Published in final edited form as:

Gut. 2009 October ; 58(10): 1363–1373. doi:10.1136/gut.2008.162842.

Anti-CTLA-4 treatment induces IL-10-producing ICOS⁺ regulatory T cells displaying IDO-dependent anti-inflammatory properties in a mouse model of colitis

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Abstract

Background and aims—Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) has been shown to act as a negative regulator of T cell function and has been implicated in the regulation of T helper 1 (Th1)/Th2 development and the function of regulatory T cells. Tests were carried out to determine whether anti-CTLA-4 treatment would alter the polarisation of naive T cells in vivo.

Methods—Mice were treated with anti-CTLA-4 monoclonal antibody (mAb) (UC10-4F10) at the time of immunisation or colonic instillation of trinitrobenzene sulfonic acid (TNBS). The cytokines produced by lymph node cells after in vitro antigenic stimulation and the role of indoleamine 2,3 dioxygenase (IDO) and of interleukin-10 (IL-10) were tested, and the survival of mice was monitored.

Results—Injection of anti-CTLA-4 mAb in mice during priming induced the development of adaptive CD4⁺ regulatory T cells which expressed high levels of ICOS (inducible co-stimulator), secreted IL-4 and IL-10. This treatment inhibited Th1 memory responses in vivo and repressed experimental intestinal inflammation. The anti-CTLA-4-induced amelioration of disease correlated with IDO expression and infiltration of ICOS^{high} Foxp3⁺ T cells in the intestine, suggesting that anti-CTLA-4 acted indirectly through the development of regulatory T cells producing IL-10 and inducing IDO.

Conclusions—These observations emphasise the synergy between IL-10 and IDO as anti-inflammatory agents and highlight anti-CTLA-4 treatment as a potential novel immunotherapeutic approach for inducing adaptive regulatory T cells.

The concept of T cell co-stimulation has greatly increased our understanding of the mechanisms controlling immunity and tolerance in vivo. Among the molecules implicated as co-stimulatory receptors or ligands for naive T cells in the past decade, CD28 and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) appear to play a crucial role. The

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► Additional methods and figures are published online only at <http://gut.bmj.com/content/vol58/issue10>

Competing interests: Declared (the declaration can be viewed on the *Gut* website at <http://www.gut.bmj.com/supplemental>).

Provenance and peer review: Not commissioned; externally peer reviewed.

monovalent homodimer CD28 is expressed on resting and activated T cells and interacts with both CD80 and CD86 on antigen-presenting cells. Its engagement essentially amplifies the transcriptional effects of T cell receptor triggering. CTLA-4, a close relative of CD28, is upregulated 2–3 days following T cell activation¹ and binds both B7 family members with an affinity about 20-fold higher than that of CD28.²

Early in vitro studies clearly suggested that CD28 and CTLA-4 had opposing effects on the response of T cells and that CTLA-4 could function as a negative regulator of T cell activation.^{3–5} The critical role of CTLA-4 in maintaining homeostasis in the immune system was illustrated in mice deficient for CTLA-4 which develop multiple lymphoproliferative disorders and die within 4 weeks after birth.^{6, 7} Another report confirmed the role of CTLA-4 in the induction and maintenance of peripheral tolerance in vivo.^{8, 9} In addition, recent evidence demonstrated that CTLA-4 could function as a regulator of T helper cell differentiation.^{10–12}

This study was initially undertaken to evaluate the role of CTLA-4 in the regulation of T helper responses by naturally occurring regulatory T cells (Tregs) in vivo. We have shown previously¹³ that CD25⁺ Tregs exerted a negative feedback mechanism on Th1 (T helper 1) responses induced by mature dendritic cells (DCs) pulsed with foreign antigens. As CTLA-4 has been shown to be constitutively expressed on Treg populations,¹⁴ we reasoned that anti-CTLA-4 treatment would alter the polarisation of naive T cells and increase Th1 priming. Surprisingly, we found that anti-CTLA-4 injection primed IL-10-producing Tregs expressing high levels of inducible co-stimulator (ICOS) receptor and displaying anti-inflammatory properties in vivo.

METHODS

Mice

Balb/c and C57BL/6 mice were from Harlan Nederland; C57BL/6 IL-10^{-/-} mice were from the Jackson Laboratory. Balb/c Indo^{-/-} mice were generated as described.¹⁵ The C57BL/6 FcγRI/III^{-/-} mice were kindly provided by Dr JS Verbeek (Utrecht, The Netherlands). All mice were housed in our pathogen-free facility and the experiments were performed in compliance with the relevant laws and institutional guidelines.

Reagents and antibodies

Keyhole limpet haemocyanin (KLH) was from Calbiochem (Leuven, Belgium). 1-Methyl-L-tryptophan (1-MT) was from Sigma-Aldrich (Bornem, Belgium). Antibodies to CTLA-4 (UC10-4F10), CD3 (145.2C11), control hamster immunoglobulin G1 (IgG1; PARSI 19) and control rat IgG2b (Lo-DNP) were produced in-house.

