Human regulatory T cells inhibit polarization of T helper cells toward antigen-presenting cells via a TGF-β-dependent mechanism

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The molecular mechanisms used by regulatory T cells (Treg) to inhibit the effector phase of adaptive immune responses are still elusive. In the present work, we investigated the possibility that Treg may interfere with a basic biological function of T helper cells (T$_H$): polarization of secretory machinery for dedicated help delivery. To address this question, we visualized by confocal microscopy different parameters of activation in T$_H$ and Treg cells interacting simultaneously with individual antigen-presenting cells (APC). Our results show that, although productive TCR engagement in T$_H$/APC conjugates was unaffected by the presence of adjacent Treg, the reorientation of T$_H$ secretory machinery toward APC was strongly inhibited. Blocking TGF-β completely reverted Treg induced inhibition of T$_H$ polarization. Our results identify a previously undescribed mechanism by which Treg inhibit effector T cells. TGF-β produced by adjacent Treg interferes with polarization of T$_H$ secretory machinery toward APC, thus affecting a crucial step of T$_H$-mediated amplification of the immune response.

confocal microscopy | immunological synapse | T cell activation

Naturally arising CD4$^+$ CD25$^+$ regulatory T cells (Treg) play a pivotal role in the maintenance of peripheral self tolerance. Deficiency of this suppressive T cell subset results in the development of autoimmune lymphoproliferative disorders in mouse models and human patients (1). Treg are also implicated in controlling immune responses against infectious agents and have been shown to be detrimental for antitumor immunity (2, 3).

Treg use different strategies to inhibit immune responses. Among these, an important mechanism involves TGF-β, which is a central cytokine in the homeostasis of the immune system (4, 5). Effector T cells in which the response to TGF-β is abrogated are resistant to Treg suppression (6, 7).

Although the role of Treg in controlling the amplitude and duration of immune responses has been thoroughly documented, and several effector molecules have been identified (8), the mechanisms by which Treg interfere with the early-activation steps of other cells of the immune system are still elusive. In particular, it is not clear whether and how Treg may interfere with the signaling leading to biological responses in effector cells, such as T helper cells (T$_H$) during their interaction with cognate antigen-presenting cells (APC).

Two key features define T$_H$ activation and effector function. The first feature is that T$_H$ form prolonged conjugates with APC and undergo sustained signaling that is required for induction of cytokine production (9, 10). The second feature is that T$_H$ polarize secretory machinery toward APC and selectively activate cognate APC (11, 12).

Although T cell activation is a slow process resulting from sustained TCR engagement and triggering (9, 10, 13), T cell polarization is a rapid and adaptable phenomenon. T cells interacting simultaneously with APC offering different antigenic stimuli rapidly remodel immunological synapses and adjust polarization of Golgi apparatus (14, 15). This mechanism en-

suring an exquisite specificity to help delivery may be instrumental to orchestrate and amplify adaptive immune responses.

In the present work, we investigated the impact that Treg may have on rapid polarization responses and on sustained signaling in autologous human T$_H$ cells.

We report that although productive TCR engagement in T$_H$/APC conjugates was unaffected by the presence of adjacent Treg, T$_H$ polarization response toward APC was impaired via a TGF-β-dependent mechanism.

By showing that Treg interfere with dedicated help delivery, we identify a mechanism by which Treg affect adaptive immune responses.

Results

Isolation, Expansion, and Characterization of Human Treg. Typical CD4$^+$ CD25$^+$ T cell purification from peripheral blood is shown in supporting information (SI) Fig. 6A. Freshly isolated CD4$^+$ CD25$^+$ T cells were mainly Foxp3$^+$ (SI Fig. 6B) and CD127$^{-/lo}$ (Fig. 6C) as described (16, 17). CD4$^+$ CD25$^+$ T cells were expanded by using beads coated with anti-CD3 and -CD28 antibodies (SI Fig. 6D). After expansion CD4$^+$ CD25$^+$ remained mainly Foxp3$^+$ and CD127$^{-/lo}$ (SI Fig. 6E). CD4$^+$ CD25$^-$ T$_H$ cells were obtained from the CD4$^+$ fraction of peripheral blood mononuclear cell (PBMC) after sorting of CD25$^+$ T cells and were expanded in parallel; they remained Foxp3$^-$ and CD127^{high} (SI Fig. 6F). To test the suppressive potential of CD4$^+$ CD25$^+$ T cells, T$_H$ cells loaded with carboxyfluorescein diacetate succinimidyl ester (CFSE) were stimulated by anti-CD3/CD28 mAb-coated beads and cocultured either in the presence or in the absence of CD4$^+$ CD25$^+$ T cells at different cell–cell ratios. As shown in SI Fig. 6 G and H, in vitro expanded CD4$^+$ CD25$^+$ T cells efficiently suppressed the proliferative response of autologous CD4$^+$ CD25$^-$ T cells (18). We also investigated whether CD4$^+$ CD25$^+$ T cells could affect T$_H$ cell proliferation and IFN-γ production after 72-h coculture with APC pulsed with a mixture of bacterial superantigens. As shown in SI Fig. 7, both IFN-γ production and T$_H$ cell proliferation were strongly inhibited in the presence of in vitro expanded CD4$^+$ CD25$^+$ T cells.

