Published in final edited form as:
Gastroenterology. 2011 June ; 140(7): 2019–2030. doi:10.1053/j.gastro.2011.02.059.

Human Colonic Myofibroblasts Promote Expansion of CD4+ CD25^{high} Foxp3+ Regulatory T Cells

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No conflicts of interest exist

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Abstract

Background & Aims—Regulatory T (Treg) cells (CD4+ $CD25^{high}$ FoxP3+) regulate mucosal tolerance; their adoptive transfer prevents or reduces symptoms of colitis in mouse models of inflammatory bowel disease (IBD). T-cell functions are regulated by mesenchymal cells. Colonic $CD90^+$ mesenchymal myofibroblasts and fibroblasts (CMFs) are abundant, non-professional antigen presenting cells in the normal human colonic mucosa that suppress proliferation of activated $CD4^+$ effector T cells. We studied CMF suppressive capacity and evaluated the ability of CMF to induce Treg cells.

Methods—Allogeneic co-cultures of $CD4^+$ T cells and CMFs, derived from normal mucosa of patients undergoing colectomy for colon cancer or inflamed colonic tissues from patients with ulcerative colitis or Crohn's disease, were used to assess activation of the Treg cells.

Results—Co-culture of normal CMF with resting or naive $CD4^+$ T cells led to development of cells with a Treg phenotype; it also induced proliferation of a $CD25^+$ $CD127^-$ $FoxP3^+$ T cells, which expressed CTLA-4, interleukin (IL)-10, and transforming growth factor-β and had suppressive activities. In contrast to dendritic cells, normal CMFs required exogenous IL-2 to induce proliferation of naturally occurring Treg cells. Induction of Treg cells in normal CMFs required MHC class II and prostaglandin E2. CMFs from patients with IBDs had reduced capacity to induce active Treg cells and increased capacity to transiently generate $CD4^+CD25^{+/-}$ $CD127^+$ T cells that express low levels of FoxP3.

Conclusions—CMFs suppress the immune response in normal colon tissue and might therefore help maintain colonic mucosal tolerance. Alterations in CMF induction of Treg cells might promote pathogenesis of IBDs.

Keywords

inflammation; TGF; immune regulation; immune response

Introduction

Gut immune responses are normally regulated to maintain a state of mucosal tolerance, which represents a balance between the need to mount protective immunity toward pathogens while not activating damaging inflammatory responses to innocuous luminal antigens (Ag)[1]. Recent studies suggested that a functionally distinct subset of regulatory T cells, $CD4^+$ $CD25^{high}$ $FoxP3^+$ cells (T_{reg}), is actively involved in the maintenance of immunological tolerance in the gastro-intestinal (GI) tract[2–3].

There are at least two types of the T_{regs}: naturally occurring ($_nTreg$) and inducible (iT_{reg}). nT_{reg} cells develop in the thymus and the majority expresses high levels of cell surface markers associated with an activated/memory T cell phenotype[2–4]. iT_{reg} cells are elicited in the periphery from naïve T cells under low-dose antigenic stimulation or by several cytokines, although the exact mechanism(s) of iT_{reg} cell induction remains unknown[4]. Forkhead transcription factor 3, Foxp3, is a master switch that controls T_{reg} cell development and function. Both types of T_{regs} down regulate immune responses to foreign and self-Ag, and contribute to the suppression of autoimmune disorders[3–5]. T_{reg} exert their effects by multiple mechanisms (e.g., cytokine deprivation, CTLA-4 signaling, IL-10 or TGB-β-production)[4–5]. It is not established which of these mechanisms is predominant, but each might have a greater importance under specific physiological/pathological conditions.

Recent studies suggest that T_{reg} cells play a major role in murine models of inflammatory bowel disease (IBD). Adoptive transfer of $CD4^+CD45RB^{high}$ T cells depleted of the T_{reg} cells into immunodeficient mice resulted in development of IBD-like chronic colitis[7–8]. In contrast, transfer of Treg cells into animals with chronic colitis led to the amelioration of the disease[7–8]. Adecrease in T_{reg} number in active IBD in humans has been documented[9]. Despite advances in understanding the role of the T_{reg} cells in the maintenance of peripheral tolerance in the intestinal mucosa, the mechanisms involved in their regulation in the intestine and what factors contribute to the alteration of the T_{reg} numbers in IBD remains unclear. It has been recently reported that small intestinal GALT associated $CD103^+$ dendritic cells (DCs), as well as small bowel epithelial cells can induce T_{regs}[10–11]. However, the origin and possibility of the expansion of the T_{regs} in the colon remain obscure.

We recently reported that colonic myofibroblasts/fibroblasts (CMFs) are novel non professional APCs and are abundant in the normal human colonic mucosa[12]. CMFs are a distinct population of mesenchymal stromal cells that are positive for CD90. Activated CMFs express α-smooth muscle actin (α-SMA, myofibroblast marker), but are negative for other cell surface markers that define conventional APCs. CMFs form a network throughout the colonic lamina propria and have been implicated in the regulation of mucosal inflammation[12–16]. Recent data from our laboratory indicates that CMFs may have a dual regulatory role on $CD4^+$ T cell activity. They induce proliferation of resting $CD4^+$ T cells in a MHC class II-dependent manner[12], but suppressing proliferation of activated $CD4^+$ effector T cells via mechanisms involving the B7-related co-inhibitors PD-L1 and PD-L2[15]. Importantly, it has been recently reported that intestinal myofibroblasts taken from chronically inflamed tissues display a fundamentally altered phenotype compared to their counterparts extracted from normal tissues at the same anatomical site[13,16]. CMFs are actively involved in the progression of the IBD associated inflammation via altered deposition of matrix, upregulation of proinflammatory cytokine production, and changes in the array of secreted, soluble immunoregulatory molecules and mediators[13,16].

In this study, we have further analyzed interactions between CMFs and $CD4^+$ T cells to determine if CMFs stimulate expansion of the T_{reg} cells. We found that CMFs contribute to the maintenance of $FoxP3^+$ phenotype of the nT_{reg} and induce generation of iT_{reg} cells from naïve $CD4^+$ T cells via mechanisms that involve MHC class II and PGE_2 signals. Moreover, we demonstrated that IBD-derived CMFs, when compared to normal CMFs, have a decreased ability to induce cells bearing the T_{reg} phenotype. Thus, our data suggests that CMFs might play a prominent role in mucosal tolerance via regulation of the Treg number, and disruption of CMF mediated regulation of the T_{reg} may contribute to IBD progression.

Materials & Methods

Antibodies and Reagents

Please see Supplemental information online at www.gastrojournal.org

Human colonic tissue and primary CMF culture

For CMF isolation, fresh human colonic mucosal sections were obtained from discarded surgical tissue in compliance with protocols approved by the UTMB Institutional Review Board. Areas of uninvolved colonic tissue from patients undergoing colectomy for colon cancer were used as the source for normal CMFs (N-CMFs). CMFs were also isolated from inflamed colonic tissues from patients with ulcerative colitis (UC) or Crohn's disease (CD) undergoing colonic resections for IBD (IBD-CMFs, CD-CMFs or UC-CMFs). Primary cultures of CMFs were generated according to the method described by Mahida *et al.*[17] and routinely used in our laboratory as previously described[12,15–16].