Purification of splenic DCs and immunisation

KLH-pulsed DCs were purified and administered at a dose of 3×10^5 cells into the footpads as described.¹³ Some groups of animals received intraperitoneal injections of 100 µg of anti-CTLA-4 monoclonal antibody (mAb) or control hamster IgG1 (PARSI 19) at days 3, 4 and 5 after immunisation. Draining lymph nodes (LNs) were analysed 6 days after immunisation. To induce memory responses, mice received a second injection of DCs 6 days after the first immunisation and draining LNs were analysed 2 days later.

KLH-specific T cell response after in vivo priming

LN cells or sorted cells were cultured in Click's medium (Sigma-Aldrich) supplemented with 0.5% heat-inactivated mouse serum and additives. Cultures were pulsed during the last 16 h with 1 µCi/well of tritium (Amersham, Brecht-St-Lenaarts, Belgium) and culture

supernatants were collected for measurement of interferon γ (IFN γ), IL-4 and IL-10 by ELISA.

Colitis model

A 1 mg aliquot of trinitrobenzene sulfonic acid (TNBS) (Sigma-Aldrich) in 50% ethanol was administered intrarectally to the anaesthetised mice via a round bottom (50.8 mm) needle (Popper, New York, USA). Mice were treated daily for 5 days after TNBS administration with 100 μ g of anti-CTLA-4 mAb or with control hamster IgG. When indicated, 500 μ g of anti-IL-10 receptor (IL-10R; 11B1.2) mAb were co-injected daily with UC10-4F10 antibody. 1-MT was given in the drinking water (5 mg/ml) starting 1–2 days before TNBS instillation. When indicated, mice were killed 5 days after TNBS administration and the colon was flushed and resected. Single cell suspensions were prepared and analysed by flow cytometry for ICOS and CD4 expression and by quantitative PCR (qPCR) for indoleamine 2,3 dioxygenase (IDO) expression.

Purification of cells from the lamina propria

Colons were treated with medium containing 10 mM gentamycin and 20 mM HEPES (Gibco, Merelbeke, Belgium) for 20 min at 37°C with constant stirring. Tissue was further digested with 5 mg/ml liberase CI (Roche, Vilvoorde, Belgium) and 0.05% DNase I (Roche), with continuous stirring at 37°C for 30 min. Digested tissue was passed through 70 and 40 μ m cell strainers. Lymphocytes were further enriched by centrifugation at room temperature at 2000 rpm for 20 min in lympholyte M (Cedarlane, Uden, The Netherlands) gradient. Cells were incubated with antibodies and assessed for the expression of ICOS, Foxp3, Ki67 and CD4.

In vitro production of cytokines

A total of 10^5 cells from the lamina propria or 5×10^5 mesenteric LN cells were cultured in uncoated wells or wells coated with 10 μ g/ml murine anti-CD3 ϵ antibody (clone 145.2C11) and soluble anti-CD28 antibody (both produced in-house). Supernatants were assayed for cytokine production 48 h later by ELISA.

Flow cytometry and cell sorting

Single-cell suspensions from spleen, LNs or colon were incubated for 20 min at 4°C with Fc-blocking antibodies and specific antibodies in staining buffer (0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)). Data were collected by multicolour flow cytometry (FacsCanto II, Becton Dickinson, Herembodegham, Belgium). For intracellular staining, cells were fixed and made permeable with the BD cytofix/cytoperm kit (BD Pharmingen, Herembodegham, Belgium). The following antibodies were used for staining: phycoerythrin-conjugated anti-CTLA-4 (clone UC10, eBiosciences, San Diego, California, USA), anti-mouse Foxp-3 (clone FJK-16s; eBiosciences), anti-CD4 (clone RM4-5, BD Pharmingen), antiglycorticoid-induced tumour necrosis factor receptor (GITR) (DTA-1, BD Pharmingen) and biotin conjugated anti-ICOS detected with peridinin chlorophyll protein–cyanin 5 (both from BD Pharmingen). For sorting, CD4⁺ cells were enriched from draining LNs by negative selection using magnetic activated cell sorting (MACS) cell separation columns (LS, Miltenyi Biotec, Utrecht, The Netherlands), labelled with anti-CD4 and anti-ICOS, and sorted with a FACSVantage (Becton Dickinson).

RT-PCR

Total RNA was extracted using TRIzol reagent (Life Technologies, Merelbeke, Belgium) and was primed with oligo(dT) for first-strand cDNA synthesis (M-MLV reverse transcriptase, Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions.

