INFLAMMATION

Proresolving lipid mediators resolvin D1, resolvin D2, and maresin 1 are critical in modulating T cell responses

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Resolution of inflammation is a finely regulated process mediated by specialized proresolving lipid mediators (SPMs), including docosahexaenoic acid (DHA)–derived resolvins and maresins. The immunomodulatory role of SPMs in adaptive immune cells is of interest. We report that D-series resolvins (resolvin D1 and resolvin D2) and maresin 1 modulate adaptive immune responses in human peripheral blood lymphocytes. These lipid mediators reduce cytokine production by activated $CD8^+$ T cells and $CD4^+$ T helper 1 (T_H1) and T_H17 cells but do not modulate T cell inhibitory receptors or abrogate their capacity to proliferate. Moreover, these SPMs prevented naïve $CD4^+$ T cell differentiation into T_H1 and T_H17 by down-regulating their signature transcription factors, T-bet and Rorc, in a mechanism mediated by the GPR32 and ALX/FPR2 receptors; they concomitantly enhanced de novo generation and function of $Foxp3^+$ regulatory T (T_{reg}) cells via the GPR32 receptor. These results were also supported in vivo in a mouse deficient for DHA synthesis ($Elovl2^{-/-}$) that showed an increase in T_H1/T_H17 cells and a decrease in T_{reg} cells compared to wild-type mice. Additionally, either DHA supplementation in $Elovl2^{-/-}$ mice or in vivo administration of resolvin D1 significantly reduced cytokine production upon specific stimulation of T cells. These findings demonstrate actions of specific SPMs on adaptive immunity and provide a new avenue for SPM-based approaches to modulate chronic inflammation.

INTRODUCTION

Acute inflammation is generally protective for the host and is mediated by a plethora of well-known chemical messengers, including cytokines, chemokines, and lipid-derived mediators (mostly produced from the essential fatty acid arachidonic acid) released by innate immune cells (*1*–*3*). Resolution of inflammation is a finely orchestrated active process governed by temporally and spatially regulated synthesis of local mediators that act in concert to reestablish tissue homeostasis after injury and phlogistic processes [for a recent review, see (*4*)]. The resolvins, protectins, maresins, and lipoxins, often referred together as specialized proresolving lipid mediators (SPMs) given their functions (*4*), are novel families of autacoids with a central role in resolving processes, which act as local mediators controlling the magnitude and extent of inflammatory events.

SPMs are produced mainly by macrophages and neutrophils via separate pathways from omega-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the former yielding E-series resolvins and the latter D-series resolvins, maresins, and protectins, as well as from omega-6 PUFA arachidonic acid, which gives rise to lipoxins, via the action of lipoxygenases ALOX-5, ALOX-12, and ALOX-15 and cyclooxygenase COX-2 (*5*–*8*). These SPMs have received considerable attention in recent years because of their ability to stereoselectively regulate and reduce inflammatory conditions in animal disease models (*4*). Thus, SPMs prevent inflammation from spreading and halt the transition from acute to chronic. Yet, published studies focus almost exclusively on acute conditions and innate immunity, and little is currently known about their possible action on the cellular components of adaptive immunity. This includes the finding that resolvin E1 induces apoptosis of activated T cells and protectin D1 reduces T cell migration (*9*, *10*). The present study investigated the selective actions of D-series resolvins and maresin 1 (MaR1), major SPMs that were recently found in human lymphoid tissues, including human spleen and lymph nodes (*11*). Hence, we focused either on circulating $CD8^+$ and $CD4^+$ T lymphocytes or on $CD4^+$ subsets, which include highly pathogenic T helper 1 (T_H1) and T_H17 cells, as well as regulatory T (T_{reg}) lymphocytes. These results document the pivotal role(s) for specific SPMs in the control of adaptive immunity, thus providing a better understanding of the impact of these potent new bioactive lipid mediators on the spectrum of immune cells and ultimately setting the standard for the potential development of new treatments for chronic inflammatory diseases.

RESULTS

Proresolving lipid mediators modulate $CD8^+$ and $CD4^+$ T cell responses

Although data on SPMs mostly focus on innate immune cells involved in the resolution of acute inflammation (*4*), we hypothesized that several SPMs, specifically resolvin D1 (RvD1), RvD2, and MaR1 (Fig. 1A), could also affect the immune responses of adaptive immune cells. To test this hypothesis, we performed initial studies to assess whether increasing concentrations of RvD1, RvD2, and MaR1 (in the 1 to 100 nM physiological range) (*12*) could affect the production of tumor necrosis factor–α (TNF-α) from human $CD8^+$ and $CD4^+$ T lymphocytes (Fig. 1B). Both T cell subsets when activated with phorbol 12-myristate 13-acetate

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(PMA)/ionomycin produced high amounts of intracellular TNF-α (Fig. 1, C and D), which was reduced upon pretreatment with all SPMs tested (Fig. 1D). Each of the specific mediators inhibited TNF-α production in a dose-dependent manner and substantially reduced cytokine production at doses as low as 10 nM. The lowest dose tested (1 nM) only showed a slight and nonsignificant reduction of TNF-α production from both T cell subsets (Fig. 1D). The same result was also observed with an epimer of another newly discovered resolvin, the aspirin-triggered resolvin D3 (AT-RvD3), which dose-dependently reduced TNF-α production from both $CD8^+$ and $CD4^+$ T cells and was significant ($P < 0.05$) at doses as low as 10 nM (fig. S1A). For this reason, in all further experiments, SPMs were used at the lowest effective concentration (10 nM). These initial results suggested that SPMs might indeed be effective in modulating adaptive immune responses.

We next ascertained the possible impact of these SPMs (at 10 nM) on the production of the specific cytokines that characterize the main proinflammatory T cell subsets, that is, cytotoxic $CD8^+$ T cells and $CD4^+$ T_H1 and T_H17 cells. PMA/ionomycin-activated $CD8^+$ T cells produced high amounts of TNF-α and interferon-γ (IFN-γ), which were strongly reduced by each of these SPMs (Fig. 2A). Furthermore, the production of both IFN-γ and interleukin-17 (IL-17) from PMA/ionomycin-activated $CD4^+$ T cells was also strongly reduced by incubation with these SPMs (Fig. 2B). The ability of the different SPMs to suppress cytokine production from $CD8^+$ and $CD4^+$ T cells was independent of the chemical class, suggesting that distinct lipid mediators can similarly modulate adaptive immune cells. Because the cytokine profile of human T cells may be differently determined depending on the assay and conditions used, we further investigated the immunomodulatory role of RvD1, RvD2, and MaR1 using a more specific and physiological stimulus for activating T cells, that is, polyclonal activation of the T cell receptor (TCR) with anti-CD3 and anti-CD28. Cytokine production after stimulation of T cells with anti-CD3/CD28 was almost identical to that of PMA/ionomycin stimulation, even if intracellular levels of cytokines were lower, as expected (Fig. 2, C and D) (*13*). In particular, RvD1, RvD2, and MaR1 significantly ($P < 0.05$) reduced the capability of $CD8^+$ T cells to produce TNF-α and IFN-γ (Fig. 2C) and that of $CD4^+$ T cells to produce TNF-α ($P < 0.05$), IFN-γ ($P < 0.01$), and IL-17 ($P < 0.01$) (Fig. 2D), overall suggesting that SPMs might regulate T_H1 and T_H17 responses.

