Immune Suppression via Glucocorticoid-Stimulated Monocytes: A Novel Mechanism To Cope with Inflammation

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*J Immunol* published online 2 July 2014
http://www.jimmunol.org/content/early/2014/07/02/jimmunol.1300891

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/07/02/jimmunol.1300891.DCSupplemental

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Glucocorticoids (GCs) are used as first-line therapies for generalized suppression of inflammation (e.g., allergies or autoimmune diseases), but their long-term use is limited by severe side effects. Our previous work revealed that GCs induce a stable anti-inflammatory phenotype in monocytes, the GC-stimulated monocytes (GCsMs) that we exploited for targeted GC-mediated therapeutic effects. We demonstrate that GCsMs interact with T cells in suppressing proliferation, as well as cytokine release of CD8+ and, especially, CD4+ T cells in vitro, and that they support generation of Foxp3+ cells. Therefore, we tested their immunosuppressive potential in CD4+ T cell–induced colitis in vivo. We found that injection of GCsMs into mice with severe colitis abolished the inflammation and resulted in significant clinical improvement within a few days.

T cells recovered from GCsM-treated mice exhibited reduced secretion of proinflammatory cytokines IFN-γ and IL-17. Furthermore, clusters of Foxp3+ CD4+ T cells were detectable at local sites of inflammation in the colon. Thus, GCsMs are able to modify T cell responses in vitro and in vivo, as well as to downregulate and clinically cure severe T cell–mediated colitis. The Journal of Immunology, 2014, 193: 000–000.
immune responses (22, 23). Therefore, we hypothesized that GCSMs also contribute to regulation of adaptive immune responses, especially those mediated by effector T cells.

In this study, we approached this hypothesis by genome-wide expression screening of murine GCSMs and ensuing functional clustering. The data indicated that GC treatment significantly affected the capacity of monocytes to interact with T cells. In agreement with this, we identified GCSMs as potent suppressors of both CD8 and CD4 effector T cell activation in vitro and in vivo. Most importantly, treatment of mice suffering from T cell-mediated transfer colitis with GCSMs (i.v.) resulted in a complete and fast cure of disease.

Materials and Methods

Mice
C57BL/6 and Rag-/- mice were kept under specific pathogen-free conditions, and experiments were performed according to approved protocols (#87-51.042010A113) of the animal welfare committee Landesamt fuer Natur Umwelt und Verbraucherschutz (Recklinghausen, Germany). Mice were used for experiments at the age of 10–12 wk. Specific pathogen–free conditions were according to the recommendation of the Federation of European Laboratory Science Association.

Abs and reagents
Anti-CD3e (145-2C11), anti-CD4 allophycocyanin (RM4-5), anti-CD8 allophycocyanin (53-67), anti-CD11b (M1/70), anti-CD28 (37.51), anti-CD80 (1G10/B7), anti-CD86 (GL1), anti-CD121b (4E2), anti-CD124 (mouse IL-4R-M1), and IFN-γ, IL-4, IL-13, and IL-17 Flex Sets were from BD Biosciences (Heidelberg, Germany). Anti-Foxp3-FTTC (FITC 16b) was from eBioscience (Frankfurt, Germany). Anti-Ly6C (HK1.4), anti-CTLA-4 PE (UC10-4B9), anti-CD274 (MH5), and anti-GTR (CD357, YGITR765) were from BioLegend. Cell culture medium generally was RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1x nonessential amino acids, 100 μg/ml Pen Streptom, and 10 μM HEPES. T cell isolation kits, anti-CD25, anti-CD19, and anti-CD90 magnetic beads were from Miltenyi Biotech (Bergisch- Gladbach, Germany). CFSE and DAPI were from Invitrogen (Karlsruhe, Germany). Medium and supplemental substances were from Biochrom (Berlin, Germany). Dexamethasone was from Sigma (Taufkirchen, Germany).

Generation of GCSMs and DCs from bone marrow
DCs from bone marrow cells were generated essentially as described earlier (24, 25). For monocyte enrichment, fresh bone marrow cells were applied to a density gradient (Ficoll) and subsequently deprived of CD11c, CD19, and CD90 cells using MACS technology. Monocytes were then cultured for 48 h with 10−7 M (40 ng/ml) dexamethasone in medium supplemented with M-CSF (50 ng/ml) using Teflon bags; they are referred to as GCSMs. Control monocytes (Ctr-Mos) were cultured identically with M-CSF (50 ng/ml) but with only the solvent (PBS; Ctrl-Mos) instead of GC. Finally, cells were washed three times and used as GCSMs (dexamethasone-treated monocytes) or Ctrl-Mos, respectively. In coculture with T cells, the ratio of T cells/monocytes was always 10:1.

Flow cytometry (FACS) analysis and cytokine measurement
Cells were surface stained with 1 μg/ml the indicated Ab for 30 min at 4˚C and washed twice with PBS/1% BSA. For intracellular staining, cells were fixed and permeabilized according to the manufacturer’s protocol (Cytofix/ Cytoperm; BD Biosciences) and subsequently incubated with 1 μg/ml of the respective Ab for 30 min at 4˚C. For intracellular cytokine measurement, cells were additionally stimulated with PMA/ionomycin and treated with GolgiStop for 6 h before fixation. After washing twice, cells were measured using a FACSCalibur or FACS Canto, and data were analyzed with FlowJo 8.7 software. Cytokines were determined from supernatants of cocultures using cytokometric bead array technology (BD Biosciences), which was performed according to the manufacturer’s instructions. Data were analyzed using FCAP Array (v1.0.1) software.

DNA microarray and statistical data analysis
In three independent experiments, total RNA from GCSMs and Ctrl-Mos was isolated and processed for microarray hybridization using Affymetrix Murine Genome (430-2.0), as reported earlier (8). Microarray data were analyzed by GCOS Software (Affymetrix), using data from corresponding control samples as baseline, and further evaluated statistically, as described previously (8). They were submitted to the Gene Expression Omnibus under accession number GSE54778.

We retained only genes that were significantly regulated in every experiment (change p value < 0.05; fold change >2, expression over background), as well as in the complete set of experiments (fold-change > 2.0, p value < 0.05, paired t test).

Functional clustering
To analyze microarray data in the context of biological functions, we used information available from the Gene Ontology (GO) consortium (http://www.geneontology.org) (8). The GO terms represent a defined vocabulary describing the biological process, cellular components, and molecular functions of genes in a hierarchical directed acyclic graph structure. Statistical analysis was performed using GO-Elite software (8).

Isolation of T cells from spleen and CFSE labeling for T cell proliferation
T cells were isolated from spleens, as described by Ahlmann et al. (26), and CD4 T cells were purified using MACS technology, according to the manufacturer’s instructions. Cells were labeled with CFSE (0.5 μM) and used in coculture assays with monocytes (T cells/monocytes = 10:1) or DC (T cells/DCs = 50:1). CFSE staining was routinely evaluated 2 h post-staining by FACS to ensure efficiency of labeling. T cell proliferation was assessed as CFSE dilution in FACS.

Repetitive stimulation of naive T cells
Naive T cells were isolated as described above and cocultured with either Ctrl-Mos or GCSM. A total of 1 × 10^6 CD4 T cells/ml or 1 × 10^5 monocytes/ml was used. After 5 d, cells were harvested and reseeded at the same ratio to fresh monocytes for three cycles. Subsequently, cells were stained intracellularly for Foxp3 expression and on the surface for CD4 (27). Cells were measured by FACS and analyzed using FlowJo 8.7 software.

Suppression assay
To induce their proliferation, T cells were stimulated with anti-CD3 and anti-CD28 Abs (5 μg/ml each) coated to 96-well round-bottom plates. A total of 1 × 10^5 splenic T cells was applied to each well. To test the suppressive activity of monocytes on stimulated T cells, 1 × 10^5 monocytes were added (triplicates for each condition) and incubated for 5 d. For controls, T cells were cultured either alone (positive control) or without stimulation by anti-CD3/anti-CD28 Abs (negative control). After 4–6 d in culture, 100 μl cell supernatants was stored until cytokine analysis was performed. Cells were harvested and stained for CD4 and CD8 and analyzed for CFSE dilution by FACS.

Transfer colitis
Syngeneic CD4+ T cells were prepared from spleen of C57BL/6 mice, and CD25+ cells were removed using MACS technology. A total of 1 × 10^4 CD4+ CD25− T cells/ml or 1 × 10^5 monocytes/ml was used. After 3 d of restimulation, T cell proliferation and cytokine production were measured. Repetitive stimulation of naive T cells
After 5 d of restimulation, T cells were cocultured with either Ctrl-Mos or GCSM. A total of 1 × 10^5 monocytes/ml was used. After 5 d, cells were harvested and reapplied at the same ratio to freshly prepared, and intraepithelial lymphocyte–containing supernatant was removed. Mice of the other group were used to prepared, and cells were labeled with CFSE for coculture with allogeneic DCs. After 5 d of restimulation, T cell proliferation and cytokine production were measured, as described previously (8).

Preparation of LPMC suspensions
LPCMs were isolated from colon of mice suffering from colitis by a standard method (31). Briefly, the colon was removed, opened longitudinally, cut into 5-mm pieces, and washed with cold Ca^2+/Mg^2+-free HBSS. The intestinal tissue specimens were transferred into HBSS with EDTA to remove intraepithelial lymphocytes. After 30 min of gentle shaking at 37˚C, the samples were vortexed, and intraepithelial lymphocyte-containing supernatant was removed. This step was repeated twice. LPCMs suspensions were prepared from the EDTA-treated de-epithelialized intestinal tissue by incubation with 100 U/ml...
collagenase and 5 U/ml DNase for 30 min at 37˚C. LPMCs were washed, re-
suspended in 44% Percoll solution (Amersham Pharmacia Biotech, Piscataway, NJ), underlain with 66% Percoll solution, and centrifuged for 30 min at 600 × g. The LPMC fraction was harvested from the interface.

FIGURE 1. Phenotype of GCsMs. Bone marrow monocytes were cultured for 48 h with dexamethasone (GCsM; 40 ng/ml) or with solvent (Ctr-Mo). Cells were surface stained for CD11b and CD124 (p < 0.05) or CD121b (p < 0.001) (A) or for CD11b and CD80, CD86, CD274, or GITR (CD357) (B). Cells were measured using FACS and analyzed by FlowJo 8.7 software. Cells were gated for CD11b+ cells and analyzed for the indicated surface molecules on CD11b+ cells. Graphs include three to five independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 2. GCsMs inhibit proliferation of CD8+ and CD4+ T cells. Splenic T cells from naive C57BL/6 mice were isolated and labeled with CFSE. A total of 1 × 10^5 bulk T cells was plated in a well of a 96-well round bottom plate that was coated with 5 µg/ml anti-CD3/anti-CD28 Abs. Where indicated, Ctr-Mos or GCsMs (1 × 10^5) were added to the T cells (at a T cell/monocyte ratio of 10:1). After 5 d of incubation at 37˚C, 5% CO2, cells were harvested and stained for either surface CD8 or CD4 and subsequently analyzed for proliferation by FACS. (A) One representative example of CD8+ proliferation by CFSE dilution. (B) Quantification of CD8+ T cell proliferation of eight independent experiments (p < 0.0001). (C) One representative example of CD4+ proliferation by CFSE dilution. (D) Quantification of CD4+ T cell proliferation of 11 independent experiments (p = 0.0001, p = 0.022). *p < 0.05, ***p < 0.001.
**Histology**

The colon was opened longitudinally, embedded as “Swiss rolls” (31) in O.C.T. compound (Tissue-Tek; Sakura Finetek, Zoeterwoude, the Netherlands), and kept frozen at −80°C until further use. Sections of 5 μm were stained with H&E. Histological analysis of the proximal, medial, and distal colon focused on epithelial hyperplasia, leukocyte infiltration and the amounts of goblet cells, ulcerations, and crypt abscesses. Mean histological damage was determined by having two experienced examiners score each colonic section in a blinded fashion using the colitis score described by Maloy (32).

**Statistical analysis**

Results are mean values ± SEM. The p values are given in the figures and/ or figure legends; *p > 0.05 was considered not significant. Statistical analysis was by the Student’s t test (two tailed and unpaired).

**Results**

**Genome-wide expression screening and phenotypic characterization**

We performed genome-wide expression screening of murine GC-stimulated monocytes to obtain more detailed information about their functional potential. Using microarray technology on murine monocytes stimulated for 16 h with GCs, we detected significant upregulation of 149 genes and downregulation of 110 genes compared with Ctr-Mos (Supplemental Tables I and II); raw data have been submitted to the Gene Expression Omnibus under access number GSE54778. Many GC-regulated genes, such as IL-10, CD163, or IL-1RII (CD121b), showed similar regulation as that for human GC-stimulated monocytes (8). Upregulation of CD121b also was confirmed at the protein level (Fig. 1A).

To gain functional insight into the pattern of regulated genes, we applied an automated unbiased functional clustering using GO-Elite software (33) and gene ontology annotations to determine which functional clusters among regulated genes were statistically overrepresented (Supplemental Table III). In addition to the GO nomenclature, we identified functional clusters among regulated genes by detailed research of published literature, analyzing the occurrence of subject headings related to the functional cluster in the National Center for Biotechnology Information’s gene-centric “Gene” database using an algorithm described previously (8). In agreement with described functional similarities between murine and human GC-treated monocytes (8, 21) we found an overrepresentation of identical or similar gene clusters among upregulated genes like “inflammatory response,” “signal transduction,” “antioxidative/ oxidative,” “scavenger receptor activity,” “antiapoptosis,” “anti-inflammatory,” and “tolerance induction” (Supplemental Table III).

Likewise, we found an overrepresentation of functional clusters like “immune response” and “IFN-gamma induced” and, strikingly, “T cell activation” and “Ag binding” among downregulated genes (Supplemental Table III). Downregulated genes that are important for T cell stimulation included genes like MHC-II and CD83. In contrast, and in agreement with our previous results (21), we confirmed upregulation of the cell surface receptor IL-4Rα (CD124) at the protein level (Fig. 1A). Expression of CD124 presents a phenotypic quality shared with a group of MDSCs. MDSCs originally were discovered in tumor-bearing mice or humans, and they are known to inhibit T cell responses (22, 34).

Therefore, we analyzed expression of molecules with functional relevance in the regulation of T cells. The decoy receptor for IL-1, CD121b (IL-1RII), was significantly upregulated on GCsMs (Fig. 1A). CD121b is expressed on myeloid cells but recently was demonstrated to be a unique cell surface marker of activated regulatory T cells (Tregs) (35). Other molecules with known functions for monocyte–T cell interactions, such as costimulatory molecules CD80, CD86, and CD274 (PD-L1), were not regulated by GC (Fig. 1B). GC-induced TNFR-related protein (GITR) is another molecule relevant for the function of immune cells (reviewed in Ref. 36). GITR is expressed on cells of the innate and adaptive immune system, but its functional role was mainly ex-

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**FIGURE 3.** GCsMs regulate cytokine production of T cells. Supernatants of anti-CD3/anti-CD28–induced T cell proliferation (black bars) and cocultures of T cells and Ctr-Mos (white bars) or GCsMs (gray bars) were harvested after 5 d of culture at 37°C, 5% CO2 and analyzed for cytokine content. (A) IFN-γ production of five independent experiments. (B) IL-17 production of five independent experiments. (C) IL-4 production of five independent experiments (*p < 0.05, **p < 0.001). (D) IL-13 production of five independent experiments (*p < 0.005, **p < 0.0005). Intracellular staining for IFN-γ (E), IL-17 (F), IL-4 (G), and IL-13 (H) in CD4+ and CD8+ T cells from cocultures in (A). Cells were stimulated with PMA/ionomycin (6 h) prior to intracellular staining. Percentage of either CD4+ or CD8+ T cells producing the respective cytokine is shown. Data are mean and SEM of four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
amined on T cells (reviewed in Refs. 36, 37). In this study, we show that GITR was downregulated on GCsMs after 48 h of culture (Fig. 1B).

Because of the phenotypic similarity between GCsMs and MDSCs, and because our functional clustering indicated that GCsMs markedly affect the capacity of monocytes to interact with T cells, we tested the potential of murine GCsMs to regulate T cells.

**T cell proliferation is suppressed by GCsMs in vitro**

We stimulated CFSE-labeled splenic T cells from naïve C57BL/6 mice with anti-CD3/anti-CD28 Abs and analyzed the influence of GCsMs on T cell proliferation. GCsMs significantly reduced the proliferation of CD8⁺ T cells (Fig. 2A, 2B) and CD4⁺ T cells (Fig. 2C, 2D). Strikingly, the proliferation of CD4⁺ T cells was diminished more by GCsMs (Fig. 2C, right panel) than was that of CD8⁺ T cells.

**Cytokine production is downregulated by GCsMs in vitro**

Next, we analyzed whether and how GCsMs influence cytokine production by activated T cells (Fig. 3). We measured the release of proinflammatory cytokines IFN-γ and IL-17, as well as that of Th2 cytokines IL-4 and IL-13, from supernatants of bulk T cells that were stimulated with anti-CD3/anti-CD28 and cocultured with the respective monocytes. Because IL-13 also can be released by monocytes/macrophages, we first measured LPS-stimulated Ctr-Mos and GCsMs. Neither unstimulated nor LPS-stimulated Ctr-Mos or GCsMs produced measurable IL-13 in our setting (data not shown). Addition of GCsMs to activated T cells resulted in a marked and significant reduction in all four cytokines (IFN-γ, IL-17, IL-4, IL-13) (Fig. 3, gray bars). Addition of Ctr-Mos also led to a reduction in IL-4 (Fig. 3C, white bar) and IL-13 (Fig. 3D, white bar), but the effect of GCsMs was significantly stronger. In contrast, release of IFN-γ (Fig. 3A, white bar) and IL-17 (Fig. 3B, white bar) was significantly reduced exclusively by GCsMs and not by Ctr-Mos. We additionally stained for intracellular cytokines, which revealed that the percentage of CD4⁺ or CD8⁺ T cells that produced IFN-γ, IL-17, IL-4, or IL-13 was not changed by GCsMs (Fig. 3E–H). This indicated that GCsMs are capable of suppressing T cell activation in terms of proliferation, which substantially leads to a reduction in total cytokine release.

To test whether the upregulated surface molecules CD124 and CD121b (Fig. 1) are responsible for regulation of T cell functions by GCsMs, we used blocking Abs to CD121b and CD124 on monocytes and subsequently tested whether this interference abolished the suppression of T cell proliferation by culture with GCsMs compared with Ctr-Mos. We did not observe differences in either T cell proliferation or cytokine production after blocking CD121b or CD124 in cocultures with GCsMs or with Ctr-Mos (data not shown).

Thus, CD121b and CD124, as well as their increased expression, do not appear to be functionally involved in the suppression of T cells by GCsMs in vitro.

**GCsMs induce Foxp³⁺ Tregs through repetitive stimulation of naïve T cells**

Other myeloid cells, such as CD40L- and IFN-γ-treated macrophages (38) and some MDSCs (39), were shown to regulate CD4⁺ T cell activation because of the induction of Tregs. Therefore, we

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**FIGURE 4.** Repetitive stimulation of CD4⁺ T cells by GCsMs generates Foxp³⁺ T cells in vitro. (A) Experimental setting for repetitive stimulation of T cells. (B) Purified splenic CD4⁺ T cells (1 × 10⁶/ml) were cocultured with the indicated monocytes at a 10:1 ratio for 5 d. Subsequently, bulk coculture cells were harvested and incubated in a second cycle with fresh monocytes at the same ratio for an additional 5 d. A third cycle was conducted in the same way. First- and third-cycle cells were collected, stained for CD4 on the surface, and stained intracellularly for Foxp3 expression. One representative example is shown for coculture of T cells and Ctr-Mos or GCsMs. (C) Percentage of Foxp³⁺ cells among CD4⁺ T cells after the third cycle of repetitive stimulation (six independent experiments). "p < 0.05.”

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analyzed whether CD4+ T cells expressed Foxp3 (Fig. 4B, upper panels) and CTLA-4 (CD152), two signature molecules of CD4+ Tregs, after 5 d of coculture with GCsMs. The percentages of Foxp3+CD4+ T cells (Fig. 4B) and CTLA-4+CD4+ T cells (data not shown) did not differ after coculture of T cells with GCsMs or Ctr-Mos. However, it was shown previously that repetitive stimulation of naive T cells with immature myeloid cells [originally reported for DCs (27)] is required to induce Tregs. We transferred this approach to our murine system and showed that repetitive stimulation of naive splenic T cells with GCsMs (Fig. 4A) indeed increased the proportion of Foxp3+ Tregs more strongly (from 7 to 41.5%) than did repetitive coculture with Ctr-Mos (from 5.9 to 20.8%) (Fig. 4B). The overall proportion of Foxp3+ CD4+ Tregs increased significantly when naive T cells were repetitively cocultured with GCsMs (Fig. 4B, 4C).

However, the generation of Foxp3+CD4+ Tregs was not mandatory for suppression of proliferation and cytokine release mentioned above, because these processes also took place without repetitive stimulation of naive splenic T cells when no Tregs were generated (Figs. 2, 4B).

**GCsMs cure CD4+ T cell–induced colitis in vivo**

Considering the marked effects, especially on CD4+ T cells in vitro, we wondered whether GCsMs similarly influence CD4+ T cells in vivo and, thus, mediate GC effects in situ. Therefore, we investigated GCsMs’ effects on CD4 cell–dependent inflammatory reactions in vivo. We chose CD4-dependent inflammatory bowel disease (32, 40–42) because, in this model, syngeneic CD4+CD25− T cells induce severe and fatal colitis when adoptively transferred into Rag2−/− mice (41). Colitis was monitored clinically by weight loss (Fig. 5). To test the influence of GCsMs on T cells already activated in vivo, we injected vehicle (PBS), Ctr-Mos, or GCsMs i.v. in a therapeutic setting [i.e., when mice had already developed severe colitis (Fig. 5A, 5B, days 19–22)]. This stage of disease is reflected by weight loss on consecutive days. For the ensuing experiments, we only used animals that fulfilled this criterion between days 19 and 22 (Fig. 5B). Mice that received GCsMs showed dramatic and significant clinical improvement over a period of 9–11 d postcell transfer (Fig. 5A, 5C). In contrast, mice that received Ctr-Mos continued to lose weight (Fig. 5B). Mice that received PBS showed moderate improvement. Histological analysis confirmed the clinical improvement (Fig. 5E). Images are representative of at least five mice for each group: healthy control (naive mouse, upper left image), colitis induced and injected with PBS (lower left image), colitis induced and treatment with Ctr-Mos (lower right image), and colitis induced and treatment with GCsMs (upper right image).

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**FIGURE 5.** Impact of GCsMs on T cell–mediated colitis in vivo. (A) Weight monitoring (% of initial weight) in model of T cell transfer colitis. On day 0, T cell transfer was performed i.v.; between days 19 and 22, monocyte transfer was done i.v. Mice were sacrificed between days 29 and 31. Colon, MLNs, and spleen were harvested for further analysis. Graph shows mean and SEM of n = 6 (PBS) and n = 10 (Ctr-Mos and GCsM) mice from three independent experiments. p < 0.05 (days 25–27), p < 0.001 (days 29–31). Ctr-Mos versus GCsM, Student t test. (B) Weight of individual Rag2−/− mice in different groups from day 0 to day 19/22 before monocyte transfer. (C) Weight of individual Rag2−/− mice in different groups from day 0 to day 19/22 before monocyte transfer. (D) Weight of individual Rag2−/− mice in different groups from day 0 to day 19/22 before monocyte transfer. (E) Mean histology score for animals in (A) was determined as described in Materials and Methods (p < 0.0001). *p < 0.05, **p < 0.01, ***p < 0.001.
weight (Fig. 5A, 5C), as did mice that received vehicle (PBS) only (Fig. 5A, 5C). In those experiments in which mice with colitis were monitored for longer periods after treatment with GCsMs, three of four animals survived, whereas three of four untreated mice died by day 60 (data not shown).

Histological analysis of colons at days 29–31 revealed dense and large inflammatory infiltrates in untreated mice with colitis that received no GCsMs (Fig. 5D, upper right image) or Ctr-Mos (Fig. 5D, lower left image) compared with naïve mice (Fig. 5D, upper left image). After injection of GCsMs, these infiltrates resolved almost completely (Fig. 5D, lower right image), reflecting the marked clinical improvement. To quantify this observation, we generated histological scores from colon sections of these mice. The scores depicted in Fig. 5E clearly emphasize the highly significant effect of GCsMs on the improvement of inflammation compared with treatment with Ctr-Mos or vehicle (PBS).

Taken together, treatment of established, severe CD4+ T cell–induced colitis by injection of GCsMs resulted in complete resolution, whereas Ctr-Mos and vehicle had no effect. Thus, GCsMs exert regulatory effects on activated CD4+ T cells, with marked effects on inflammation in vivo.

Mechanisms of GCsMs on CD4+ T cells in vivo: suppression of IL-17 and IFN-γ

To investigate whether inhibition of colitis by GCsMs was due to alteration or downregulation of activated T cells, we examined LPMCs, MLN cells, and splenocytes from mice with colitis. Because naïve Rag-/- mice do not harbor intrinsic T cells, all T cells derive from initially transferred cells. The yield of CD4+ T cells in lamina propria (LPMCs), MLNs, and spleen was very low, and we used cell suspensions of these for restimulation with anti-CD3/anti-CD28 Abs. Prior to stimulation, we ascertained the presence of cultured LPMCs, MLN cells, and splenic T cells were analyzed by FACS (data not shown). After 3 d of stimulation, supernatants of cultured LPMCs, MLN cells, and splenocytes were analyzed for cytokine production. Unstimulated cultures revealed no detectable cytokine production (data not shown). IFN-γ production from LPMCs, MLN cells (Fig. 6), and splenic T cells (Supplemental Fig. 1C) was significantly reduced in GCsM-treated animals. Also, IL-17 production was strongly downregulated in MLN cells (as well as in T cells from spleen; Supplemental Fig. 1) but not in cells from lamina propria (LPMCs). Other than in the in vitro system, production of IL-4 and IL-13 was not influenced in single-cell suspensions from LPMCs, MLN cells and splenic T cells after treatment of mice with injection of GCsM (Fig. 6, Supplemental Fig. 1). In addition, IL-10 was determined from restimulated LPMCs and MLN cells, but it was below the detection limit of the assay system in all cases (data not shown).

Thus, therapeutic transfer of GCsMs resulted in alteration of the cytokine pattern released by T cells from mucosal lamina propria, MLN cells, and spleen, exhibiting suppression of IFN-γ by all populations and suppression of IL-17 by T cells from MLNs and spleen.

Mechanisms of GCsMs on CD4+ T cells in vivo: inducing differentiation of Foxp3+CD4+ Tregs

To explore whether downregulation of colitis by GCsMs in vivo would also encompass differentiation of T cells into Tregs (as seen in vitro after repetitive interactions), we analyzed Foxp3 expression ex vivo and in situ. First, we analyzed Foxp3 expression in CD4+ T cells from lamina propria (LPMCs) and MLNs, all obtained from mice suffering from colitis. Although the T cells transferred into mice on day 0 were negative for Foxp3 (data not shown), a certain percentage of Foxp3+ cells emerged physiologically in all treatment groups (Fig. 7A), and a small amount already was detectable on the day of treatment (days 19–22, data not shown) when colitis had fully developed. However, as shown in Fig. 7A, both the numbers and percentages of Foxp3+ CD4+ T cells in LPMCs and MLNs were not different between the groups tested. Histological analysis of Foxp3+ cells in situ in the colon, the organ primarily affected in inflammatory bowel disease, revealed that, although few Foxp3+ cells (Fig. 7B) were detected in LPMCs by FACS, they were not found in cryosections of naive mice (left panel). In colon sections of mice that developed colitis and were treated with Ctr-Mos, few single Foxp3+ cells were detectable (middle panel). However, colon sections of mice that received GCsMs (right panel) contained clusters of Foxp3+ cells, which

![FIGURE 6. CD4+ T cells are regulated by GCsMs in vivo. MLN and LPMC single-cell suspensions from mice used in colitis experiments (Fig. 5) were prepared as described in Materials and Methods. LPMC and MLN single cells (1 × 10^5/well of a 96-well plate) were restimulated with precoated anti-CD3/anti-CD28 Abs (5 μg/ml each) for 3 d, and supernatants were harvested. Cytokines of supernatants were measured by FACS using cytometric bead array technology. Data are mean ± SEM of 4–10 individual mice from three independent experiments. *p < 0.05, **p < 0.001.](http://www.jimmunol.org/Downloadedfrom http://www.jimmunol.org)}
apparently developed locally at the site of inflammation. To quantify Foxp3 expression, clusters (defined as more than five Foxp3+ cells) were analyzed from eight sections for each group (Fig. 7C). Colon sections of GCsM-treated animals displayed significantly more Foxp3+ clusters compared with colons of mice treated with Ctr-Mos. To identify the type of cell that presented with Foxp3 expression, we performed double staining of tissue sections for fluorescence microscopy (Fig. 7D).

Taken together, these data indicate that CD4+Foxp3+ Tregs accumulated locally but did not expand in draining lymph nodes (MLNs) and LPMCs in animals treated with GCsMs.

Discussion

In this study, we demonstrate that GCsMs regulate activated CD8+ and, especially, CD4+ T cells by reducing their proliferation and, consequently, their total cytokine release in vitro. Also, repetitive encounter between CD4+ T cells and GCsMs induces Foxp3+ Tregs in vitro.

In vivo injection of GCsMs into mice suffering from CD4+ T cell–induced colitis resulted in clinical healing. This remarkable downregulation was associated with altered release of IFN-γ and IL-17 in vivo. Thus, GCsMs are capable of downregulating an already established severe immune response in vivo.

Because GCsMs share the expression of CD11b, Gr-1, and CD124 (21) with monocytic MDSCs, which are known suppressors of T cell activation (30, 37), and because genes involved in T cell interaction were overrepresented among GC-regulated genes, we wondered whether GCsMs would suppress T cell activation. We demonstrated that GCsMs suppress activation of CD8+ T cells in vitro, as was described for MDSCs (39, 43–45). Interestingly, GCsMs were even more effective suppressors of CD4+ T cell responses. GCsMs inhibited proliferation of polyclonally activated CD4+ T cells and substantially reduced their cytokine production in vitro.

It was proposed that GCs downregulate Th1 responses and induce a Th2 shift (by a combination of direct effects on T cells and indirect effects on APCs) (42), but it only has been shown that GCs reduce the release of IL-12 after classical activation of monocytes or macrophages (46, 47) and favor Th2 responses in this setting (42, 47). In our study, regulation of cytokine production of T cells by GCsMs did not result in a Th2 shift in vitro or in vivo. In vitro, GCsMs inhibited activation of naïve T cells and suppressed all cytokines examined. However, in vivo, the inflammatory cytokines IFN-γ and IL-17 released by activated T cells appeared to be regulated selectively by GCsMs.
Hence, we now describe a direct effect of GC on naive monocytes that results in a subtype that actively modifies T cell responses.

To explore the mechanisms of T cell regulation by GCsMs, we used neutralizing Abs against the immunosuppressive cytokine IL-10 and the surface molecules CD80, CD121b, and CD124 in cocultures of GCsMs and T cells. There are conflicting data about the mechanisms of MDSC-mediated regulation. The IL-4Rα-chain (CD124) was proposed to be involved in the suppressive activity of MDSCs toward CD8+ T cells (44), but those results were called into question by a study that demonstrated that CD124 is not involved in the regulatory function of MDSCs (48). Furthermore, CD80 was described to be used by MDSCs to inhibit IFN-γ production and proliferation of T cells (49). In addition, CD121b was reported to be a possible marker for Tregs that could be involved in their suppressive capacity on effector T cells by sequestering IL-1 from the environment (35). We did not observe any influence of IL-10, CD80, CD121b, or CD124 on the GCsM-mediated regulation of T cell activation in vitro (data not shown).

For human DCs, it was shown that GC treatment, together with either vitamin D (50) or CD40 ligation (51), generates tolerogenic DCs that require high IL-10 or CD274 (PD-L1) expression to induce Tregs. We were unable to detect increased IL-10 protein production (data not shown) or CD274 on GCsMs. Nevertheless, we explored the possibility that inhibition of activated CD4+ T cells was also indirect and mediated by the induction of Foxp3+ Tregs. When we analyzed Foxp3 expression in T cells after 5 d of coculture with GCsMs, we did not observe an increase in the Foxp3+ Treg population, although suppressive activity was fully developed (Figs. 2, 3).

However, GCsMs are able to differentiate Foxp3+ Tregs from naive T cells in vitro when stimulated repetitively (Fig. 4), as was described for immature APCs (27).

Expression of GITR protein is implicated in costimulation of cells of the innate and adaptive immunity (52). In the setting of transfer colitis, GITR expression on APCs was critical for the development of Treg function (52). Therefore, we analyzed the expression of GITR on GCsMs; however, GITR was significantly downregulated on GCsMs, indicating that it is not involved in GCsM-mediated mechanisms on T cells.

Taken together, these facts point to an as yet unidentified mechanism that is used by GCsMs to suppress T cell activation in vitro. It exerts its effects faster than Foxp3+ Tregs and prior to their appearance. It is noteworthy that GCsMs confer their regulatory activity at a 10:1 ratio (T cell/monesocyte) and, thus, are far more effective than Tregs, which need ratios of 2:1 (T cell/Treg) to be inhibitory in vitro.

The marked capability of these GCsMs to suppress CD4+ T cells responses in vitro prompted us to investigate whether they are able to suppress established T cell responses in vivo. Transfer of GCsMs led to rapid amelioration, and even healing, of T cell–induced colitis. The clinical effect correlated with an inhibited secretion of the inflammatory cytokines IFN-γ and IL-17 by CD4+ T cells from corresponding LPMCs (for IFN-γ), MLNs, and spleens ex vivo. IL-10 does not seem to play a role systemically because it was not detectable upon restimulation of LPMCs and MLN cells (data not shown). Because Rag2−/− mice do not have intrinsic T cells (28), T cells that were regulated by GCsMs were the colitogenic CD4+CD25+ T cells that were transferred i.v. on day 0.

Because we observed that GCsMs have the potential to generate Tregs in vitro (although they were not required for the short-term suppressive effects) and because Tregs can arise locally at the site of the immune response (53), we investigated whether Tregs would be generated in colonic mucosa. Examination of CD4+Foxp3+ T cells from lamina propria (LPMCs) and MLNs clearly showed that Tregs generate intrinsically (see PBS group) as a natural mechanism to counter-regulate inflammation. None of the monocytic subtypes transferred into colitis mice changed the amount of Tregs in the LPMCs and MLNs. However, the presence of CD4+ Foxp3+ T cell clusters in colonic epithelium after treatment with GCsMs provides evidence for local accumulation of Tregs and, possibly, for local activation at the site of inflammation. Hence, we speculate that GCsMs may be able to induce local accumulation of Tregs in vivo at sites of inflammation while directly suppressing activated T cells. Whether this generation of Tregs at sites of inflammation might also contribute to the initial control of established colitis or whether it would induce a longer-lasting state of tolerance has to be explored. Because the in vitro effect of GCsMs to induce Tregs takes some time, they might not be involved in the initial control of inflammation, but they may be important for maintaining control.

Taken together, we suggest that GCsMs use at least two distinct mechanisms to control and regulate CD4+ T cells. One is a fast and very efficient (due to a T cell/GC ratio of 10:1) direct inhibition of activated T cells without the participation of Tregs (as shown directly in vitro). The other mechanism is their capacity to induce Tregs in vitro and locally at sites of inflammation in vivo. Thus, it is tempting to speculate that GCsMs have the unique dual ability to actively inhibit acute inflammation mediated by CD4+ T cells (proliferation and cytokine pattern) and simultaneously induce a longer-lasting state of tolerance via Tregs.

Moreover, GCsMs could be a promising novel target of therapeutic strategies to exploit the efficacy of GCs without their adverse effects and, in this context, to support distinctly active and long-lasting suppression of T cell–mediated inflammation through the innate immune system.

Acknowledgments

We thank Andrea Stadtbäumer, Eva Nattkemper, and Tarek Chehab for excellent technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References