A qPCR core kit for SYBR Green (Eurogentec, Seraing, Belgium) and a GeneAmp 5700 Sequence Detection System (PE Applied Biosystems, Lennik, Belgium) were used for RT-PCR. The following primer pairs were used: RPL13, forward 5'-GGCACCAGTCAGACCGATAT-3', and reverse 5'-CAGGATCTGGCCCTTGAAC-3'; INDO, forward 5'-GGACGGACTGAGAGGACA-3', and reverse 5'-CACACATACGCCATGGTGAT-3'; and TaqMan probe, 5'-CTGGCAC ACCTGGCCCTGG-3'.

Transcript amounts were normalised to those of the gene encoding ribosomal protein L13 as housekeeping gene.

Histological scores

Mice were killed 3–5 days after colonic instillation of TNBS. Colons were flushed and fixed with 4% formaldehyde solution (Merck, Leuven, Belgium) before being stained with H&E. Slides were scored by a pathologist “blinded” to sample identity with the histological scoring system.¹⁶ Total lesion score ranged from 0 to 6 and was obtained by summing scores for inflammation and damage/necrosis (0 = none, 1 = mild, 2 = moderate, 3 = severe).

Statistical analysis

Bar graphs represent the mean (SD) for 3–5 individual mice.

Statistical significance was determined with the Mann–Whitney test for two-tailed data. A *p* value <0.1 was considered significant. Survival curves were generated using the Kaplan–Meier method and the significance of the difference in survival rate was determined by the log-rank test.

RESULTS

Injection of anti-CTLA-4 mAb during priming drives the generation of IL-10-producing cells and results in impaired memory Th1 responses

We first evaluated the expression of CTLA-4 on CD4⁺ T cells during priming. KLH-pulsed DCs were injected into the footpads of syngeneic animals and draining LNs analysed by fluorescence-activated cell sorting (FACS). A time course analysis consistently showed an increase in the proportion of CD4⁺ T cells expressing CTLA-4 from 3 days after immunisation (not shown).

Next, we tested whether anti-CTLA-4 treatment (UC10-4F10⁵) would affect T cell priming. Mice were injected with KLH-pulsed DCs (day 0) and treated with 100 µg anti-CTLA-4 mAb at days 3, 4 and 5. The immune response was analysed at day 6 in the draining LNs, as shown previously.¹⁷ The data in fig 1A indicate that anti-CTLA-4 injection did not alter T cell priming, as assessed by KLH-dependent proliferation in culture, and revealed a strong increase in IL-10 production in >30 independent experiments. IL-4 production was enhanced by anti-CTLA-4 treatment, whereas IFNγ secretion was partially decreased. A similar increase in IL-10 production was noted after anti-CTLA-4 treatment in mice immunised by the same antigen in adjuvant (not depicted).

We next determined the effect of anti-CTLA-4 injection on recall responses. Mice were primed and boosted with KLH-pulsed DCs with or without UC10-4F10 treatment at the first immunisation. Of note, analysis of the memory responses 2 days after boost showed that IFNγ production was strongly decreased or abrogated in all experiments performed, whereas IL-4 and IL-10 remained unchanged (fig 1B and data not shown). These results show that anti-CTLA-4 treatment during priming induced the development of cells producing IL-4 and IL-10, resulting in impaired memory Th1 responses.

The anti-CTLA-4 mAb seems to act as antagonist

Previous studies have shown that the UC10-4F10 mAb may act as antagonist and, if cross-linked, as an agonist. We therefore used mice deficient for the two activating receptors FcγRI and FcγRIII and showed that anti-CTLA-4 treatment similarly induced the development of cells producing high levels of IL-4 and IL-10 (fig 1C).

IL-10-producing T cells express high levels of ICOS receptor and display a Th2-like phenotype

Several observations prompted us to analyse ICOS expression on cell populations producing IL-10, as high expression of ICOS was linked to IL-10 secretion.¹⁸ We performed flow cytometry on LN cells from mice injected or not with anti-CTLA-4 and showed that the proportion of CD4⁺ T cells expressing high levels of ICOS was increased by about threefold in anti-CTLA-4-treated mice (fig 2A). For comparison, the proportion of ICOS^{high} among CD4⁺ T cells reached about 1% in all naive mice tested.

RT-PCR analysis of ex vivo sorted populations confirmed that mRNA for IL-10 was expressed at higher levels in CD4⁺ ICOS^{high} T cells (Supplementary fig 1). The analysis of several factors selectively affecting T helper development revealed that ICOS^{high} T cells displayed enhanced expression of mRNA for IL-4, and the transcription factors GATA-3 and cMAF, but lower expression of IFNγ and T-bet (a Th1-specific T box transcription factor), as compared with ICOS^{low} T cells (Supplementary fig 1). Finally, we compared the effect of anti-CTLA-4 treatment in wild-type versus STAT6 (signal transducer and activator of transcription 6)-deficient mice and found that lack of Th2-prone transcription factor STAT6 impaired the development of ICOS^{high} T cells (Supplementary fig 2). Collectively, these results suggest that UC10-4F10 injection during priming favours the generation of Th2-type cells.