SPMs regulate IL-2 production from T cells without affecting their viability

The immunomodulatory activity of D-series resolvins and MaR1 on T cell responses was also demonstrated by the reduction of the crucial

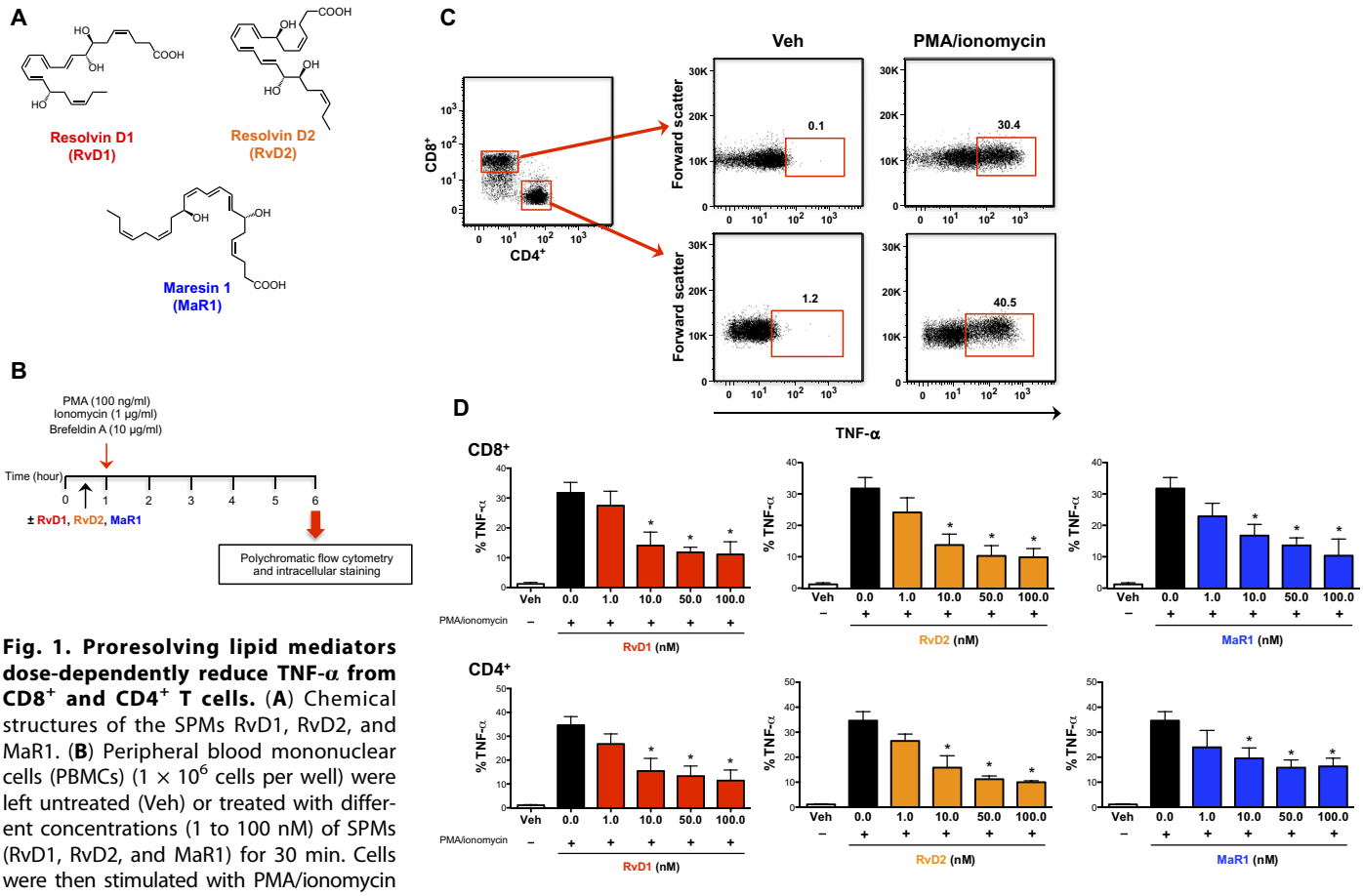

Fig. 1. Proresolving lipid mediators dose-dependently reduce TNF-α from $CD8^+$ and $CD4^+$ T cells. (**A**) Chemical structures of the SPMs RvD1, RvD2, and MaR1. (**B**) Peripheral blood mononuclear cells (PBMCs) (1×10^6 cells per well) were left untreated (Veh) or treated with different concentrations (1 to 100 nM) of SPMs (RvD1, RvD2, and MaR1) for 30 min. Cells were then stimulated with PMA/ionomycin for 6 hours, stained at the cell surface and intracellularly, and analyzed by flow cytometry. (**C**) Representative cytofluorimetric plot of the gating strategy for TNF-α evaluation from $CD8^+$ and $CD4^+$ T cells. PBMCs were appropriately gated according to physical parameters. (**D**) Percentages of intracellular cytokine production in both $CD8^+$ and $CD4^+$ T cells. Data are means ± SEM of four independent experiments. *$P < 0.05$ [one-way analysis of variance (ANOVA)].

growth factor IL-2 compared with IL-2–producing anti-CD3/CD28–activated $CD8^+$ and $CD4^+$ T cells (Fig. 3A). This effect was not due to an induction of cell death, as assessed by annexin V staining that was used as a marker for apoptosis in combination with propidium iodide (PI), to distinguish between apoptotic and necrotic cells. As expected, resting T cells were all annexin V–negative and PI-negative, whereas ~20% of anti-CD3/CD28–activated T cells were apoptotic (annexin V–positive) (Fig. 3B). Almost no variation in live cells (annexin V–negative and PI-negative), early apoptosis (annexin V–positive and PI-negative), late apoptosis (annexin V–positive and PI-positive), or necrosis (annexin V–negative and PI-positive) could be detected in RvD1-, RvD2-, and MaR1-treated activated T cells, either on the total $CD3^+$ T cell population (Fig. 3B, upper panel) or in $CD8^+$ or $CD4^+$ T cells (Fig. 3B, lower panel). Even after 24 hours of treatment, no significant increase in the proportion of total apoptotic cells was observed in both $CD8^+$ and $CD4^+$ T cells treated with the different SPMs compared to activated cells, although general cell viability showed a ~50% decrease (fig. S1B). SPM treatment of resting T cells did not induce apoptosis, ruling out a potential cytotoxic role of these SPMs (fig. S1C). The decrease in IL-2 did not result in a significant decrease in T cell proliferation (Fig. 3C) nor was associated with an altered cell surface expression of several inhibitory receptors, including Fas ligand (FasL), programmed cell death protein 1 (PD-1), and cytotoxic T lymphocyte–associated protein 4 (CTLA-4) (Fig. 3D).

SPMs critically affect T_H1/T_H17 and T_{reg} differentiation

T_H1 and T_H17 subsets in peripheral blood are both derived from naïve $CD4^+$ T cells upon antigen stimulation and specific cytokine skewing (*14*). Because both classes of SPMs dampened the inflammatory response of TNF-α– and IFN-γ–producing T_H1 cells and of IL-17–producing T_H17 cells from PBMCs, we next investigated whether RvD1, RvD2, or MaR1 could directly affect their differentiation from naïve $CD4^+$ T cells into T_H1 and T_H17 lineages. To this aim, a standard naïve $CD4^+$ T cell differentiation assay was performed by polyclonal stimulation with anti-CD3/CD28 and specific polarizing cytokines in the presence of RvD1, RvD2, or MaR1 (Fig. 4A).

Under specific polarizing conditions, highly purified naïve $CD4^+$ T cells displayed significantly higher amounts of intracellularly produced and extracellularly released IFN-γ (T_H1) ($P < 0.05$) and IL-17 (T_H17) ($P < 0.01$), as compared to nonpolarized (T_H0) cells (Fig. 4, B and C). Furthermore, T_H17 cells produced less IFN-γ, whereas T_H1 cells produced very low levels of IL-17, confirming previous data showing that the T_H17 cytokine profile overlaps with T_H1 cells (*15*). RvD1, RvD2, and MaR1 all affected T_H1 and T_H17 polarization (Fig. 4, B and C). In particular, in nonskewed T_H0 cells, which produce very low levels of both IFN-γ and IL-17, RvD1, RvD2, and MaR1 induced a slight and nonsignificant decrease in both cytokines (fig. S2), whereas they significantly reduced T_H1 and T_H17 generation, acting on both intracellular production (Fig. 4B) and extracellular release (Fig. 4C) of IFN-γ ($P < 0.05$) from T_H1 cells and that of IL-17 ($P < 0.01$) and IFN-γ ($P < 0.05$) from T_H17 cells. To address whether T_H1 and T_H17 polarization was associated with the acquisition of their typical features, we also measured the mRNA encoding for the transcription factors known to be critical for their differentiation, T-bet and RORc, respectively. As expected, T_H1 and T_H17 conditions induced the highest expression of their specific transcription factors. The presence of RvD1, RvD2, or MaR1 during T_H1/T_H17 polarization led to decreased T-bet in T_H1 cells and RORc in T_H17 cells, with D-series resolvins being more effective than MaR1 (Fig. 4D). These findings support a pivotal role for SPMs in hindering de novo T_H1/T_H17 differentiation. Notably, these SPMs also slightly increased intracellular IL-4 production (Fig. 4B), but not its extracellular release (Fig. 4C), and they decreased the signature T_H2 transcription factor GATA-3 (Fig. 4D).

