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The Vitamin D Analog, TX527, Promotes a Human CD4+CD25highCD127low Regulatory T Cell Profile and Induces a Migratory Signature Specific for Homing to Sites of Inflammation

Femke Baeke,*1 Hannelie Korf,*1 Lut Overbergh,* Annemieke Verslype,* Lieven Thorrez, ‡ Leentje Van Lommel, ‡ Mark Waer, ‡ Frans Schuit, ‡ Conny Gysemans,* and Chantal Mathieu*

The use of hypocalcemic vitamin D analogs is an appealing strategy to exploit the immunomodulatory actions of active vitamin D in vivo while circumventing its calcemic side effects. The functional modulation of dendritic cells by these molecules is regarded as the key mechanism underlying their ability to regulate T cell reactivity. In this article, we demonstrate the capacity of the vitamin D analog, TX527, to target T cells directly. Microarray analysis of purified human CD3+ T cells, cultured in the presence of TX527, revealed differential expression of genes involved in T cell activation, proliferation, differentiation, and migratory capacity. Accordingly, functional analysis showed a TX527-mediated suppression of the T cell proliferative capacity and activation status, accompanied by decreased expression of effector cytokines (IFN-γ, IL-4, and IL-17). Furthermore, TX527 triggered the emergence of CD4+CD25highCD127low regulatory T cells featuring elevated levels of IL-10, CTLA-4, and OX40 and the functional capacity to suppress activation and proliferation of effector T cells. Moreover, the vitamin D analog profoundly altered the homing receptor profile of T cells and their migration toward chemokine ligands. Remarkably, TX527 not only modulated skin-homing receptors as illustrated for the parent compound, but also reduced the expression of lymphoid organ-homing receptors (CD62L, CCR7, and CXCR4) and uniquely promoted surface expression of inflammatory homing receptors (CCR5, CXCR3, and CXCR6) on T cells. We conclude that TX527 directly affects human T cell function, thereby inhibiting effector T cell reactivity while inducing regulatory T cell characteristics, and imprints them with a specific homing signature favoring migration to sites of inflammation.

vitamin D, in particular its active metabolite 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], has been identified as a potentially interesting immunomodulator based on immune effects observed in vitro and in vivo (1, 2). Most reports on 1,25(OH)₂D₃ put forward its actions on APCs, especially dendritic cells (DCs), as the key feature underlying the immunomodulatory properties of this compound (3, 4). Active vitamin D alters the phenotype and functionality of DCs, locking them in a nonmature state, with features of tolerogenic DCs (3). The altered DCs are CD14⁺ and characterized by reduced expression of costimulatory molecules (e.g., CD40, CD80, and CD86), as well as of the T7 framework Program of the European Union with SAVEBETA as well as of the T7 framework Program of the European Union with Natural Immunomodulators as Novel Immunotherapies for Type 1 Diabetes, and the Juvenile Diabetes Research Foundation Center for β Cell Therapy in Diabetes (Grant 4-2005-1327).

The microarray data presented in this article have been submitted to the Gene Expression Omnibus under accession number GSE3984.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: 7-AAD, 7-aminocoumarin-10-carboxylic acid; CLA, cutaneous lymphocyte-associated Ag; DC, dendritic cell; GADD45α, growth arrest and DNA damage-inducible α; GITR, glucocorticoid-induced TNFR-related protein; KEGG, Kyoto Encyclopedia of Genes and Genomes; ND, not detected; nTreg, naturally occurring regulatory T cell; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; Treg, regulatory T cell; VDR, vitamin D receptor.

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greatly exceed the levels seen in physiological circumstances. One solution could be topical application of 1,25(OH)_{2}D_{3}, but even this administration route leads to hypercalcemia and bone decalcification (15). Only the use of synthetic analogs of 1,25(OH)_{2}D_{3}, in which calcemic and immunomodulatory actions have been dissociated, can lead to a possible in vivo application, topically or systemically. In this study, we explored the ability of TX527 [19-nor-14,20-bisepi-23-yne-1,25(OH)_{2}D_{3}], a hypocalcemic vitamin D analog with established immunoregulatory properties (16), to directly interfere with T cell phenotype and functionality. By using a synchronized system of T cell activation, we demonstrate direct effects of TX527 on human T lymphocytes, both at the phenotypic and functional level. Analysis of surface markers suggests the promotion of a Treg population (CD4^{+}CD25^{hi}CD127^{lo}), with differential alterations in cytokine production in CD4^{+} and CD8^{+} T cells. Finally, TX527 alters the chemokine receptor profile of T lymphocytes, inducing a specific homing signature, which would direct migration to skin (CCR4 and CCR10) and, in particular, to sites of inflammation (CCR5, CXCR3, and CXCR6).

Materials and Methods

Materials

RPML 1640 medium with Glutamax-I, FCS, and antibiotics (penicillin and streptomycin) were purchased from Invitrogen (Merelbeke, Belgium). Purified anti-CD3 mAb (clone UCHT1) and anti-CD28 mAb (clone 37A4) were obtained from R&D Systems (Minneapolis, MN). Human rIL-2 was purchased from PeproTech (London, U.K.). Human rCCL27, rCXCL12, and rCXCL16 were obtained from R&D Systems. The vitamin D analog TX527 [19-nor-14,20-bisepi-23-yne-1,25(OH)_{2}D_{3}] was synthesized by M. Vandewalle and P. De Clercq (University of Ghent, Ghent, Belgium) and obtained from Théramex (Monaco, France).

T cell isolation and culture

Fresh whole blood samples were collected from healthy donors, and PBMCs were isolated by Ficoll-gradient centrifugation (Axis-Shift Poc, Oslo, Norway). CD3^{+} T cells were further purified by a negative selection method using the Pan T cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer’s recommendations (purity routinely >96%). CD3^{+} T cells (0.7 × 10^{6} cells/ml) were seeded in 24-well culture plates coated with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) in RPML 1640 medium, supplemented with Glutamax-I, 10% heat-inactivated FCS, penicillin (100 IU/ml), and streptomycin (100 IU/ml). Where indicated, naturally occurring Tregs (nTregs) were depleted from the starting CD3^{+} T cell fraction, using CD25-microbeads II (Miltenyi Biotec) according to the protocol provided by the manufacturer. Every second day, cells were split and supplemented with fresh medium containing human rIL-2 (12.5 ng/ml). The cells were treated with TX527 (10^{-8} M) or vehicle (ethanol) every other day starting at day 2. This treatment schedule is chosen based on previous results showing an optimal induction of VDR expression levels in human T cells on day 2 following activation (17). In some experiments, CD4^{+} T cells were further separated from the total CD3^{+} T cell fraction after the 10-d culture period by negative selection (CD4^{+} T cell Isolation Kit II, Miltenyi Biotec, purity >95%). Also, the CD4-depleted cell fraction, which contained >85% CD8^{+} T cells, was retained for further analysis. In some experiments, CD4^{+}CD25^{hi}CD127^{lo} T cells from vehicle-treated and TX527-treated T cell populations were isolated by flow cytometric cell sorting on a FACSVantage (BD Biosciences, San Jose, CA).

Microarray analysis

RNA samples were prepared from five independent experiments using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany), and the quality was verified on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA (500 ng) was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) with oligo(dT)_{18} primers containing a T7 RNA polymerase promoter site. cDNA was further in vitro transcribed to cRNA and biotin labeled, according to Affymetrix user manual 702646Rev.8 (Affymetrix). Biotinylated cRNA was purified and fragmented. The quality of labeled and fragmented cRNA, respectively, was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies).

Fragmented biotinylated cRNA (10 μg) was hybridized overnight to the Human Genome U133 Plus 2.0 Array (Affymetrix). The arrays were washed and stained with streptavidin-PE on an automated Affymetrix Fluidics Station and scanned using the Affymetrix 3000 GeneScanner (Affymetrix). The image files were analyzed using Affymetrix GCOS Software (Affymetrix), and the RMA algorithm was used to calculate the signal intensities for each probe cell. The microarray datasets are deposited into the public database Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE23984. Gene probes displaying a fold change of >1.5 combined with p < 0.01 were considered as differentially expressed. The biological function analysis tool of the Ingenuity Pathway Analysis Software (Ingenuity Systems, Redwood City, CA; http://www.ingenuity.com) was used to identify the principal biological processes that were most significantly associated with the set of differentially expressed genes. Heat maps were constructed by hierarchical clustering with Functional Treeview (18) using log_{2} transformed expression values centered around zero.

RNA isolation and real-time RT-PCR

Total RNA was routinely isolated using the High Pure RNA Isolation Kit (Roche) or the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) in the case of sorted cells. The RNA samples (0.5–1 μg) were reverse transcribed using 100 U Superscript II Reverse Transcriptase (Invitrogen) and 5 μM oligo(dT)_{18} at 42°C for 80 min. Real time RT-PCR conditions were as previously reported (19, 20) using the MyQycler (Bio-Rad, Hercules, CA) and TaqMan systems (Applied Biosystems, Foster City, CA). Primer and probe sets for β-actin, VDR, 24-hydroxylase, IFN-γ, IL-4, IL-10, and IL-17 were the same as reported previously (17, 19, 21, 22). cDNA plasmid standards were used to quantify mRNA expression levels (19). For normalization, RT-PCR mRNA target copy numbers were divided by β-actin copy numbers, as a stably expressed housekeeping gene among all experimental conditions.

Proliferation assay

On day 8 of the cultures, CD3^{+} T cells were labeled with 2.5 μM CFSE (Molecular Probes, Leiden, The Netherlands) and replated in 24-well plates (0.7 × 10^{5} well). Cells were replenished with fresh medium supplemented with IL-2 (12.5 ng/ml) with or without TX527 (10^{-8} M) and cultured for an additional 48 h (until day 10). For analysis, cells were labeled with mAbs against CD4 and CD8, and the percentage of dividing cells (determined by a decrease of CFSE-signal) in each T cell subset was determined by flow cytometry. Dead cells were excluded from analysis by 7-aminoactinomycin D (7-AAD) staining (Bioscience, San Diego, CA).