ICOS^{high} T cells are Tregs

We next examined the phenotype of cell populations expressing various levels of ICOS by FACS analysis during memory responses. The IL-10-producing ICOS^{high} T cells were found to express higher levels of Foxp3, GITR and CTLA-4, three molecules involved in negative signalling, as compared with ICOS^{low} and ICOS^{neg} cell populations (fig 2B), suggesting that ICOS^{high} cells may belong to the regulatory lineage. Of note, the percentage of Foxp3⁺ cells among CD4 T cells was increased after anti-CTLA-4 treatment (14.1% (0.3%) vs 11.3% (0.3%) in mice injected with DCs pulsed with KLH only; 8.6% (0.7%) in naive mice), showing that anti-CTLA-4 induced the development of regulatory-type cells.

To determine whether these T cells are immunosuppressive, purified ICOS^{high} and ICOS^{low} T cells were sorted and stimulated in vitro with DCs pulsed with KLH. Figure 2C shows that ICOS^{high} T cells produced IL-10 (left panel) upon antigenic stimulation, whereas ICOS^{low} cells secreted IFNγ (right panel) in the same conditions. The capacity of ICOS^{high} T cells to suppress IFN production was tested directly by stimulating ICOS^{low} cells with KLH-pulsed DCs in the presence of decreasing numbers of ICOS^{high} T cells. IFNγ production was strongly inhibited in a dose-dependent manner (fig 2C, right panel), whereas IL-4 was not affected (not shown). Collectively, these results indicate that ICOS^{high} T cells generated by anti-CTLA-4 treatment are immunosuppressive and inhibit Th1 responses in vitro.

To investigate whether IL-10 was involved in immunosuppression, neutralising antibodies to IL-10R were added into the co-cultures. Anti-IL-10R blocked the inhibitory effect of ICOS^{high} cells on IFNγ production by ICOS^{low} cells, indicating that the suppression in vitro was mediated by IL-10 (fig 2D). The role of IL-10 in vivo was assessed using IL-10 knockout (KO) mice as recipients. We injected KLH-pulsed DCs from C57BL/6 mice into

wild-type or IL-10-deficient mice with or without anti-CTLA-4 and tested the memory immune response after a second immunisation. IFN γ production was down-regulated in wild-type but not in IL-10 KO recipients when animals were treated with anti-CTLA-4 at priming (fig 2E). Although this experiment may be difficult to interpret because of the increased IFN γ production in IL-10 KO mice, these results suggest that ICOS^{high} T cells suppress Th1-type responses by secreting IL-10 in vitro and in vivo. Of note, similar numbers of ICOS^{high} T cells were detected in anti-CTLA-4-treated wild-type and IL-10 KO mice (not depicted), showing that IL-10 is not required for their differentiation.

Next, we tested whether depletion of naturally occurring Tregs¹³ would impact the development of ICOS^{high} cells and showed that anti-CTLA-4-induced differentiation of IL-10-producing cells was unaffected by CD25⁺ cell depletion, suggesting that ICOS^{high} T cells are distinct from the natural Treg population (Supplementary fig 3).

Anti-CTLA-4 treatment ameliorates TNBS-induced colitis

The inhibitory effect of anti-CTLA-4 treatment on Th1 responses prompted us to determine whether induction of ICOS^{high} Tregs may control Th1-mediated colitis.^{19, 20} C57BL/6 mice were given 1 mg of TNBS intrarectally and treated or not daily for 4–5 days with 100 μ g of UC10-4F10. Administration of anti-CTLA-4 clearly ameliorated the disease, as assessed by increased survival and decreased weight loss (fig 3A,B). Macroscopic and histological examination of TNBS-injected mice (fig 3C,D) revealed a thickening of the colon wall, with a predominant inflammatory infiltrate in the lamina propria, and necrosis extending deeply into the muscular and serosal layers. Of note, UC10-4F10 treatment decreased colitis severity and reduced inflammatory lesions (fig 3C,D). In contrast, this treatment had no effect in mice with oxazolone colitis (Supplementary fig 4).

Finally, we tested the cytokine profile of the mesenteric lymph nodes after in vitro stimulation and showed that anti-CTLA-4 treatment favoured the production of Th2-type cytokines (Supplementary fig. 5).

