CTLA-4 (CD152) blockade does not cause a pro-inflammatory cytokine profile in regulatory T cells

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Abstract

Objective

The activation of T cells is closely regulated. One cell intrinsic mechanism is based on the expression of inhibitory molecules; another is mediated by regulatory T (Treg) cells. The co-regulatory molecule CTLA-4 is constitutively expressed by Treg cells and up-regulated in effector-T-cells after activation. Recently, it was described that Treg cells can display an unstable phenotype and convert into pathogenic pro-inflammatory cytokine secreting cells. Here we have analysed the role of CTLA-4 in the regulation of cytokine production by T-helper (Th) cells with a special focus on Treg cells.

Methods

Proliferation of unstimulated CTLA-4 knock-out and wild-type cells as well as their activation status and the impact of CTLA-4 blockade on proliferation of Treg and effector T cells under stimulation were analysed by flow cytometry. Furthermore, the cytokine concentrations were analysed by a multiplex suspension assay.

Results

CTLA-4 knock-out T cells proliferated without stimulation and displayed an activated phenotype ex vivo. Proliferation of effector but also that of Treg cells was controlled by CTLA-4. The blockade of CTLA-4 led to an increased secretion of GM-CSF, IL-1β, IL-2, and IFN-γ by Th cells. However, the blockade of CTLA-4 in Treg cells did not cause any conversion into pathogenic pro-inflammatory T cells, since the non-cytokine secreting phenotype remained unchanged.

Conclusion

These results have major implications on therapies targeting the CTLA-4-system, e.g. by CTLA4-Ig or anti-CTLA-4-antibodies, as the blockade of CTLA-4 did not unlock the stability of Treg cells.

Key words

CTLA-4, cytokines, co-stimulation, regulatory T cell

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The study was supported by grants SFB650 and SFB854.

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Received on August 29, 2010; accepted in revised form on December 15, 2010.

Competing interests: none declared.

Introduction

In order to ensure optimal functionality of the immune system, both the activation as well as the down-regulation of immune responses have to be regulated as well as possible. Several systemic as well as cellular mechanisms have evolved to guarantee the appropriate response of immune cells. Cytotoxic T lymphocyte antigen 4 (CTLA-4, CD152) plays a central role in systemic and cellular regulation of immune responses and is expressed constitutively in Foxp3$^+$ regulatory T cells (Treg cells) as well as in conventional T cells upon activation (1-3).

CTLA-4 is the major down regulatory molecule of immune responses (4, 5). While the expression of CTLA-4 is induced after the activation of effector T cells, its homologue CD28 is constitutively expressed on T cells and mediates positive co-stimulation (6). Both molecules share the same ligands – CD80 and CD86 – but CTLA-4 exhibits a 30-fold higher affinity (7, 8) allowing the replacement of CD28 in the binding to CD80 and CD86 (3, 9). CTLA-4 crosslinking leads to the inhibition of IL-2 transcription and cell cycle arrest (10). Additionally, CTLA-4 triggers CD80 and CD86 signalling which induces indoleamine-2,3-dioxygenase (IDO) in dendritic cells and, thus, tryptophan degradation causing the inhibition of T cell proliferation (11-13).

CTLA-4 deficient mice die at the age of 2–5 weeks due to a massive lymphoproliferative disease (14-16). In CTLA-4$^{-/-}$ mice, lung, liver, pancreas, heart and the intestine are infiltrated by T cells (15, 17, 18). Recently, it was shown that these activated and tissue infiltrating T cells are indeed autoreactive and that CTLA-4$^{-/-}$ mice, therefore, constitute a model of a very aggressive form of an autoimmune disease (19). Additionally, in humans, polymorphisms in the genomic region of CTLA-4 have been shown to be associated with several autoimmune diseases and a general susceptibility to autoimmune pathologies (14, 15, 20).

Another important mechanism to protect the organism from prolonged or autoimmune-driven immune responses are Foxp3-expressing CD4$^+$CD25$^+$ regulatory T cells which constitutively express CTLA-4 (2, 21, 22). In the periphery, it is 5-10% CD4$^+$CD25$^+$ regulatory T cells which are found among the CD4$^+$ T cell population (23). Natural CD4$^+$CD25$^+$ regulatory T cells arise in the thymus, while adaptive CD4$^+$CD25$^+$ regulatory T cells are induced in the periphery, *e.g.* via a CTLA-4 dependent TGF-β effect (24, 25). CTLA-4 is constitutively expressed in CD4$^+$CD25$^+$ regulatory T cells (2, 21, 22), and blockade of CLTA-4 *in vitro* leads to a reduced suppressive capacity (1, 2, 26). *In vivo* CTLA-4 blockade abrogates the ability of regulatory T cells to inhibit colitis (27), and the Treg cell mediated transplant acceptance is reduced when CTLA-4 is blocked (28). Moreover, the life span of CTLA-4$^{-/-}$ mice treated with wild-type (WT) Treg cells was significantly prolonged underlining the importance of CTLA-4 for the function of T cells (1).

Because of this overwhelming evidence of the necessity of T cells to maintain an intact organism, it was natural that the adoptive transfer of T cells was also used in preclinical trials to treat autoimmune diseases (29). However, recent studies have pointed out a potential drawback of this promising therapy as it was shown that T cells may be phenotypically unstable and are able to differentiate into cytokine secreting, pro-inflammatory cells potentially representing pathological T cells (30). How this detrimental conversion is regulated, remains as yet not fully understood.

Because of the important role of CTLA-4 for the regulation of T cell effector function, this molecule is clearly a candidate for the prevention of conversion of those cells to circumvent unwanted pro-inflammatory responses. In addition, CTLA4-Ig treatment of patients suffering from rheumatic diseases not only inhibits CD28 signalling on effector T cells but also blocks CTLA-4 signalling in T helper cells as well as Treg cells. Furthermore, blockade of CTLA-4 used in cancer therapies induces effective anti-tumour immune responses. Therefore, the present study addressed the question as to how the blockade of CTLA-4 on regulatory T cells affects the phenotype and cytokine expres-

sion of themselves in comparison to their target cells. Our results show that CTLA-4 blockade is not involved in the conversion of Treg cells into pro-inflammatory pathogenic T cells, and that this underlines an important safety aspect of antagonistic CTLA-4 approaches for treatments in autoimmune diseases.

Materials and methods

Mice

C57Bl/6 mice at the age of 5–7 weeks were used. CTLA-4$^{-/-}$ mice (10) were the offspring of CTLA-4$^{+/-}$ mice. They were genotyped by PCR using the following oligonucleotides: CTLA-4, 5'-Tgg AgT CCT TCA TAg TTA gg-3´, 5'-AAC ATC ACA CTC ACC ATC TTg C, neo 5'-CAT AgT TAg gTC TgT gAT gC-3´, and 5'-gCC TTC TAT CgC CTT CTT gAC g-3´and were used at the age of three weeks. All mice had a C57Bl/6 background and were bred under specific pathogen-free conditions in the Bundesinstitut für Risikobewertung (BfR) Berlin, Germany.

Antibodies and reagents

αCD62L (MEL-14, DRFZ), αCTLA-4 (UC10-4F10, DRFZ) and as isotype (560-31.1B9), αCD69 (H1.2F3, BD Pharmingen, USA), αCD25 (7D4, BD Pharmingen), αCD25 (pC61.5, DRFZ), αCD3 (145-2C11, BD Pharmingen), αCD4 (RM4-5, BD Bioscience), αCD4 (GK-1.5, DRFZ), and αMHC-II (M5/114, DRFZ) were used in their respective forms as FITC, PE, PerCp and Cy5 conjugates. αCTLA-4 Fab fragments and control Fab fragments were prepared with the Immunopure Fab preparation kit (Pierce Chemicals Co.) and used at 200 µg/ml. Magnetic microbeads αBio, αCD4, αFITC, αMHC-II and αFITC multisort beads (Miltenyi Biotech, Bergisch Gladbach) were used.

Cell cytometry

Cytometric analysis was performed using FACS Calibur and CellQuest software (BD Biosciences) or FlowJo (Tree Star, Ashland OR). Dead cells were excluded according to forward and sideward scatter and staining with propidium iodide (0.4 µg/ml).

Cell isolation

Isolation of different T cell subsets was performed using magnetic cell separation (MACS) according to the manufacture's instructions. CD4$^+$ T cells were isolated to a purity of >99%, CD4$^+$CD25$^+$ T cells to >92% using αBio-microbeads, and CD4$^+$CD25$^-$ T cells were isolated by an additional depletion of CD25$^+$ cells to a purity of >99%. MHC-II$^+$ APCs were isolated to a purity of >99% using αMHC-II-microbeads (irradiated 3000 rad). T cells were stained with CFSE (Molecular Probes, USA) at 5 µM for 7 min. After washing, cells were resuspended in RPMI 1640 (PAA, Pasching, Austria) containing 10% FCS (Sigma-Aldrich, Steinheim), 100 U/ml penicillin (PAA, Pasching, Austria), 100 U/ml streptomycin (PAA, Pasching, Austria), Glutamax-I (2 mM Glutamin, Invitrogen/Life Technologies, USA), 50 µM 2-Mercaptoethanol (Invitrogen/Life Technologies, USA).

Cell culture

Cell cultures were set up using 4x10^6 cells/ml in complete RPMI. T cells were stimulated with αCD3 at 1 µg/ml (145-2C11, BD Pharmingen) and APC: The T cell/APC ratio was 1:2. The CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells were cultured with APC alone or

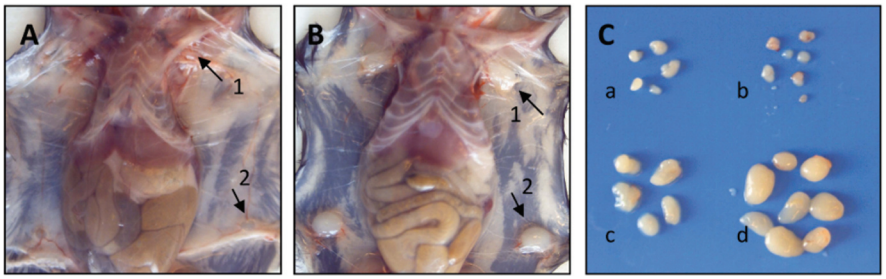
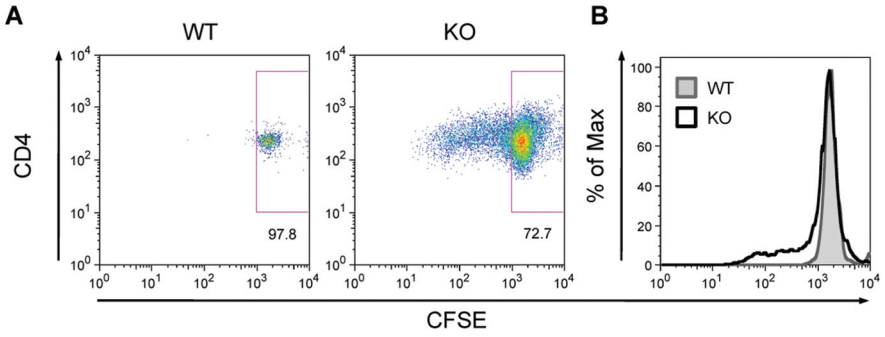


Fig. 1. CTLA-4 knock-out (KO) mice exhibit a lymphoproliferative disease. Three-week old CTLA-4 WT (**A**) and CTLA-4 KO (**B**) siblings are shown. Axillary (1) and inguinal (2) lymph nodes are indicated by arrows. (**C**) Direct comparison of mesenteric (a) and peripheral (b) lymph nodes of a CTLA-4 WT and mesenteric (c) and peripheral (d) lymph nodes of a CTLA-4 KO mouse.



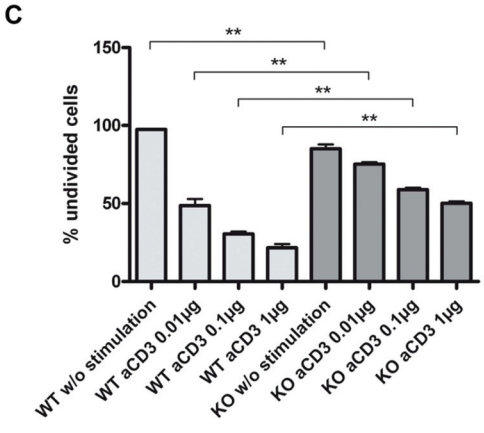


Fig. 2. CTLA-4 KO T cells are less responsive to proliferation signals. (**A**) Spleen cells of CTLA-4 WT and KO mice were cultured without stimulation. CD4$^+$ T cells were analysed for proliferation on day three as indicated by CFSE dilution. (**B**) An overlay of CTLA-4 WT and KO CD4$^+$ T cells indicates the different proliferation behaviour in the absence of a stimulus. One representative result is shown for n=6. (**C**) Spleen cells of CTLA-4 WT and KO mice were cultured without stimulation or in the presence of the anti-CD3 (aCD3) amounts indicated. CD4$^+$ T cells were analysed for proliferation on day three as indicated by CFSE dilution. n=6, Mann-Whitney test, $^{**}p<0.01$.

co-cultured at a ratio of 1:1. Proliferation was determined by flow cytometry using CFSE labelling on day 3.

Multiplex suspension array

Culture supernatants were frozen immediately and stored at -80°C until analysis. IL-1β, IL-2, IL-4, IL-5, IL-10, GM CSF, IFN-γ and TNF-α were quantified by multiplex suspension array (Bio-Rad Laboratories, Munich).

Statistical analysis

The Wilcoxon signed rank test was used for analysis of paired (dependent) samples and the Mann Whitney test was used for analysis of unpaired (independent) samples. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ were considered to be significant.

Results

The impact of CTLA-4 deficiency on the size of secondary lymphoid organs is illustrated in Figure 1. The phenotype of CTLA-$4^{-/-}$ mice is especially marked by a massive increase in the size of all lymph nodes. Axillary (1) and inguinal (2) lymph nodes in wild-type (Fig. 1A) and CTLA-4 deficient mice (Fig. 1B) are represented. A direct comparison of the size of lymph nodes from wild-type and CTLA-4 knock-out mice reflects the massive lymphoproliferative disease in CTLA-4 knock-out mice (Fig. 1C). The size of mesenteric lymph nodes (a, c) as well as that of peripheral lymph nodes (b, d) is drastically increased in mice lacking CTLA-4. This phenotype exhibits a central role of CTLA-4 for the homeostasis of peripheral T cells, part of which is also regulatory T cells.

The altered functionality of CTLA-4 deficient T helper cells (Th cells) is clearly illustrated by the spontaneous proliferation induced in about 30% of peripheral $CD4^+$ Th cells (Fig. 2A and B) in the absence of any extrinsic stimulus. However, in contrast the induction of proliferation of CTLA-4 knock-out cells by stimulation with anti-CD3 is significantly reduced compared to wild-type Th cells (Fig. 2C). The dose-dependent increase in the numbers of non-dividing cells after stimulation of different stimulatory strengths unexpectedly indicates a reduced responsiveness of CTLA-4 deficient $CD4^+$ T cells.

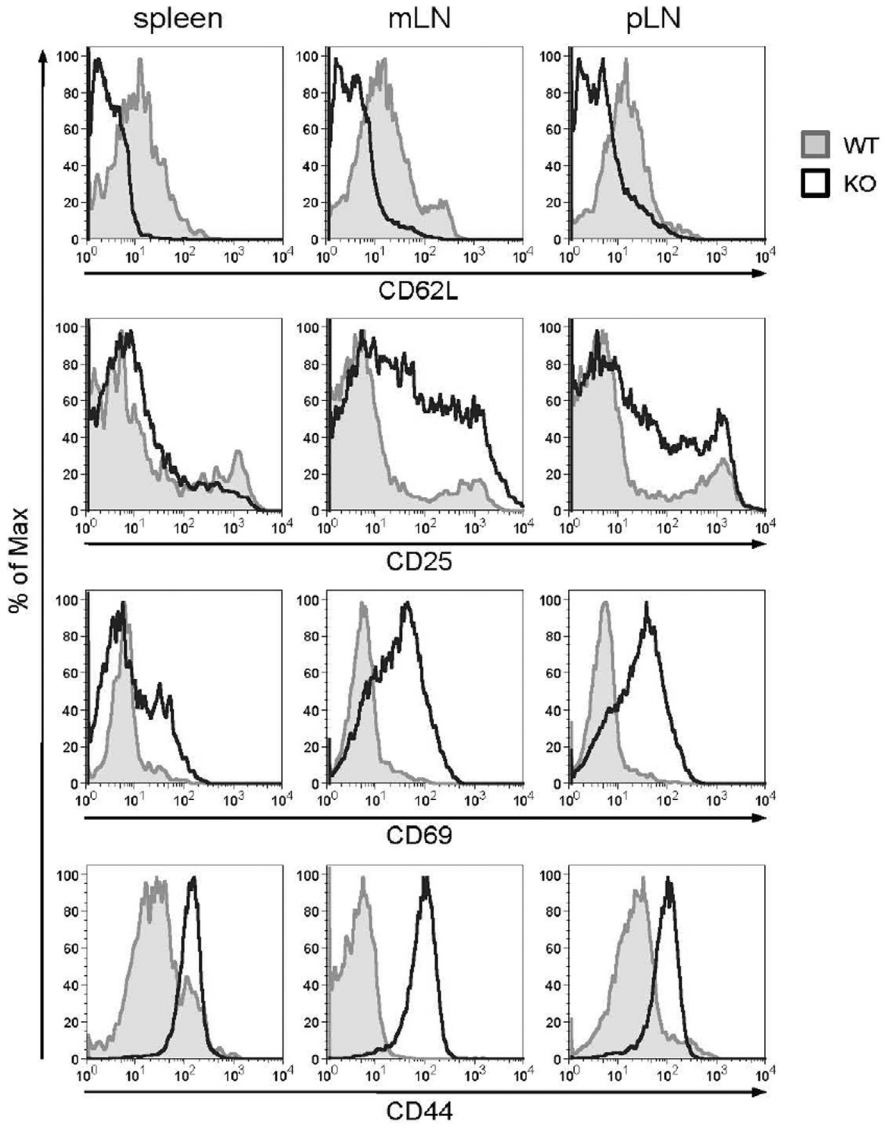


Fig. 3. CTLA-4 knock-out (KO) T cells show a high *in vivo* activation. CTLA-4 WT and KO mice were sacrificed at the age of three weeks. $CD4^+$ T cells from spleen, mesenteric lymph nodes (mLN) and peripheral lymph nodes (pLN) were analysed *ex vivo* for the expression of CD62L, CD25, CD69 and CD44. CTLA-4 WT $CD4^+$ T cells are shown as filled gray graphs, CTLA-4 KO $CD4^+$ T cells are shown empty black graphs. One representative result is shown for n=5.

To analyse the effect of CTLA-4 deficiency on Th cells in more detail, we conducted a phenotypic characterisation of $CD4^+$ T helper cells from wild-type and CTLA-4 knock-out mice. As shown in Figure 3 Th cells from CTLA-4 deficient mice are marked by a down-regulation of CD62L in spleen, mesenteric and peripheral lymph nodes. This is accompanied by an up-regulation of the activation-induced surface molecules CD25, CD69 and CD44. This result clearly illustrates that the absence of CTLA-4 in $CD4^+$ T cells leads to an activation of almost all of these T cells.

Since the activated phenotype of $CD4^+$ T cells from CTLA-4 knock-out mice could be due to CTLA-4 deficiency in conventional T cells as well as in regulatory T cells, we focused on the role of CTLA-4 for the functionality of natural Treg cells. For that reason we blocked CTLA-4 induced signalling by the addition of anti-CTLA-4 Fab-fragments. The addition of anti-CTLA-4 Fab-fragments to $CD4^+$ T cells from wild-type mice led to a routinely monitored increase in prolifera-

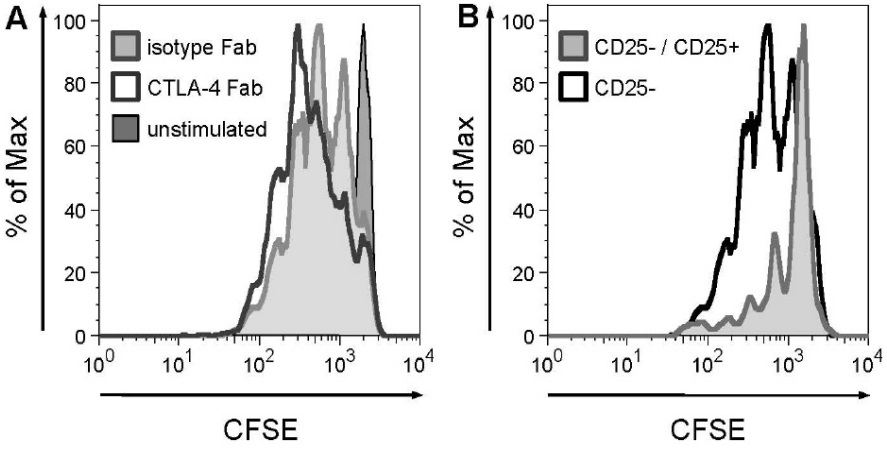


Fig. 4. Assay system: CTLA-4 blocking leads to enhanced proliferation of T cells, and CD4+CD25+ Treg cells suppress the proliferation of CD4+CD25- T cells. **(A)** CD4+CD25- T cells were isolated and left unstimulated or stimulated by anti-CD3 and APC. Control Fab fragments (isotype Fab) or blocking anti-CTLA-4 Fab fragments (CTLA-4 Fab) were added to the cultures. On day 3, the proliferation was analysed by CFSE dilution, n=6. **(B)** CD4+CD25- T cells and CD4+CD25+ Treg cells were isolated and CD4+CD25- T cells were cultured alone or co-cultured with CD4+CD25+ Treg cells. The cells were stimulated by anti-CD3 and APC. On day 3, the proliferation of CD4+CD25- T cells was analysed by CFSE dilution, n=6.

tion as indicated by an enhanced CFSE dilution (Fig. 4A). