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Human Anti-Inflammatory Macrophages Induce Foxp3$^+$GITR$^+$CD25$^+$ Regulatory T Cells, Which Suppress via Membrane-Bound TGFβ-1[1]

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CD4$^+$ T cell differentiation and function are critically dependent on the type of APC and the microenvironment in which Ag presentation occurs. Most studies have documented the effect of dendritic cells on effector and regulatory T cell differentiation; however, macrophages are the most abundant APCs in the periphery and can be found in virtually all organs and tissues. The effect of macrophages, and in particular their subsets, on T cell function has received little attention. Previously, we described distinct subsets of human macrophages (pro- and anti-inflammatory, mφ1 and mφ2, respectively) with highly divergent cell surface Ag expression and cytokine/chemokine production. We reported that human mφ1 promote, whereas mφ2 decrease, Th1 activation. Here, we demonstrate that mφ2, but not mφ1, induce regulatory T cells with a strong suppressive phenotype ($T_{m\varphi2}$). Their mechanism of suppression is cell-cell contact dependent, mediated by membrane-bound TGFβ-1 expressed on the regulatory T cell (Treg) population since inhibition of TGFβ-1 signaling in target cells blocks the regulatory phenotype. $T_{m\varphi2}$, in addition to mediating cell-cell contact-dependent suppression, express typical Treg markers such as CD25, glucocorticoid-induced TNF receptor (GITR), and Foxp3 and are actively induced by mφ2 from CD25-depleted cells. These data identify mφ2 cells as a novel APC subset capable of inducing Tregs. The ability of anti-inflammatory macrophages to induce Tregs in the periphery has important implications for understanding Treg dynamics in pathological conditions where macrophages play a key role in inflammatory disease control and exacerbation. *The Journal of Immunology,* **2008, 181: 2220–2226.**

Studies over the past 10 years have attempted to determine mechanisms of induction as well as modes of suppression by regulatory CD4$^+$ T cell subsets. To date there are four widely accepted CD4$^+$ T regulatory cell (Treg)[3] types: 1) naturally occurring (nTreg), 2) inducible (Tr1), 3) anergic T cells, and 4) Th3 Tregs.

nTregs are thymic-derived CD4$^+$CD25^{high}Foxp3$^+$ cells which require cell-cell contact for suppression. Although not yet fully elucidated, mouse models have shown that suppression may be mediated by either membrane-bound TGFβ-1 (1, 2), lymphocyte activation gene-3/CD223 (3), or glucocorticoid-induced TNF receptor (GITR) (4). A recent report showed that human CD4$^+$CD25$^+$Foxp3$^+$ Tregs are not solely derived from the thymus, but rather from the CD4$^+$ memory pool, and that they have limited proliferative ability, eliminating the possibility of self-renewal of the Treg population. These observations suggest a model whereby the stimulation of memory CD4$^+$ cells in the periphery leads to the continuous generation and renewal of Foxp3$^+$ Tregs (5).

Inducible (Tr1) Tregs are typically induced in vitro by the presence of IL-10 (6), and their suppressive ability is mediated by IL-10 secretion. Anergic T cells have been shown to have a suppressive phenotype that is cell-cell contact dependent and not IL-10 or TGFβ-1 mediated (7, 8). Typically, T cell anergy is induced by Ag stimulation of the TCR/CD3 complex in the absence of costimulatory signals. Interestingly, anergic T cells are also capable of rendering APC tolerogenic (9), which may be one mechanism of sustaining suppression.

The induction of Th3 cells is tightly linked with the induction of mucosal tolerance: mucosal administration of relevant Ag can prevent the onset of autoimmune diseases such as experimental allergic encephalitis, diabetes, and allergy in mice. The regulatory property of Th3 cells is thought to be mediated by soluble, secreted TGFβ-1, as the suppression can generally be reversed by administration of TGFβ-1-blocking Abs (reviewed in Ref. 10). Interestingly, the role that TGFβ-1 plays in both nTreg and Th3-mediated suppression is drawing the two cell types closer together than previously thought (11).

We and others have previously described diametrically opposed macrophage subsets, derived from the same CD14$^+$ monocytic precursor population, which we designated mφ1 and mφ2 (12, 13). mφ1 secrete high levels of IL-23 (IL-12p40/IL-23p19) upon stimulation, and in the presence of IFN-γ initiate transcription of IL-12p35, leading to the secretion of IL12p70 as well. mφ2 cells, on the other hand, do not secrete IL-12p70 or IL-23, but rather secrete IL-10 in response to LPS. The expression of HLA-DR at the cell surface of mφ1 and mφ2 cells is low compared with mature

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[3] Abbreviations used in this paper: Treg, regulatory T cell; GITR, glucocorticoid-induced TNF receptor; LAP, latency-associated peptide; mφ1/2, pro- and anti-inflammatory macrophages, respectively; mTGFβ-1, membrane TGFβ-1; nTreg, naturally occurring T regulatory cell; T_{Ind}, indicator responder T cell; $T_{m\varphi1/2}$, allogeneic T cells raised against mφ1/2 cells.

dendritic cells, and in the case of mφ2 is further reduced upon TLR stimulation (12). mφ2 cells express the scavenger receptor CD163 and are highly phagocytic, a trait ascribed to macrophages resident at mucosal surfaces (14) and to peritoneal macrophages (15). Microarray analysis of mφ1 and mφ2 cells revealed exclusive expression of another scavenger receptor, stabilin 1 (STAB1) in mφ2 cells, and also found to be expressed in alternatively activated macrophages (Ref. 16 and our unpublished observations). Upon activation, mφ2 secrete an abundance of chemokines but very few proinflammatory cytokines (17). The TLR-induced down-regulation of T cell stimulatory molecules such as HLA-DR, CD80, and CD86, the absence of proinflammatory cytokines (IL-23, IL-12), and the high levels of IL-10 secreted by mφ2 cells prompted us to investigate whether mφ2 cells, despite the absence of TLR stimulus, could induce the differentiation of Tregs and to discern which subset of Tregs these cells may belong to.