Anti-CTLA-4 treatment ameliorates TNBS-induced colitis in an IL-10- and IDO-dependent manner

We first tested the role of IL-10 in the amelioration of colitis. Our data in fig 4 indicate that administration of neutralising anti-IL-10R mAb prevented amelioration of disease, as assessed by survival (fig 4A) and weight loss changes (fig 4B), showing that IL-10 was critical. Histological analysis revealed a more extended zone of inflammation in the colon of anti-IL-10R-treated mice (not shown). We next analysed the mucosal production of cytokines by cells from the lamina propria stimulated with anti-CD3 and anti-CD28 mAbs in vitro. The data in fig 4C indicate that anti-CTLA-4 treatment decreases the production of IFN γ in mice displaying normal colonic tissues (but not in the mouse marked* showing macroscopic signs of strong inflammation). IL-4 and IL-10 were not detected (not shown). Neutralisation of IL-10 in vivo strongly increased the mucosal production of IFN γ , showing that anti-CTLA-4 treatment inhibits the ongoing Th1 response of the intestine through the production of IL-10. Of note, this finding correlates with a decreased expression of IL-12p40 mRNA in the colon (not depicted).

Because previous data showed that Tregs had the capacity to induce IDO production by DCs, we addressed the role of IDO in vivo using the pharmacological inhibitor 1-MT and IDO KO mice¹⁵ (table 1). Of note, IDO-deficient mice and mice treated with 1-MT were not rescued from lethal colitis by anti-CTLA-4 treatment, suggesting that IDO was involved in anti-CTLA-4-induced protection.

Finally, we examined whether amelioration of disease by anti-CTLA-4 treatment was associated with expression of IDO and Treg infiltration in the colon of TNBS-instilled mice. We assayed the expression of IDO using real-time RT-PCR. The group of anti-CTLA-4-treated mice was divided according to intestinal pathology: “inflamed” referring to altered intestinal transit, thickening of the colon, focal hyperaemia and intestinal ulceration. IDO expression was significantly increased in anti-CTLA-4-treated mice displaying normal colonic tissues, but not in mice showing macroscopic signs of inflammation (fig 5). These data illustrate an inverse correlation between intestinal pathology and local IDO expression. Next, flow cytometric analysis was performed to determine the percentage of Foxp3⁺ (fig 6A) and ICOS^{high} Foxp3⁺ (fig 6B–D) among CD4⁺ T cells. The proportion of Foxp3⁺ cells was enhanced in the mesenteric LNs and in the colon after anti-CTLA-4 treatment (fig 6A). Furthermore, the data revealed increased numbers of ICOS^{high} Foxp3⁺ among CD4⁺ T cells in the mesenteric LNs (fig 6B, see right panel for a summary of all experiments) and the colon (fig 6D, left panel) of mice treated with anti-CTLA-4 mAb. Of note, analysis of Ki67 expression showed an increased proliferation rate of ICOS^{high} Foxp3⁺ among CD4⁺ cells in the mesenteric LNs (fig 6C, see right panel for a summary of all experiments) and the colon (fig 6D, right panel) of mice injected with UC10-4F10, as compared with mice treated with TNBS only.

DISCUSSION

The main finding of this work is that treatment of primed mice with anti-CTLA-4 antibodies induces the development and/or expansion of adaptive Tregs expressing high levels of ICOS and producing IL-4 and IL-10. These cells inhibit memory Th1 responses *in vitro* and *in vivo*, and repress experimental intestinal inflammation by a mechanism involving IL-10 and IDO. The amelioration of disease was associated with expression of IDO and Treg infiltration in the colon.

Our results are consistent with an antagonistic effect of anti-CTLA-4 mAb which blocks negative signalling and promotes CD28/B7 interaction and differentiation of naïve T cells to “unbridled” Th2 cells. The importance of CD28 co-stimulation in the differentiation of Th2 cells was supported by several studies.^{21–23} Accordingly, CTLA-4 blockade enhanced Th2 priming,^{24–26} and CTLA-4-deficient T cells exhibited a dramatic tendency to differentiate into Th2 populations *in vitro*^{12, 27} and *in vivo*.¹⁰ In our study, the exact phenotypic alterations that UC10-4F10 injection induces in responding T cells remain to be determined: the critical role of STAT6 and the selective expression of GATA3 and cMAF in ICOS^{high} T cells, as well as the increased production of IL-4 and IL-10 by cells from mediastinal LNs in mice treated with TNBS and anti-CTLA-4, would be consistent with a Th2 phenotype. However, these cells express the transcription factor Foxp3 which is the hallmark of Tregs, suggesting that fully differentiated Th2 cells may further develop into Tregs, possibly upon the synergistic effect of high affinity T cell receptor engagement,²⁸ IL-4,²⁹ IL-10 and/or STAT6.³⁰ Consistent with this notion, low-dose CD28 superagonist treatment appears selectively to increase the pool of Tregs *in vivo*.³¹

Whether the suppression of Th1 immunity is due to Th2 or to Tregs is still a matter of speculation. It is interesting that very recent observations suggest that commitment to T cell lineages is more plastic than previously appreciated^{32, 33} (for comment see Rowell and Wilson³⁴). In particular, Tregs can be “destabilized”, lose Foxp3 expression and become effector cells (³⁵ and JA Bluestone *et al*, unpublished). We would conclude from our study that, at a certain time point, CD4⁺ ICOS^{high} T cells induced upon anti-CTLA-4 treatment display a regulatory function: (1) they express Foxp3, the transcription factor required for the development and function of Tregs (fig 2B); (2) they inhibit IFN γ production by effector cells *in vitro* in an IL-10-dependent manner (fig 2C,D); (3) they induce IDO expression by

DCs in vitro (not shown) and in vivo (fig 5 and table 1); and (4) their number correlates with the control of inflammation in vivo (fig 6).