Generation of human DCs from peripheral blood mononuclear cells

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CMF: T cell allogeneic cocultures

Unless otherwise indicated, peripheral blood mononuclear cells (PBMC) were prepared from the blood of healthy donors by density gradient centrifugation over Ficoll-Paque™ Plus according to the manufacturer's instructions. Naïve, resting and regulatory human $CD4^+$ T cells were purified from these PBMC using commercially available kits that negatively select $CD4^+$ $CD45RA^+$ T cell, human $CD4^+$ resting T cell and positively select $CD4^+$ $CD25^+$ regulatory T cells, respectively (Miltenyi Biotec., Auburn, CA). The purity of isolated T cells (>98%) was confirmed by flow cytometry. CMF primary cultures were stimulated with IFN-γ (100 U/ml) for 7 days prior to study in order to induce optimal MHC class II expression as previously described[12]. Cells were then rested for at least 24 h prior to use in the experiments. CMFs or freshly generated DC (obtained as described above) were cocultured with allogeneic T cells at a ratio 10:1, respectively, and incubated for 5–7 days at 37°C with 5% CO_2. In some CFSE proliferation experiments, human recombinant IL-2 (eBioscience) was added to the CMFs: T co-cultures at a concentration of 10 ng/mL.

Flow cytometry

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IL-10, TGF-β and PGE_2 ELISA

Conditioned media were collected from the CMF: T cell, CMF or T cell culture wells at 24h, 5 and 7 days and analyzed for the production of the IL-10 and TGF-β using ELISA kits (BD Bioscience) and PGE_2 using a R&D Systems kit, according to the manufacturer's instructions.

Real Time RT-PCR

The real time RT-PCR was carried out as described previously[15]. Please see Supplemental information online at www.gastrojournal.org.

CFSE proliferation assays

Please see Supplemental information online at www.gastrojournal.org

T Cell Suppression Assays

The T cell suppression experiments were performed as described previously[24]. For details, please see Supplemental information online at www.gastrojournal.org.

Statistical analysis

Unless otherwise indicated, the results were expressed as the mean ± SE of data obtained from at least three independent experiments done with triplicate sets in each experiment. Differences between means were evaluated by ANOVA using Student's *t*-test for multiple comparisons. Values of $P < 0.05$ were considered statistically significant.

Results

CMFs stabilize FoxP3 expression in nT_{reg} and induce their proliferation in presence of IL-2

We showed previously that CMFs induce proliferation of resting $CD4^+$ T cells isolated from peripheral blood [12], which is also known to contain $CD4^+$ $CD25^{high}$ $FoxP3^+$ nT_{reg} cells

(nT_{reg}). Thus, we investigated the interaction of the T_{reg} and CMFs isolated from normal colonic mucosa (N-CMFs).

Previously, we reported that, in culture, MHC class II expression by CMF drastically decreases when compare to that on acutely isolated cells and the high levels demonstrated in situ[12]. Thus, in all experiments primary CMFs were stimulated with IFN-γ (100 U/ml) prior to use in order to restore optimal MHC class II expression as described in the Methods. Theability of N-CMFS to induce generation of T_{reg} in seven day allogeneic co-cultures of the CMFs with CFSE-labeled resting $CD4^+$ T cells were studied. A significant increase in the percentage of the $CD25^{high}FoxP3^+$ T cells in the dividing fraction of $CD4^+$ T cells co-cultured with N-CMFs was observed (Gate P3, Figure 1A) and represented ~31.4 ± 5.8 % of the dividing T cells (Figure S1, see supplement online at www.gastrojournal.org). This coincides with increased expression of the suppressive cytokines IL-10 and TGF-β1 by T cells co-cultured with N-CMFs (Figure 1B). The majority of proliferating $CD4^+CD25^{high}$ T cells derived from CMFs-T cell co-cultures that were positive for FoxP3 did not express CD127, the IL-7 α chain receptor, and, thus, correspond to the true T_{reg} phenotype (Figure 1B). A moderate increase in of the $FoxP3^+CD127^+$ T cell fraction corresponding to the FoxP3 transiently expressing $CD4^+$ effector T cells was also noted in the CMF-T cell co-cultures (Figure 1B). In contrast to T_{reg} cells the expression of FoxP3 by T effector cells reported to be low, and was not sufficient to suppress expression of CD127 maker and increase the production of suppressive cytokines produced by the T_{reg}[18].

Next, we analyzed how N-CMFs affect FoxP3 expression and proliferation of nTreg purified from peripheral mononuclear cells. When purified nT_{reg} were cultured alone, their FoxP3 expression was reduced, whereas those in co-culture with N-CMFs maintained FoxP3 expression (Figure 1C). Analysis of purified nT_{reg} induced by CMFs demonstrated that, in contrast to classical APCs such as BM-derived DCs, co-culturing of N-CMFs with nT_{reg} did not induce significant proliferation of nT_{reg} cells (Figure 1D). IL-2 is reported to be essential for the physiological expansion of nT_{reg} in humans and rodents[19–20]. Thus, we analyzed whether addition of IL-2 to the N-CMFs-nT_{reg} co-cultures resulted in proliferation of the nT_{reg}. Figure 1D demonstrates that addition of IL-2 to these co-cultures resulted in strong proliferation of the nT_{reg} comparable to that induced by BM-derived DCs.

CMFs induce generation of iT_{reg} cells from naïve $CD4^+$ $CD45RA^+$ T cells

Next, we sought to determine the capacity of CMFs to generate iT_{reg} cells from naïve $CD4^+$ $CD45RA^+$ T (Th_0) cells. Allogeneic Th_0 cells were incubated with MHC class II expressing CMFs at ratios of 10:1 for up to 12 days. A significant increase in the expression of FoxP3 mRNA was observed at day seven of the co-cultures (Figure 2A) and in the frequency of the iT_{reg} cells (Figure 2B). The percentage of the induction of $FoxP3^+$ T cells in CMF primed Th_0 cells (10.2±3.6%) was comparable to that in the cocultures with DCs (12.3 +− 3.9 %) (Figure 2B). The iT_{regs} generated from CMFs-primed Th_0 cells did not express surface CD127 (Figure 3A). Since downregulation of the CD127 is associated with acquisition of regulatory function by T cells and inversely correlated with FoxP3 expression[21–23], our data suggests that normal CMFs can contribute to the generation of the iT_{reg} from naive $CD4^+$ T cells at a capacity comparable to that of classical APCs, such as DC.

The T_{reg} cells induced by CMFs exhibit an activated phenotype

Increased expression of CTLA-4 is associated with an activated phenotype of T_{reg} cells and is involved in T_{reg} suppressive function[4–5]. Thus, we analyzed whether CMF-induced iT_{reg} cells express CTLA-4. The $CD4^+$ $CD25^{high}$ $CD127^-$ T cells sorted from the CMFs:Th_0 cell co-cultures. (Figure 3A) demonstrated that the cells express both intracellular CTLA-4 (Figure 3B) and FoxP3 mRNA (Figure 3C). Since production of IL-10 and TGF-β1 by T_{reg}

cells is central to their suppressive function[4–5], expression of these cytokines was measured by real-time RT-PCR in $CD4^+$ $CD25^{high}$ $CD127^-$ T cells (iT_{reg}) sorted from the seven day Th_0:CMFs cell co-cultures. CMF-induced iT_{reg} cells express moderate levels of the IL-10 and high levels of TGF-β1 (Figure 3C), but not TGF-β2 and TGF-β3 (data not shown). Taken together these results indicate that the CMF-induced iT_{reg} cells have the elements needed to exert suppressive function.

The iT_{reg} cells induced by CMFs inhibit activated T effector cell proliferation

The main function of T_{reg} cells is to negatively regulate responses of immune cells, including activated T effector cells[2,4–5]. Thus, we analyzed the ability of CMF-induced T_{reg} to suppress proliferation of CD3/CD28 preactivated syngeneic $CD45RA^+CD4^+$ naïve T cells (T effector, T_{eff}). T cells from the seven day CMF:Th_0 cell cocultures were sorted into $CD4^+$ $CD25^{high}$ and $CD4^+CD25^{low}$ populations (Figure 4A). These populations were incubated separately for 96 h with syngeneic, preactivated T_{eff} in various T_{reg}:T_{eff} ratios. The CMF-induced $CD4^+$ $CD25^{high}$ T cells induced a significant (60%) decrease in the proliferation of preactivated $CD45RA^+CD4^+$ naïve T helper cells (Figure 4B) at a 1: 8 T_{reg}:T_{eff} ratio, which was comparable with the percentage of suppression by circulating $CD4^+$ $CD25^{high}$ T cells isolated from PBMC of the same donors (Figure 4C). Thus, our data demonstrated that CMFs induce functionally active iT_{reg}. Surprisingly, a minor level of suppression by CMF-induced $CD4^+$ $CD25^{low}$ T cell fraction has been also observed (Figure 4C), perhaps due to the production of the immunoregulatory cytokines by $CD4^+CD25^{low}$ T cell fraction sorted from CMF-$CD4^+$ T cell co-cultures.