Flow cytometric analysis of T cell phenotype

For analysis of surface protein expression, CD3^{+} T cells were harvested at the end of the 10-d culture term and stained with directly conjugated mAbs against CD4, CD8, CD25, CD69, CCR5 (eBioscience), cutaneous lymphocyte-associated Ag (CLA), integrin β_{2}, glucocorticoid-induced TNFR-related protein (GITR), Biologic, Sandiego, CA), CCR4, CCR6, CCR9, CCR10, CXCR6 (R&D Systems), CXCR3, CXCR5, CXCR4, CCR7, slectin (CD62L), CD40L, IL-7Rα (CD127), OX40 (CD134/TNFRSF4), ICOS (CD278) (BD Biosciences), or matching isotype controls. Dead cells were excluded from analysis using 7-AAD (eBioscience). Intracellular staining with mAbs against FOXP3 and CTLA-4 (both BD Biosciences) or matching isotype controls. Samples were acquired on an FACSComp instrument (BD Biosciences), and the analysis was performed using the FACSComp software (BD Biosciences). Overlay graphs were constructed using the FlowJo software (Tree Star, Ashland, OR).

Suppression assay

Autologous CD4^{+}CD25^{+} responder T cells, isolated from PBMCs, were labeled with eFluor 670 Proliferation Dye (eBioscience). PBMCs, depleted from CD19^{+} B cells, M. Vanderbilt, Gladbach, Germany) according to the manufacturer’s recommendations (purity routinely >96%). CD3^{+} T cells (0.7 × 10^{6} cells/ml) were seeded in 24-well culture plates coated with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) in RPML 1640 medium, supplemented with Glutamax-I, 10% heat-inactivated FCS, penicillin (100 IU/ml), and streptomycin (100 IU/ml). Where indicated, naturally occurring Tregs (nTregs) were depleted from the starting CD3^{+} T cell fraction, using CD25-microbeads II (Miltenyi Biotec) according to the protocol provided by the manufacturer. Every second day, cells were split and supplemented with fresh medium containing human rIL-2 (12.5 ng/ml). The cells were treated with TX527 (10^{-8} M) or vehicle (ethanol) every other day starting at day 2. This treatment schedule is chosen based on previous results showing an optimal induction of VDR expression levels in human T cells on day 2 following activation (17). In some experiments, CD4^{+} T cells were further separated from the total CD3^{+} T cell fraction after the 10-d culture period by negative selection (CD4^{+} T cell Isolation Kit II, Miltenyi Biotec, purity >95%). Also, the CD4-depleted cell fraction, which contained >85% CD8^{+} T cells, was retained for further analysis. In some experiments, CD4^{+}CD25^{hi}CD127^{lo} T cells from vehicle-treated and TX527-treated T cell populations were isolated by flow cytometric cell sorting on a FACSVantage (BD Biosciences, San Jose, CA).

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crowding in proliferation assays, equal numbers of unlabeled responder T cells were added to the cultures.

Chemotaxis assay
T cell migration was evaluated using 3-µm pore polycarbonate filters in 24-well transwell chambers (Corning, Lowell, MA). Control and TX527-treated CD3+ T cells (5 x 10^5 cells in 100 µl RPMI 1640 + 0.5% BSA) were added to the upper chamber and allowed to migrate toward the lower chambers, containing 600 µl RPMI + 0.5% BSA supplemented with human recombinant CXCL12 (100 ng/ml), recombinant CXCL16 (150 ng/ml), or recombinant CCL27 (2 µg/ml). A control condition, without chemokines, was included to determine background migration. Following incubation for 2 h at 37°C, cells in the lower compartment were collected and counted using CountBright Absolute Counting Beads (Molecular Probes). The percentage of migrating cells was calculated as a ratio between the number of migrating cells and the initial number of cells. Specific migration was calculated by subtracting the mean number of spontaneously migrating cells (migration to medium only) from the mean number of cells that migrated in response to the chemokine.

Statistical analysis
Functional enrichment scores were calculated by the Benjamini-Hochberg corrected Fischer’s exact test. All other statistical analysis was performed using the Student t test. Significance was defined as p < 0.05.

Results
TX527 regulates transcription of immune-related genes in purified human CD3+ T cells
Affymetrix GeneChip Arrays (Affymetrix) were performed on peripheral blood CD3+ T cells that were cultured as described in Materials and Methods for 10 d in the presence or absence of TX527. Of ∼54,000 probe sets analyzed, 936 (including 846 with an assigned gene identifier) were differentially expressed in TX527-treated T cells compared with vehicle-treated T cells (>1.5-fold regulated; p < 0.01). Within this group, 473 (50.5%) showed an increase in expression, and 463 (49.5%) showed a decrease in expression.

To provide evidence that our experimental system generated reliable data, we first verified differential expression of VDR- and known vitamin D-regulated genes among the two experimental conditions. As expected, upregulation of VDR and direct vitamin D target genes, such as 24-hydroxylase (known as CYP24A1) and also carbonic anhydrase II (CA2), demonstrate a direct regulation by ligand (TX527)-bound VDR in T cells (Supplemental Fig. 1A, Table I). Differential gene expression of VDR and 24-hydroxylase was confirmed by real-time RT-PCR (Supplemental Fig. 1B). Separation of CD3+ T cells into CD4+ and CD8-enriched T cell fractions prior to real-time RT-PCR analysis revealed a comparable degree of TX527-mediated regulation of these genes in both T cell subsets, suggesting that CD4+ and CD8+ T cells are equally responsive to the analog (Fig. 1A). These results indicate that this hypocalcemic vitamin D analog shares important metabolic-related actions with the parent compound.