In agreement with our observations, Kavanagh *et al*³⁶ have examined the effects of anti-CTLA-4 antibody in cancer patients and reported an expansion of CD4⁺ Foxp3⁺ Tregs displaying suppressor function. In contrast, data from Powrie and colleagues^{37, 38} have shown that anti-CTLA-4 administration abrogated inhibition of colitis by CD25⁺CD45RB^{low} cells, providing evidence for a role for CTLA-4 in the suppressive function of Tregs. This difference could be due to the nature of Tregs (ICOS^{high} vs CD25⁺) and/or to the protocol (three injections at the time of priming vs administration on alternate days for 6 weeks).

The similar phenotype in FcγRI/III-deficient mice is consistent with an antagonistic effect of the anti-CTLA-4 mAb, as the agonist effect would most probably require UC10-4F10 cross-linking.

Reports in the literature showed that injection of anti-CTLA-4 may enhance Th1-type immunity and²⁵ antitumour responses,^{39, 40} and accelerate acute rejection of cardiac allografts.⁴¹ Our own observations suggest that anti-CTLA-4 treatment may have a different effect depending on the cell populations (CD4 vs CD8, which differ in sensitivity to CTLA-4 negative signalling).⁴² Indeed, DC-induced cytotoxic responses were strongly enhanced following anti-CTLA-4 treatment (Supplementary fig 6). Collectively, these observations suggest that this treatment results in increased Th2/Treg and CTL responses but decreased Th1 responses. In agreement with our observations, Watanabe *et al* have reported that CTLA-4 blockade within the first week of *Helicobacter pylori* infection skewed the response towards Th2 and inhibited the development of gastric inflammation.²⁷

Our observations suggest that ICOS–ICOS ligand (ICOSL) interaction is not required for the development of Tregs but is critical for their production of IL-10 (not shown). Of note, a recent report⁴³ has revealed the existence of two functional subsets of human Foxp3⁺ Tregs, including ICOS⁺ cells which produced high amounts of IL-10.

There is evidence that reverse signalling through B7 after engagement of CTLA-4 on Tregs initiates the immunoregulatory pathway of tryptophan catabolism in DCs. The mechanisms by which IDO downregulates immune responses are still elusive and may involve multiple pathways.^{44–46} In our experiments, anti-CTLA-4 treatment similarly induced ICOS^{high} T cells in wild-type and IDO-deficient mice (not shown), suggesting that IDO did not act by triggering the differentiation of IL-10-producing Tregs but rather at the effector cell level. Of note, there is evidence that naïve CD4 T cells and Th2 cells were resistant to apoptosis induced by kynurenins,⁴⁷ an observation in line with our results showing that only the Th1-type memory response is downregulated following anti-CTLA-4 treatment.

IDO appears critical for the regulation of TNBS colitis upon CTLA-4 engagement, as the beneficial effect of anti-CTLA-4 treatment was lost in IDO-deficient mice. In contrast, the absence of IDO did not alter the course of inflammation in mice injected with TNBS only, an unexpected finding⁴⁸ which suggests that IDO-dependent counter-regulation requires other factors/cells in addition to IDO production and T cell activation by TNBS, possibly activated Tregs, producing IL-10 and expressing high levels of CTLA-4, ICOS and Foxp3. The respective role of IL-10 and IDO, both required for Th1 suppression in this model, is still unclear. Of note, our unpublished data confirm previous data^{49, 50} showing that IL-10 is critical for IDO production by DCs in vitro, suggesting that IL-10 may act upstream by modulating the expression of IDO. As IDO expression was detected in the colon but not in the spleen or mesenteric LNs, we postulate that IDO may act as anti-inflammatory agent in

the intestine and that IL-10 may inhibit Th1 activation in the lymphoid organs and enhance IDO expression in the gut.

An interesting question is whether ICOS^{high} T cells play a role as a counter-regulatory mechanism in the steady state—that is, to prevent inflammatory responses to gut flora. Our unpublished data reveal a higher proportion of ICOS^{high} T cells in Peyer's patches and mesenteric lymph nodes as compared with spleen and LNs, and suggest that bacterial colonisation is critical for the generation of ICOS^{high} Tregs, which were found to be absent in axenic mice. These observations suggest a critical role for this population in the control of inflammatory responses to intestinal flora. In contrast, we and others⁵¹ found that commensal bacteria were not required for the development of natural Tregs.

