing γδ T cells by Mycobacterium tuberculosis: requirement for Th1-type CD4 T cell help and inhibition by IL-10. J. Immunol. 152: 4984–4992.

29. Gett, A. V., and P. D. Hodgkin. 2000. A cellular calculus for signal integration by T cells. Nat. Immunol. 1: 239–244.

30. Deenick, E. K., A. V. Gett, and P. D. Hodgkin. 2003. Stochastic model of T cell proliferation: a calculus revealing IL-2 regulation of precursor frequencies, cell cycle time, and survival. J. Immunol. 170: 4963–4972.

31. Salot, C., S. Laplace, S. Siaigah, S. Berczyage, I. Tenaud, A. Cassignais, F. Romagne, B. Dreno, and J. Tiollier. 2007. Large scale expansion of γδ T lymphocytes: Immuncell γδ T cell therapy product. J. Immunol. Methods 326: 63–75.
50. Peng, M. Y., Z. H. Wang, C. Y. Yao, L. N. Jiang, Q. L. Jin, J. Wang, and B. Q. Li. 2008. Interleukin 17-producing γδ T cells increased in patients with active pulmonary tuberculosis. *Cell. Mol. Immunol.* 5: 203–208.

51. Fenoglio, D. A., P. Oggi, S. Catellani, F. Battaglia, A. Ferrera, M. Setti, G. Mordacca, and M. R. Zocchi. 2009. V61 T lymphocytes producing IFN-γ and IL-17 are expanded in HIV-1-infected patients and respond to Candida albicans. *Blood* 113: 6611–6618.

52. Thomas, D. A., and J. Massagué. 2005. TGF-β directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell* 8: 369–380.

53. Smyth, M. J., M. W. Teng, J. Swann, I. Kyparissoudis, D. I. Godfrey, and R. T. Abraham. 1990. Regulatory effects of transforming growth factor-β on CD4+CD25+Foxp3+ T regulatory cells in mycobacterial T cell responses. *J. Immunol.* 144: 1446–1455.

54. Bukowski, J. F., C. T. Morita, Y. Tanaka, B. R. Bloom, M. B. Brenner, and D. J. McKean. 1990. Regulatory effects of transforming growth factor-β on CD4+CD25+Foxp3+ T regulatory cells in mycobacterial T cell responses. *J. Immunol.* 144: 1446–1455.

55. Peng, M. Y., Z. H. Wang, C. Y. Yao, L. N. Jiang, Q. L. Jin, J. Wang, and B. Q. Li. 2008. Interleukin 17-producing γδ T cells increased in patients with active pulmonary tuberculosis. *Cell. Mol. Immunol.* 5: 203–208.

56. Cipriani, B., G. Borsellino, F. Poccia, R. Placido, D. Tramonti, S. Bach, L. Battistini, and C. F. Brossan. 2000. Activation of C-C β-chemokines in human peripheral blood γδ T cells by isopentenyl pyrophosphate and regulation by cytokines. *Blood* 95: 59–47.

57. Wesch, D., S. Marx, and D. Kabellitz. 1997. Comparative analysis of αβ and γδ T cell activation by Mycobacterium tuberculosis and isopentenyl pyrophosphate. *Eur. J. Immunol.* 27: 952–956.

58. Sireci, G., E. Espinosa, C. Di Sano, F. Dieli, J. J. Fournié, and A. Salerno. 2001. Differential activation of human γδ cells by isopentenyl phosphoantigens. *Eur. J. Immunol.* 31: 1076–1086.

59. Becknell, B., and M. A. Caligiuri. 2005. Interleukin-2, interleukin-15, and their roles in human natural killer cells. *Adv. Immunol.* 86: 209–239.

60. Ma, A., R. Koka, and P. Burkett. 2006. Diverse functions of IL-2, IL-15, and IL-7 in lymphoid homeostasis. *Annu. Rev. Immunol.* 24: 657–679.

61. Kehrl, J. H., L. M. Wakefield, A. B. Roberts, S. Jakowlew, M. Alvarez-Mon, G. Murdaca, and M. R. Zocchi. 2009. Vγδ T lymphocytes producing IFN-γ and IL-17 are expanded in HIV-1-infected patients and respond to Candida albicans. *Blood* 113: 1628–1635.

62. Cipriani, B., G. Borsellino, F. Poccia, R. Placido, D. Tramonti, S. Bach, L. Battistini, and C. F. Brossan. 2000. Activation of C-C β-chemokines in human peripheral blood γδ T cells by isopentenyl pyrophosphate and regulation by cytokines. *Blood* 95: 59–47.

63. Wesch, D., S. Marx, and D. Kabellitz. 1997. Comparative analysis of αβ and γδ T cell activation by Mycobacterium tuberculosis and isopentenyl pyrophosphate. *Eur. J. Immunol.* 27: 952–956.

64. Poccia, F., B. Cipriani, S. Vendetti, V. Colizzi, G. Sireci, A. Salerno, and F. Dieli. 1999. In vivo γδ T cell priming to mycobacterial antigens by primary *Mycobacterium tuberculosis* infection and exposure to non-peptidic ligands. *Mol. Med.* 5: 471–476.

65. McKarns, S. C., R. H. Schwartz, and N. E. Kaminiski. 2004. Sma3 is essential for TGF-β1 to suppress IL-2 production and TCR-induced proliferation, but not IL-2-induced proliferation. *J. Immunol.* 172: 4275–4284.

66. Poccia, F., M. Malkovský, A. Pollak, V. Colizzi, G. Sireci, A. Salerno, and F. Dieli. 1999. In vivo γδ T cell priming to mycobacterial antigens by primary *Mycobacterium tuberculosis* infection and exposure to non-peptidic ligands. *Mol. Med.* 5: 471–476.