More importantly, TX527 also affected transcription of previously described immune-related vitamin D targets, such as IFN-γ, CCR10, and growth arrest and DNA damage-inducible α (Table I), suggesting direct immunomodulatory actions on T cells and adaptive immune responses. Ingenuity Pathway Analysis (Ingenuity Systems, http://www.ingenuity.com), a literature-based online annotation tool, was used to identify the interactions and biological significance of the affected genes. The data revealed a preferential regulation of genes involved in cellular growth and proliferation, cell death, cellular development, cellular movement, and cell-to-cell signaling and interaction. These groups of genes represented the top five biological networks most significantly affected by the vitamin D analog (Fig. 1B, 1C; Supplemental Table I). First, we identified targets involved in cell growth, proliferation, and cell death, already described for the parent compound, 1,25(OH)2D3. In addition to these described pathways, TX527 uniquely regulated the expression of a large set of genes (n = 140) involved in cellular movement (Fig. 1D) and cell-to-cell signaling of T cells.

TX527 inhibits proliferation of CD4+ and CD8+ T cells
We next investigated whether the transcriptional regulation of genes involved in cell growth and proliferation by TX527 cor-

Table I. Microarray analysis reveals TX527-mediated gene regulation in purified human CD3+ T cells

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Title</th>
<th>Fold Change by TX527</th>
<th>UniGene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA2</td>
<td>Carbonic anhydrase II</td>
<td>+2.9</td>
<td>Hs.155097</td>
</tr>
<tr>
<td>CYP24A1 (24-hydroxylase)</td>
<td>Cytochrome P450, family 24, subfamily A, polypeptide 1</td>
<td>+17.4</td>
<td>Hs.89663</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D (1,25-dihydroxyvitamin D3) receptor</td>
<td>+2.0</td>
<td>Hs.524368</td>
</tr>
<tr>
<td>IL2RA (CD25)</td>
<td>IL-2R, α</td>
<td>+2.4</td>
<td>Hs.231367</td>
</tr>
<tr>
<td>TNFRSF4 (OX40)</td>
<td>TNFR superfamily, member 4</td>
<td>+2.4</td>
<td>Hs.129780</td>
</tr>
<tr>
<td>CD40LG</td>
<td>CD40L</td>
<td>−2.2</td>
<td>Hs.592244</td>
</tr>
<tr>
<td>CD69</td>
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<td>Hs.208854</td>
</tr>
<tr>
<td>IL7R (CD127)</td>
<td>IL-7R</td>
<td>−1.8</td>
<td>Hs.635723</td>
</tr>
<tr>
<td>CCR10</td>
<td>Chemokine (C-C motif) receptor 10</td>
<td>+5.5</td>
<td>Hs.278446</td>
</tr>
<tr>
<td>CXCR3</td>
<td>Chemokine (C-X-C motif) receptor 3</td>
<td>+1.6</td>
<td>Hs.198252</td>
</tr>
<tr>
<td>CXCR6</td>
<td>Chemokine (C-X-C motif) receptor 6</td>
<td>+4.2</td>
<td>Hs.34526</td>
</tr>
<tr>
<td>FUT7</td>
<td>Fucosyltransferase 7 α (1, 3 fucosyltransferase)</td>
<td>−2.0</td>
<td>Hs.457</td>
</tr>
<tr>
<td>IFNG</td>
<td>IFN-γ</td>
<td>−6.0</td>
<td>Hs.856</td>
</tr>
<tr>
<td>IL4</td>
<td>IL-4</td>
<td>−1.6</td>
<td>Hs.73917</td>
</tr>
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</table>

Gene expression profiles of human CD3+ T cells, treated with vehicle or TX527, were monitored by microarray analysis. Gene probes displaying a fold change >1.5 combined with p < 0.01 were considered as differentially expressed. A selection of genes of interest is listed. When multiple probe sets of a gene were differentially regulated, the average fold change is displayed.

GADD45A, growth arrest and DNA damage-inducible α.
related with the functional ability to inhibit T cell proliferation upon activation. The data indicated that, although CD4+ and CD8+ T cells did proliferate at the end 10-d culture term, cell division of both T cell populations was significantly inhibited by TX527 (Fig. 2). Importantly, there was no significant difference in the viability of T cells cultured with TX527 compared with vehicle-treated cells, indicating that the compound is not inherently toxic to T cells at this concentration (data not shown).

TX527 differentially modulates cytokine profiles in CD4+ and CD8-enriched T cell subsets
As microarray analysis revealed a TX527-mediated regulation of genes involved in cellular development, we analyzed the direct effects of TX527 on T cell differentiation by measuring the cytokine profile of activated human T cells postexposure to the vitamin D analog. For this purpose, CD3+ T cells were cultured as described above, but further separated into a CD4+ and a CD8-enriched T cell population, and mRNA-expression levels of 24-hydroxylase and VDR in the different T cell fractions were analyzed by quantitative real-time RT-PCR. The results shown represent the mean values ± SEM from one out of four independent experiments. The biological function analysis tool of the Ingenuity Pathway Analysis Software identified the most significant biological processes that were associated with the set of TX527-regulated genes, obtained by microarray analysis. The x-axis of the bar plot depicts the significance score of the top five of functional categories regulated by TX527. The statistical threshold is indicated by the dashed line. C. Pie chart displaying the number of TX527-regulated genes for each of the top five biological processes most significantly affected by the analog. D. Heat map showing the gene expression profiles of TX527-regulated genes (n = 140) that are involved in cellular movement. As indicated, the expression levels are mapped to a color gradient from low (green) to high expression (red), and the individual columns represent expression profiles of five independent donors. *p < 0.05; **p < 0.001 versus ctr.
tion and a CD4-depleted fraction (consisting out of >85% CD8+ T cells) at the end of the culture term, allowing a specific analysis of the cytokine profiles of the distinct T cell fractions.