The data presented herein describe the induction of T cells into a strong Treg subset only after being stimulated once by mφ2, but not by mφ1, cells. The mechanism of suppression of the mφ2-activated T cells ($T_{mφ2}$) was found to require cell-cell contact and was mediated by membrane-bound TGFβ-1. In addition to surface-expressed TGFβ-1, these $CD4^+$ Tregs were $CD25^+$, $GITR^+$, and $Foxp3^+$ and were induced from $CD25^-$ cells. Given the abundance of macrophages in the peripheral immune system and in virtually all tissues (resident macrophages), it is exceedingly important to understand how macrophage subsets influence and shape the T cell repertoire in homeostatic and inflammatory situations.

Materials and Methods

Reagents

Abs for FACS analysis were purchased from BD Pharmingen (CD1a, CD4, CD14, CD25, CD80, CD86, CD163, CTLA-4, HLA-DR), R&D Systems (GITR, latency-associated peptide (LAP)), IQ Products (TB21-TGFβ-1), and from eBioscience (FoxP3). Anti-CD3 (OKT-3) and anti-CD28 (B-T3) were obtained from Leiden University Medical Center (LUMC) Pharmacy and Sanquin, respectively. Insect cell-derived, 97% pure, carrier-free recombinant LAP was purchased from R&D Systems. Goat polyclonal blocking Abs to TGFβRII and isotype control goat IgG were obtained from Abcam and used at 20 μg/ml. ALK inhibitor (TGFβRI kinase inhibitor SB431542) was purchased from Sigma-Aldrich and used at 0.1 μM. GITR agonist Ab (clone 110416) and isotype control were obtained from R&D Systems and used at 10 μg/ml. ELISA Ab kits were obtained from Invitrogen-BioSource (IL-12p40 and IL-10) and R&D Systems (MIP1-β) and the human 17-plex assay was performed using Bio-Rad Multiplex kits and equipment. CFSE and LPS were all obtained from Sigma-Aldrich and used according to the manufacturer's recommendations.

Cell culture

All macrophages were generated as previously described (12). Briefly, monocytes from anonymous healthy blood donors' buffy coats were enriched for CD14 expression with MACS microbeads (Miltenyi Biotec) and cultured in RPMI 1640 medium, L-glutamine, and 10% FCS in the presence of 5 ng/ml GM-CSF (Invitrogen-BioSource) or 50 ng/ml M-CSF (R&D Systems) for 6 days to generate mφ1 and mφ2, respectively. Hallmark cytokine secretion assays were performed by activating the macrophages with 100 ng/ml LPS for 16 h and performing ELISAs for IL-12p40 and IL-10 on supernatants. FACS analysis for CD1a, CD14, CD163, CD80, CD86, and HLA-DR was performed to validate the purity of the macrophage types. T cell lines were generated by adding 10^6 PBMC to 2×10^5 APC (either mφ1 or mφ2) in 24-well plates and adding exogenous IL-2 (25 U/ml) and IL-15 (10 ng/ml) to all cultures on day 3 and splitting as necessary until day 13 in Iscove's medium supplemented with L-glutamine, penicillin/streptomycin, and 5% pooled human serum. $T_{mφ1/2}$ cell lines were rested in IL-2-free medium for 48 h before FACS and 30 h before coculture assays. $T_{mφ1/2}$ cultures were subjected to consecutive adhesion steps (24 h and 2 h) to remove any macrophages from the culture before the coculture assays. Coculture assays were performed by adding 5×10^3 or 10^4 rested $T_{mφ1/2}$ live cells (donor A anti-donor B) to an "indicator" proliferation assay (10^4 T cells (donor A anti-mature dendritic cell donor B) + 5×10^4 irradiated PBMC donor B). Proliferation was determined by CFSE dilution. Briefly, cells (indicator responder T cells (T_{Ind}) or $T_{mφ1/2}$) were labeled with 1 μM CFSE according to Sigma-Aldrich instructions on day 0, cocultured for 4 days as described above, stained for CD4, and analyzed on a FACSCalibur (BD Biosciences). Generation of supernatants for the supernatant transfer experiments was done by incubating $T_{mφ1/2}$ cell lines with plate-bound anti-CD3 and anti-CD28 (1 μg/ml each) for 24 h. Cell-free supernatants were subsequently added to the indicator described above.

Live cell sorting was performed at the dedicated LUMC flow cytometry facility on a FACSAria (BD Biosciences) cell sorter.

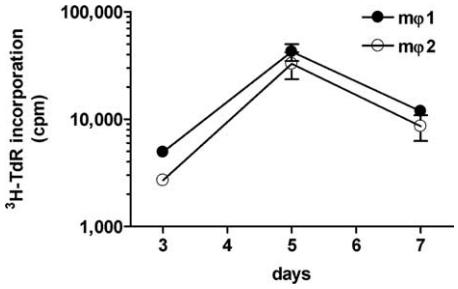

FIGURE 1. mφ1 and mφ2 induce similar allogeneic T cell proliferation. PBMC (1×10^5) were incubated with 2×10^4 non-activated allogeneic mφ1 or mφ2 for a period of 7 days. Proliferation was assessed by tritiated thymidine incorporation of triplicate data points. There are no significant differences between proliferation of T cells responding to allogeneic mφ1 and mφ2 cells (two-way ANOVA). Results are representative of three individual experiments.

Results

mφ1 and mφ2 induce proliferative allogeneic responses

To first determine whether mφ1 and mφ2 cells differed quantitatively in their capacity to present Ag, we tested and compared their ability to induce allogeneic T cell responses. Based on the assumption that allogeneic Ag density on mφ1 and mφ2 would be comparable and homogeneously expressed on all cells in each population tested in the absence of TLR stimulus as described previously (12), PBMC were cultured in the presence of HLA-mismatched mφ1 or mφ2 over a period of 7 days. Similar patterns of proliferation, both in intensity and kinetics, were induced by both subtypes of macrophages, which peaked at day 5 (Fig. 1).

mφ2 induce differentiation of allogeneic T cells with regulatory properties

Due to the phenotype of mφ2 (TLR-mediated secretion of IL-10, low expression of HLA-DR, CD80, and CD86, poor Ag presentation capacity in Th1 recall responses) we investigated whether T cells (donor A) having encountered mφ2 (donor B) could regulate the response of autologous T_{Ind} cells (donor A anti-donor B) to irradiated PBMCs (donor B). T_{Ind} cells were generated by incubating PBMC (donor A) to LPS-matured dendritic cells (donor B) for a period of 13 days. Fig. 2, *B* and *C*, shows that titration of T cells that have encountered $T_{mφ2}$ into the coculture assay (containing T_{Ind} cells + irradiated PBMC (donor B)) are capable of reducing T_{Ind} cell proliferation to less than half maximal value as determined by CFSE dilution, even at the low ratio of 1:1. In contrast, the addition of similar numbers of T cells that had encountered allogeneic $T_{mφ1}$ was incapable of reducing T_{Ind} cell proliferation (Fig. 2, *A* and *C*), showing that mφ2, but not mφ1, are capable of inducing T cells with a regulatory function. To determine the proliferative status of the $T_{mφ1/2}$ cells in the coculture assays, bulk $T_{mφ1/2}$ cultures were labeled with CFSE before addition to the coculture and assayed for dilution on day 4. Fig. 2*D* shows that in the absence of allogeneic PBMC (donor B) the $T_{mφ1/2}$ cells do not proliferate; however, in the presence of Ag, $T_{mφ1}$ proliferate more extensively than $T_{mφ2}$.

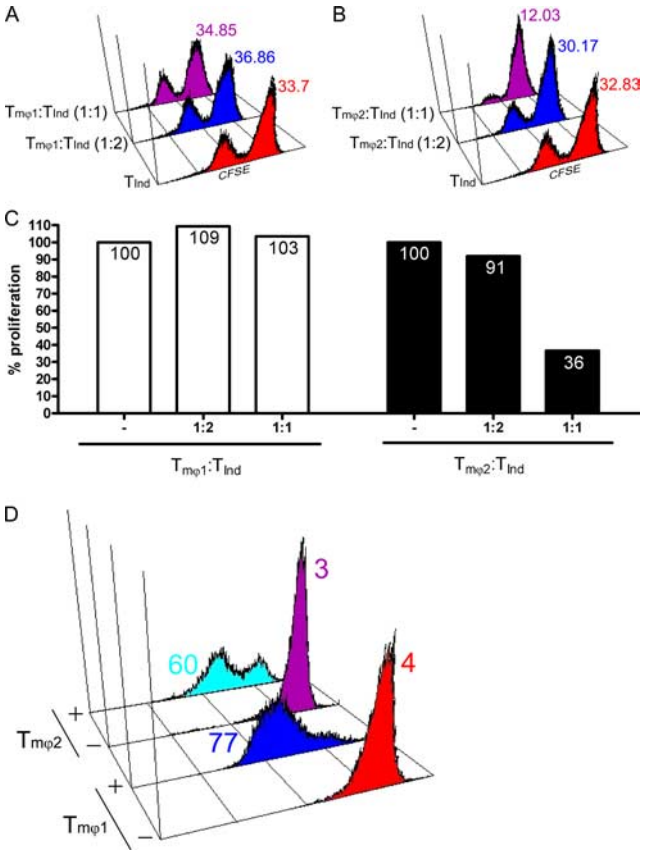

FIGURE 2. mφ2 induce differentiation of T cells ($T_{mφ2}$) with regulatory properties. CFSE dilution coculture assay showing proliferation of 10^4 T_{Ind} cells in the presence of increasing numbers (5×10^3 and 5×10^4; 1:2 and 1:1 ratios, respectively) of $T_{mφ1}$ (*A*) or $T_{mφ2}$ (*B*) cells per well (multiple wells pooled for FACS analysis). Addition of $T_{mφ1}$ cells did not reduce the proliferation of T_{Ind} cells, whereas addition of $T_{mφ2}$ did. Numbers above histograms indicate percentage of $CD4^+$ T_{Ind} cell population that divided. Histograms shown are representative of duplicate experiments. *C*, Graphical representation of data obtained in *A* and *B*, with T_{Ind} cell proliferation set to 100%. *D*, Histograms showing CFSE dilution of $T_{mφ1}$ and $T_{mφ2}$ cells in the coculture assay in the presence (+) or absence (−) of allogeneic PBMC (donor B). Numbers above histograms indicate percentage of $CD4^+$ $T_{mφ1/2}$ population that divided. All histograms were gated on $CD4^+$ live cells.

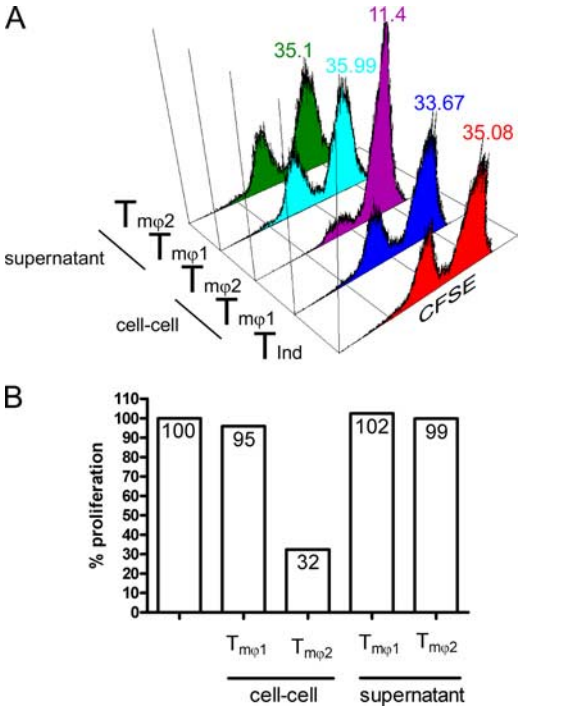

FIGURE 3. $T_{mφ2}$ cells regulate by cell-cell contact. *A*, CFSE dilution coculture assay showing proliferation of 10^4 T_{Ind} cells in the absence or presence of 10^4 $T_{mφ1}$ or $T_{mφ2}$ (1:1 ratio) per well (multiple wells pooled for FACS analysis). Addition of supernatants from the same $T_{mφ1}$ or $T_{mφ2}$ cells activated with anti-CD3/CD28 for 24 h shows no suppression of T_{Ind} cells. Numbers above histograms indicate percentage of $CD4^+$ T_{Ind} population that divided. All histograms were gated on $CD4^+$ live cells. *B*, Representation of data obtained in *A*, with T_{Ind} cell proliferation set to 100%.

$T_{mφ2}$ regulate by cell-cell contact

To discern the mechanism of inhibition, we stimulated $T_{mφ1}$ and $T_{mφ2}$ cell lines with plate-bound anti-CD3 and anti-CD28 for 24 h. Additionally, we purified $T_{mφ2}$ cells into $CD25^+$ and $CD25^-$ populations (as in Fig. 6*A*) and stimulated them with immobilized Abs. The supernatants of these cultures were analyzed for various cytokines by Multiplex and ELISA. Interestingly, in bulk cultures, all cytokines, with the exception of TNF-α, were secreted at high levels by $T_{mφ2}$ cells, including the immunosuppressive cytokines IL-10 and CCL4 (18) (Table I). Furthermore, in purified populations, we observed elevated levels of IL-6, IL-8, IL-13, and CCL4 in $CD25^+$ $T_{mφ2}$ cells (Table I). ELISAs for soluble TGFβ-1 on unmodified and acidified supernatants (acidification is necessary for TGFβ-1 detection) revealed no differences between the $T_{mφ1}$ and $T_{mφ2}$ cell subsets (data not shown). The bulk supernatants were transferred to CFSE-labeled T_{Ind} cells proliferating in response to exposure to irradiated stimulatory PBMC. Unexpectedly, elevated concentrations of cytokines including IL-10 or CCL4 were not inhibitory, while the cells producing them maintained a regulatory phenotype when given physical access to the proliferating T cells (Fig. 3). Furthermore, addition of neutralizing anti-IL-10 and/or anti-CCL4 and isotype Abs to the cell-cell contact experiments did not reverse the suppressive phenotype of the $T_{mφ2}$ cells (data not shown). The lack of suppression from $T_{mφ2}$ cell supernatants was observed in all experiments performed regardless of the T_{Ind} cell source. Testing several T cell lines and clones minimized the chances of encountering artifacts due to selective receptor expression by specific T_{Ind} cell lines.

Table I. *Cytokine concentrations (pg/ml) secreted by $T_{mφ1}$ and $T_{mφ2}$*[a]

	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-13	IFN-γ	TNF-α	CCL4	GM-CSF
$T_{mφ1}$ bulk ($n = 3$)	3,063	208	110	289	3,242	125	9,882	8,364	2,053	14,000	3,572
$T_{mφ2}$ bulk ($n = 8$)	5,023	445	681	446	4,048	431	21,628	14,597	1,759	80,000	4,737
$T_{mφ2}$ $CD25^-$ ($n = 5$)	1,608	36	15	10	52	9	968	1,349	304	2,450	299
$T_{mφ2}$ $CD25^+$ ($n = 5$)	409	67	193	503	3,864	49	10,010	1,875	712	8,383	1,567

[a] Cells were either left as bulk cultures or in the case of $T_{mφ2}$ further sorted based on CD25 expression and subsequently stimulated with anti-CD3/CD28 for 1 day. Supernatants were tested with Bio-Rad Multiplex with the exception of CCL4 (ELISA).

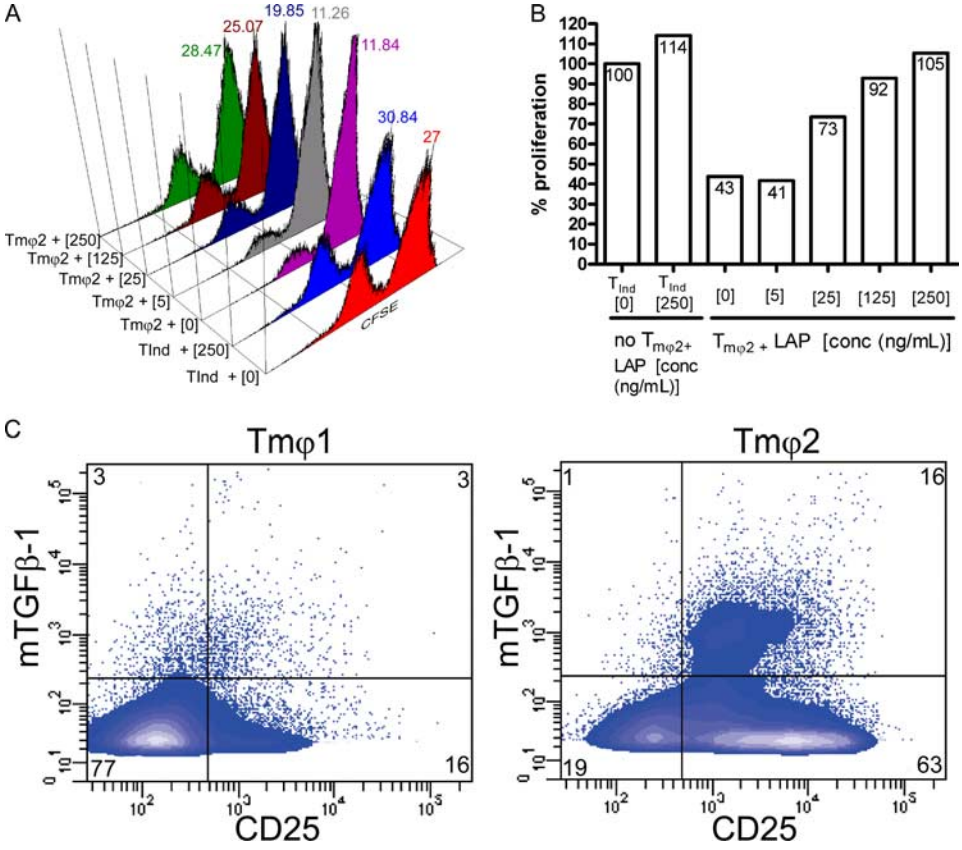

FIGURE 4. $CD4^+$ $T_{m\varphi2}$ cells express CD25 and membrane-bound TGFβ-1, the inhibition of which reverses their suppressive ability. *A*, CFSE dilution coculture assay showing proliferation of $CD4^+$ 10^4 T_{Ind} cells in the absence or presence of 10^4 $T_{m\varphi2}$ (1:1 ratio) per well (multiple wells pooled for FACS analysis). LAP peptide titration from 5 to 250 ng/ml to the coculture reverses $T_{m\varphi2}$ induced suppression, while addition of 250 ng/ml to T_{Ind} increased proliferation by only 14%. *B*, Representation of data obtained in *A*, with T_{Ind} cell proliferation set to 100%. *C*, FACS analysis of CD25 and mTGFβ-1 expression on live, $CD4^+$, $T_{m\varphi1}$, and $T_{m\varphi2}$ cells 13 days after encountering mφ1 or mφ2 cells, respectively, and a further 48 h with IL-2 and IL-15 retraction to establish a resting population.

$T_{m\varphi2}$ cells express membrane bound TGFβ-1, which mediates their suppressive ability

Since the regulatory attribute of the $T_{m\varphi2}$ cells was cell-cell contact dependent, we tested the expression of active TGFβ-1 (membrane TGFβ-1 (mTGFβ-1)) at the cell surface by flow cytometry. By using the TB21 Ab, we determined that a population of $CD4^+$ $T_{m\varphi2}$ cells expressed high levels of CD25 as well as active mTGFβ-1 long after initial stimulation (day 13). By contrast, $T_{m\varphi1}$ cells showed few cells expressing CD25 and little mTGFβ-1 (Fig. 4*C* and see Fig. 6*A*). To determine whether TGFβ-1 was directly involved in $T_{m\varphi2}$-mediated regulation, we titrated TGFβ-1 LAP into the cocultures. TGFβ-1 is typically expressed in a latent form due to its non-covalent association with LAP. Removal of LAP leads to presentation of bioactive TGFβ-1 (reviewed in Ref. 19). Titration of commercially available exogenous LAP dose dependently reversed the $T_{m\varphi2}$ cells' ability to suppress (Fig. 4, *A* and *B*). Staining $T_{m\varphi1}$ and $T_{m\varphi2}$ cells with anti-LAP Ab revealed similar high levels of inactive TGFβ-1 on both populations (data not shown). This observation, taken together with the lack of soluble factors accountable for the suppression (Fig. 3), supports the notion that active but not latent mTGFβ-1 is the mechanism of regulation used by $T_{m\varphi2}$ cells.

Next, we sorted the $T_{m\varphi2}$ cells into $CD25^{low}$ and $CD25^{high}$ populations by flow cytometry (as shown in Fig. 6*A*). Subsequently, we determined the suppressive ability of these cells. Only the $T_{m\varphi2}$ $CD25^+$ cells were the population capable of inhibiting the proliferation of T_{Ind} cells at a ratio of 1:1 (Fig. 5). To further test the involvement of TGFβ-1 in suppression, we opted to target the TGFβ-1 signaling pathway at various levels. Typically, the TGFβ-1 signaling cascade is initiated by the binding of TGFβ-1 to TGFβ-receptor complex, which comprises two subunits, RI and RII. Ligand binding leads to heterodimerization and clustering of receptor subunits. In turn, this results in TGFβRI-mediated phosphorylation of secondary messengers called SMADs, namely SMAD2 and SMAD3. These then translocate to the nucleus to regulate transcription directly or interact with other transcription factor subunits. Addition of LAP, which inactivates TGFβ-1 as described before, reversed the inhibition induced by $T_{m\varphi2}$ cells (Fig. 4, *A* and *B*). To further interfere with the ligand binding, we added neutralizing goat anti-human TGFβRII polyclonal Abs (ab10853) to our suppression assay. This inhibited the suppressive effects of $CD25^+$ $T_{m\varphi2}$ cells, while addition of isotype control polyclonal Abs had no effect. Anti-TGFβRII had no effect on proliferation of the controls where $CD25^-$ $T_{m\varphi2}$ cells were added to the assay (Fig. 5*A*). GITR, a cell surface TNFR family member, is temporarily up-regulated on effector T cells upon activation, and triggering GITR signaling has been shown to abrogate suppressive effects mediated by TGFβ-1 (20). Indeed, addition of cross-linking GITR mAb (110416) to the same assays also abrogated the suppressive effect, whereas the isotype control and $CD25^-$ $T_{m\varphi2}$ cells had no effect on T_{Ind} cell proliferation (Fig. 5*B*). Next, we targeted the TGFβ-1 signaling pathway farther downstream by interrupting the kinase activity of TGFβRI with SB431542-hydrate, known to inhibit phosphorylation of SMAD2 and SMAD3. Addition of SB431542-hydrate reversed the inhibition induced by $CD25^+$ $T_{m\varphi2}$ cells, while control proliferation remained unaffected (Fig. 5*C*). Taken together, these data show that mφ2 cells induce a $T_{m\varphi2}$ $CD4^+$ population that expresses high levels of CD25 as well as mTGFβ-1, which is able to suppress in a TGFβ-1-dependent manner since blocking the TGFβ-1 signaling pathway reversed suppression.

$T_{m\varphi2}$ cells are $CD25^+$, $Foxp3^+$ and $GITR^+$

More detailed FACS analysis on sorted populations (Fig. 6*A*) showed that $T_{m\varphi2}$ $CD4^+CD25^+$ cells maintained very high levels of GITR at the cell surface long after stimulation as compared to

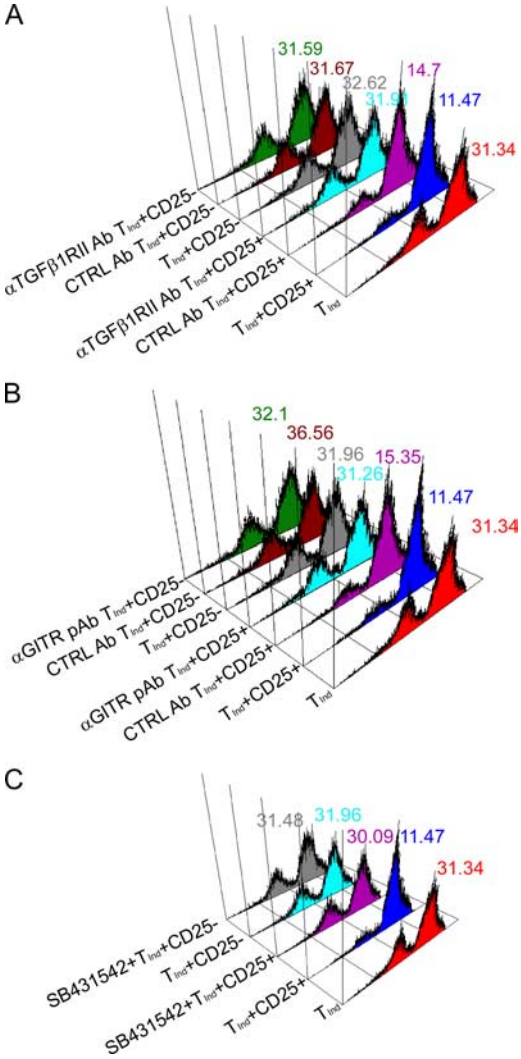

FIGURE 5. Inhibition of TGFβ-1 signaling and GITR activation suppresses $CD25^+$ $T_{m\varphi2}$ cell suppression. $T_{m\varphi2}$ cells were sorted based on CD4 and CD25 expression (as in Fig. 6) and added to a CFSE-labeled T_{Ind} cell coculture assay. Only $CD4^+CD25^+$ cells inhibit $CD4^+$ T_{Ind} cell proliferation. *A*, Addition of anti-TGFβ-1RII Ab but not isotype controls inhibited suppression observed by $CD4^+CD25^+$ $T_{m\varphi2}$ cells on $CD4^+$ T_{Ind} cells. *B*, Addition of anti-GITR polyclonal Ab but not isotype control Ab reversed the observed $CD4^+CD25^+$ $T_{m\varphi2}$-mediated suppression on $CD4^+$ T_{Ind} cells. *C*, Addition of ALK5 inhibitor (SB431542), thus inhibiting TGFβ-1RI enzyme activity, reversed the suppression on $CD4^+$ T_{Ind} cells induced by $CD4^+CD25^+$ $T_{m\varphi2}$.

the $CD25^-$ population (Fig. 6*B*). Expression of GITR has been associated with $CD4^+CD25^+$ Tregs (reviewed in Ref. 20). $T_{m\varphi2}$ cells also expressed high levels of FoxP3 (Fig. 6*C*). FoxP3 has long been identified as a transcription factor associated with Tregs (21, 22). In general, the phenotypic analysis of the $T_{m\varphi2}$ cells reveals expression of typical Treg markers that have previously been reported, with the exception of CTLA-4, which $T_{m\varphi2}$ cells did not express at all (Fig. 6*D*).

mφ2 cells induce $T_{m\varphi2}$ cells from $CD25^-$ populations

To eliminate the possibility that mφ2 cells were merely expanding pre-existing naturally occurring $CD4^+CD25^+$ Tregs present at the initiation of culture, we depleted the PBMC of $CD25^+$ cells (donor A) before addition to macrophages (donor B) and expansion using the established protocol. $T_{m\varphi2}$ cells established from a $CD25^-$ starter population retained the ability to suppress T_{Ind} cell proliferation whereas mφ1 cells have no suppressive effect on T_{Ind} populations (Fig. 7). These data therefore strongly support the ability of mφ2 cells to be able to induce Tregs from $CD4^+CD25^-$ cells

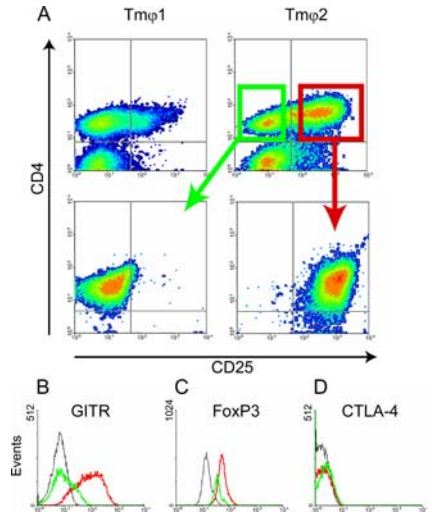

FIGURE 6. $T_{m\varphi2}$ cells are $CD4^+$, $CD25^+$, $GITR^+$, $FoxP3^+$, and $CTLA\text{-}4^-$. *A*, $T_{m\varphi2}$ cells were enriched to >95% by flow cytometry based on CD4 and CD25 expression for coculture assays (Fig. 5) and also subsequently stained with Treg-associated markers. *Upper left panel*, CD4 and CD25 expression on $T_{m\varphi1}$ bulk cultures after 13 days protocol followed by a 48-h resting period in the absence of IL-2 and IL-15. *Upper right panel*, Same staining for cells exposed to mφ2 cells following the same expansion protocol. *B*, GITR expression is maintained high on $CD25^+$ $T_{m\varphi2}$ cells. *C*, FoxP3 is expressed at higher levels in $CD25^+$ $T_{m\varphi2}$ cells. *D*, CTLA-4 was not detected in either $CD25^+$ or $CD25^-$ $T_{m\varphi2}$ cell populations by intracellular staining. Green histograms indicate $CD4^+CD25^-$; red histograms, $CD4^+CD25^+$; black histograms, isotype control. Plots are representative of three independent experiments.

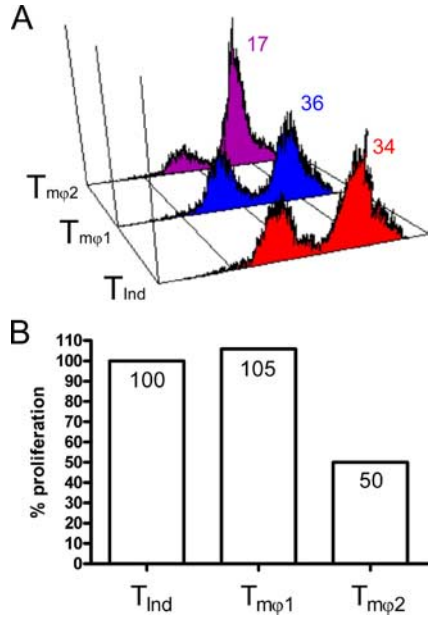

FIGURE 7. mφ2 cells induce $T_{m\varphi2}$ suppressive cells from CD25-depleted PBMC. *A*, PBMCs from donor A were depleted of $CD25^+$ cells by MACS-positive selection. Cells were subsequently put through the $T_{m\varphi1/2}$ differentiation protocol and tested in a T_{Ind} cell CFSE coculture assay. Histograms show CFSE dilution coculture assay showing proliferation of $CD4^+$ 10^4 T_{Ind} cells in the absence or presence of 10^4 $T_{m\varphi1/2}$ (1:1 ratio) per well (multiple wells pooled for FACS analysis). *B*, Representation of data obtained in *A*, with T_{Ind} cell proliferation set to 100%.

and may therefore have important implications in regulation of the immune system in the periphery.

Discussion

Macrophages are the principal sentinel phagocytes resident in tissues and are distributed throughout the body. For years, laboratories have described the ability of macrophages to phagocytose microbial pathogens and present Ag to T cells, subsequently initiating a Th response. More recently, the discovery of various human macrophages subsets, namely mφ1 and mφ2 (pro- and anti-inflammatory, respectively), has prompted us to investigate the impact of these macrophage subsets on T cell activation. Herein we have studied the type of T cell responses these various subsets can elicit. We have previously reported that mφ1 cells can support Th1 cell proliferation and function, whereas mφ2 failed to do so (12). The characteristic features of TLR-activated mφ2 (secretion of IL-10, down-regulation of HLA-DR and costimulatory molecules upon activation) prompted us to investigate whether mφ2 cells could support Treg differentiation. Using an allogeneic model of T cell activation, we observed that non-TLR-stimulated mφ1 and mφ2 cells could initiate equally good allogeneic T cell responses. These results differ from those obtained by others, who reported absence of proliferation to allogeneic macrophages (23). The precise nature and phenotype of the macrophages used in that study, however, were not described, making it difficult to compare with results obtained in our laboratory. In any case, the $T_{m\varphi1}$ and $T_{m\varphi2}$ cells we generated did not display anergic phenotypes and were capable of proliferating to similar extents in subsequent stimulations (to anti-CD3 and anti-CD28) in the absence of exogenous IL-2 (data not shown). In the absence of TLR stimulus, HLA-DR and T cell costimulatory molecules such as CD80 and CD86 are comparable between mφ1 and mφ2 cells (12).

Addition of $T_{m\varphi1/2}$ cells to autologous responder T cells showed that only $T_{m\varphi2}$, but not $T_{m\varphi1}$, cells have a strong suppressive functional phenotype. Analysis of cytokine production in the $T_{m\varphi}$ subsets indicated that despite anti-inflammatory cytokines being secreted by $T_{m\varphi2}$ cells, the suppression of proliferation was most likely due to a cell-cell contact mechanism rather than to secreted cytokines. Although the presence of immunoregulatory cytokines such as CCL4 (18) and IL-10 did not account for the regulatory ability of T cells in our system, they were nonetheless a good correlate. Interestingly, $T_{m\varphi2}$ cells also secreted elevated quantities of inflammatory cytokines IL-6, IL-8, IL-13, and IFN-γ, indicating an ability to recruit additional inflammatory cells to the site of activation while maintaining an ability to suppress T cell responses via cell-cell contact. While IL-13 secretion has previously been ascribed proinflammatory properties in acute graft-vs-host disease (24), it has also recently been associated with anti-inflammatory processes (25). Significantly, IL-13 can increase TGFβ-1 secretion in vivo (26).

Our data show that $T_{m\varphi2}$ cells express mTGFβ-1 at their cell surface and that inhibition of TGFβ-1 signaling could abrogate their suppressive abilities. The expression of mTGFβ-1 at the $T_{m\varphi2}$ cell surface, however, appeared to be extremely labile since simple manipulations such as cell sorting (via CD25) led to a complete loss of distinguishable mTGFβ-1 at the end of the procedure (data not shown), whereas a distinct small population was observed before the sort (Fig. 4*C*). Typically, we detected only 3–16% of the $T_{m\varphi2}$ $CD4^+CD25^+$ population to be mTGFβ-1^+ by FACS, and yet strong suppression was always observed at 1:1 ratios, which could be reversed by targeting the TGFβ-1 pathway, and thus we suspect that our observations may be a gross underestimation of the real mTGFβ-1 expression due to the labile nature of the protein. Further experimentation, in particular bioassays, will need to be designed to determine the levels of mTGFβ-1 on the surface of resting $T_{m\varphi2}$ cells with minimal interference from manipulations. The fickle properties of mTGFβ-1 detection therefore made it impossible for us to FACS sort sufficient cells based on active mTGFβ-1 expression. Staining $T_{m\varphi1}$ and $T_{m\varphi2}$ cells with anti-LAP (latent TGFβ-1) Ab revealed similar high levels of inactive TGFβ-1 on both populations (data not shown). We therefore conclude that the expression of active, but not latent, TGFβ-1 as depicted by TB21 staining (Fig. 4*C*) is the causative factor in suppressing bystander proliferation.

Interestingly, Oida et al. (33) reported that $CD4^+CD25^+$ cells are specifically capable of converting small amounts of latent TGFβ-1 into active form. Although expression of active mTGFβ-1 at the cell surface has been described before on T cells (1), there remain doubts as to the precise mechanism of signaling (27). In our case, however, we successfully reversed inhibition by three different TGFβ-1 targeting mechanisms, namely addition of exogenous LAP, neutralizing TGFβRII Ab, and inhibition of SMAD phosphorylation. Addition of anti-TGFβ-1 (TB21) Ab directly to suppression assays had little effect in our study, even at the high concentrations reported to be necessary for inhibition (50 μg/ml). Moreover, we constantly observed isotype effects at these Ab concentrations and therefore discounted these results. We suspect that the lack of TB21 blocking may be due to several factors, including the labile properties of surface expression of active TGFβ-1 in combination with requirement of tight apposition of membranes required for ligand/receptor signaling to occur, thus sterically inhibiting access of TB21 Ab to newly expressed TGFβ-1. TGFβRII expression may not fluctuate so readily and therefore may be more susceptible to Ab-mediated blocking. The possible mechanisms of reversal of inhibition by addition of GITR Ab remain unclear, as this could either directly play a role in TGFβ-1 signaling by inhibiting SMAD2/3 phosphorylation on T effector cells (reviewed in Ref. 28) or provide a secondary stimulus activating necessary MAPK in effector T cells necessary to overcome the suppressive effects of Tregs. Alternatively, GITR signaling could affect the Treg population directly as reported previously by Valzasina et al. (29 and reviewed in Ref. 20) since $T_{m\varphi2}$ expressed elevated levels of GITR. Interestingly, interference of GITR signaling can abrogate suppression in naturally occurring Tregs (30). Collectively, these data clearly show that a subset of $T_{m\varphi2}$ cells expresses mTGFβ-1; we unambiguously demonstrate that the mechanism of suppression to be TGFβ-1 dependent since interference of the TGFβ-1/TGFβR ligand interaction as well as inhibiting downstream phosphorylation events reversed the suppressive phenotype.

CD25, FoxP3, GITR, and CTLA-4 are all up-regulated on most T cells upon stimulation; therefore, our experiments we purposefully designed to assess expression of these markers long after stimulus (13 days) and after a 48-h retraction of exogenous IL-2/IL-15. This enabled us to distinguish activation-induced kinetic expression from sustained expression as reported on Tregs. $T_{m\varphi2}$ cells expressed much higher levels of typical Treg markers than do $T_{m\varphi1}$, including FoxP3 transcription factor, with the exception of CTLA-4. FoxP3 expression on $CD4^+CD25^-$ appeared higher than expected, and this may be due to the presence of TGFβ-1 (originating from $CD4^+CD25^+$ $T_{m\varphi2}$) cells, which has been shown to induce higher FoxP3 expression in $CD4^+CD25^-$ cells (31).

Vukmanovic-Steijc et al. (5) recently described the rapid proliferative state of human $CD4^+CD45RO^+Foxp3^+CD25^+$ T cells in vivo, and they determined that a principal source of Tregs may be the $CD4^+$ memory T cell pool. Stimulation of a memory $CD4^+$ T cell population in peripheral tissues, where macrophages are the primary APC, leads to the generation of Tregs (5). Depletion of

naturally occurring Tregs ($CD4^+CD25^+$ cells) from our starting culture material led to the expansion of functional $T_{m\varphi2}$ cells capable of suppressing T_{Ind} cell proliferation, thus negating the possibility of expanding pre-existing Tregs. It would be interesting to establish whether mφ2 play a role in the expansion of such cells in vivo. It is conceivable that given the large concentrations of M-CSF in human serum, the default differentiation path for circulating monocytes is to an anti-inflammatory mφ2 phenotype. Only in the presence of additional stimuli will the default pathway then be skewed toward the differentiation of inflammatory cells. The presence of mφ2 in humans has been described (14, 15), and Ags at mucosal surfaces are renowned for being capable of inducing a tolerogenic state despite constant antigenic challenge (32). Therefore, the presence of such macrophages may well be responsible for maintaining a Treg population in the periphery until an inflammatory stimulus occurs to shift the balance toward an inflammatory response.

In conclusion, we show that after encountering anti-inflammatory type 2 macrophages (mφ2), $CD4^+$ T cells adopt a $CD25^+FoxP3^+mTGF\beta\text{-}1^+$ functional suppressor phenotype. This, while widely accepted to be compatible with a thymic nTreg phenotype, has more recently been described as a population emerging from memory $CD4^+$ T cells after encountering Ag in the periphery. Additional experiments are required to determine the mechanism of type 2 macrophage induction/maintenance of nTreg and Treg turnover in humans, including the induction mechanism as well as the effect on $CD8^+$ T cells.

These studies shed new light on the complex mechanisms and function of Treg generation in humans, an intensively studied but yet unresolved area. These data have important implications in deciphering states of immunopathology and infection as well as tumor biology where the balance of proinflammatory and anti-inflammatory activities is thought to be critical.

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Disclosures

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