De novo generation of $CD4^+$ $CD25^{high}$ $FoxP3^+$ T_{reg} cells by CMFs is MHC class II- and PGE_2- dependent

We have previously shown that MHC class II is essential for CMF-induced resting $CD4^+$ T cell proliferation[12]. Others have demonstrated that MHC class II-TcR interactions are involved in the development of the $FoxP3^+$ polyclonal T_{reg}[11]. Thus, we examined the role of MHC class II expression by human CMFs in the induction of iT_{reg} cells. Allogeneic co-cultures of CMFs with Th_0 cells were established in the presence of the anti-class II MHC antibodies (anti- HLA-DR, -DQ and -DP cocktail, clones L243 and IVA12). A significant decrease in FoxP3 mRNA expression and CMF induction of iT_{reg} cells in the CMF:T_{reg} cell co-cultures (up to 50%) was observed in the presence of anti-MHC class II Abs, but not isotype controls (Figure 5A–B). The fact that CMF-induced iT_{regs} were only partially reduced in the presence of anti-MHC class II Abs suggested that this induction requires other mediators as well.

Since it has been demonstrated that peripheral induction of regulatory T cells may involve PGE_2[24] and because CMFs are avid producers of these molecules [16–17], we investigated involvement of this mediator in CMF-induced generation of the T_{regs}. Addition of a PGE_2 synthesis inhibitor indomethacin (INT 10 μM) to the CMF: Th_0 cell co-culture decreased in the induction of iT_{regs} (5.92±1.8 %), when compared to 10.86±2.6% in the controls (Figure 6A). Additionally, a strong upregulation of indomethacin-inhibitable PGE_2 production was observed in the CMF: naïve T helper cell co-culture (Figure 6B). Moreover, a significant decrease of the FoxP3 mRNA expression in the presence of INT was observed (Figure 6C). Taken together these data suggest the importance of the MHC class II- and PGE_2 mediated signals in the colonic induction of the T_{reg} cells.

IBD-derived CMFs have a decreased capacity to induce T_{reg} cells

CMFs are important contributors to the progression of IBD and, when isolated from chronically inflamed IBD tissues, they display a fundamentally altered phenotype/function compared with cells extracted from normal tissues[13,16]. Thus, we compared the capacity of

IBD- and N- CMFs to induce a T_{reg} cell phenotype from Th_0 cells. Allogeneic Th_0 cells were co-cultured with MHC class II expressing CD-, UC- or N-CMFs at a ratio of 10:1 for up to 12 days. No significant difference in the generation of the $FoxP3^+CD25^{high}$ cells by CMF derived from IBD vs those derived from normal controls was observed (Figure S2 at www.gastrojournal.org). IL-7 receptor α-chain or CD127 has been shown to be inversely correlated with the suppressive activity of the $CD4^+CD25^{high}$ T cells[21–22]. Moreover, IL-7/IL-7R signaling has been suggested to be implicated in the development and persistence of murine chronic colitis[23]. Thus, we analyzed whether the combination of CD127 with CD4, FoxP3 and CD25 identified by multi-color flow cytometry analysis would lead to the identification of a difference in the generation of the T_{reg} by CMFs derived from IBD compared to those isolated from normal controls (Figure 7A and B). A decrease in the generation of the $CD4^+CD127^-FoxP3^+$ T cells and increase $CD4^+CD127^+FoxP3^+$ T was observed when Th_0 cells were primed with IBD-derived CMFs when compare to N-CMFs (Figure 7A). Up to 50% of IBD-induced $FoxP3^+$ T cells were positive for CD127. A significant decrease in the induction of the true T cells bearing the true T_{reg} phenotype was observed (a.k.a. $FoxP3^+$ $CD25^{high}$, but negative for the CD127) as shown on Figure 7B. Instead we found increased generation of $CD4^+CD25^{+/-}$ $FoxP3^+$ cells that were positive for CD127 when Th_0 cells were primed by IBD-derived CMFs. T cells derived from the co-culture of IBD-CMFs with Th_0 cells had reduced expression of TGF-β1 and did not produce IL-10, cytokines associated with active T_{reg} (Figure 7C). While the mechanisms implicated in this impairment of IBD-derive CMF are currently unknown, our data suggest that in contrast to normal CMFs, IBD-derived CMFs have diminished capacity to induce “true” T_{reg} phenotype generation from Th_0 cells.

Discussion

APCs play a critical role in maintaining the balance between tolerance and inflammation in the gut[1,10–12]. Role of professional APCs, such as DCs and macrophages, in the differentiation of T cells and regulation of their activity has been well investigated[1,10–12,25]. Little is known about the role of intestinal stromal cells in these processes. It has been established that stromal cells are important contributors to immune homeostasis and function[12–16,26–27]. Despite the fact that the term “stromal” encompasses a broad range of stationary cells with various developmental origins, the $CD90^+$ fibroblast-like cells which are replenished in part from bone marrow mesenchymal stem cells form a major stromal component that is ubiquitously found in a close association with T cells in peripheral lymphoid organs, including mesenteric lymph nodes[26,27].

We recently reported that human colonic stromal $CD90^+$ myofibroblast/fibroblast cells are abundant in the normal human colonic lamina propria and can act as non professional APCs[12]. Low level expression of B7.1/2 molecules and relatively higher expression of B7-related co-inhibitor B7-H1 (PD-L1) and B7-DC(PD_L2)[15] led us to hypothesize that CMFs are local “suppressors” of activated T cell responses in normal colon contributing to mucosal tolerance. The present study supports our hypothesis and suggests that in the normal colon, CMFs can also indirectly contribute to the suppression of active inflammation by supporting expansion of the T_{reg} cells.

As has been previously reported for the mesenchymal stem cells[28] (probable progenitor cells for CMFs), our experiments indicate that N-CMFs may contribute to the maintenance of $FoxP3^+$ phenotype of nT_{reg}. Although the exact mechanisms involved in CMF’s beneficial effect on the maintenance of nTreg phenotype are still to be determined, an effect on both survival and FoxP3 expression stability is likely. Stromal cells are known to produce IL-7, Il-15, retinoic acid and TGFβ[16,29] and these mediators contribute to T_{reg} maintenance[4,16,29]. We demonstrated that CMFs can support nT_{reg} proliferation, but in contrast to professional

APCs such as DCs, N-CMFs require the presence of exogenous IL-2. IL-2 is essential for the physiological expansion the nTreg cells in humans and rodents. TCR engagement on the nT_{reg} cells in combination with IL-2 was shown to be sufficient to overcome anergic properties of these cells resulting in the nT_{reg} proliferation[19–20,30]. Activated $CD4^+$ T effector cells were demonstrated to contribute to the proliferation/survival of nT_{reg} by secreting IL-2 *in vitro* and *in vivo*[31]. Neutralization of the IL-2 reduces nT_{reg} numbers *in vivo*[20].

While this is the first report demonstrating that human normal colonic $CD90^+$ stromal cells support nT_{reg} expansion, it is not without precedent. Other mesenchymal cells such as dermal fibroblasts[32] and rheumatoid synovial fibroblasts[29] have been shown to induce proliferation of nT_{reg} by an IL-15-dependent mechanism. Further, other members of the fibroblast family such as hepatic stellate cells have been shown to be capable of expanding nTreg when supplemented with IL-2[33].