To obtain some in vivo evidence for the role of SPMs in reducing T_H1 and T_H17 responses, we analyzed the ability of these cells to express IFN-γ and IL-17 in mice deficient for elongase 2 ($Elovl2^{-/-}$), the key enzyme involved in the synthesis of DHA (the precursor of D-series

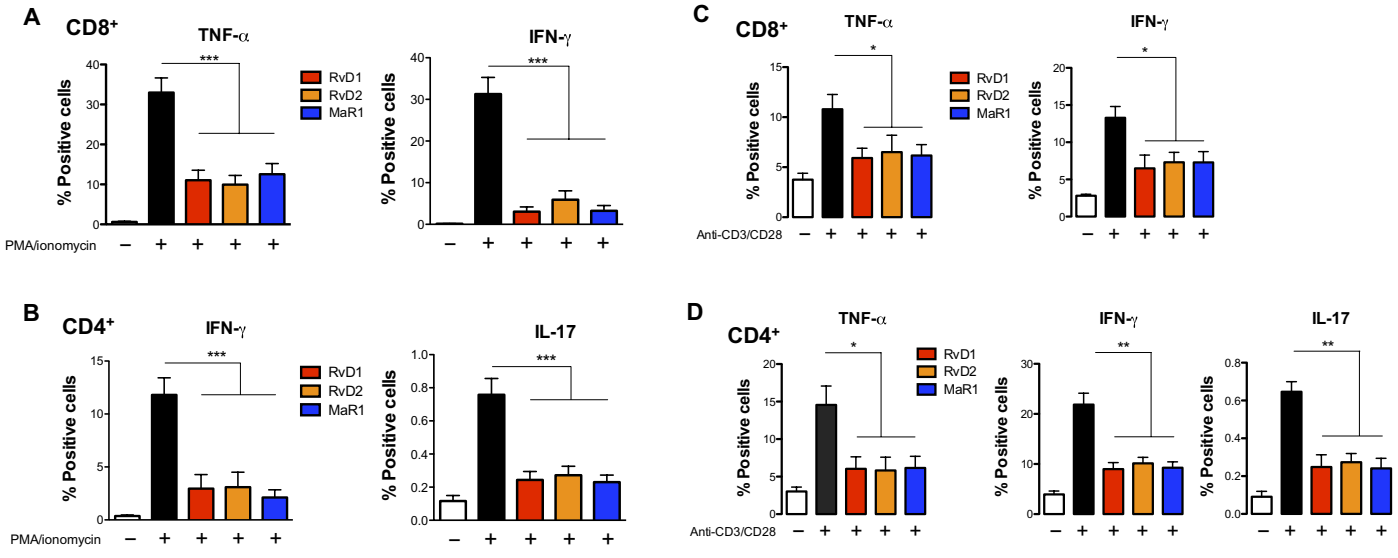

Fig. 2. Proresolving lipid mediators reduce $CD8^+$ and $CD4^+$ T cell responses. (**A** to **D**) PBMCs (1×10^6 cells per well) were left untreated or treated with RvD1, RvD2, and MaR1 (10 nM) for 30 min. Cells were then stimulated with PMA/ionomycin for 6 hours [(A) and (B)] or with anti-CD3/CD28 beads [(C) and (D)], stained at the cell surface and intracellularly, and analyzed by flow cytometry, as detailed in Materials and Methods. Percentages of intracellular production of TNF-α and IFN-γ from $CD8^+$ and of TNF-α, IFN-γ, and IL-17 from $CD4^+$ T cells are shown as means ± SEM of eight independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (one-way ANOVA).

resolvins and maresins) from EPA. Elovl2$^{-/-}$ mice have significantly reduced DHA levels and increased levels of DHA precursors, including EPA (*16*). As shown in Fig. 4E, splenic T cells produce higher amounts of IFN-γ and IL-17 in Elovl2$^{-/-}$ mice compared to wild-type control mice and this was reversed after DHA supplementation, suggesting that, in the absence of the precursor of D-series resolvins and maresins, T_H1 and T_H17 responses are exacerbated. To further corroborate the in vivo role of SPMs in reducing T cell activation, intraperitoneal administration of RvD1 (100 ng per mouse) together with anti-CD3 (50 μg per mouse) significantly reduced the percentage of IFN-γ and IL-17 production ($P < 0.05$) from peripheral blood CD4$^+$ T cells (Fig. 4F) in both wild-type and Elovl2$^{-/-}$ mice, whereby the latter showed increased cytokine production. Together, these results suggest that anti-inflammatory actions of SPMs at physiological doses on T_H1 and T_H17 cells are also demonstrable in vivo.

In light of the role of SPMs in resolving inflammation and because T_{reg} cells are an important cell subset involved in modulating and maintaining self-regulation of the immune system, we also investigated whether SPMs could affect the generation of induced T_{reg} (iT_{reg}) cells. This cell subset develops from naïve CD4$^+$ T cells upon antigen stimulation and transforming growth factor–β (TGF-β) exposure (*17*). To this aim, highly purified naïve CD4$^+$ T cells were cultured under T_{reg}-inducing conditions in the presence of RvD1, RvD2, and MaR1 (all at 10 nM) (Fig. 5A). We found that each of these SPMs potentiated iT_{reg} differentiation, with the lipids significantly enhancing Foxp3 expression compared to control iT_{reg} cells ($P < 0.05$ for RvD2 and $P < 0.01$

