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Regulatory T Cells Target Chemokine Secretion by Dendritic Cells Independently of Their Capacity To Regulate T Cell Proliferation

Sara Morlacchi,* Valentina Dal Secco,* Cristiana Soldani,* Nicolas Glaichenhaus,† Antonella Viola,*‡ and Adelaida Sarukhan*†

The online version of this article contains supplemental material.

The clinical manipulation of regulatory T cells (Tregs) represents a promising strategy for the regulation of unwanted immune responses. It is now becoming clear that Tregs exert multiple effects on different cell targets under particular conditions; however, the interplay between these different factors remains unclear. Using mouse Tregs of known Ag specificity, we report in this study two different levels of Treg-mediated suppression: one that targets T cell proliferation and one that targets dendritic cell-mediated proinflammatory chemokine (CCL3 and CCL4) production. These two effects can be dissociated, and whereas modulation of T cell proliferation depends on the strength of the antigenic stimulus, modulation of chemokine production by dendritic cells does not. We also provide evidence that the bystander effect of Tregs on immune responses observed in vivo may be in great part explained by a decrease in the recruitment of target T cells, and therefore in the magnitude of the response, rather than by a direct effect on their priming or proliferation. Overall, our results shed some light on the different aspects that need to be considered when attempting to modulate Tregs for clinical purposes. The Journal of Immunology, 2011, 186: 6807–6814.
purchased from The Jackson Laboratory and back-crossed onto the TCR-HA and TCR-HA × pgk-HA mice. WT15 mice, transgenic for a TCR that recognizes an immunodominant peptide of Leishmania Ag (LACK) in the context of MHC I-A^d, and on a RAG-deficient background, have been described elsewhere (17). Bone marrow from BALB/c-transgenic (NF-κB-RE-luc)-Xen mice (Caliper Life Sciences) carrying a transgene containing six NF-κB responsive elements and a modified firefly luciferase cDNA were used to derive DCs as previously described (14).


**Abs and reagents**

The clonotypic 6.5 mAb, which recognizes the transgenic TCR-HA, was produced in our laboratory and was used to couple to biotin or PE. All other Abs for flow cytometry were purchased from BD Pharmingen. Cells were analyzed on a flow cytometer (FACSCanto; Becton Dickinson). FACS data were analyzed using Diva software and FlowJo software. LPS (Escherichia coli 026:B6) was purchased from Sigma-Aldrich. The HA peptide (SVSSFERFEIFPK) and LACK peptide (ICFSPSLEHPIVVSGSWD) were purchased from Invitrogen.

Vybrant CFDA-SE cell tracer kit (Invitrogen) and CellVue Maroon (Molecular Targeting Technologies) were used according to the manufacturer’s instructions. Luciferase experiments were performed using the Briteplate plus kit (PerkinElmer) according to the manufacturer’s instructions.

**In vitro proliferation and suppression assays**

For in vitro experiments, total LN and spleen suspensions from TCR-HA single-transgenic or TCR-HA × pgk-HA double-transgenic mice were stained with CD4 and 6.5 Abs and sorted on a FACSaria for the obtention of HA-specific 6.5+ T conventional (Tc) cells (CD4+ 6.5+ GFP+ ) or 6.5+ Tregs (CD4+ 6.5+ GFP- ), respectively. In some experiments, CD4 cells were sorted by gating on 6.5+ and CD8-, CD19-, CD11c-, CD11b- cells. DCs were obtained from the spleens of BALB/c mice by positive selection with anti-CD11c microbeads (Miltenyi Biotec).

All experiments were performed in complete DMEM (Lonza), supplemented with 2-mercaptoethanol (Invitrogen) and 10% FCS. Tc cells (2.5 × 10^5) were incubated with DCs (1.5 × 10^6) in flat-bottom 96-well plates in the absence or presence of Tregs at a Tc cell/Treg ratio of 1:1 or 2:1.

After 2 d of culture, supernatants were collected for the quantification of cytokines, and 1 μCi [3H]methyl-thymidine was added for an additional 16 h. All experiments were performed in triplicate.

**Cytokine and chemokine detection**

Cytokine and chemokine concentrations were quantified from supernatants of in vitro cultures using the ELISA duoset kits (R&D Systems) according to the manufacturer’s instructions.

**mRNA quantification**

RNA was purified using a RNeasy Mini Kit (Qiagen) and retro-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR analysis was performed with TaqMan Gene Expression MasterMix (Applied Biosystems). The following primers and probes were used: for CCL3 (MIP-1α), a custom designed TaqMan Gene Expression Assay (TaqMan MGB probes, FAM dye-labeled) (Applied Biosystems) validated by the manufacturer; for β-ACTIN, TaqMan Gene Expression Assay (Mm00607939_s1; Applied Biosystems).

**Adoptive transfers**

For in vivo experiments, 5 × 10^5 to 7 × 10^6 CFSE-labeled wt15 cells (CD4+, Thy1.1-) were adoptively transferred by i.v. injection into BALB/c recipient mice that either had received or had not received 4 × 10^6 6.5+ GFP+ Tregs 18 h earlier. Recipients were then immunized with soluble LACK plus HA peptide by s.c. injection into one footpad and with the LACK peptide alone in the other footpad, together with 50 μg LPS. Alternatively, the first population of wt15 cells injected was not CFSE-labeled, and a second wave of CFSE-labeled wt15 cells was injected 18 h after immunization. Immunized mice were sacrificed after 3 d, and the corresponding popliteal draining LNs were collected and treated with 1.5 mg/ml collagenase IV (Sigma-Aldrich) and 0.2 mg/ml DNase (Roche) at 37°C for 30 min. Cells were washed in PBS, counted, and stained with specific Abs. Axillary LNs were used as control nondraining LNs.

For the results shown in Fig. 6C, BALB/c bone marrow-derived DCs were labeled with CMTMR, loaded for 2 h with LACK peptide (5 μg/ml) or LACK and HA peptides (5 μg/ml each), washed, and injected s.c. into the footpads of BALB/c mice having received 4 × 10^6 6.5+ GFP+ T cells and 5 × 10^5 wt15 cells. Popliteal LNs were recovered 20 h later and frozen in OCT. CCL3 quantification in DCs was performed as previously described (14). Briefly, fixed 10-μm cryostat sections were incubated with the primary CCL3 (MIP-1α) Ab (1:20; R&D Systems) and revealed with Alexa 467-anti-goat secondary Ab (Molecular Probes). Acquisition of

**FIGURE 1.** Suppression of HA-specific T cell proliferation but not chemokine production is dependent on the strength of the antigenic stimulus in vitro. A, FACS-sorted 6.5+GFP+ cells from TCR-HA × pgk-HA mice were used as source of HA-specific Tregs for all experiments reported in this study. FACS-sorted 6.5+GFP+ cells from TCR-HA mice were used as source of HA-specific Tc cells. Percentage of GFP+ 6.5+ cells in the CD4+ gate or in the 6.5+ gate (in parentheses) are shown. B, 6.5+GFP+ Tc cells isolated from single-transgenic TCR-HA mice were coincubated with splenic-derived DCs and different doses of HA peptide, in the absence or presence of 6.5+ Tregs. Supernatants were recovered 48 h later for chemokine measurement, and thymidine was added for an additional 16 h for determination of proliferation. Data represent the mean of triplicate wells and one representative experiment out of three. Tr, Treg.
images was made by confocal microscopy (Fluoview FV1000; Olympus, Tokyo, Japan) using laser excitation at 405, 543, and 633 nm. Images were acquired with an oil immersion objective ($\times 60$, 1.4 NA Plan-Apochromat; Olympus) with a resolution of 800 x 800 dots per inch.

A region of interest on CMTMR+ DCs was drawn and the parameter “percentage of material colocalized,” which includes both the number of voxels and their intensities, was calculated using the “Colocalization” module of Imaris 5.0.1, 64-bit version (Bitplane AG, Saint Paul, MN). For each data set, 15 individual cells were analyzed for colocalization.

**Statistical analysis**

Results are expressed as means ± SD. For the experiments shown in Fig. 6, groups were compared by using paired Student $t$ test.

**FIGURE 2.** Treg-mediated suppression affects CCL3 mRNA transcription by the DC. A, Splenic DCs that were loaded or not (uDC) with 1 µg/ml peptide were coincubated with 6.5°Tc cells, 6.5°Tregs, or both. CCL3 protein in the supernatant was determined by ELISA 24 or 48 h after coincubation. In parallel, total cells from the cultures were recovered 24 h after and analyzed for CCL3 mRNA levels. B, Total CD4°GFP° (Tc) and GFP° (Treg) cells were sorted from a Foxp3-GFP knock-in mouse. Tc cells were stimulated with coated anti-CD3 and soluble CD28 or with splenic DCs with soluble anti-CD3 in the absence (solid bars) or presence (striped bars) of Tregs. IL-2 and CCL3 protein in the supernatant were determined 48 h after coculture. C, Splenic DCs and 6.5° cells from a TCR-HA mouse were sorted by MACS and coincubated with 1 µg/ml HA peptide. Twenty-four hours later, cells were sorted based on CD11c and CD4 markers, and CCL3 mRNA levels were determined. Experiments were repeated twice with similar results. Tr, Treg.

**FIGURE 3.** Suppression of chemokine production but not of T cell proliferation in a non-antigen-specific system. 6.5°GFP°Tregs were coincubated with 6.5°Tc cells (HA-specific) or with wt15 cells (LACK-specific) together with DCs and increasing doses of both peptides. Supernatants were collected after 48 h for chemokine measurement, and thymidine was added for an additional 16 h. Two independent experiments gave similar results. Tr, Treg.
Results

Chemokine production, but not T cell proliferation, is inhibited by Tregs upon strong Ag stimulation

We have previously reported that Tregs can suppress proinflammatory chemokine production within reactive LNs (14). In particular, using an adoptive transfer model in which naive HA-specific T cells are transferred into BALB/c recipients in the absence or presence of Tregs of the same Ag specificity, we found that CCL2 and CCL3 chemokine production in the LN was significantly reduced when HA-specific Tregs were coinjected. Curiously, proliferation of the transferred naive T cells was not affected by the presence of Tregs under the stimulation conditions used (HA peptide plus LPS). These results suggest the existence of two levels of suppression by Tregs, one resulting in chemokine suppression and one resulting additionally in inhibition of T cell proliferation, and that these levels may depend on the strength of the antigenic stimulus.

To confirm this observation and to eliminate possible contributions of other cell populations or complex interactions present in vivo, we performed the same kind of experiments in vitro. As source of Tregs we used TCR-HA pgk-HA mice, which express the HA-specific transgenic TCR and the HA Ag on thymic epithelium, back-crossed to Foxp3-GFP knock-in mice. This permitted us to sort a pure population of bona fide HA-specific Tregs (6.5+Tregs) that were positive for GFP and for the transgenic TCR (Fig. 1A). As source of naive, conventional T cells, we used GFP-negative 6.5+ cells from TCR-HA Foxp3-GFP knock-in mice (6.5+Tc cells). As APCs, we chose to use splenic-derived DCs to better reflect the different populations susceptible to presenting Ag in vivo (18).

When we incubated 6.5+Tregs together with 6.5+Tc cells, we observed suppression of T cell proliferation at low peptide doses (0.1 μg/ml) but not at high peptide doses (1 μg/ml). However, secretion of CCL3 and CCL4 proinflammatory chemokines in the supernatant of such cultures remained strikingly abrogated even at high peptide doses (Fig. 1B). Thus, these data confirm that Treg-mediated inhibition of T cell proliferation is abrogated upon high Ag concentrations, whereas suppression of proinflammatory chemokine secretion is a Treg feature that prevails even upon strong antigenic stimulation.

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FIGURE 4. Proliferation of Tregs does not abrogate chemokine production. A, wt15 cells (Thy1.1+) and 6.5+GFP+ cells were stained with CFSE and CellVue Maroon, respectively, and coincubated together with increasing amounts of both peptides. Seventy-two hours later, cell proliferation was determined by FACS, and cells were gated according to CD4 and Thy1.1 expression. B, After 48 h, the supernatant from wt15 cells alone (solid circles) or with 6.5+Tregs (open circles) was taken and tested for IL-2 and CCL3 protein levels. The experiment was repeated twice, with similar results. Tr, Treg.

FIGURE 5. Modulation of HA-specific but not LACK-specific cells in the same cytokine environment. wt15 and 6.5+Tc cells were stained with CFSE and CellVue Maroon, respectively, and coincubated together with increasing amounts of both peptides. Seventy-two hours later, cell proliferation was determined by FACS, and cells were gated according to CD4 and Thy1.1 expression. A, Proliferation in response to a low dose of both peptides was determined for each of the three T cell populations after staining with CD4 and Thy1.1 Abs. B, IL-2 and CCL3 levels in the supernatant were tested for both Tc cells incubated in the absence (solid circles) or presence (open triangles) of Treg cells. Tr, Treg.
DCs are the main targets of Treg-mediated CCL3 suppression

We had previously shown that, within reactive LNs, CCL2 is produced by DCs only, whereas CCL3 is detectable in both T cells and DCs (14). To identify Treg targets in our experimental system, it was important to identify the main source of CCL3 in vitro. First, we tried to determine whether the decrease of CCL3 in supernatants of cocultures with Tregs was due to a decrease in gene transcription rather than to a decrease in secretion or an increase in its consumption. For this, we coincubated splenic DCs, either unloaded (as control) or loaded with HA peptide, together with 6.5+Tc cells, 6.5*Tregs, or both, and measured CCL3 production by ELISA and real-time PCR (Fig. 2A). The concentration of CCL3 increased significantly in the supernatant of 48-h HA–DC–6.5+Tc cell cocultures, but very low levels of chemokine were detected in the presence of 6.5*Tregs. Real-time PCR results confirmed an increase of CCL3 mRNA when loaded DCs were incubated with 6.5+Tc cells but not when incubated with 6.5*Tregs or with both, indicating that the suppression of CCL3 occurs at the mRNA level.

To determine which cell was the main source of CCL3 (i.e., DC or Tc cell), we stimulated Tc (GFP+ cells) with anti-CD3 in the absence or presence of DCs. In some wells, we added GFP+ Tregs for suppression. Tc cell activation worked efficiently in both conditions and induced comparable IL-2 secretion that was abrogated by the addition of GFP+ Tregs (Fig. 2B). However, CCL3 was only detected in the supernatant of Tc cells stimulated in the presence of DCs, and, again, chemokine production was suppressed by Tregs. These results strongly suggest that, in our experimental system, DCs are the main source of CCL3 and that chemokine production by DCs is a main target of Treg-induced suppression. To prove formally that CCL3 is secreted by DCs in DC–Tc cell cocultures, we evaluated CCL3 mRNA expression in the two FACS-sorted cell populations after 24-h coincubation with HA peptide. CCL3 mRNA was only detected among the CD11c+ DCs.
population corresponding with DCs and not among the stimulated CD4+Tc cells.

Altogether, these results indicate that, in conditions of strong antigenic stimulation, chemokine production by DCs is a preferential target of Treg inhibition.

It has been recently shown that cognate interaction with T cells induces NF-κB activation in DCs, promoting their survival (19). Because CCL3 and CCL4 have been shown to be NF-κB target genes (20), we analyzed the effects of Treg activity on NF-κB activation in DCs, using bone marrow-derived DCs from mice transgenic for the luciferase gene under a promoter containing six responsive NF-κB elements. Luciferase expression, measured after overnight cell cocultures, showed NF-κB activation in DCs upon cognate interaction with 6.5°Tc cells (Supplemental Fig. 1). Coincubation of 6.5°Tregs with loaded DCs resulted in weak luciferase expression, similar to that observed in the unloaded DC control. However, when both Tc cells and Tregs were cocultured with HA–DCs, no suppression of DC luciferase expression was observed, suggesting that the signaling pathway involved in the Treg targeting of CCL3 mRNA transcription either does not involve NF-κB or is downstream of NF-κB. These results also suggest that Tregs do not affect NF-κB–mediated survival signals delivered to the DC upon cognate interaction with Tc cells.

Suppression of chemokine production, but not of Tc cell proliferation, is independent of the Treg Ag-specificity

We had previously reported that HA-specific Tregs were not capable of affecting the proliferation of Tc cells specific for another Ag, in vitro or in vivo, even when both Ags were presented by the same APC (21, 22). However, several studies show that Tregs, if activated by their cognate Ag, can modulate immune responses toward other unrelated Ags in vivo (23). Based on the results obtained above with the HA system, it is also possible that the different TCRs involved in recognizing different Ags may simply vary in terms of affinity and signal strength, making them more or less susceptible to Treg-mediated suppression. We thus asked ourselves whether the dissociation between the inhibitory action of Tregs on DC and Tc cell proliferation could also be observed with Tc cells specific for another Ag. To address this question, we isolated Tc cells from wt15 mice, which are transgenic for the TCR that recognizes an immunodominant peptide of the Leishmania Ag (LACK), and we tested the ability of 6.5°Tregs to modulate Tc cell proliferation in vitro versus chemokine production. As expected, coincubation of 6.5°Tregs and 6.5°Tc cells and DCs loaded with both HA and LACK peptides resulted in suppression of Tc cell proliferation at low Ag dose and in suppression of chemokine production at both low and high Ag doses. When 6.5°Tc cells were replaced with LACK-specific wt15 Tc cells, CCL3 and CCL4 production was suppressed at both high and low doses of peptide (Fig. 3), indicating that Tregs can suppress chemokine production even in an Ag-unrelated context. In contrast, no suppression of Tc cell proliferation was observed, confirming our previous data (21). This result was confirmed also with total LACK protein (Supplemental Fig. 2), excluding the possibility that Tregs are exerting broader effects on DCs, such as decreasing their Ag processing capacity. We also obtained the same results when scaling down the LACK peptide while keeping the HA peptide concentration constant (Supplemental Fig. 3). The stronger proliferation observed when wt15 Tc cells were cocultured with 6.5°Tregs was due, at least in part, to considerable proliferation of the 6.5°Tregs (Fig. 4A), which proliferated only when the HA peptide was present. This Treg proliferation was surely due to IL-2 secreted by the wt15 Tc cells, but the proliferating Tregs retained their capacity to suppress CCL3 production (Fig. 4B). Finally, we performed an in vitro experiment where all three types of cells (wt15, 6.5°Tc cells, 6.5°Tregs) were present in the same well and stimulated with low doses of peptide. The results shown in Fig. 5A confirmed that 6.5°Tc cell proliferation at low antigenic doses remains suppressed even when in the same cytokine environment as the wt15 T cells (Fig. 5B).

These results confirm that T cell proliferation and chemokine production by DCs represent two targets of Treg-mediated suppression with different sensitivity to the strength of the stimulus, which in turn depends on the antigenic dose and the affinity of the TCR expressed by the target Tc cell.

Tregs inhibit in vivo recruitment but not proliferation of Tc cells

Based on the in vitro results, we asked whether Tregs could exert their bystander effect in vivo by altering recruitment of Tc cells via suppression of chemokine production, rather than by directly affecting their proliferative capacity. We examined this possibility by adoptively transferring wt15 T cells into BALB/c mice that had previously received or not received 6.5°GFP+ Tregs and by immunizing the recipients with LACK peptide in one footpad and both LACK and HA peptides in the other footpad. Low quantities of LPS were coadministered together with the peptides, based on our previous work where such immunization protocol allowed efficient T cell activation but did not abrogate suppression of chemokine production by Tregs (14). Wt15 cells were labeled with CFSE and distinguished from GFP+ Tregs by Thy1.1 staining. Only in mice that received 6.5°Tregs in addition to the wt15 cells was a significant reduction in total cellularity and in the absolute number of Thy1.1° Tc cells observed in the popliteal LN that received both peptides, this compared with the contralateral LN, which received only the LACK peptide (Fig. 6A). As expected, the percentage of GFP+ (6.5°Treg) cells increased only in the LNs that received both peptides (Fig. 6A), confirming their capacity to proliferate in vivo upon cognate Ag stimulation (16, 24, 25). Importantly, the presence of 6.5°Tregs did not affect the proliferative capacity of the wt15 Tc cells (Fig. 5A, lower row). These results indicate that Tregs decrease the magnitude of the in vivo immune response by affecting T cell recruitment as well as recruitment of other immune cells such as APCs. Indeed, the total number of DCs was also dramatically decreased in the LNs receiving both peptides compared with that in the LNs receiving only the LACK peptide (2.36 ± 2.1 × 10^4 versus 9.95 × 10^4 ± 3.9, p = 0.04).

To confirm that the Tregs were inhibiting recruitment of T cells to the LN rather than accelerating their exit from such structures,
we performed similar adoptive transfer experiments as those described earlier, except that the wt15 cells transferred before immunization were not labeled with CFSE. Eighteen hours after immunization, recipient mice received another injection of wt15 cells, this time CFSE labeled. Draining popliteal LN were analyzed 6 h later. Again, only in mice that were transferred with 6.5 Tregs in addition to the wt15 Tc cells was the absolute number of Thy1.1+ cells and CFSE+ wt15 cells markedly decreased in the LNs having received both peptides, this compared with the contralateral LN, which received only the LACK peptide (Fig. 6B).

Finally, to confirm that this decrease in recruitment was due to a decrease in DC-mediated CCL3 production in vivo, we injected CMTMR-labeled DCs that were loaded with the LACK peptide or with both HA/LACK peptides into the footpads of mice adoptively transferred with 6.5 Tregs and wt15 cells. CCL3 staining on frozen LN sections was performed, and the amount of percentage of CCL3 colocalization with CMTMR was determined. Ag-frozen LN sections was performed, and the amount of percentage of CCL3 colocalization with CMTMR was determined. Ag-presenting DCs within LNs where both Tregs and wt15 cells were activated contained significantly less CCL3 cells compared with that of DCs where only wt15 cells were activated (Fig. 6C).

Overall, these results confirm two levels of Treg-mediated suppression, where abrogation of chemokine production by DCs is less sensitive to increasing antigenic doses or TCR affinities than abrogation of T cell proliferation (Fig. 7).

**Discussion**

Strong antigenic stimulation, LPS-mediated maturation of DCs, or enhanced costimulation have all been reported to abrogate suppression of target T cell proliferation by Tregs in vitro (26–31). Furthermore, in vivo experiments performed with HA-specific T cells showed that under weak antigenic stimulation, polyclonal Tregs affected T cell proliferation and differentiation, whereas under strong stimulation conditions, only T cell differentiation remained partially suppressed (32). However, the magnitude of an immune response during the initiation phase is not only dictated by the amount of T cell proliferation on a per cell basis but also by the total number of T cells that enter and proliferate within the reactive LN. Whereas the former depends mainly on the strength of the TCR signaling, the latter depends in great part on the release of proinflammatory chemokines. In this study, we have shown that Treg-mediated suppression involves not only T cell proliferation but also production of proinflammatory chemokines, in accordance with our previous results (13, 14). Furthermore, our results indicate that, under conditions of strong TCR signaling, suppression of proinflammatory chemokine production is maintained even if suppression of T cell proliferation is abrogated, confirming the notion that Tregs act at different levels and on different targets.

It has been shown that, in vitro, both T cells and DCs can produce CCL3 upon cognate Ag interaction (33). However, in our experimental conditions, DCs were the main cellular source of this chemokine. During immune responses, DCs are known to produce high levels of chemokines, such as CCL2, CCL3, CCL4, CCL5, CXCL8, and CXCL10, which sustain the recruitment of circulating, immature DCs, monocytes, and T cells to inflamed tissue (34). We and others had already shown that Tregs inhibit DC chemokine production in vivo, although the molecular mechanism by which Tregs down-modulate chemokine transcription remains to be determined. The fact that Tregs can modify other aspects of DC biology such as expression of costimulatory molecules (35, 36) or IL-6 production (37) could be suggestive of targeting of common signaling pathways downstream of activating receptors, such as NF-κB. However, our experiments suggest that NF-κB activation is not a target of Treg-mediated suppression and that other pathways are involved. The action of Tregs on DCs may indeed be complex and involve suppression of specific genes and activation of others, such as IL-10 (37).

A controversial point regarding Treg suppression is whether it is Ag specific or not. Although several reports indicate that Tregs can mediate bystander suppression (38–42), it has been reported that Ag-specific Tregs are much more efficient in suppressing autoimmune diseases such as thyroiditis (43), diabetes (44), or experimental autoimmune encephalitis (42, 45) than their polyclonal counterparts. However, this may be explained by the fact that Tregs have to be activated in an Ag-specific manner and does not exclude that, once activated, Tregs can efficiently suppress immune responses that are antigenically unrelated. In contrast, it is possible that Tregs act at multiple levels of the immune response with different mechanisms. The results presented in this study show that, although Tregs do not abrogate proliferation of Tc cells under conditions where the strength of the stimulus is high, they are very efficient in suppressing CCL3 and CCL4 up-regulation by DCs and, thereby, recruitment of immune cells to the LN. This suggests that the bystander effect of Tregs on immune responses directed toward unrelated Ags observed in vivo may, at least in part, be explained by a decrease in the recruitment of such T cells and therefore in the magnitude of the response, rather than by a direct effect on their priming or proliferation.

We would like to propose a multilevel model of Treg functions, whereby regulation of T cells is most profound at conditions of low signal strength, as it operates on proliferation of T cells and on chemokine production by DCs. As the strength of the TCR signaling starts increasing and as T cells with other Ag specificities and higher TCR affinities are involved, suppression of T cell proliferation is abrogated but the magnitude of the immune response is still down-modulated because chemokine production by DCs remains suppressed (Fig. 7). This conclusion is supported by our in vivo experiments showing that recruitment and not proliferation of LACK-specific T cells was affected by the presence of activated HA-specific Tregs. Moreover, several other studies have observed that the effect of Tregs during the first days of the immune response consists in the reduction of T cell numbers within reactive LNs and target tissues, rather than their proliferation, independently of their Ag specificity (16, 46, 47).

Overall, our results shed some light onto the different aspects that need to be considered when attempting to modulate polyclonal versus monospecific Tregs for clinical purposes.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


Figure S1
Figure S2
Supplemental Figure 1: Tr do not induce NFκB activation in DCs but do not suppress its activation by Tc upon cognate antigen interaction. BM-derived DCs expressing the luciferase transgene under the control of the NFκB responsive element were loaded with HA peptide (1µg/ml) and coincubated with 6.5+Tc, 6.5+Tr or both. Luciferase expression after overnight culture was determined as described in material and methods and the fold-increase respect to unloaded DCs is shown. DCs loaded with peptide but not incubated with T cells or DCs stimulated with LPS were also done as control. The experiment represents the average of triplicate wells, and represents one out of two similar experiments.

Supplemental Figure 2: Tr suppress DC-mediated chemokine production without affecting their antigen processing capacity. DCs were incubated with LACK protein (200µg/ml) and used to stimulate wt15 cells in the absence or presence of 6.5+Tr. Culture supernatants were tested by ELISA for chemokines and cell proliferation was determined by thymidine incorporation after 64h of coculture.

Supplemental Figure 3: Tr do not inhibit proliferation of LACK-specific T cells, independently of the antigenic dose. wt15 and 6.5+Tr were coincubated at a constant dose of HA peptide and decreasing doses of LACK peptide. Proliferation was determined by thymidine incorporation after 64h of coculture.