Conclusion

Our results demonstrate that anti-CTLA-4 treatment during priming may cause a qualitative change in the immune response. Injection of UC10-4F10 inhibited Th1 memory responses and conferred significant protection against disease in TNBS-induced colitis. Our data in vivo support the notion that the anti-CTLA-4 acts indirectly through the differentiation of adaptive Tregs producing IL-10 and inducing IDO production in the intestine. Our observations highlight anti-CTLA-4 treatment as a potential novel immunotherapeutic approach for inducing adaptive Tregs and treating autoimmune and inflammatory disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank O Leo for interesting discussions; F Andris, S Denanglaire, S Meghari and M Vermeersch for valuable help; and P Veirman for animal care.

Funding: The Laboratory of Animal Physiology is supported by grants of the Fonds National de la Recherche Scientifique (FNRS)/Télévie, by the Belgian Programme on Interuniversity Poles of Attraction initiated by the Belgian State, by the Programme of Excellence (CIBLES) initiated by the Walloon Region, by European Grants (DC-THERA, Cancerimmunotherapy) and by the Belgian Cancer Foundation. CC and GO have fellowships from the FNRS; MM is Research Director from the FNRS.

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Figure 1.

(A, B) Injection of anticytotoxic T lymphocyte-associated antigen 4 (CTLA-4) monoclonal antibody (mAb) increases interleukin-4 (IL-4) and IL-10 production and inhibits T helper 1 (Th1) memory responses. Keyhole limpet haemocyanin (KLH)-pulsed dendritic cells (DCs) were injected into the footpads of syngeneic C57BL/6 mice that were further treated with 100 μg of control mAb or anti-CTLA-4 mAb at days 3, 4 and 5 after immunisation. (B) Six days after priming, mice were challenged with KLH-pulsed DCs. Draining lymph nodes (LNs) were harvested 6 days after priming (A) or 2 days after boost (B) and cultured with graded doses of KLH. Proliferation and cytokine production were measured. (C) Fc γRI and III are dispensable for UC10-4F10-induced IL-4 and IL-10 production. C57BL/6 wild type (WT) and Fc $\gamma\text{RI/III}$ knockout (KO) mice were primed with KLH-pulsed WT DCs with or without treatment with anti-CTLA-4 mAb. LNs were harvested 6 d later and cells cultured with graded doses of KLH. IL-4 and IL-10 were measured in the supernatants. The critical role of activating Fc γRI and III to cross-link hamster mAb was demonstrated by the failure of soluble anti-CD3 mAb to activate Fc γRI - and Fc γRIII -deficient spleen cells in vitro (not

shown). (A–C) Data are shown as mean (SD) of at least three individual mice in each group and are representative of 30 (A), 12 (B) and 3 (C) independent experiments. IFN, interferon.

Figure 2.

CD4⁺ ICOS^{high} T cells display a regulatory phenotype. (A) Lymph node cells from C57BL/6 naïve or primed mice treated or not with control or anticytotoxic T lymphocyte-associated antigen 4 (CTLA-4) monoclonal antibody (mAb) were analysed ex vivo by flow cytometry for CD4 and inducible co-stimulator (ICOS) expression. Data are represented as cytometric dot plots of individual mice representative of three mice. The gate was set arbitrarily to calculate the proportion of cells expressing high levels of ICOS, and the numbers in the upper-right corners of the dot plots represent the percentage of cells in the quadrant. Data are from one experiment representative of 30. (B) C57BL/6 mice were primed with keyhole limpet haemocyanin (KLH)-pulsed dendritic cells (DCs) and treated with anti-CTLA-4, and further boosted in vivo with KLH-pulsed DCs. Lymph nodes were harvested 2 days later and analysed ex vivo by flow cytometry. Cells were triple stained for CD4, ICOS and Foxp3, glucocorticoid-induced tumour necrosis factor receptor (GITR) or CTLA-4 expression. Data are represented as cytometric dot plots of Foxp3, GITR or CTLA-4 staining on CD4⁺ cells gated according to ICOS expression. Data are of individual mice representative of three experiments. The numbers in the upper-right corners of the dot plots represent the percentage of cells in the quadrant. Data are from one experiment

representative of 12. (C, D) CD4⁺ T cells from lymph nodes of 10 C57BL/6 mice primed with KLH-pulsed DCs and treated with anti-CTLA-4 were pooled and sorted into two fractions expressing low or high levels of ICOS. The capacity of ICOS^{high} cells to suppress interferon γ (IFN γ) production was tested by stimulating 10⁵ ICOS^{low} cells with 2 $\times$ 10⁵ KLH-pulsed DCs in the presence of decreasing numbers of ICOS^{high} cells. IFN γ and interleukin-10 (IL-10) were measured in supernatants. When indicated, 10 μ g of α IL-10R mAb were added in the culture. (C) The bars represent the mean (SD) of three independent experiments. (D) Data are from one experiment representative of three. (E) C57BL/6 wild-type (WT) or IL-10 knockout (KO) mice were primed with KLH-pulsed DCs from WT or IL-10 KO mice and treated with anti-CTLA-4, and further boosted in vivo with KLH-pulsed DCs. Lymph nodes were harvested 2 days later and cultured with graded doses of KLH. Proliferation and cytokine production were measured. Data are shown as the mean (SD) of at least three individual mice in each group and are representative of three independent experiments.

Figure 3.

Anticytotoxic T lymphocyte-associated antigen 4 (CTLA-4) treatment ameliorates intestinal inflammation. C57BL/6 mice were instilled with trinitrobenzene sulfonic acid (TNBS; 1 mg/mouse) intracolonic and treated or not with daily injections of 100 μ g control or anti-CTLA-4 monoclonal antibody (mAb). (A) Kaplan–Meier plots of cumulative mice surviving vs time (10 mice per group). (C, D) Histopathology of colons from mice instilled with ethanol or TNBS (day –5) and treated with control or anti-CTLA-4 mAb. Sections were scored in a blind fashion in accordance with the inflammation and the damage/necrosis, as described in the Methods section. (B) Body weight change of groups of mice instilled with ethanol or TNBS, and treated or not with anti-CTLA-4.

Figure 4.

Interleukin-10 (IL-10) is critical for disease amelioration. C57BL/6 mice were instilled with trinitrobenzene sulfonic acid (TNBS; 1 mg/mouse) intracolonicallly and treated or not with daily injections of 100 μ g anticytotoxic T lymphocyte-associated antigen 4 (CTLA-4) monoclonal antibody (mAb) or control mAb. When indicated, 500 μ g of anti-IL-10R (11B1.2) mAb were co-injected daily with UC10-4F10 antibody. (A) Kaplan–Meier plots of cumulative mice surviving vs time. The difference in survival of mice treated or not with anti-CTLA-4 was very significant ($p = 0.0099$); the difference in survival of mice treated with anti-CTLA-4 with or without anti-IL-10R was significant ($p = 0.0197$), as determined by the log-rank test. (B) Body weight change of groups of 10 mice instilled with ethanol or

TNBS, and treated or not with anti-CTLA-4 and/or anti-IL-10R. Numbers in parentheses represent the number of surviving mice. (C) Lamina propria cells were stimulated in culture with 10 μ g of α CD3 and α CD28. Cytokine production was measured in supernatants 48 h later. Data are pooled from two experiments. IFN, interferon.

Figure 6.

Increased numbers of inducible co-stimulator (ICOS)^{high} T regulatory cells in the mesenteric lymph nodes (LNs) and the colon of anticytotoxic T lymphocyte-associated antigen 4 (CTLA-4)-treated mice. C57BL/6 mice were treated as in fig 5 and LNs and colons were harvested 3 days after trinitrobenzene sulfonic acid (TNBS) instillation. (A) The percentage of Foxp3⁺ T cells among CD4⁺ T cells was quantified in the mesenteric LNs and the colons of different groups of mice. (B, C) The proportion of ICOS^{high} Foxp3⁺ among CD4⁺ (B and D, left panel) and of proliferating (Ki67⁺) among ICOS^{high} Foxp3⁺ CD4⁺ (C and D, right panel) cells was quantified in the mesenteric LNs (B, C) and the colon (D) of mice treated or not with control or anti-CTLA-4 monoclonal antibody (mAb). Data are from one experiment representative of three (A, B and C, left panels) or are pooled from three experiments (B, C right panels and D). Symbols represent individual mice (B, C) or the mean of two (D) mice.

Table 1

IDO is required for disease amelioration

Mice	Untreated	α CTLA-4 treated	
Balb/c WT	13.3% (n = 15)	56.0% (n = 16)	p = 0.0273
Balb/c IDO KO	26.6% (n = 15)	20.0% (n = 15)	p = 0.8016
C57BL/6 + H ₂ O	7.3% (n = 26)	64.0% (n = 24)	p = 0.0004
C57BL/6 + 1-MT	14.9% (n = 26)	9.6% (n = 24)	p = 0.9536

Mice were instilled intracolonicallly with TNBS and treated or not daily with α CTLA-4 monoclonal antibody.

Data are presented as percentage of surviving mice.

α CTLA-4, anticytotoxic T lymphocyte-associated antigen 4; IDO, indoleamine 2,3 dioxygenase; KO, knockout; 1-MT, 1-methyl-L-tryptophan; TNBS, trinitrobenzene sulfonic acid; WT, wild type.