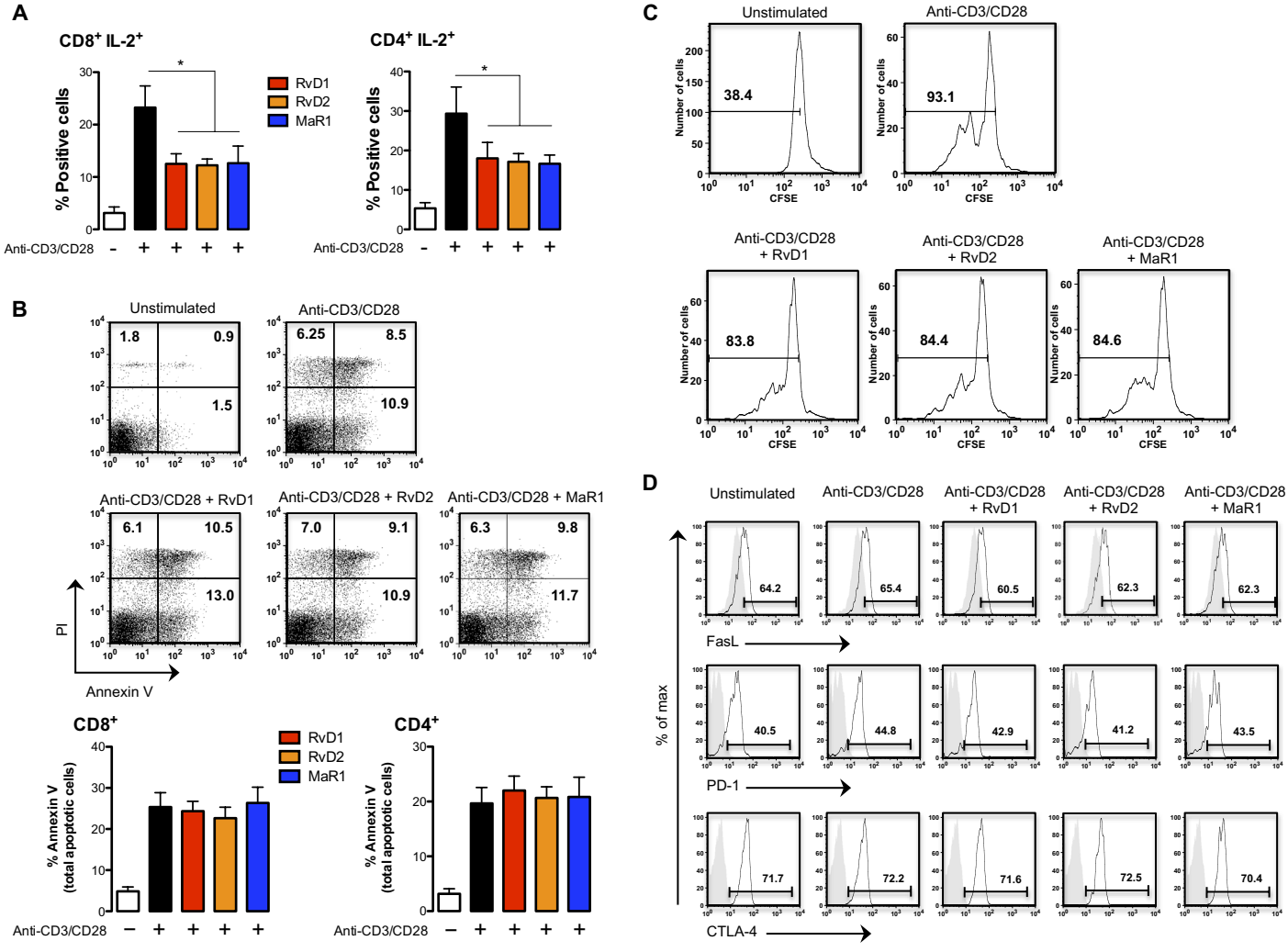

Fig. 3. Proresolving lipid mediators inhibit IL-2 production from TCR-activated CD8$^+$ and CD4$^+$ T cells without affecting their viability. PBMCs (1×10^6 cells per well) were left untreated or treated with RvD1, RvD2, and MaR1 (10 nM) for 30 min. Cells were then stimulated with anti-CD3/CD28 for 8 hours, stained at the cell surface and intracellularly, and analyzed by flow cytometry. (**A**) Percentages of intracellular production of IL-2 from CD8$^+$ and CD4$^+$ T cells are shown as means ± SEM of six independent experiments. *$P < 0.05$ (one-way ANOVA). (**B**) Cell death of CD8$^+$ and CD4$^+$ T cells after stimulation with anti-CD3/CD28 beads through staining for annexin V and PI flow cytometry analysis. The percentage of annexin V–positive/PI-negative cells (early apoptotic cells) and annexin V–positive cells (total apoptotic cells) is reported in the cumulative graph. Data are means ± SEM of four independent experiments. (**C**) Cell proliferation of CD3$^+$ T cells after stimulation with anti-CD3/CD28 beads (day 4) shown by carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution. (**D**) Surface expression of FasL, PD-1, and CTLA-4 in CD3$^+$ T cells after stimulation with anti-CD3/CD28. A representative experiment (of four independent experiments) of receptor expression is shown (the isotype is shown in gray).

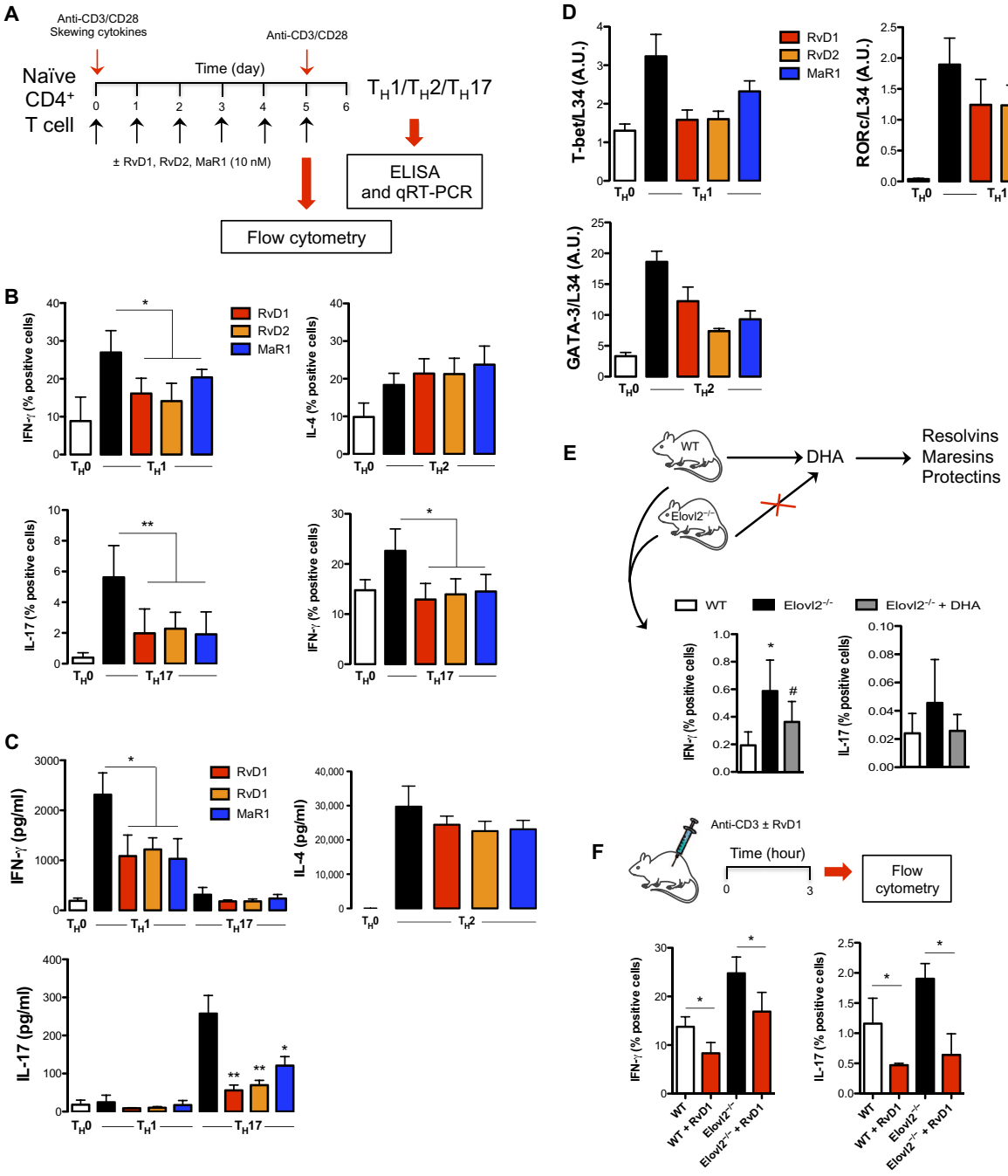

Fig. 4. Proresolving lipid mediators affect T_H cell polarization. (**A**) Schematic representation of T_H1, T_H17, and T_H2 generation. ELISA, enzyme-linked immunosorbent assay. (**B**) Percentages of intracellular cytokine production from polarized T_H1, T_H17, and T_H2 cells in the presence or absence of RvD1, RvD2, or MaR1 (10 nM), assessed after 6 hours of restimulation with anti-CD3/CD28. Data are means ± SEM of six independent experiments. *$P < 0.05$, **$P < 0.01$ (one-way ANOVA). (**C**) ELISA of IFN-γ, IL-17, and IL-4 in supernatants of T_H1, T_H17, and T_H2 cells polarized in the presence or absence of RvD1, RvD2, or MaR1 (10 nM), measured after 24 hours of restimulation with anti-CD3/CD28. Data are means ± SEM of six independent experiments. *$P < 0.05$, **$P < 0.01$ (one-way ANOVA). (**D**) Real-time quantitative polymerase chain reaction (qRT-PCR) analysis of the expression of T-bet, RORc, and GATA-3 in T_H1, T_H17, and T_H2 cells. Cycling threshold values are normalized to those of mRNA encoding ribosomal protein L34, and data are normalized to the maximum value obtained for each donor. Data are expressed as arbitrary units (A.U.) and are means ± SEM of four independent experiments. (**E**) Percentages of intracellular production of IFN-γ and IL-17 from $CD4^+$ T cells of splenocytes obtained from wild-type (WT), Elovl2 knockout (Elovl2$^{-/-}$), and Elovl2$^{-/-}$ + DHA mice. Data are means ± SEM of four different mice per experimental group and performed in duplicate. *$P < 0.05$ versus WT, $^{\#}P < 0.05$ versus Elovl2$^{-/-}$ (one-way ANOVA). (**F**) Percentages of intracellular production of IFN-γ and IL-17 from peripheral blood $CD4^+$ T cells obtained from WT and Elovl2 knockout (Elovl2$^{-/-}$) mice injected intraperitoneally with 100 ng of RvD1 for 15 min and then with 50 μg of anti-CD3 for 3 hours. Data are means ± SEM of four different mice. *$P < 0.05$ versus WT or Elovl2$^{-/-}$ (*t* test).

for RvD1 and MaR1) (Fig. 5B). SPM-induced de novo generation of T_{reg} cells was also paralleled by their capacity to increase their suppressive marker CTLA-4 and IL-10 release ($P < 0.05$), although they were incapable of modulating granzyme B production (Fig. 5B), suggesting that SPMs affect not only T_{reg} induction but also specific functional properties. This action was further supported by in vivo evidence that Elovl2$^{-/-}$ mice have significantly lower levels ($P < 0.05$) of Foxp3$^+$ T_{reg} cells (identified as shown in fig. S3) compared to wild-type mice, levels that were restored in DHA-supplemented mice (Fig. 5C).