Having characterized the regulatory function of in vitro expanded CD4$^+$ CD25$^+$ T cells (Treg), we used these cells to study their impact on the dynamics of T$_H$ interaction with APC in all of the further experiments described in this study.
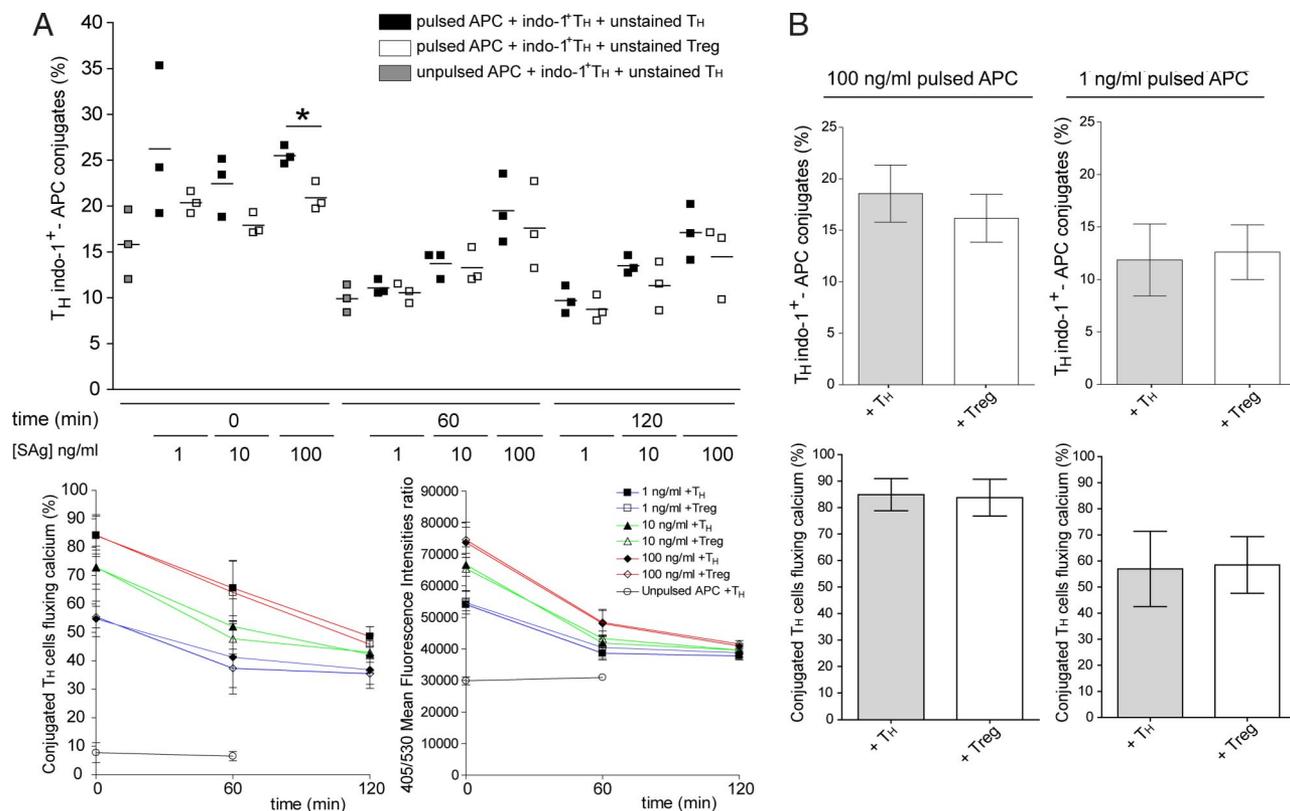
Fig. 1. Sustained $[Ca^{2+}]_i$ increase in T_H cells is unaffected in the presence of Treg. Indo-1-loaded T_H cells were conjugated with EBV-B cells in the presence of either unstained T_H or unstained Treg. The following cellular ratio was used 1:1:1 (T_H Indo-1^+ : APC : Treg or control T_H). (*A*) APC were either unpulsed or pulsed with 100 ng/ml, 10 ng/ml, or 1 ng/ml superantigens. (*Upper*) Percentage of Indo-1-loaded T_H cells in conjugate at the indicated time points. (*Lower Left*) Percentage of T_H in conjugate that exhibit $[Ca^{2+}]_i$ increase. (*Lower Right*) Mean fluorescence intensity of 405/530 emission ratio in T_H cells at different time points after conjugate formation. (*B*) APC pulsed with either 100 ng/ml or 1 ng/ml superantigens were precultured with Treg or control T_H for 2 h 30 min. Indo-1-loaded T_H were conjugated at the ratio 1:1:1 (T_H Indo-1^+ : APC : Treg or control T_H). (*Upper*) Percentage of Indo-1-loaded T_H cells in conjugate. (*Lower*) Percentage of T_H in conjugate that exhibit $[Ca^{2+}]_i$ increase. Data are from three independent experiments performed by using cells from two different donors. Bars represent SD values. Statistical significance of differences between groups was evaluated by an unpaired Student's *t* test with GraphPad Prism software. *, $P < 0.05$.