Exposure of the cells to TX527 almost completely abrogated the Th1 cytokine function of CD4+ T cells, as demonstrated by the near basal levels of IFN-γ mRNA (Fig. 3A). In contrast to IFN-γ, CD4+ control cells showed rather low levels of the Th2 and Th17 cytokines, IL-4, and IL-17, respectively. Nevertheless, a similar TX527-mediated inhibition of IL-4 and IL-17 could be observed (Fig. 3A). Importantly, TX527 did not completely inhibit T cell cytokine production altogether because a specific upregulation of the immunoregulatory cytokine IL-10 was observed within the CD4+ T cell subset (Fig. 3A), whereas expression of IL-2 and TGF-β remained unchanged (data not shown). Analysis of CD8-enriched T cell fractions revealed similar results for all cytokines analyzed, except for IL-10, as TX527 treatment failed to upregulate the expression of this cytokine (Fig. 3B), indicating that the IL-10-inducing capacity of this synthetic VDR-agonist is restricted to CD4+ T cells.

TX527 alters the activation status of CD4+ and CD8+ T cells and promotes a Treg phenotype

We next investigated whether the TX527-mediated inhibition of T cell proliferation and cytokine responses is in agreement with a decreased activation status of the cells, because the microarray data revealed decreased mRNA expression levels of the T cell activation markers CD69 and CD40L in CD3+ T cells exposed to TX527 (Table I). To verify these inhibitory effects of TX527 on T cell activation, surface expression levels of CD69 and CD40L were measured by flow cytometry. By gating on the CD4+ T cell subset, the data revealed that, despite the 10-d time lapse since the initial T cell activating signal, CD4+ T cells from vehicle-treated cell cultures still featured a substantial number of CD69+ (36.1%) and CD40L+ T cells (26.8%) (Fig. 4A). In accordance with the reduced proliferative capacity, TX527 prevented CD4+ T cells to become fully activated, because only a minimal proportion of the cells stained positive for CD69 (21.0%; \( p < 0.005 \)) and CD40L (7.5%; \( p < 0.05 \)). Similar results for CD69 were obtained on the gated CD8+ T cell population (\( p < 0.05 \)), whereas CD40L was almost not expressed by this population (Fig. 4A).

Remarkably, microarray analysis revealed that TX527 enhanced the expression of IL-2RA by human CD3+ T cells, also known as

FIGURE 2. TX527 reduces the proliferative capacity of CD4+ and CD8+ T cells. Purified human CD3+ T cells were activated with anti-CD3/anti-CD28 and repeatedly treated with vehicle (ctr) or TX527 (10⁻⁸ M) every second day as described before. On day 8, cells were harvested, CFSE-labeled, and seeded out again in the presence or absence of TX527 (10⁻⁸ M). The cultures were supplemented with IL-2 (12.5 ng/ml) on the usual 2-d intervals to maintain T cell growth. On day 10, T cells were harvested and stained with mAbs directed against CD4 and CD8 before flow cytometric analysis was performed. The proliferating cell fraction was identified by a decrease of CFSE signal. Dead cells were excluded from analysis using 7-AAD staining. Mean values ± SEM of the percentages of proliferating cell fractions from one representative experiment are shown. A total of three independent donors were tested. *\( p < 0.05 \); **\( p < 0.005 \) versus ctr.

FIGURE 3. Cytokine responses in CD4+ and CD8-enriched T cell fractions are differentially regulated by TX527. Human CD3+ T cells, activated by anti-CD3/anti-CD28 and treated with vehicle (ctr) or TX527 (10⁻⁸ M) as described earlier, were further separated into a CD4+ and a CD8-enriched T cell populations following the 10-d culture period. The relative mRNA levels of IFN-γ, IL-17, IL-4, and IL-10 in the different T cell fractions were analyzed by quantitative real-time RT-PCR. The values depicted are the mean ± SEM of one representative experiment out of two to four independently tested donors. *\( p < 0.05 \) versus ctr. ND, not detected.
CD25, another surface marker implicated in T cell activation (Table I). Importantly, CD25 is, next to its status as an activation marker, also a marker for the identification of human nTregs, which were originally defined as CD4+CD25high T cells. Furthermore, the gene expression profile of TX527-treated T cells showed a decreased expression level of IL-7R, also known as CD127low T cells upon TX527 treatment resulted from an increased IL-7R expression level. Together, these data indicate that TX527 diminishes T cell activation while favoring the emergence of T cells with a regulatory phenotype.

**TX527 promotes Treg conversion from naive T cells with functional suppressive capacity**

To assess whether the increased abundance of CD4+CD25high CD127low T cells upon TX527 treatment resulted from an expansion of pre-existing nTregs or, instead, Treg conversion of naive CD4+ T cells was promoted by the analog, the starting population was depleted of nTregs prior to exposure to the analog (Fig. 5A). TX527 treatment of nTreg-depleted T cells induced the appearance of the characteristic CD4+CD25highCD127low population at the end of the culture period, suggesting that the analog induces a Treg phenotype rather than expanding nTregs already present in the starting culture (Fig. 5A).