The actions of SPMs on T cells are mediated by GPR32 and ALX/FPR2 receptors

To verify whether SPM-induced effects were associated with a higher CD4$^+$ T cell response and to ascertain the molecular mechanism behind the immunomodulatory role of these lipid mediators on T cells, we sought to investigate the involvement of SPM receptors in the effects we observed on T_H1, T_H17, and iT_{reg} cells. Because we still have limited information regarding the full spectrum of receptors engaged by the different classes of SPMs, we focused on the known receptors for D-series resolvins GPR32 and ALX/FPR2 (*18*, *19*). In Fig. 6A (left panels), we show that T_H1 cells displayed the highest mRNA expression of both GPR32 and ALX/FPR2 compared to T_H0 cells, whereas T_H17 and T_H2 cells showed similar levels of GPR32 and lower levels of ALX/FPR2. Furthermore, iT_{reg} cells expressed very low levels of both receptors. The levels of GPR32 were higher in all the T cell subsets compared to those of ALX/FPR2, whereas iT_{reg} cells showed a similar expression of both receptors. Immunoblotting analysis demonstrated that all T_H subsets express both GPR32 and ALX/FPR2, showing an actual SPM

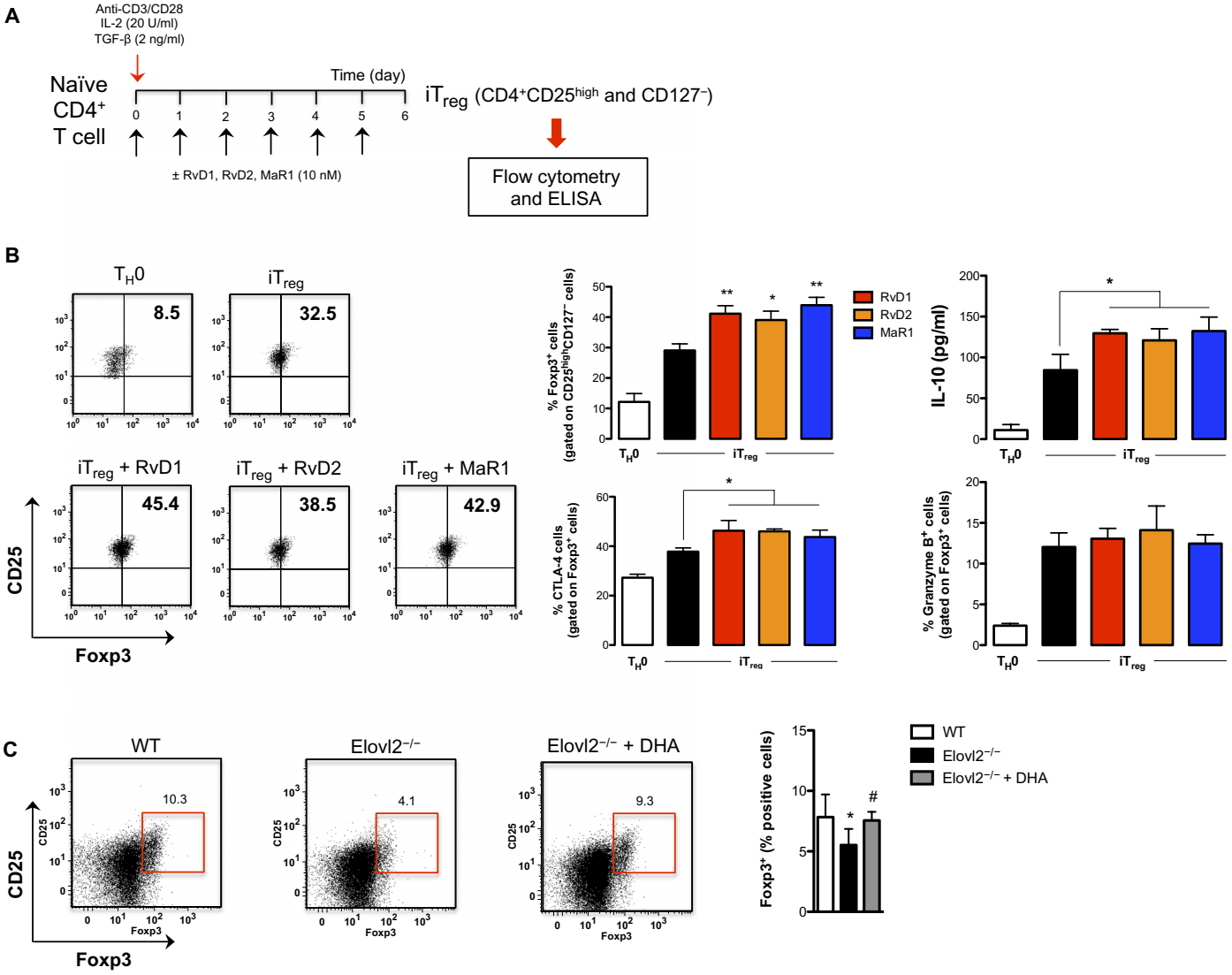

Fig. 5. Proresolving lipid mediators promote de novo generation of Foxp3-expressing T_{reg} cells. (**A**) Schematic representation of iT_{reg} generation. (**B**) Flow cytometry analysis of iT_{reg} cells gated on CD4$^+$CD25^{high} and CD127$^-$ cells and expressing Foxp3, CTLA-4, and granzyme B, and ELISA of IL-10 in supernatants of iT_{reg} cells. Data are means ± SEM of four independent experiments. *$P < 0.05$ versus iT_{reg}, **$P < 0.01$ versus iT_{reg} (one-way ANOVA). (**C**) Percentages of intracellular expression of Foxp3 in CD4$^+$CD25^{high} T cells of splenocytes obtained from WT, Elovl2 knockout (Elovl2$^{-/-}$), and Elovl2$^{-/-}$ + DHA mice. Data are means ± SEM of five different mice. *$P < 0.05$ versus WT, $^{\#}P < 0.05$ versus Elovl2$^{-/-}$ (one-way ANOVA).

receptor protein expression on T cells. In particular, T_H1, T_H17, and iT_{reg} cells showed significantly higher ($P < 0.05$) levels of GPR32 with respect to T_H0, whereas the expression of this receptor in T_H2 cells was similar to that of T_H0 control group (Fig. 6A, middle panels). Conversely, ALX/FPR2 showed no differential expression and remained unchanged among all T cell subsets. Flow cytometry analysis also revealed increased expression of both receptors on CD3/CD28-activated total peripheral CD3 T cells (Fig. 6A, right panels).

Because these two G protein (heterotrimeric guanine nucleotide–binding protein)–coupled receptors are currently the only known receptors for RvD1, we next sought to verify their possible role as mediators of the observed effects of this proresolving lipid on T cells. Preincubation with anti-GPR32 or anti-ALX/FPR2 neutralizing antibodies alone or in combination abrogated the suppressive activity of RvD1 on T_H1 and T_H17, as well as its enhancing activity on iT_{reg} lymphocytes. In particular, the single neutralization of either GPR32 or ALX/FPR2 significantly counteracted the inhibitory action of RvD1 on both PBMC-derived (Fig. 6B) and de novo generated T_H1 and T_H17 cells ($P < 0.05$) (Fig. 6C), whereas the inactivation of both receptors was more potent, completely restoring the intracellular levels of IFN-γ and IL-17 as to those of activated T cells (Fig. 6, B and C), suggesting that the role of these two receptors might be additive. Conversely, the RvD1-induced de novo generation of $Foxp3^+$ iT_{reg} cells was specifically and significantly counteracted when neutralizing only GPR32 ($P < 0.05$) and not ALX/FPR2 (Fig. 6C), confirmed by the evidence that neutralization of both receptors was not additive.

DISCUSSION

Since their first identification 15 years ago, SPMs, which include resolvins, protectins, and maresins, have proven to act as initiators of resolution programs of acute inflammation, thereby reducing granulocyte trafficking and the production of cytokines and extracellular reactive oxygen species, as well as lowering the magnitude of the overall inflammatory response by enhancing macrophage-mediated clearance of cellular debris and invading microbes (*4*). Although the role of each of the SPMs is highly associated with the resolution of acute inflammation operated by cells of innate immunity, it is becoming increasingly clear that these bioactive lipids might also take part in the control of chronic inflammation, possibly via acting on cells of adaptive immunity (*20*–*23*). In this regard, little systematic evidence for a direct role of these resolving mediators on the distinct adaptive cell populations has been assessed (*24*, *25*).

We interrogated the adaptive immune responses to specific SPMs that have not been addressed earlier. Cytotoxic $CD8^+$ T cells eliminate neoplastic, infected, or damaged cells mainly through the release of cytotoxins (perforin, granzymes, and granulysins) and potentiate innate immune responses [macrophages and natural killer (NK) cells] through the release of cytokines, such as IFN-γ and TNF-α (*26*). The observed ability of SPMs to dampen cytokines from $CD8^+$ cells suggests that their role in resolving inflammation is exerted not only directly by clearing and blunting the responses of those innate immune cells during acute inflammation but also indirectly by avoiding further recruitment or activation of innate cells, thus avoiding the onset of chronic inflammation or immune-mediated damage. Furthermore, $CD8^+$ cells can also prime naïve and restimulate experienced $CD4^+$ T cells to release high levels of helper cytokines (*27*). T_H cells develop from naïve $CD4^+$ T cells and differentiate into specialized T_H subsets after encountering foreign or autoantigens (*14*, *28*). However, persistent or uncontrolled T_H cell responses are often associated with pathological states and tissue damage. In particular, excessive and/or abnormal T_H1 and T_H17 cell responses are involved in chronic inflammation and mediate several autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, and psoriasis (*29*).

Our present results reveal that specific SPMs, namely, RvD1, RvD2, and MaR1, not only can directly modulate the inflammatory responses of already existing and activated T_H1 and T_H17 cells but also can critically prevent their generation from naïve $CD4^+$ T cells acting on their transcription factor–induced activation programs. Additionally, SPMs can enhance the differentiation of $CD4^+$ T cells into T_{reg} cells. Because T_{reg} cells typically serve to dampen excessive immune responses, these cells play an important role in preventing the overactivation of T_H1 and T_H17 cells. This result is in line with the very recent report of MaR1 in engaging T_{reg} cells in mice to promote resolution of lung inflammation (*30*). Although further studies are needed to demonstrate the existence of an indirect modulation of T_H1 and T_H17 cells by SPM-mediated sustained induction of T_{reg} cells, these findings suggest that SPMs might modulate inflammatory responses via several selective mechanisms on specific adaptive immune cells. This hypothesis is conceivable, also in light of the recent discovery that T_H17 cells transdifferentiate into T_{reg} cells during resolution of inflammation (*31*), where SPMs might be possible key players of such T_H17 instability and plasticity.

T_H2 lymphocytes were relatively unresponsive to SPM treatment under the conditions tested, in that none of the proresolving mediators tested regulated the generation of mature effector cells or cytokine release. These results are aligned with recent papers reporting that, in T_H2-driven pathologies and mouse models, DHA-derived SPMs like RvD1 and PD-1 do not affect IL-4 release and might ameliorate clinical outcome by acting on different targets than T_H2 cells (*32*–*35*). Conversely, RvE1, which is derived from EPA, facilitates resolution of T_H2-mediated asthmatic airway inflammation and atopic dermatitis by reducing T_H2 cytokines (*29*, *36*, *37*). Our findings are also supported by the in vivo evidence that mice incapable of producing DHA-derived SPMs have higher numbers of hyperactive T_H1 and T_H17 cells and concomitantly reduced levels of T_{reg} cells. In this genetic background, either DHA supplementation or in vivo injection of RvD1 in anti-CD3–treated mice reduced the activation of T_H1 and T_H17 cells and restored T_{reg} cell numbers. In line with this, it has also been shown that deletion or pharmacological inhibition of SPM-generating 12/15-lipoxygenase regulated murine and human dendritic cell maturation and activation, favoring T_H17 differentiation of $CD4^+$ T cells (*38*), thus highlighting the critical role for SPMs in modulating T_H responses. The recent identification of SPMs in human secondary lymphoid organs (*11*), where most naïve-to-effector or iT_{reg} differentiation happens, provides in vivo relevance of our findings. Notably, the expression of GPR32 and ALX/FPR2, the RvD1 receptors, by all T cell subsets suggests that these receptors are involved in DHA-derived SPM-induced effects on cell-mediated adaptive immunity. Both receptors seemed to be involved in RvD1 signaling on both T_H1 and T_H17 cells independently of their relative expression, whereas GPR32 appeared to specifically mediate the effects of RvD1 on T_{reg} cells. On the other hand, T_H2 cells, which were not modulated by SPMs, were the only T cell subset to display no variation in the expression of any of these receptors compared to their T_H0 precursors. Furthermore, the finding that SPMs preserve cell viability and proliferation while modulating proinflammatory responses is notable because it rules out the possibility that observed immunomodulation of T cells is caused by SPM-induced cell death.

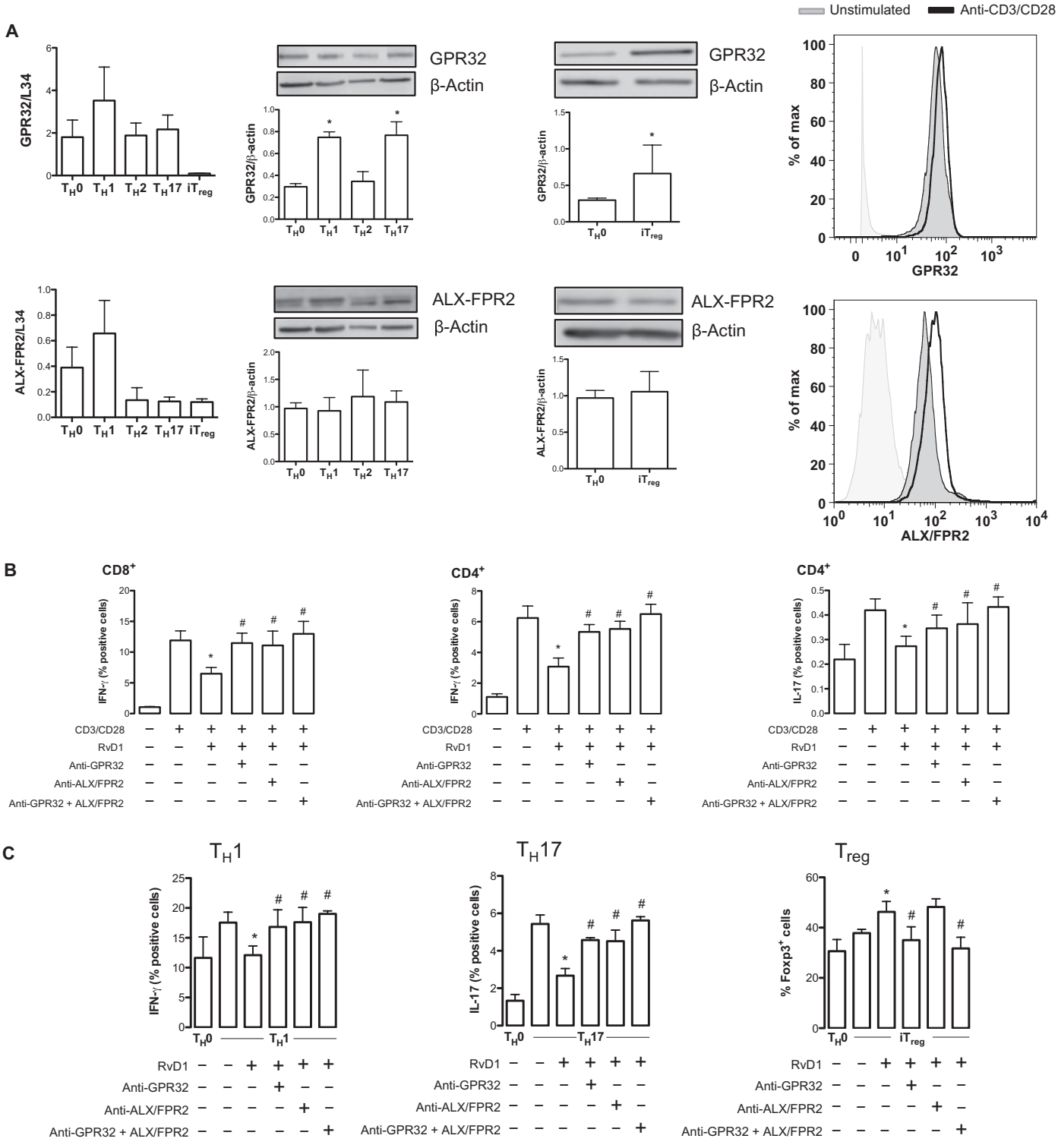

Fig. 6. The effects of proresolving lipid mediators on T cells are mediated by GPR32 and ALX/FPR2 receptors. (**A**) qRT-PCR analysis and immunoblotting of GPR32 and ALX/FPR2 in polarized T_H0, T_H1, T_H2, T_H17, and iT_{reg}, as detailed in Materials and Methods. Data are means ± SEM of four independent experiments. *$P < 0.05$ versus T_H0 (one-way ANOVA for T_H1, T_H2, and T_H17, and *t* test for iT_{reg}). A flow cytometry representative analysis of GPR32 and ALX/FPR2 is shown in resting and anti-CD3/CD28–activated total peripheral $CD3^+$ T cells. (**B** and **C**) Intracellular cytokine production from $CD8^+$ and $CD4^+$ T cells, as well as from polarized T_H1, T_H17, and iT_{reg}, pretreated with neutralizing antibodies against GPR32 or ALX/FPR2 (alone or in combination; 2 μg/ml) in the presence of RvD1 and after anti-CD3/CD28 stimulation for 8 hours. Data are means ± SEM of four independent experiments. *$P < 0.05$ versus anti-CD3/CD28–activated cells, #$P < 0.05$ versus RvD1-treated cells (one-way ANOVA).

Current clinical research is increasingly directed to the possibility of interfering with the functions of T_H cells. Thus, the finding that natural endogenous mediators like SPMs (that is, RvD1, RvD2, and MaR1) exert a noncytotoxic regulatory role on cells central to induction of autoimmunity represents a promising beginning for a new avenue of research. These results suggest that SPMs might possibly act on the balance between pathogenic T_H1/T_H17 and tolerogenic T_{reg} cells, which is typically altered during chronic inflammatory and autoimmune diseases. This study, which is schematically summarized in fig. S4, extends the original paradigm, whereby the SPMs found in the resolving exudates not only stimulate signs of resolution, terminating acute inflammation and restoring homeostasis (*4*), but, as reported herein, can also modulate adaptive immunity.

MATERIALS AND METHODS

Study design

This is an experimental laboratory study performed with human blood samples ($n = 40$) and animals ($n = 26$). The objective was to study the role of specific SPMs (RvD1, RvD2, and MaR1) on activation and differentiation of T cell subsets with the translational perspective to gain insights into the possibility that such bioactive lipids could regulate adaptive immunity and thus control T cell–mediated chronic inflammatory processes. All the healthy donors gave their written informed consent to the study. In vivo studies were carried out with ethical permission from the Animal Ethics Committee of the North Stockholm region, Sweden. The number of replicates is indicated for each experiment in the respective figure legends. Mechanistic studies on cells from healthy blood donors were performed with in vitro assays without blinding or randomization.

Materials

RvD1 (7*S*,8*R*,17*S*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid), RvD2 (7*S*,16*R*,17*S*-trihydroxy-4*Z*,8*E*,10*Z*,12*E*,14*E*,19*Z*-docosahexaenoic acid), AT-RvD3 (4*S*,11*R*,17*R*-trihydroxy-5*Z*,7*E*,9*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid), and MaR1 (7*R*,14*S*-dihydroxy-4*Z*,8*E*,10*E*,12*Z*,16*Z*,19*Z*-docosahexaenoic acid) were prepared by total organic synthesis as essentially described (*39*–*42*) or purchased from Cayman Chemical.

Peripheral blood cell isolation and purification of naïve $CD4^+$ T lymphocytes

PBMCs were isolated after venous puncture from healthy donors and were separated by density gradient over Ficoll-Hypaque (Amersham Biosciences). $CD4^+$ T lymphocytes were purified by immunomagnetic depletion with the human $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec) and by means of negative selection through autoMACS Pro Separator (Miltenyi Biotec). Briefly, effector and memory T cells, NK cells, B cells, dendritic cells, and granulocytes were labeled using a cocktail of biotin-conjugated antibodies and anti-biotin magnetic microbeads. Highly purified unlabeled naïve $CD4^+$ T cells ($CD4^+CD45RA^+CD27^+CD45RO^-$) were obtained by depletion of the magnetically labeled cells and had a purity of more than 95%, which was confirmed by flow cytometry.

Flow cytometry

To measure the intracellular cytokine levels, secretion was inhibited by adding brefeldin A (1 μg/ml) (Sigma-Aldrich) 5 hours before the end of stimulation with either PMA/ionomycin or Dynabeads CD3/CD28 T Cell Expander (one bead per cell; Invitrogen) (*16*). At the end of the incubation, cells were stained at the cell surface with e780-conjugated anti-CD3 (eBioscience), PerCP5.5-conjugated anti-CD4 (eBioscience), and Brilliant Violet–conjugated anti-CD8 (BioLegend), made permeable with Cytofix/Cytoperm reagents (BD Biosciences), and then stained intracellularly with phycoerythrin (PE)–Cy7–conjugated anti–TNF-α (eBioscience), allophycocyanin (APC)–conjugated anti–IFN-γ (eBioscience), PE-conjugated anti–IL-17 (eBioscience), anti-PercP5.5–conjugated anti–IL-2 (BioLegend), Brilliant Violet 421–conjugated IL-4 (eBioscience), and fluorescein isothiocyanate (FITC)–conjugated anti–granzyme B in 0.5% saponin at room temperature for 30 min. In some experiments, cells were also stained at the cell surface with PE-conjugated anti-FasL (Miltenyi Biotec), APC-conjugated anti–PD-1 (eBioscience), and PE-conjugated anti–CTLA-4 (Miltenyi Biotec). Intracellular cytokines were analyzed by flow cytometry in a fluorescence-activated cell sorter (FACS) (CyAN ADP, Beckman Coulter). For each analysis, at least 300,000 events were acquired by gating on Pacific Orange–conjugated Live/Dead negative cells. In some experiments, PBMCs were preincubated for 30 min with anti-GPR32 (2 μg/ml; clone GTX71225, GeneTex) and/or anti-FPR2/ALX (2 μg/ml; clone FN-1D6-A1, Genovac) before incubation with vehicle or RvD1 (10 nM) and stimulation with Dynabeads CD3/CD28 T Cell Expander (one bead per cell; Invitrogen). The list of antibodies used for flow cytometry and their dilution is detailed in table S1.

Detection of apoptotic and necrotic cells

Apoptotic and necrotic cells were detected using annexin V–FITC and PI staining (eBioscience), respectively. Cells were washed twice in phosphate-buffered saline (PBS) followed by resuspension in binding buffer (Annexin V Kit; eBioscience) and then incubated with 5 μl of annexin V–FITC for 15 min at room temperature. Cells were then extensively washed with binding buffer, 5 μl of PI was added to the cells, and cells were analyzed within 2 hours on a FACS (Cyan ADP, Beckman Coulter).