Treg Do Not Interfere with $[Ca^{2+}]_i$ Increase in T_H Cells Conjugated with APC. We initially investigated whether Treg could affect T_H conjugation with APC or sustained signaling in T_H cells. We measured $[Ca^{2+}]_i$ increase in T_H cells at different time points after conjugation with APC pulsed with different concentrations of superantigens (either in the presence of bystander Treg or of bystander T_H, as a control).

As shown in Fig. 1*A* at the different superantigen concentrations used Treg either did not affect or only marginally affected T_H/APC conjugation. Interestingly, the fraction of conjugated T_H cells undergoing calcium mobilization and the intensity of $[Ca^{2+}]_i$ increase in conjugated T_H cells were unaffected in the presence of Treg (Fig. 1*A*).

Preincubation of Treg with APC for 2 h 30 min did not inhibit the number of T_H/APC conjugates nor the fraction of conjugated T_H cells undergoing calcium mobilization (Fig. 1*B*).

Taken together the above results indicate that Treg do not inhibit productive TCR engagement and signaling initiation in T_H cells conjugated with APC.

Polarization of T_H Secretory Machinery Is Impaired in the Presence of Treg. Because in our cellular system Treg did not block early signaling in T_H/APC conjugates, we investigated whether they could interfere with additional steps of the T_H/APC cognate interaction. A key function of T_H cells is the rapid and selective polarization of secretory machinery toward APC (12, 14, 15, 19). To investigate whether Treg may affect this basic function of T_H cells, we visualized intracellular IFN-γ in T_H cells conjugated either with EBV-

transformed-B (EBV-B) cells or with autologous mature dendritic cells (DC) pulsed with bacterial superantigens.

After 2 h 30 min of incubation (a time sufficient to activate T_H for IFN-γ production) (20), cells were stained with anti-IFN-γ mAb. T cell/APC conjugates were visualized by using confocal microscopy.

Images depicting three-cell conjugates in which one APC simultaneously interacted with a T_H and a Treg were registered. Conjugated T_H cells were scored for IFN-γ accumulation beneath the cell–cell contact site (14).

In the absence of Treg a major fraction of T_H conjugated with APC exhibited polarized IFN-γ toward the APC (both EBV-B cells and autologous DC) in agreement with reported data (12, 14, 15) (Fig. 2). Interestingly, in the presence of Treg a profound inhibition of IFN-γ polarization was observed in T_H cells interacting with EBV-B cells as well as DC (Fig. 2). To further quantify this inhibitory effect we measured in individual T_H/APC conjugates the distance between IFN-γ accumulation and the center of the immunological synapse (IS) using the Profile function of the Zeiss software. This analysis showed that the distance between IFN-γ intracellular accumulation, and the IS was significantly increased in the presence of Treg (SI Fig. 8 *A* and *B*).

To investigate whether Treg could affect IFN-γ production by T_H during the 2-h 30-min coculture, we measured intracellular IFN-γ by FACS analysis. As shown in SI Fig. 9, 2-h 30-min coculture with Treg resulted only in a partial reduction of IFN-γ production in T_H cells.
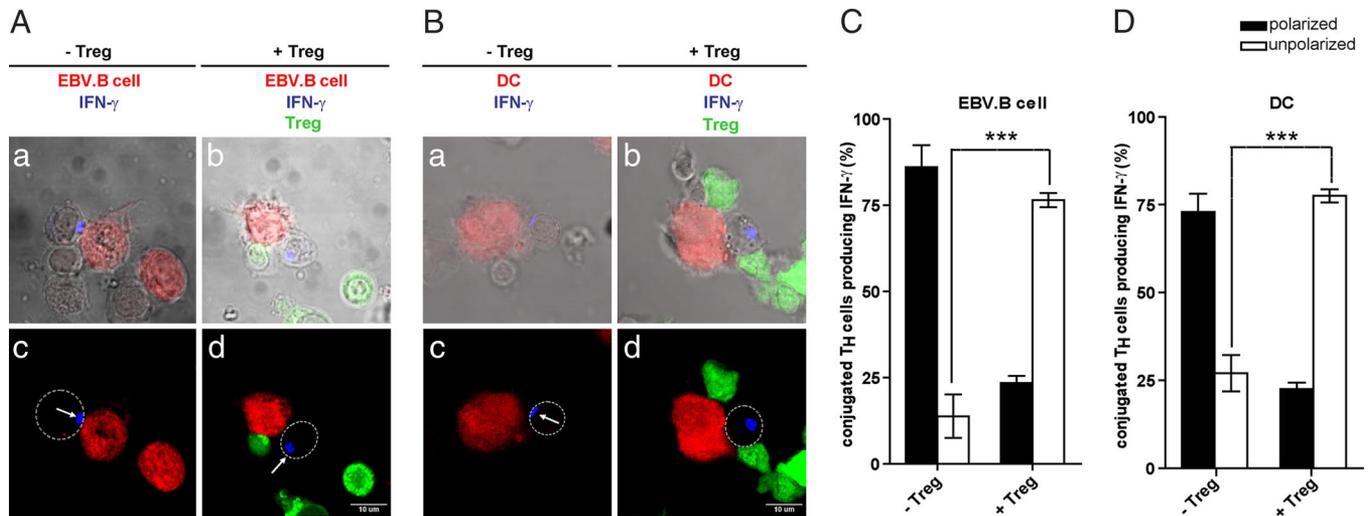
Fig. 2. Treg inhibit polarization of IFN-γ toward APC. (*A* and *B*) EBV-B cells (red, *A*) or mature autologous DC (red, *B*) were conjugated with either T$_H$ alone (unstained *a*, *c*) or together with Treg (green *b*, *d*) at 1:1:1 ratio. After 2-h 30-min incubation at 37°C, cells were stained for IFN-γ. (*B*) White arrows indicate IFN-γ accumulation; white circles mark T cell contour. (*C* and *D*) Quantification of T$_H$ IFN-γ polarization toward EBV-B cells (*C*) or toward DC (*D*) by visual inspection. In *C*, 72 three-cell conjugates plus Treg and 75 conjugates without Treg were scored. In *D*, 43 conjugates plus Treg and 46 conjugates without Treg were scored. The histograms represent the mean +/− SD values of three independent experiments using cells from three different donors. Statistical significance of difference between groups was evaluated by an unpaired Student's *t* test using the GraphPad Prism software. ∗∗∗, *P* < 0.001.

Taken together, these results highlight a mechanism used by Treg to interfere with the early steps of T$_H$ cell effector function. During the first few hours of cell–cell interaction, Treg reduce only IFN-γ production. Yet by affecting polarization toward APC, they avoid dedicated help delivery.

Treg Acquire Their Inhibitory Potential in a Time-Dependent Fashion. Activation of IFN-γ production requires a sustained signaling and cannot be detected after a few minutes of cell–cell conjugation (20). Yet T$_H$ cell polarization is a very rapid phenomenon accomplished within a few minutes after T$_H$/APC encounter (14, 15). To determine whether Treg could affect swift polarization

responses we investigated the effect of Treg on the polarization of additional markers of the secretory machinery such as tubulin cytoskeleton and Golgi apparatus that are known to relocate very rapidly toward the T$_H$/APC contact site (14, 15).

T$_H$/APC were cocultured for either 15 min or 2 h 30 min (either in the presence or absence of Treg), conjugates were stained with either anti-β tubulin mAb or a rabbit polyclonal Ab against the GM130 protein (to visualize Golgi apparatus). Images depicting three-cell conjugates in which one APC simultaneously interacted with a T$_H$ and a Treg were registered. Conjugated T$_H$ cells were scored for Microtubule Organization Center (MTOC) or Golgi apparatus polarization.
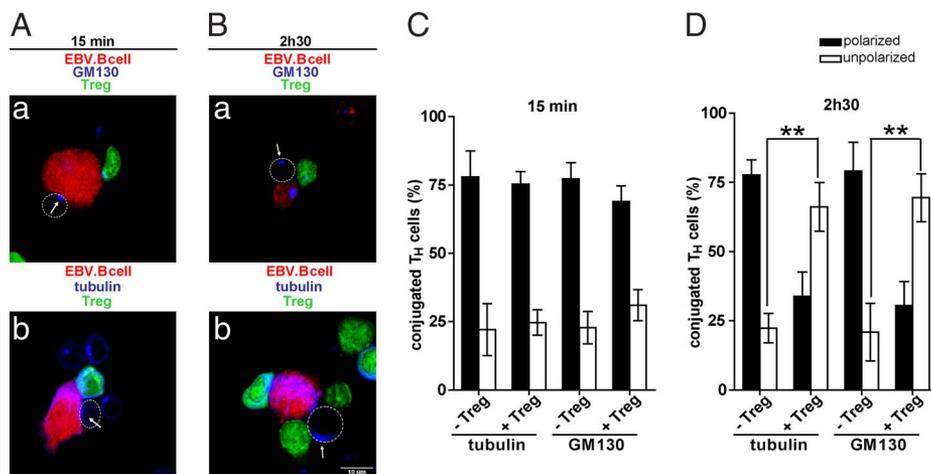

Fig. 3. Inhibition of secretory machinery polarization occurs in a time-dependent fashion. EBV-B cells were conjugated with T$_H$ for different times either in the presence or in the absence of Treg. (*A* and *B*) EBV-B cells (red) were conjugated with T$_H$ (unstained) in the presence of Treg (green) at 1:1:1 ratio. After 15-min incubation at 37°C (*A*) or 2-h 30-min incubation at 37°C (*B*), cells were stained for either GM130 (*Aa* and *Ba*) or tubulin (*Ab* and *Bb*). White arrows indicate localization of MTOC or GM130; white circles mark T cell contour. (*C* and *D*) Quantification of T$_H$ GM130 and tubulin polarization toward APC after 15 min incubation (*C*) or after 2-h 30-min incubation (*D*). In *C*, 66 three-cell conjugates plus Treg and 85 conjugates without Treg were scored for GM130; 60 three-cell conjugates plus Treg and 63 conjugates without Treg were scored for tubulin. In *D*, 82 three-cell conjugates plus Treg and 60 conjugates without Treg were scored for GM130; 88 three-cell conjugates plus Treg and 91 conjugates without Treg were scored for tubulin. The histograms represent the mean and SD values of three independent experiments using cells from three different donors. Statistical significance of differences between groups was evaluated by an unpaired Student's *t* test using the GraphPad Prism software. ∗∗, *P* < 0.01.
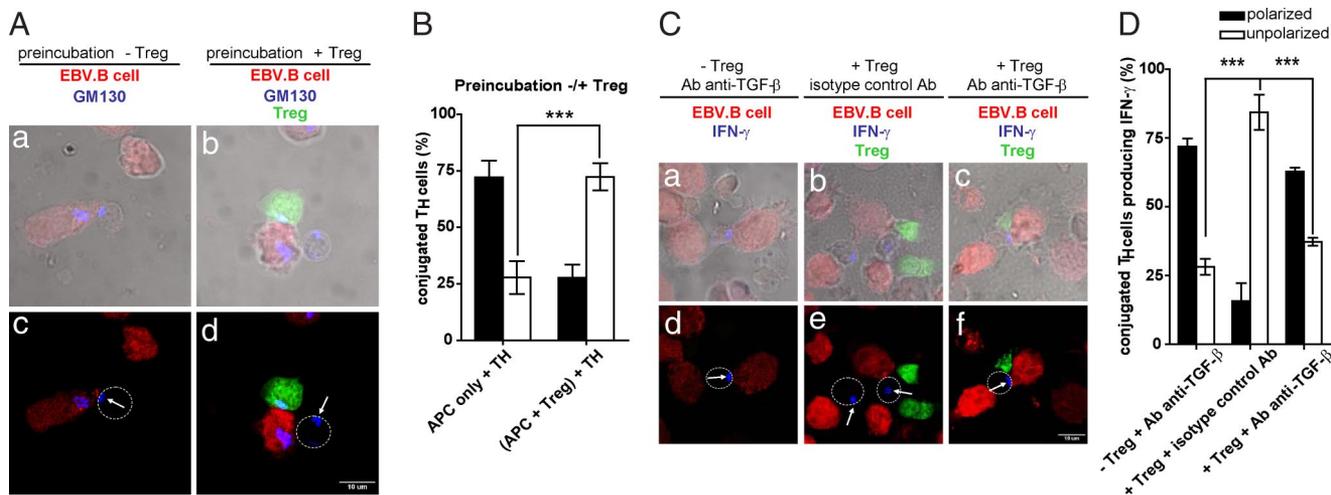
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Fig. 4. Inhibition of polarized IFN-γ secretion in T_H cells is mediated by TGF-β production. (*A*) EBV-B cells (red) were either kept in culture alone (*a, c*) or conjugated with Treg (green, *b, d*) for 2 h 30 min. T_H (unstained) were added for an additional 15 min at 1:1:1 ratio. Cells were stained for GM130. White arrows indicate GM130 localization; white circles mark T cell contour. (*B*) Quantification of T_H GM130 polarization toward APC in the culture conditions described in *A*. A total of 48 three-cell conjugates (plus Treg) and 48 conjugates (without Treg) were scored. (*C*) EBV-B cells (red) were conjugated with either T_H alone (unstained, *a, d*) or with T_H plus Treg (green, *b, c, e, f*) at 1:1:1 ratio. After 2-h 30-min incubation at 37°C, cells were stained for IFN-γ. In *a, d, c*, and *f*, cells were treated with anti-TGF-β mAb at time 0 of conjugate formation. In *b* and *e*, cells were treated with an isotype control Ab. White arrows indicate IFN-γ accumulation; white circles mark T cell contour. (*D*) Quantification of T_H IFN-γ polarization toward APC in the culture conditions described in *C*. Forty-four conjugates T_H alone plus anti-TGF-β Ab, 43 conjugates T_H plus Treg plus the isotype control Ab, and 41 conjugates T_H plus Treg and plus anti-TGF-β Ab were scored. The histograms represent the mean and SD values of three independent experiments using cells from three different donors. Statistical significance of differences between groups was evaluated by an unpaired Student's *t* test using the GraphPad Prism software. ***, $P < 0.001$.

As shown in Fig. 3 and SI Fig. 8, in the absence of Treg, a large fraction of T_H cells polarized tubulin cytoskeleton and Golgi apparatus toward superantigen pulsed EBV-B cells both at early and at late times after conjugation (Fig. 3 *C* and D and SI Fig. 8 *C* and *D*). This result further supports the notion that T_H rapidly polarize toward APC and stay polarized for a sustained time (12, 14, 15). Interestingly, whereas 15 min after conjugation, polarization of the secretory machinery was unaffected in the presence of Treg (Fig. 3 *A* and *C* and SI Fig. 8*C*), at 2 h 30 min, polarization of both tubulin cytoskeleton and Golgi apparatus was impaired (Fig. 3 *B* and *D* and SI Fig. 8*D*).
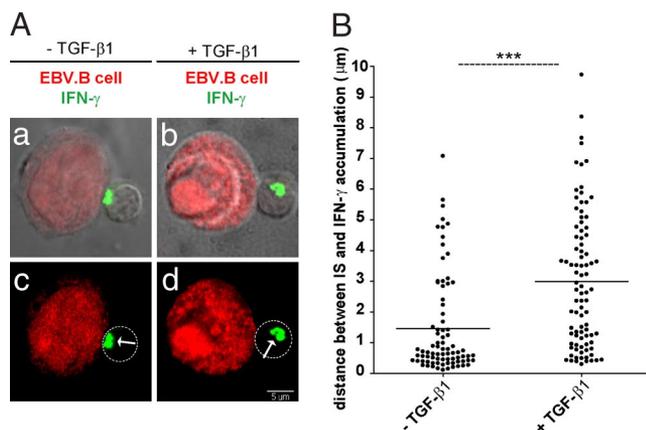

Fig. 5. Exogenous TGF-β1 inhibits polarization of antigen specific T cells. (*A*) Peptide-pulsed EBV-B cells (red) were conjugated with either 6396p5.1.2 cells in the absence (*a, c*) or presence of 20 ng/ml TGF-β (*b, d*). After 2-h 30-min incubation at 37°C, cells were stained for IFN-γ (green). (*B*) Distances of IFN-γ from the center of the T cell/APC contact site were measured by using the Profile function of the Zeiss software. Each dot corresponds to a T_H/APC conjugate. Ninety conjugates plus TGF-β and 81 conjugates without TGF-β were scored. Statistical significance of differences between groups was evaluated by an unpaired Student's *t* test by using the GraphPad Prism software. ***, $P < 0.001$.

These results indicate that the inhibitory effect of Treg on T_H polarization is observed only after a sustained interaction with APC. This delayed effect may be due to a time delay required for Treg activation. Alternatively, Treg may need to condition APC so that interaction of conditioned APC with T_H results in a failure of effector cells to polarize.

To address these questions, we first investigated whether APC could be conditioned during interaction with Treg by measuring polarization responses in T_H interacting with chemically fixed APC either in the presence or in the absence of Treg. SI Fig. 10 *A* and *B* show that, in these conditions, Treg exhibited an inhibitory effect on T_H polarization. These results suggest that in our cell system the APC mainly play the role of cellular platforms for T_H/Treg encounter and are not implicated in mediating the observed Treg inhibition.

We next investigated whether preactivation of Treg resulted in a rapid inhibition of polarization responses in T_H cells. To so, EBV-B cells were either cultured alone or cocultured with Treg for 2 h 30 min. T_H cells were then added to the culture for an additional 15 min. In these conditions, a profound effect of Treg on the polarization of T_H Golgi apparatus was observed in three-cell conjugates even though the cells were cocultured for only 15 min (Fig. 4 *A* and *B* and SI Fig. 8*E*). In T_H/APC, conjugates without Treg a moderate inhibition of T_H polarization was observed (SI Fig. 8*E*).

Together the above results suggest that preactivation of Treg may elicit the secretion of a soluble factor acting in a paracrine fashion.

TGF-β Mediates Treg-Induced Inhibition of T_H Polarization. It has been shown that Treg inhibit T_H and CTL effector function via a mechanism that requires functional TGF-β signaling in effector cells (6, 7, 21). We investigated the possibility that TGF-β may mediate the observed inhibitory effect on T_H polarization response.

We included in our experimental approach a blocking mAb against TGF-β. Conjugated T_H cells were scored for IFN-γ polarization. As shown in Fig. 4 *C* and *D* and SI Fig. 8*F*,

anti-TGF-β mAb alone did not affect the polarization of T_H cells. Conversely, addition of the anti-TGF-β mAb to the cultures containing Treg markedly reverted the inhibitory effect exerted by Treg. In control experiments the anti-TGF-β used in this study inhibited TGF-$\beta 1$ mediated induction of Foxp3 expression in human T cells (data not shown).

To investigate whether TGF-β could affect polarization of secretory machinery also in antigen-specific T cells, cloned $T_H 1$ cells were conjugated with MHC-matched EBV-B cells (pulsed with the antigenic peptide) in the presence of 20 ng/ml soluble TGF-$\beta 1$. As shown in Fig. 5, treatment with soluble TGF-$\beta 1$ resulted in a clear inhibition of T cell polarization toward cognate APC, thus further supporting the role of this cytokine in affecting dedicated help delivery.

Taken together, the above results indicate that Treg inhibit polarization in T_H cells via a TGF-β-dependent mechanism.

Discussion

Although the suppressive role of Treg in controlling immune responses has been thoroughly investigated, the molecular mechanisms used by Treg to control the activation of the different cells of the immune system are still elusive. In particular, how Treg may affect the biological function of effector T cells is still an unresolved question. In the present work, we provide a stepping stone to address this challenging question. Using Treg isolated from human peripheral blood and expanded in culture, we investigated whether and how these cells may affect T_H cell polarization toward APC. Our results show that Treg inhibit polarization of T_H cells toward APC via a TGF-β-dependent mechanism.

In a recent study Sumoza-Toledo *et al.* (22) investigated the effect of murine Treg on $CD4^+$ T cells IS formation. They found that although synaptic PKCθ recruitment was altered in T cells in the presence of Treg, polarization of MTOC toward APC was unaffected (22). However, in this study, polarization of tubulin cytoskeleton was investigated only a short time after conjugate formation. Therefore, our results extend these previous observations.

We show that T_H undergo conjugate formation and sustained signaling when interacting with the APC in the presence of Treg. This indicates that, in our system, Treg do not interfere with the early steps of T_H activation, but they rather affect downstream pathways. Accordingly, it has been recently shown, using *in vitro* time-lapse video microscopy, that $[Ca^{2+}]_i$ increase at the single-cell level in murine T cells conjugated with APC is not affected by Treg (23). Taken together, our results and those published results indicate that regulation does not directly affect TCR signaling.

Previous studies investigated the role of Treg in inhibiting the stop signal *in vivo* in naïve murine T cells. They showed that in the presence of Treg, the length of T cell/APC interactions was reduced (24, 25). Our present results are not in contrast with those previous findings. Indeed, in our experimental approach *in vitro* expanded human $CD4^+$ T cells were conjugated by centrifugation with APC to measure the extent of calcium responses by FACS analysis. Therefore, our experimental approach did not address the question of whether Treg affect the length of the stop signal in migrating T_H cells.

A key function of T_H cells is their capacity to rapidly polarize their secretory machinery toward APC. This may allow T_H cells to provide dedicated help to other actors of the immune response such as B lymphocytes or macrophages (11, 12, 26). Via this mechanism immune cells that display the strongest antigenic stimulus for T cells can be selectively activated, resulting in a progressive amelioration of specific immune responses (11, 14, 15).

Our results, by showing that Treg rapidly inhibit T_H polarization, shed light on the biology of these cells. We show that, in addition to other previously described mechanisms used by Treg

to inhibit adaptive immune responses (8), TGF-$\beta 1$ inhibits polarization responses.

Our morphological quantifications of three-cell conjugates in which one APC was simultaneously in contact with one Treg and one T_H show that, although the proximity between Treg and T_H facilitates inhibition (SI Fig. 8E), a direct contact between Treg and regulated cells is not required (SI Fig. 11). This observation is in agreement with previously reported studies based on two-photon microscopy approaches in which it was shown that Treg do not need to enter in contact with T_H to deliver their inhibitory signals (24, 25). In addition, the observation that anti-TGF-β blocking antibody reverts Treg inhibition of T_H polarization and that soluble TGF-$\beta 1$ mimics this Treg inhibitory effect contributes to support a model of Treg function in which Treg act (independently of cell–cell contact) via the secretion of soluble mediators.

Nevertheless, it should be noted that in our study [and also in the above mentioned previous studies (24, 25)] undetected short-lived contacts between Treg and regulated T cells could contribute to the observed inhibition.

Our observations are in apparent contrast with studies showing that *in vitro* suppression by Treg of T cell proliferation and cytokine production requires cell–cell contact and is normally independent of soluble factors (27). We also observed that the anti-TGF-β used in this study did not reverse suppression of T_H proliferation and IFN-γ production in 72-h cultures (data not shown). Conversely and interestingly when the same expanded Treg population were used in short time cocultures in which T_H polarization toward the APC was investigated, the Treg inhibition was TGF-β-dependent (Fig. 4 *C* and *D*).

We propose that our results identify an additional mechanism used by Treg to interfere with the effector function of T_H cells. Although at late times Treg block T_H cell proliferation and cytokine production, during the first few hours of cell–cell interaction they affect T cell polarization toward APC. TGF-β secretion appears to be necessary and sufficient to mediate this last inhibitory effect, whereas other Treg derived inhibitory mechanisms may be responsible of the observed inhibition of T_H proliferation *in vitro* (28).

What could be the biological function of the observed inhibition of T_H polarization toward APC? An initial answer to this challenging question comes from the observation that the addition of TGF-$\beta 1$ to T_H/APC conjugates inhibited T_H induced increase of CD40 and ICAM-1 expression on the surface of APC, showing an inhibitory effect of TGF-β on APC function (SI Fig. 12). These results also suggest that, in the course of an immune response, our presently described mechanism may contribute to alter the stop signal in migrating T cells, in agreement with reported measurements of cellular dynamics in living tissues (24, 25).

It is tempting to speculate that by inhibiting polarization of T_H cells toward APC Treg would limit T_H mediated APC activation. This mechanism would not be relevant to control T_H activation *in vitro*; on the contrary, it would affect *in vivo* the function of APC during further encounters with new T cells, thus contributing to the remarkably efficient control of immune responses exerted by Treg. A scheme presented in SI Fig. 13 summarizes our interpretation of the results.

A recent *in vivo* study demonstrated a central role for T cell-produced TGF-$\beta 1$ in controlling differentiation of T_H cells and induction of inflammatory diseases (29). Our *in vitro* observations are compatible with this recent study: we suggest that in the course of an *in vivo* response, the effect of TGF-β on T_H polarization may be by instrumental in fine tuning APC activation to avoid an uncontrolled amplification of the immune response leading to immunopathological lesions.

In conclusion, our results unveil an unknown strategy used by Treg to interfere with the development and sustenance of adaptive immune responses.

Although the inhibitory effect of Treg on the activation of naïve T cells to become effectors has been thoroughly documented, much less is known about the mechanisms by which Treg may efficiently outcompete the T_H-mediated amplification of immune responses. Here, we show that Treg can suppress this pivotal T_H cell function. By impairing dedicated help delivery, Treg affect a crucial early step of the T_H-dependent immune responses (*SI Text*).

Materials and Methods

T Cells and APC. T cells were isolated from the whole blood of healthy donors (Centre de Transfusion Sanguine, Centre Hospitalier Universitaire Purpan, Toulouse, France). The $CD4^+$ $CD25^+$ enriched T cell fraction was sorted from PBMC by using magnetic separation (Miltenyi Biotec). $CD4^+$ T cells were isolated by negative depletion followed by positive selection using anti-CD25 coated beads. Two cellular fractions were obtained, the $CD4^+$ $CD25^-$ fraction and the $CD4^+$ $CD25^+$ fraction. Cell purity was assessed by FACS analysis (Facscan, Becton Dickinson) using PE-labeled anti-CD25 mAb (clone M-A251, BD PharMingen) and FITC-labeled anti-CD4 mAb (clone RPA-T4, BD PharMingen). $CD4^+$ $CD25^+$ fractions were routinely 91–98% pure. Cells were assessed for CD127 expression using PE-labeled anti-CD127 mAb (clone R34.34, Immunotech) and/or for Foxp3 using FITC-labeled anti-Foxp3 mAb (clone PCH101, eBioscience).

DC were derived from autologous PBMC. Adherent monocytes were cultured in RPMI medium 1640 10% FCS supplemented with IL-4 (1,000 units/ml) and GM-CSF (50 ng/ml, both from R&D Systems) (30). The percentage of $CD11c^+$ cells in monocyte-derived DCs was checked by FACS after 15 days of culture and was routinely 90% pure. EBV-transformed B cells (LG2) were cultured as described (9).

T Cell Expansion. Freshly isolated T cell populations were cultured and expanded in RPMI medium 1640, 5% human serum, IL-2 (150 units/ml) in the presence of anti-CD3/CD28 mAb-coated Dynabeads (Invitrogen). For $CD4^+$ $CD25^+$ T cells, IL-15 (5 ng/ml, from R&D Systems) was added to the culture medium. Bead: cell ratio was 2:1 for the $CD4^+$ $CD25^+$ T cells and 1:1 for the $CD4^+$ $CD25^-$ T cells (31).

Suppression Assay. CFSE-loaded (5×10^4) $CD4^+$ $CD25^-$ cells per well were cultured in 96 U-bottom plates with $CD4^+$ $CD25^+$ T cells at different ratios.

Cells were stimulated with CD3/CD28 mAb-coated beads (18). Cells were cocultured for 72 h, $CD4^+$ $CD25^-$ cell proliferation was measured by FACS analysis.

Confocal Microscopy. EBV-B cells or DC were pulsed with a mixture of bacterial superantigens (TSST-1, SEE, SEB, 100 ng/ml, Toxin Technology) for 1 h at 37°C. During the last 15 min, cells were loaded with 0.5 μM CMTMR-Orange (Molecular Probes). The superantigens not bound to APC were removed by washing. In some experiments APC loaded with CMTMR-orange were fixed with 1% paraformaldehyde before pulsing with superantigens. In parallel, $CD4^+$ $CD25^+$ Treg were loaded with 0.5 μM CMFDA-green (Molecular Probes) for 15 min at 37°C. Each T cell subset (10^5) and 2×10^5 APC were conjugated as described (14). At different time points after conjugation, cells were fixed and permeabilized either with 0.1% Triton X-100 (for GM130 staining) or with 0,1% saponin for (IFN-γ and tubulin staining) as described (14). The following primary antibodies were used: anti-GM130 (either rabbit polyclonal Ab, kind gift of A. De Matteis, Istituto Mario Negri Sud, Italy, or mouse mAb, clone 35, from BD PharMingen); anti-IFN-γ monoclonal Ab (clone B27, BD PharMingen); anti-β tubulin mAb (clone TUB 2.1, Sigma). Primary Ab were followed by either goat anti-mouse or goat-anti-rabbit polyclonal Ab labeled with Alexafluor 633 (Molecular Probes). In some experiments anti-TGF-β mAb (clone 2G7) (32) or isotype control Ab (BD PharMingen) were added to the culture medium. In some experiments, a DRB1*0101-restricted T_H1 cell clone (6396p5.1.2) specific for the measles virus fusion protein peptide F254–268 and DR-matched EBV-B cells were used. Cells were cocultured either in the presence or in the absence of 20 ng/ml recombinant human TGF-β1 (from R&D Systems). The samples were mounted and examined using a Zeiss LSM 510 (Zeiss) confocal microscope with a 63 Plan-Apochromat objective (1.4 oil), electronic zoom 3, as described (14). Snapshots depicting overlapping of differential interference contrast images with green, red, and blue fluorescence are unprocessed images. In snapshots depicting green, red, and blue fluorescence only, background subtraction and color saturation was applied to the whole image using Adobe Photoshop software (Adobe).

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured in T_H cells conjugated with APC, as described (9).

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