To verify whether the TX527-induced Tregs exhibit functional capacity to suppress T cell responses, we tested their ability to counteract effector T cell activation and proliferation. In this regard, CD4+CD25+ T cell fractions, isolated from 10-d cultured vehicle- or TX527-treated T cells, were cocultured with Cell Proliferation Dye-labeled CD4+CD25− responder T cells in a standard suppression assay. T cell-depleted PBMCs and anti-CD3 provided the costimulatory and TCR stimuli, respectively. Of note, in line with the ability of TX527 to promote a Treg phenotype from naive T cells, clear elevated yields of CD4+CD25+ T cells were recovered from TX527-treated cultures (7.3% from vehicle-treated versus 16.5% from TX527-treated cultures; p < 0.005). More importantly, TX527-conditioned CD4+CD25+ T cells effectively prevented full activation of effector T cells as evidenced by the reduced expression of early activation marker, CD69, on labeled responder T cells (72.1% on activated responder T cells alone versus 60.4% in the presence of TX527 suppressors; p < 0.05) (Fig. 5B). Corroborating these findings, activation-induced responder T cell proliferation was inhibited when cultured in the presence of TX527 CD4+CD25+ T cells (72.8% proliferation of responder T cells alone versus 25.2% in the presence of TX527 suppressors; p < 0.05) (Fig. 5C). However, although the vitamin D analog promoted elevated Treg numbers displaying functional activity, TX527-conditioned T cells did not exhibit a superior suppressive function, because equal cell numbers of control CD4+CD25+ T cells showed a comparable ability to reduce expression of early activation markers and proliferation of responder T cells (Fig. 5B, 5C, lower panels).

In an attempt to unravel the mechanism by which TX527-induced Tregs are able to suppress effector T cell responses, we first tested their ability to produce secreted regulatory mediators. As IL-10 represents an important hallmark cytokine of various Treg subsets, we investigated whether the TX527-induced CD4+CD25highCD127low fraction was responsible for the increased IL-10 mRNA levels observed in the total CD4+ T cell fraction upon exposure to the analog (Fig. 3A). CD4+ T cells derived from vehicle-treated and TX527-treated cell cultures were sorted into a CD4+CD25highCD127low fraction (Treg fraction) and a remaining (non-Treg) fraction. FACS-sorted Treg fractions of TX527-treated T cells displayed elevated IL-10 mRNA levels compared with control Treg counterparts (Fig. 5D). However, also the TX527-treated non-Treg fractions featured increased IL-10 copy numbers compared with the control non-Tregs (data not shown).

Thus, the ability of TX527 to trigger IL-10 production could not be exclusively linked to the observed CD4+CD25highCD127low cell fraction. In the same line of investigation, we verified TX527-mediated regulation of other Treg-associated molecules, including OX40, ICOS, GITR, FOXP3, and CTLA-4, as possible contributors to the suppressive activity of the cells. Flow cytometric
analysis of the CD4+ T cell population revealed that continuous exposure to TX527 resulted in the inhibition of GITR expression, whereas FOXP3 and ICOS levels remained unaffected by the treatment. In contrast, the clear elevated expression of CTLA-4 and OX40 by CD4+ T cells upon exposure to the analog may implicate them as possible mediators in the suppressive function of TX527 Tregs (Fig. 5E).

TX527 induces a migratory signature in T cells specific for homing to skin and sites of inflammation

Next to the effects of TX527 on T cell activation, proliferation, and differentiation, a significant overrepresentation of genes involved in cellular movement (n = 140; Fig. 1D) was found among the set of differentially expressed genes. Remarkably, this group of cellular movement genes comprised 17 genes directly acting in the chemokine signaling pathway (Kyoto Encyclopedia of Genes and Genomes [KEGG]: 04062) (Fig. 6A). Furthermore, also various genes participating in other aspects of cell motility were found to be affected by TX527, including members of the pathways regulating focal adhesion (n = 11; KEGG: 04510), cell adhesion molecules (n = 10; KEGG: 04514), and actin cytoskeleton (n = 9; KEGG: 04810).

To gain insight into the role of TX527 as a potential modulator of T cell movement, we first analyzed the surface expression levels of a broad range of homing receptors. Our search comprised a selection of those receptors affected by TX527 at the mRNA level (Fig. 6A, Table I), as well as a large set of other tissue- or environment-specific lymphocyte trafficking molecules, including skin-homing receptors (CCR4, CCR6, CCR10, and CLA), receptors regulating T cell trafficking to the gut (integrin β7 and CCR9), homing receptors for secondary lymphoid organs (β2-selectin/CD62L, CCR7, and CXCR7), and receptors directing T cells to sites of inflammation (CCR5, CXCR3, and CXCR6) (Fig. 6B). In accordance with data reported for 1,25(OH)2D3, TX527 conditioning of the cells induced expression of CCR10 on CD4+ (p < 0.001) and CD8+ T cells (p < 0.005), whereas expression of CLA showed a decreasing trend, which was most pronounced in CD8+ T cells (CD4+: NS; CD8+: p < 0.05).
TX527-responsive skin-homing receptor, as the analog strongly elevated the number of cells expressing CCR4 ($p < 0.05$), especially within the CD8$^+$ T cell compartment, whereas expression of CCR6, another receptor proposed to be involved in skin homing, was not altered by TX527. Gut-homing receptors CCR9 and integrin $\beta_7$ were not affected by the analog. Of interest, TX527 impaired the expression of receptors involved in T cell homing to secondary lymphoid organs within CD4$^+$ and CD8$^+$ T cell populations, including CD62L (CD4$^+$: $p < 0.0005$; CD8$^+$: $p < 0.005$), CCR7 (CD4$^+$: $p < 0.005$; CD8$^+$: $p < 0.01$; CD8$^+$: NS). Instead, TX527 uniquely triggered elevated expression of multiple chemokine receptors guiding T cells to sites of inflammation in both T cell subsets, including CCR5 (CD4$^+$: $p < 0.01$; CD8$^+$: $p < 0.05$), CXCR3 (CD4$^+$: $p < 0.001$; CD8$^+$: $p < 0.05$), and CXCR6 (CD4$^+$: $p < 0.005$; CD8$^+$: $p < 0.05$).