Our data indicate that priming of naïve $CD4^+$ T cells with allogeneic N-CMFs can induce generation of iT_{reg} cells at a rate comparable with BM-derived DCs. Although FoxP3 expression is mainly associated with regulatory T cell phenotype, FoxP3 may possibly be transiently expressed by activated $CD4^+$ T cells[34]. We demonstrate here that $FoxP3^+$ iT_{reg} induced by human CMFs have an activated phenotype with strong expression of CTLA-4, TGF-β1 and IL-10, and are capable of suppressing the proliferation of activated syngeneic effector T cells. In contrast to rodent studies, it has been difficult to convert human peripheral blood derived naïve $CD4^+$ T cells into $FoxP3^+$ iT_{reg} cells with potent and stable suppressive ability; only transient FoxP3 expression in the TCR activated human naïve T cells occurred in the presence of TGFβ1[35]. Further, this type of stimulation was not sufficient to confer to these $FoxP3^+$ cells significant anergic and suppressive capacities. More recently it have been reported that the presence of additional factors, such as *trans*-retinoic acid, PGE_2, PD-L1 signaling, might be required for the generation of active iTreg with suppressive capacity[10,24,36–387]. All of these factors are known to be produce by human fibroblasts/myofibroblasts[14–16]. We demonstrate herein that induction of iT_{reg} from N-CMF primed naive $CD4^+$ T cells depends on both cell-contact mediated interactions (MHC class II-TCR signaling) and production of a soluble factor (PGE_2). PGE_2 reported topromote the conversion of $CD4^+CD25^-$ cells to T_{reg} cells[24]. However, to our knowledge, this is the first report suggesting the importance of the CMF-derived PGE_2 in the regulation of T_{reg} cells and, consequently, effector T cell behavior in the colon. It is not clear which of the various potential iT_{reg} induction mechanisms is of greater importance under specific physiological conditions, and this will be an important question for future.

It has been suggested that fibroblast-like stromal cells can modify the quality, quantity and duration of inflammatory responses[38]. The direct immunosuppressive, PD-L1-mediated effect of CMFs has been previously demonstrated by us[15]. It is still unclear whether PD-L1 or T_{reg} mediated immune suppressive mechanisms predominate during a particular inflammatory event. However, it is likely that disruptions in these anti-inflammatory, immunoregulatory functions may prevent the proper transition from acute to resolving inflammation leading to the establishment of chronic inflammation. There is ample substantiation that intestinal fibroblasts/myofibroblasts taken from the chronic disease tissues display a fundamentally altered phenotype compared with these cells from normal tissues at the same anatomical site: changes in TNF-α, TGFβ and MMP production were identified in the IBD-derived myofibroblasts and it is thought that these cells contribute to the IBD-associated fibrosis and disruption of immunoregulation[39–41]. Here we present additional evidence that CMFs may play a role in immunopathogenesis of IBD: IBD-derived CMFs have a decreased capacity to induce active T_{reg} cells capable of producing suppressive cytokines TGFβ1 and IL-10. Moreover, a shift in the balance in IBD-CMF –

primed T cells from "true" T_{reg} phenotype ($CD4^+CD25^{high}CD127^-$ $FOXP3^+$) toward FoxP3 expressing $CD127^+$ T cells ($CD4^+CD25^{high}CD127^+$ $FOXP3^+$) suggests that during IBD progression CMFs may promote the persistence of $CD127^+$ colitogenic $CD4^+$ T cells that only transiently express FoxP3. It is unclear how the disruption of this immunoregulatory function of the CMFs occurs during the switch from acute to chronic inflammation associated with IBD, but in rheumatoid arthritis an alteration in synovial fibroblasts capacity to regulate T responder/T_{reg} balance has been recently reported to implicate IL-15 expression by the disease derived fibroblasts[29]. Further studies are clearly necessary to understand the role of the CMF-diminished capacity to induce T_{reg} in the progression of IBD.

Our study adds human CMFs to the list of mesenchymal stromal cells (e.g., dermal and synovial fibroblasts, hepatic stellate cells and mesenchymal stem cells) [40–42,45], that may contribute to the regulation of the T_{reg} cells and, thus, to the maintenance of peripheral tolerance. Further, CMFs appear to be local contributors to the maintenance of mucosal tolerance in the colon via at least two independent mechanisms: (1) expression of co-inhibitors of the B7 family (reference) and (2) support of T_{reg} cells expansion. These findings taken together with previous reports[12,15] indicate that CMF are relevant to normal inflammatory responses and that alteration of their regulatory function might be involved in IBD immunopathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by grants from the NIDDK (DK55783), American Gastroenterology Association, the John Sealy Memorial Endowment Fund, the UTMB Gastrointestinal Research Interdisciplinary Program, the James W. McLaughlin Endowment Fund, and Crohn's & Colitis Foundation of America

References

1. Annacker O, Powrie F. Homeostasis of intestinal immune regulation. Microbes Infect. 2002; 4:567–574. [PubMed: 11959513]
2. Makita S, Kanai T, Nemoto Y, et al. Intestinal lamina propria retaining CD4+CD25+ regulatory T cells is a suppressive site of intestinal inflammation. J Immunol. 2007; 178:4937–4946. [PubMed: 17404275]
3. Allez M, Mayer L. Regulatory T cells: peace keepers in the gut. Inflamm Bowel Dis. 2004; 10:666–676. [PubMed: 15472533]
4. Tang Q, Bluestone JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. Nat Immunol. 2008; 9:239–244. [PubMed: 18285775]
5. von Boehmer H. Mechanisms of suppression by suppressor T cells. Nat Immunol. 2005; 6:338–344. [PubMed: 15785759]
6. Gad M. Regulatory T cells in experimental colitis. Curr Top Microbiol Immunol. 2005; 293:179–208. [PubMed: 15981481]
7. Powrie F, Leach MW, Mauze S, et al. Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. Int Immunol. 1993; 5:1461–1471. [PubMed: 7903159]
8. Mottet C, Uhlig HH, Powrie F. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. J Immunol. 2003; 170:3939–3943. [PubMed: 12682220]
9. Maul J, Loddenkemper C, Mundt P, et al. Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease. Gastroenterology. 2005; 128:1868–1878. [PubMed: 15940622]