Proliferation assay

$CD3^+$ T cells were isolated through positive selection with autoMACS Pro Separator, and 1×10^6 cells were labeled with CFSE at a final concentration of 5 μM for 10 min at 37°C in agitation. Cells were washed twice with PBS/10% fetal bovine serum, suspended in culture medium, and analyzed immediately on a FACS (Cyan ADP, Beckman Coulter). Analysis of cells immediately after CFSE labeling indicated a labeling efficiency higher than 99%. Cell proliferation was followed by flow cytometry at day 2 and 4 upon stimulation with Dynabeads CD3/CD28 T Cell Expander in the presence or absence of RvD1, RvD2, and MaR1 (10 nM).

T_H cell differentiation assay

For T_H1 and T_H17 polarization of T cells, highly purified naïve $CD4^+$ T cells were cultured in round-bottom 96-well plates (Falcon) at a density of 5×10^4 cells at 37°C in 200-μl final volumes of X-VIVO 15 medium with Dynabeads CD3/CD28 T Cell Expander and under T_H1-, T_H2-, and T_H17-polarizing conditions in the presence or absence of RvD1, RvD2, or MaR1 (10 nM). The following human recombinant cytokines were used: for T_H1 polarization, IL-12 (10 ng/ml) (Miltenyi Biotec); for T_H17 polarization, IL-1β (10 ng/ml), IL-6 (20 ng/ml), IL-23 (100 ng/ml), and TGF-β (1 ng/ml) (Miltenyi Biotec). After 5 days, cells were collected and washed extensively, and their viability was determined by trypan blue exclusion. Cells (1×10^6/ml) were restimulated

for 6 hours (for flow cytometry) or 24 hours (for ELISA and qRT-PCR) with Dynabeads CD3/CD28 T Cell Expander (one bead per cell). Cultures were supplemented with RvD1, RvD2, or MaR1 every other day for 5 days for flow cytometry analysis and then were restimulated with CD3/CD28 beads for 24 hours (for ELISA and qRT-PCR). For T_H2 polarization, naïve $CD4^+$ T cells were kept under polarizing conditions using IL-4 (25 ng/ml) (Miltenyi Biotec) and Dynabeads CD3/CD28. Cells were extensively washed and restimulated with CD3/CD28 beads every 3 days with fresh medium plus IL-4 and IL-2 (20 ng/ml) (Miltenyi Biotec) up to day 12. After 12 days, cells were collected for ELISA or qRT-PCR or restimulated for 6 hours with PMA/ionomycin (100 nM) for intracellular staining. In some experiments, T_H1, T_H17, and iT_{reg} cells were incubated for 30 min with anti-GPR32 (2 μg/ml) and anti-FPR2/ALX (2 μg/ml) antibodies (alone or in combination), washed with complete medium, subsequently treated with vehicle or RvD1 (10 nM), and then stimulated with CD3/CD28 beads.

Generation of iT_{reg} cells

Highly purified naïve $CD4^+$ T cells were cultured in round-bottom 96-well plates (Falcon) at a density of 5×10^4 cells at 37°C in 200-μl final volumes of X-VIVO 15 medium in the presence of Dynabeads CD3/CD28 T Cell Expander (one bead per cell; Invitrogen), TGF-β (2 ng/ml; Miltenyi Biotec), and IL-2 (20 U/ml) in the presence or absence of RvD1, RvD2, or MaR1 (10 nM). Cultures were supplemented with SPMs every other day for 5 days.

Enzyme-linked immunosorbent assay

Cytokine content was determined by standard two-site sandwich ELISA, using available commercial kits for IFN-γ and IL-17 (eBioscience), as previously reported (*43*), and through multiplex bead–based Luminex assay for measurement of IL-4 and IL-10 (R&D Systems).

Real-time quantitative polymerase chain reaction

Total RNA was extracted with an RNeasy Micro Kit (Qiagen). A mixture containing random hexamers, oligo(dT)15 (Promega), and SuperScript II Reverse Transcriptase (Invitrogen) was used for complementary DNA synthesis. Transcripts were quantified by qRT-PCR on an ABI PRISM 7900 sequence detector (Applied Biosystems) with Applied Biosystems predesigned TaqMan Gene Expression Assays and Absolute QPCR ROX Mix (Thermo Fisher Scientific). The following probes were used (Applied Biosystems; assay identification numbers are in parentheses): T-bet (Hs00203436_m1), RORc (Hs01076112_m1), GATA-3 (Hs00231122), GPR32 (Hs01102536_s1), and FPR2 (Hs02759175_s1). For each sample, mRNA abundance was normalized to the amount of ribosomal protein L34 (Hs00241560_m1).

Immunoblotting

Purified and polarized T_H0, T_H1, T_H2, T_H17, and iT_{reg} cells were lysed with lysis buffer, and cell homogenates were subjected to 10% SDS–polyacrylamide gel electrophoresis (50 μg per lane) under reducing conditions. Gels were then electroblotted onto 0.45-μm nitrocellulose filters (Bio-Rad) and were incubated with primary anti-GPR32 polyclonal mouse antibody (1:500; clone GTX71225, GeneTex), anti-ALX/FPR2 monoclonal rabbit antibody (1:500; clone FN-1D6-A1, Genovac), or anti–β-actin monoclonal mouse antibody (1:10,000; Bio-Rad) and then with secondary goat anti-rabbit polyclonal antibody (1:2000; Santa Cruz Biotechnology) for GPR32 and goat anti-mouse polyclonal antibody (1:2000 for ALX and 1:10,000 for β-actin).

Elovl2 knockout animals and in vivo experiments

$Elovl2^{-/-}$ mice were generated as described previously (*16*). All animals were housed at room temperature and maintained on a 12-hour light/dark cycle. Adult mice were fed standard chow DHA-free diet (10% kcal fat; D12450H, Research Diets) or DHA-enriched diet (10% kcal fat; 14% DHA; D13021002, Research Diets) according to the experimental groups. All animals were fed ad libitum and had free access to water. At the end of the study, animals were euthanized with CO_2 and cervical dislocation. For in vivo experiments, mice were pretreated intraperitoneally with 100 ng of RvD1 for 15 min and then injected with 50 μg of anti-CD3 (BioLegend; Armenian hamster IgG, clone 145-2C11). Blood samples were recovered 3 hours after antibody injection, and a cell suspension was prepared for flow cytometry analysis.

Statistical analysis

All data were expressed as means ± SEM. Differences between groups were compared using Student's *t* test (two groups) or one-way ANOVA (multiple groups) followed by a post hoc Bonferroni test. The criterion for statistical significance was $P < 0.05$ or less. All statistical analyses were performed with GraphPad Prism. FACS analysis was performed using the FlowJo analysis program (Tree Star).

SUPPLEMENTARY MATERIALS

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Fig. S1. AT-RvD3 dose-dependently reduces TNF-α, and RvD1, RvD2, and MaR1 do not affect long-term cell death in both $CD8^+$ and $CD4^+$ T cells.

Fig. S2. RvD1, RvD2, and MaR1 do not affect T_H0 cells.

Fig. S3. Gating strategy for T_{reg} identification.

Fig. S4. Schematic representation of SPM effects on T cell subsets.

Table S1. Antibodies used for T cell surface staining and for analysis of intracellular cytokine production.

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Proresolving lipid mediators resolvin D1, resolvin D2, and maresin 1 are critical in modulating T cell responses

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Naïve T cells nudged by lipid mediators

Specialized proresolving lipid mediators are known to modulate the innate immune system, and here, Chiurchiù *et al*. report that some of these lipid mediators can also inhibit human T cell activation. Incubation with lipid mediators increased naïve $CD4^+$ T cell differentiation into regulatory T cells and suppressed differentiation of T_H1 or T_H17 cells. A mouse model that is unable to generate precursors for these lipid mediators also had an increase of T_H1 and T_H17 cells and relatively fewer regulatory T cells than wild-type mice. These results identify a potentially useful avenue of immunomodulation for steering T cell responses in inflammation or autoimmunity.

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