Next, the functional relevance of the unique chemokine receptor profile was assessed by measuring the chemotactic response of TX527-conditioned CD3$^+$ T cells to three chemokines: CXCL12,

FIGURE 6. TX527 imprints T cells with a homing receptor signature specific for migration to inflammatory sites and alters their responsiveness to chemokine ligands. T cells, activated by anti-CD3/anti-CD28 and cultured in the presence of TX527 or vehicle, were screened for altered expression of genes and proteins involved in cellular movement and altered migratory capacity. A, Heat map showing the microarray gene expression levels, mapped to a color gradient from low (green) to high expression (red), of those genes acting in the chemokine signaling pathway (KEGG: 04062) and regulated by TX527 ($n = 17$). Genes marked with an asterisk were further analyzed at the protein level. B, T cell homing receptor expression was measured by flow cytometry. Histogram overlays of CD4$^+$ or CD8$^+$ subsets of vehicle-treated (solid lines) versus TX527-treated (dashed lines) T cells depict surface expression of receptors directing T cells to skin (CCR4, CCR6, CCR10, and CLA), gut (CCR9 and integrin $\beta_7$), secondary lymphoid organs (CD62L, CCR7, and CXCR4), or to inflammatory sites (CCR5, CXCR3, and CXCR6). 7-AAD was used to exclude dead cells from analysis. The results are representative of three to eight independent experiments. C, The functional ability of CD3$^+$ T cells, conditioned with TX527 (10$^{-8}$ M) or vehicle (ctr) to migrate toward the chemokines CXCL12 (100 ng/ml), CXCL16 (150 ng/ml), and CCL27 (2 $\mu$g/ml) was investigated using a transwell tissue culture system (with a 3-µm pore size). The results shown are representative of four independent experiments and depict the net migration after subtraction of background migration. *$p < 0.005$ versus control.
CXCL16, and CCL27, being the ligands for CXCR4 (secondary lymphoid organ homing), CXCR6 (homing to inflammatory sites), and CCR10 (skin-homing), respectively. Cells that had been pre-exposed to TX527 exhibited a substantial decrease in their migratory capacity to the chemokine CXCL12, whereas chemotaxis toward CXCL16 and CCL27 was enhanced compared with vehicle-treated T cells (Fig. 6C). Overall, these data demonstrate that TX527 dramatically alters the migratory behavior of human T cells in response to chemokine ligands by modulating their homing receptor signature, as well as by targeting important signaling pathways underlying cellular movement processes.

Discussion

Analogs of vitamin D have been developed to allow in vivo exploitation of the anticancer and immunomodulatory effects of vitamin D with reduced calcemic side effects (23). Vitamin D affects the immune system in a wide variety of ways, and in animal models, the therapeutic potential of analogs in the modulation of graft survival or autoimmunity has been demonstrated (2). The main target for vitamin D and its analogs is thought to be the DC, thereby indirectly altering T cell responses, but effects involving direct modulation of T cell responses by these compounds remain less well characterized. In this report, we investigated the ability of a hypocalcemic vitamin D analog, TX527, to directly interfere with proximal pathways involved in human T cell activation at a transcriptional, phenotypical, as well as functional level. We demonstrated that the synthetic VDR agonist exerted inhibitory actions on T cell proliferation and effector cytokine production, functions that are shared with the parent compound. However, TX527 also exclusively triggered the induction of a functional CD4+CD25+CD127low Treg population in the absence of any other cytokines. Particularly, the ability of 1,25(OH)2D3, acting in synergism with IL-2, to induce CD4+CD25+ and/or CD4+CD25+Foxp3+ Tregs upon 1,25(OH)2D3 or analog treatment in vivo (34, 35). Until now, this effect is proposed to rely on the ability of VDR agonists to induce myeloid DCs with tolerogenic properties, because in vitro 1,25(OH)2D3-treated DCs favor the emergence of CD4+FOXP3+ Tregs (7). The results presented in this study strongly indicate that TX527 can directly modulate T cell signaling pathways to promote a CD4+CD25+CD127low Treg phenotype, underlining the capacity of this agonist to affect T cell responses in the absence of APCs and APC-derived factors. In support of our data, others have reported upregulation of CD25 on human T cells when simultaneously exposed to IL-2 and 1,25(OH)2D3 (36). In addition, a recent study demonstrated the ability of 1,25(OH)2D3, acting in synergism with IL-2, to induce IL-10–producing CD4+FOXP3+ Tregs from human purified CD4+ peripheral blood T cells in vitro (14). The fact that we did not observe FOXP3 induction at day 10 could possibly be linked to its transient expression pattern, peaking at 4 d and decreasing thereafter, as shown in the presence of 1,25(OH)2D3 or analog treatment in vivo (14). Also important to note here is that the Tregs induced by 1,25(OH)2D3 and IL-2 that were implemented for demonstration of suppressive function were originally activated in the presence of monocytes at the start of the culture (14). This is in sharp contrast with TX527-conditioned Tregs, which were never in contact with APCs during the initial 10-d culture term. Considering the importance of costimulatory signals for Treg homeostasis and function (37), these crucial differences in experimental setup in the above-mentioned study and the current one may explain the failure of the analog to promote FOXP3 induction and superior suppressive function.

Besides the ability to induce functional Tregs, TX527 also preferentially regulated 140 genes involved in cellular movement, representing one of the top five biological processes most significantly affected by TX527. Several of them encode chemokine receptors that are not uniquely involved in the regulation of T cell homing to cutaneous sites, strongly arguing for a much broader role of vitamin D analogs in T cell trafficking than currently assumed. In agreement with previous studies (38, 39), our results demonstrated a similar TX527-mediated regulation of CCR10 and CLA. In addition, we identified the skin-homing receptor CCR4 as an essential component for 1,25(OH)2D3-mediated inhibition of experimental autoimmune encephalomyelitis (32). Interestingly, in an experimental setup comparable to ours, a combination of 1,25(OH)2D3 and dexamethasone was shown to induce IL-10–producing CD4+ Tregs in vitro, which were able to suppress autoimmune demyelination in vivo in an Ag-specific way (27, 31). In accordance with our data, these induced Tregs did not produce IFN-γ and IL-4, nor did they express Foxp3 (27, 33).

The concept of VDR agonists favoring the development of T cells with regulatory properties is further strengthened by the presence of CD4+CD25+CD127low T cells upon TX527 treatment. Our data suggest the de novo conversion of naïve CD4+ T cells into CD4+CD25+CD127low T cells rather than expansion of existing Tregs, because depletion of CD4+CD25+ cells from the starting culture still reproduced the characteristic Treg phenotype following TX527 treatment. More importantly, TX527-induced Tregs exhibited the functional capacity to suppress activation as well as proliferation of effector T cells. However, although the analog clearly triggered increased Treg numbers, their suppressive functions remained comparable to those of CD4+CD25+ T cells isolated from control T cell cultures. Nevertheless, TX527-induced Tregs featured elevated levels of regulatory mediators such as IL-10 as well as CTLA-4 and OX40, which might be relevant for increased suppressive function in more relevant in vivo settings. Different groups have previously reported increased numbers of CD4+CD25+ and/or CD4+CD25+Foxp3+ Tregs upon 1,25(OH)2D3 or analog treatment in vivo (34, 35). Until now, this effect is proposed to rely on the ability of VDR agonists to induce myeloid DCs with tolerogenic properties, because in vitro 1,25(OH)2D3-treated DCs favor the emergence of CD4+FOXP3+ Tregs (7). The results presented in this study strongly indicate that TX527 can directly modulate T cell signaling pathways to promote a CD4+CD25+CD127low Treg phenotype, underlining the capacity of this agonist to affect T cell responses in the absence of APCs and APC-derived factors. In support of our data, others have reported upregulation of CD25 on human T cells when simultaneously exposed to IL-2 and 1,25(OH)2D3 (36). In addition, a recent study demonstrated the ability of 1,25(OH)2D3, acting in synergism with IL-2, to induce IL-10–producing CD4+FOXP3+ Tregs from human purified CD4+ peripheral blood T cells in vitro (14). The fact that we did not observe FOXP3 induction at day 10 could possibly be linked to its transient expression pattern, peaking at 4 d and decreasing thereafter, as shown in the presence of 1,25(OH)2D3 or analog treatment in vivo (14). Also important to note here is that the Tregs induced by 1,25(OH)2D3 and IL-2 that were implemented for demonstration of suppressive function were originally activated in the presence of monocytes at the start of the culture (14). This is in sharp contrast with TX527-conditioned Tregs, which were never in contact with APCs during the initial 10-d culture term. Considering the importance of costimulatory signals for Treg homeostasis and function (37), these crucial differences in experimental setup in the above-mentioned study and the current one may explain the failure of the analog to promote FOXP3 induction and superior suppressive function.

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CCR4+CD8+ T cells were identified as an immature memory treated CD4+ T cells toward CXCL12 and CCL21, the ligands of effector T cell functions while inducing increased numbers of 1,25(OH)2D3, but only when applied in combination with TNF-

References
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References


**Supplemental Figure 1. TX527 triggers VDR-dependent signaling pathways in human activated T cells.** Purified human CD3+ T cells were activated by anti-CD3/anti-CD28. From day 2 onwards, cells were repeatedly treated with vehicle (ctr) or TX527 (10^{-8}M) every second day. On day 10, mRNA-expression profiles of TX527-treated and vehicle-treated T cells were compared using microarray analysis and real-time RT-PCR. A) Heat map showing individual expression levels of VDR, 24-hydroxylase and CA2 of 5 independent donors, mapped to a color gradient from low (green) to high expression (red). B) mRNA expression levels of 24-hydroxylase and VDR were confirmed by quantitative real-time RT-PCR. The results shown are mean values ± SEM of 1 representative experiment from 4 independent donors (*, p<0.05; ***,p<0.005 vs ctr).