10. Siddiqui KR, Powrie F. CD103^{+} GALT DCs promote Foxp3^{+} regulatory T cells. Mucosal Immunol. 2008; (Suppl1):S34–S38. [PubMed: 19079226]
11. Westendorf AM, Fleissner D, Groebe L, et al. CD4+Foxp3+ regulatory T cell expansion induced by antigen-driven interaction with intestinal epithelial cells independent of local dendritic cells. Gut. 2009; 58:211–219. [PubMed: 18832523]
12. Saada JI, Pinchuk IV, Barrera CA, et al. Subepithelial myofibroblasts are novel nonprofessional APCs in the human colonic mucosa. J Immunol. 2006; 177:5968–5979. [PubMed: 17056521]
13. Andoh A, Bamba S, Brittan M, et al. Role of intestinal subepithelial myofibroblasts in inflammation and regenerative response in the gut. Pharmacol Ther. 2007; 114:94–106. [PubMed: 17328956]
14. Pinchuk IV, Beswick EJ, Saada JI, et al. Monocyte chemoattractant protein-1 production by intestinal myofibroblasts in response to staphylococcal enterotoxin a: relevance to staphylococcal enterotoxigenic disease. J Immunol. 2007; 178:8097–8106. [PubMed: 17548648]
15. Pinchuk IV, Saada JI, Beswick EJ, et al. PD-1 ligand expression by human colonic myofibroblasts/fibroblasts regulates CD4+ T-cell activity. Gastroenterology. 2008; 135:1228–1237. [PubMed: 18760278]
16. Pinchuk IV, Mifflin RC, Saada JI, et al. Intestinal mesenchymal cells. Curr Gastroenterol Rep. 2010; 12:310–318. [PubMed: 20690004]
17. Mahida YR, Beltinger JJ, Makh S, et al. Adult human colonic subepithelial myofibroblasts express extracellular matrix proteins and cyclooxygenase-1 and -2. Am J Physiol. 1997; 273:G1341–G1348. [PubMed: 9435560]
18. Allan SE, Crome SQ, Crellin NK, et al. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. Int Immunol. 2007; 19:345–54. [PubMed: 17329235]
19. Wuest TY, Willette-Brown J, Durum SK, et al. The influence of IL-2 familycytokines on activation and function of naturally occurring regulatory T cells. J Leukoc Biol. 2008; 84:973–80. [PubMed: 18653463]
20. Setoguchi R, Hori S, Takahashi T, et al. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. J Exp Med. 2005; 201:723–35. [PubMed: 15753206]
21. Liu W, Putnam AL, Xu-Yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med. 2006; 203:1701–1711. [PubMed: 16818678]
22. Michel L, Berthelot L, Pettré S, et al. Patients with relapsing-remitting multiple sclerosis have normal Treg function when cells expressing IL-7 receptor alpha-chain are excluded from the analysis. J Clin Invest. 2008; 118:3411–3419. [PubMed: 18769633]
23. Totsuka T, Kanai T, Nemoto Y, et al. IL-7 Is essential for the development and the persistence of chronic colitis. J Immunol. 2007; 178:4737–4748. [PubMed: 17404253]
24. English K, Ryan JM, Tobin L, et al. Cell contact, prostaglandin E(2) and transforming growth factor beta 1 play non-redundant roles in human mesenchymal stem cell induction of CD4+CD25(High) forkhead box P3+ regulatory T cells. Clin Exp Immunol. 2009; 156:149–160. [PubMed: 19210524]
25. Brandtzaeg P. Nature and function of gastrointestinal antigen-presenting cells. Allergy. 2001; 56 (Suppl67):16–20. [PubMed: 11298000]
26. Mueller SN, Germain RN. Stromal cell contributions to the homeostasis and functionality of the immune system. Nat Rev Immunol. 2009; 9:618–29. [PubMed: 19644499]
27. Haniffa MA, Wang XN, Holtick U, et al. Adult human fibroblasts are potent immunoregulatory cells and functionally equivalent to mesenchymal stem cells. J Immunol. 2007; 179:1595–604. [PubMed: 17641026]
28. Di Ianni M, Del Papa B, De Ioanni M, et al. Mesenchymal cells recruit and regulate T regulatory cells. Exp Hematol. 2008; 36:309–18. [PubMed: 18279718]
29. Benito-Miguel M, García-Carmona Y, Balsa A, et al. A dual action of rheumatoid arthritis synovial fibroblast IL-15 expression on the equilibrium between CD4+CD25+ regulatory T cells and CD4+CD25- responder T cells. J Immunol. 2009; 183:8268–79. [PubMed: 20007590]

30. Hoffmann P, Eder R, Kunz-Schughart LA, et al. Large-scale in vitro expansion of polyclonal human CD4(+)CD25high regulatory T cells. Blood. 2004; 104:895–903. [PubMed: 15090447]

31. Yu A, Malek TR. Selective availability of IL-2 is a major determinantcontrolling the production of CD4+CD25+Foxp3+ T regulatory cells. J Immunol. 2006 Oct 15; 177(8):5115–21. [PubMed: 17015695]

32. Clark RA, Kupper TS. IL-15 and dermal fibroblasts induce proliferation of natural regulatory T cells isolated from human skin. Blood. 2007; 109:194–202. [PubMed: 16968902]

33. Jiang G, Yang HR, Wang L, et al. Hepatic stellate cells preferentially expand allogeneic CD4+ CD25+ FoxP3+ regulatory T cells in an IL-2-dependent manner. Transplantation. 2008; 86 :1492–502. [PubMed: 19077880]

34. Merlo A, Tagliabue E, Menard S, et al. Matured human monocyte-derived dendritic cells (MoDCs) induce expansion of CD4(+)CD25(+)FOXP3(+) T cells lacking regulatory properties. Immunol Lett. 2008; 117:106–113. [PubMed: 18295349]

35. Tran DQ, Ramsey H, Shevach EM. Induction of FOXP3 expression in naive humanCD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. Blood. 2007 Oct 15; 110(8):2983–90. [PubMed: 17644734]

36. Beswick EJ, Pinchuk IV, Das S, et al. Expression of the programmed death ligand 1, B7-H1, on gastric epithelial cells after Helicobacter pylori exposure promotes development of CD4+ CD25+ FoxP3+ regulatory T cells. Infect Immun. 2007; 75:4334–4341. [PubMed: 17562772]

37. Wang J, Huizinga TW, Toes RE. De novo generation and enhanced suppression of human CD4+CD25+ regulatory T cells by retinoic acid. J Immunol. 2009; 183:4119–26. [PubMed: 19717521]

38. Flavell SJ, Hou TZ, Lax S, Filer AD, Salmon M, Buckley CD. Fibroblasts as novel therapeutic targets in chronic inflammation. Br J Pharmacol. 2008; 153 (Suppl 1):S241–S246. [PubMed: 17965753]

39. McKaig BC, Hughes K, Tighe PJ, et al. Differential expression of TGF-beta isoforms by normal and inflammatory bowel disease intestinal myofibroblasts. Am J Physiol Cell Physiol. 2002; 282:C172–C182. [PubMed: 11742810]

40. Di Sabatino A, Pender SL, Jackson CL, et al. Functional modulation of Crohn's disease myofibroblasts by anti-tumor necrosis factor antibodies. Gastroenterology. 2007; 133:137–149. [PubMed: 17631138]

41. Di Sabatino A, Jackson CL, Pickard KM, et al. Transforming growth factor beta signalling and matrix metalloproteinases in the mucosa overlying Crohn's disease strictures. Gut. 2009; 58:777–789. [PubMed: 19201776]

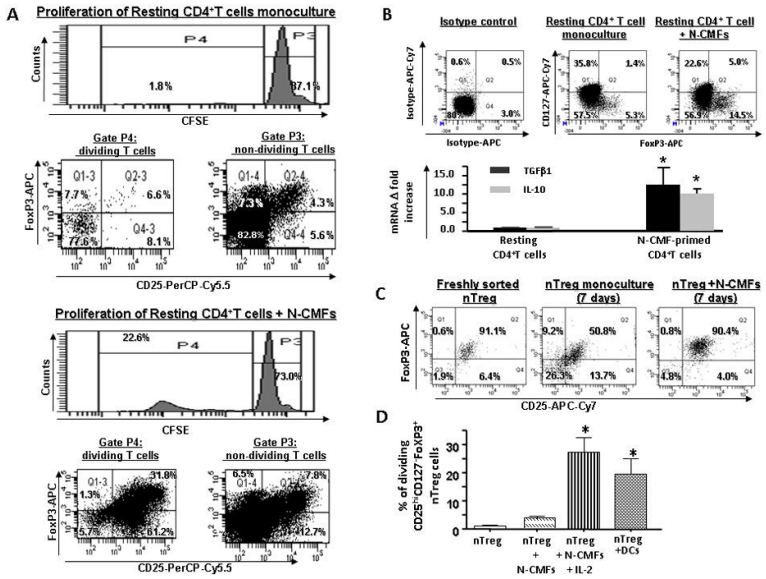

Figure 1.

Normal (N) CMFs contribute to the maintenance of nT_{reg} phenotype. CFSE-labeled resting $CD4^+$ T cells were cultured without or with allogeneic N-CMFs at a ratio 1:10 for 7 days in 24 well plates. T cell from these co-cultures were subjected to surface CD4 and CD25, CD127 and intracellular FoxP3 staining following by flow cytometry. (A) Live events were gated in P1 and percentage of CD25+FoxP3+ T cells in non dividing (P3) and dividing (P4) fractions of $CD4^+$ resting T cells growing in monoculture or cocultured with N-CMFs was evaluated. A representative experiment is shown (n=5 allogeneic donor pair, two experimental replicate each) (B) Distribution of CD127 and FoxP3 (FACS analysis) and expression of TGFβ1 and IL-10 (real-time RT-PCR) in the $CD4^+$ resting T cells growing in monoculture or cocultured with N-CMFs was evaluated. A representative experiment is shown (n=4 allogeneic donor pair, two experiment replicate each) (D) The surface CD25 and intracellular FoxP3 expression in the freshly isolated and seven day monoculture of nTreg, and N-CMF primed nTreg was analyzed. A representative experiment is shown (n=5 allogeneic donor pair, two experiment replicate each). (D) Percentage of the dividing $CD4^+CD25^{high}Cd127^-FoxP3^+$ nT_{reg} in response to the allogeneic stimulation with N-CMF in presence/absence of IL-2 (10 ng/mL) or BM-derived DC was measured in 7 day co-culture as described above. Results are calculated as the mean value of the percentage of the dividing cells for three independent allogeneic pair of N-CMFs and $CD4^+$ T cells healthy donors ± SD. Each assay was conducted in duplicate. * $p < 0.05$.

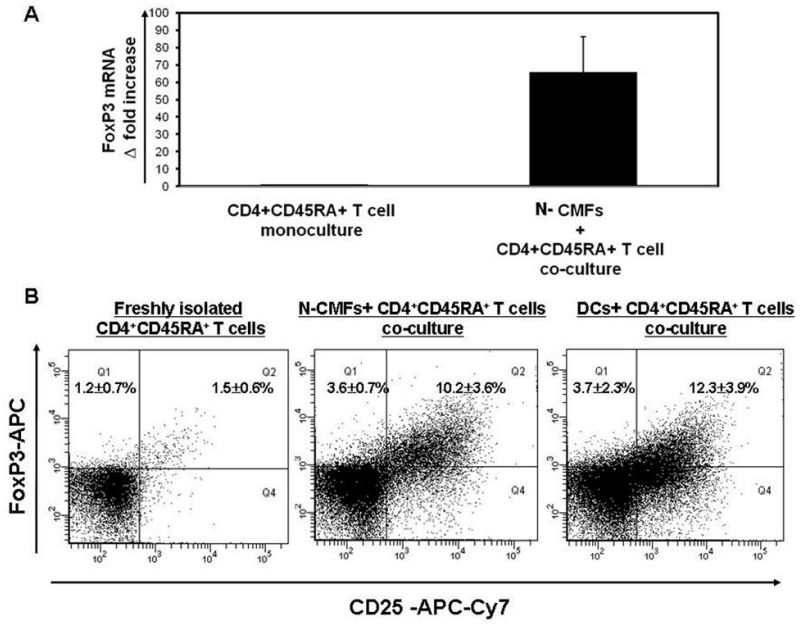

Figure 2.

$CD4^+$ $CD25^{high}$ $FoxP3^+$ T_{reg} cells (iT_{reg}) are induced from naive $CD4^+CD45RA^+$ T helper (Th_0) cells primed with N-CMFs. Th_0 cells were cultured without or with allogeneic N-CMFs at a ratio 1:10 for one week. (A) The RNA from the N-CMF primed Th_0 cells was analyzed for FoxP3 mRNA expression using real time RT-PCR. The mRNA levels for FoxP3 was normalized to 18S. Data represent mean of mRNA Δ fold increase ± SEfrom duplicates in four experiments (n=8) **P*<0.01. (B) The surface CD25 and intracellular FoxP3 expression in the allogeneic N-CMF or BM-derived DC primed $CD4^+$ T cells was analyzed using flow cytometry.. The appropriate isotype controls were included in the experiments. A representative experiment is shown (n=5). **p*<0.01.

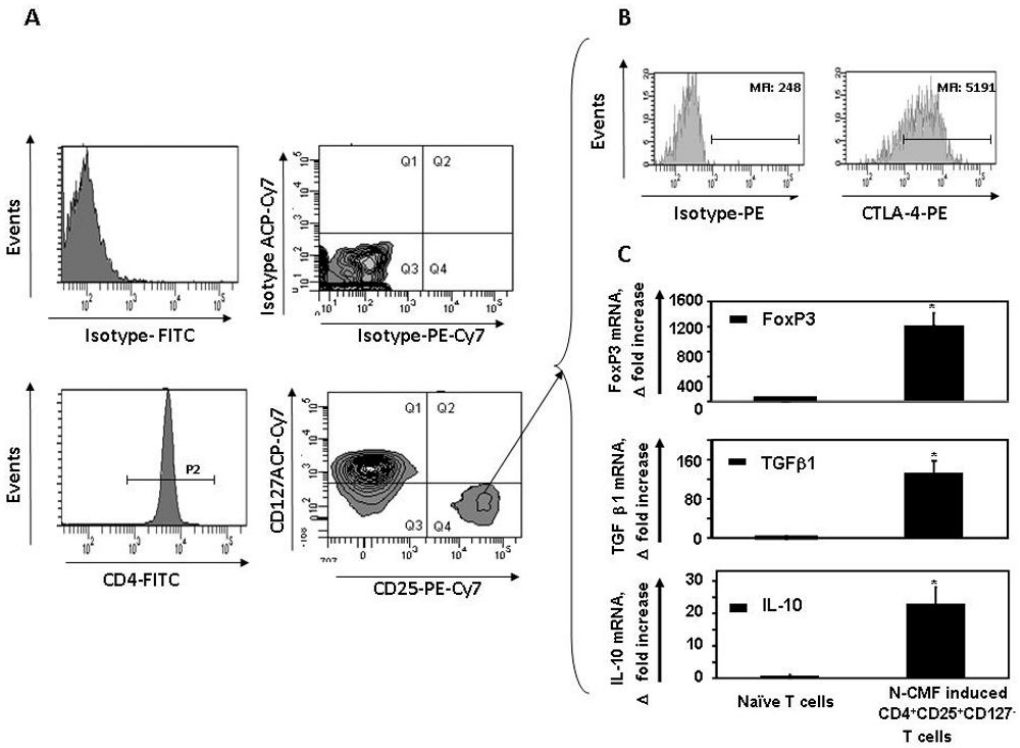

Figure 3.

$CD4^+$ $CD25^{high}$ $FoxP3^+$ iTreg induced by allogeneic N-CMFs exhibit an activated phenotype. Th_0 cells were cultured with allogeneic N-CMFs at ratio 1:10. Harvested T cells (day 7) were stained with anti-CD4 FITC, anti-CTLA-PE, anti-CD25PE-Cy7 and anti-CD127 mAbs or appropriate isotype controls. Live events were gated in P1, then (A) $CD4^+$ T cells were gated in P2 and analyzed for CD25 and CD127 expression. The $CD4^+$ T cells bearing $CD25^+CD127^-$ phenotype (Q4 quadrant) were (B) analyzed for CTLA-4 expression and sorted for further analysis. A representative experiment is shown (n=5). The RNA was extracted from sorted $CD4^+$ $CD25^+CD127^-$ T cells was analyzed for (C) FoxP3, TGF-β1 and IL10 mRNA expression using real time RT-PCR. The mRNA levels for each gene of interest were normalized to 18S. Data represent mRNA Δ fold increase mean ± standard errors from duplicates in five experiments (n=10) *p<0.01.

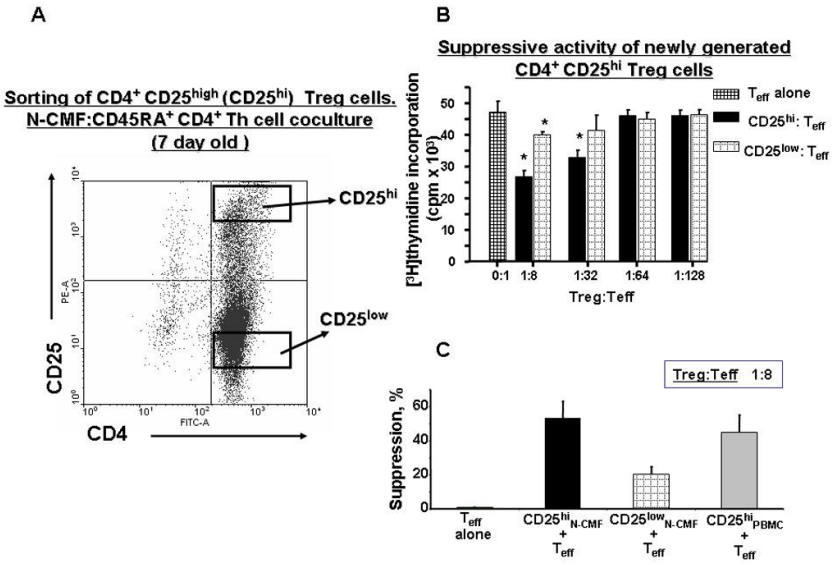

Figure 4.

$CD4^+CD25^{high}$ iT_{reg} cells induced by N-CMFs suppress proliferation of syngeneic, activated $CD4^+$ T effector cells. (A) $CD4^+CD25^{high}$ T cells induced by co-culturing of Th_0 with CMFs for 7 days were sorted by FACSAria sorter and $CD4^+CD25^{high}$ or $CD4^+CD25^-$T cells were co-cultured with CD2/CD3/CD28-activated $CD45RA^+CD4^+CD25^-$ effector (Teff) cells for 4 days. Eighteen hours prior to the end of the co-culture, the cells were pulsed with [3H]-methyl-thymidine. (B) Mean counts per minute (c.p.m) ± standard error (SE) of triplicate cultures of $CD4^+$ T cells isolated from one donor are shown here in a representative experiment of three. (C) The percent suppression of Teff is shown and represents a decrease in the proliferative response (c.p.m.) of $CD4^+$ Teff cells when compared to activated Teff cells alone. The means are shown as the results of triplicates in 3 experiments, n=9 (* $p < 0.05$).

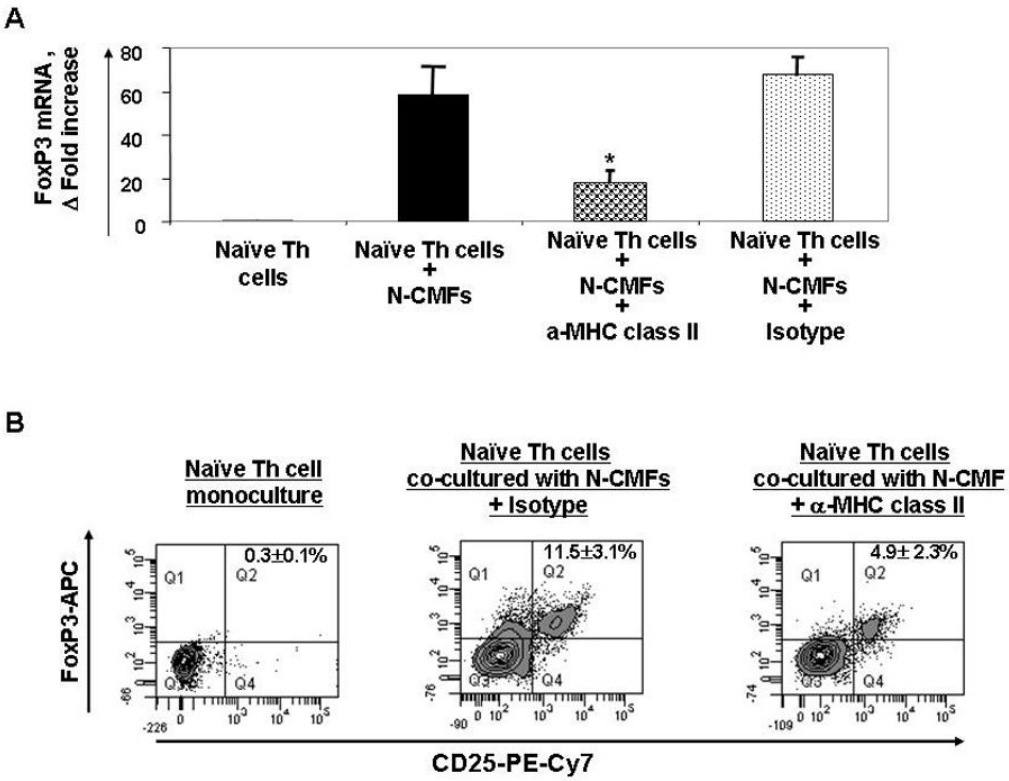

Figure 5.

Induction of the iT_{reg} from naïve $CD4^+CD45RA^+$ T cells (Th_0) by allogeneic N-CMFs depends on MHC class II. T cells were cultured without or with allogeneic CMFs at a ratio 1:10 for 7 days in 24 well plates in presence/absence of anti-MHC class II mAb mix (anti-HLA-DR, -DQ and -DP cocktail, clones L243 and IVA12) or isotype mix control. (A) The RNA from the N-CMF primed T cells was analyzed for FoxP3 mRNA expression by using real time RT-PCR. The mRNA level for FoxP3 was normalized to 18S. Data represent mean of mRNA Δ fold increase ± SEfrom duplicates in three experiments (n=6) **P*<0.01. (B) The surface CD25 and intracellular FoxP3 expression in the N-CMF primed T cells was analyzed using flow cytometry. Harvested T cells were stained with anti-CD4 FITC, anti-CD25PE-Cy7, and anti-FoxP3-APC mAbs. The appropriate isotype controls were included in the experiments. A representative experiment is shown (n=5). **p*<0.01.

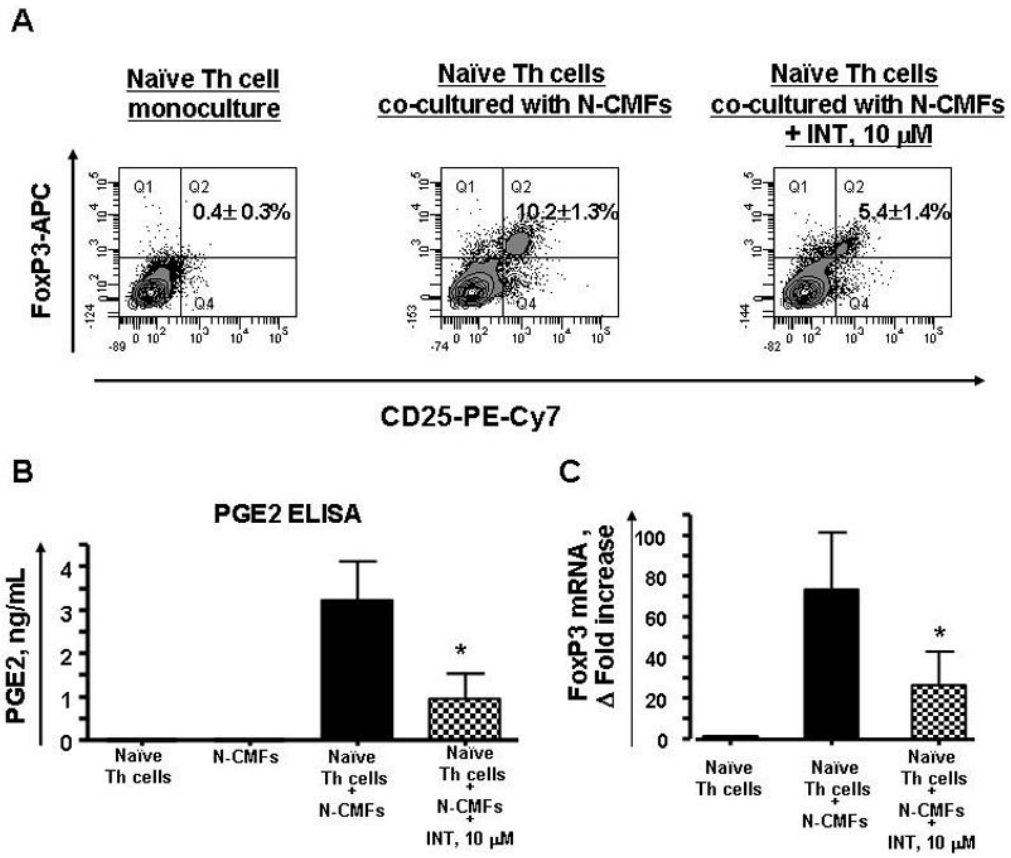

Figure 6.

Induction of iT_{reg} from naïve $CD4^+CD45RA^+$ T (Th_0) cells by allogeneic N-CMFs involves PGE_2. T cells were cultured without or with allogeneic N-CMFs at a ratio 1:10 for 7 days in 24 well plates in presence/absence of indomethacin, 10 μM. (A) The surface CD25 and intracellular FoxP3 expression in the allogeneic N-CMF primed T cells was analyzed using flow cytometry. The appropriate isotype controls were included in the experiments. A representative experiment is shown (n=6). **P*<0.05. (B) The PGE2 production in culture supernatant was measured using ELISA. Data represent mean ± SEfrom duplicates in three experiments (n=6). (C) The RNA from the N-CMF primed T cells were analyzed for FoxP3 mRNA expression using real time RT-PCR. The mRNA level for FoxP3 was normalized to 18S. Data represent mean of mRNA Δ fold increase ± SEfrom duplicates in three experiments (n=6). * *P*< 0.05.

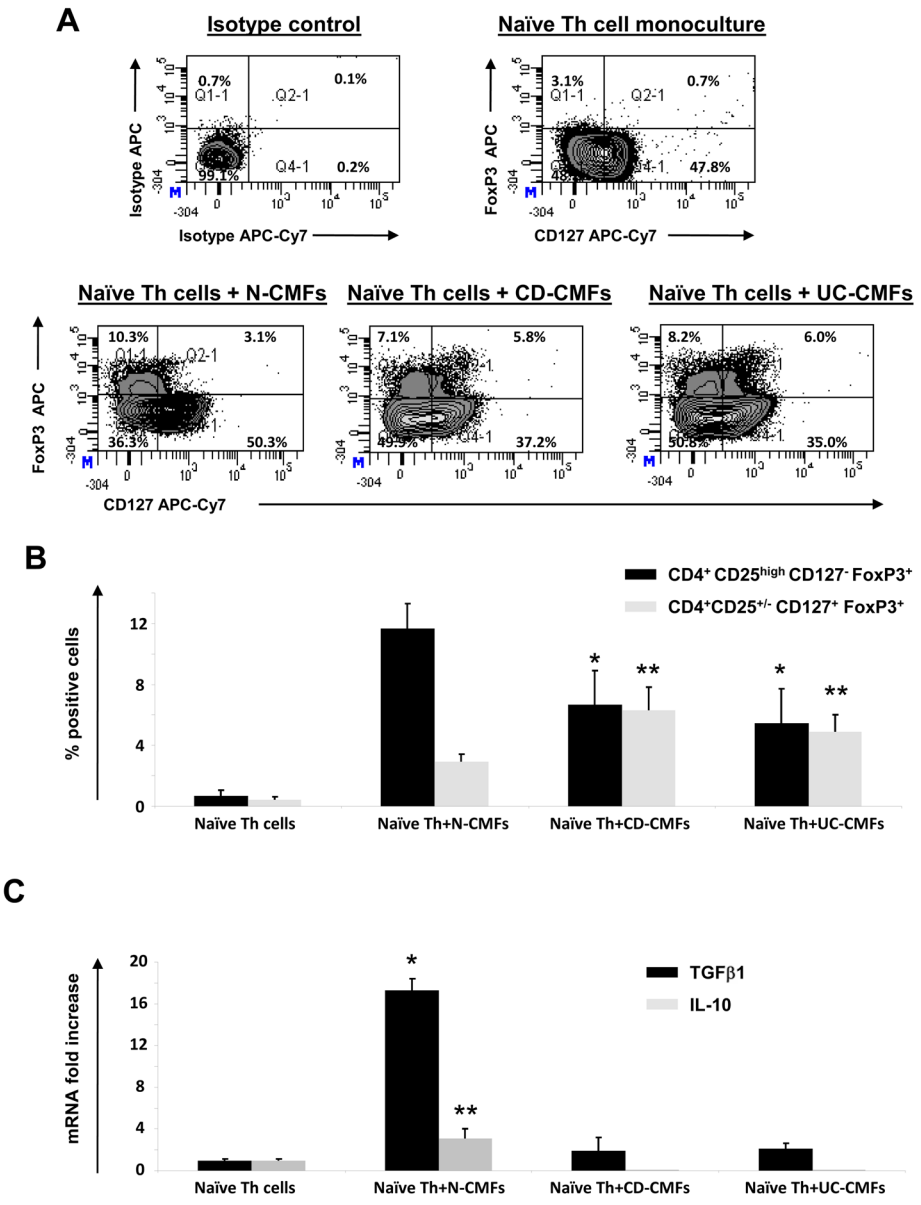

Figure 7.

IBD-derived CMFs have reduced capacity to induce the T_{reg} cells from $CD4^+CD45RA^+$ T helper (Th_0) cells and promote the expression of FoxP3 by $CD127^+$ T cells. Th_0 cells were cultured without or with allogeneic CMFs at a ratio 1:10 up to 12 days in 24 well plates. (A) The surface CD127 and intracellular FoxP3 expression in the N-, CD or UC-CMF primed $CD4^+$ T cells was analyzed using flow cytometry. The appropriate isotype controls were included in the experiments. A representative experiment is shown (n=9). (B) Distribution of the iTreg ($CD4^+CD25^{high}CD127^-FoxP3^+$) and transitory FoxP3 expressing cells ($CD4^+CD25^{+/-}CD127^+FoxP3^+$) in the N-, CD or UC -primed T cells. The results are shown as mean ± standard errors from nine independent experiments. The ANOVA one way variance analysis combined with Bonferroni's multiple comparison post test was used to analyze the significance of the variation between the experimental groups. *A significant decrease ($p<0.05$) in the generation of the iTreg ($CD4^+CD25^{high}CD127^-FoxP3^+$) by CD- and UC-CMFs when compare to N-CMF was observed. **A significant increase ($p<0.05$) in

the generation of the $CD4^{+}CD25^{+/-}CD127^{+}FoxP3^{+}$ by CD- and UC-CMFs when compare to N-CMF was observed. (C) TGF-β1 and IL-10 mRNA expression by T cells derived from the N-, CD or UC-CMF primed T cells was analyzed using relative quantitative real time RT-PCR. The mRNA levels for TGF-β1 and IL-10 was normalized to 18S. Data represent mean of mRNA Δ fold increase ± SEfrom duplicates in four experiments (n=8). The Bonferroni-corrected ANOVA variance analysis revealed a significant increase in TGF-β1 and IL-10 production when Th0 cells were primed by N-CMFs (*$p<0.001$ and **$p<0.05$, respectively), but not when the Th0 cells were primed with CD or UC-CMFs.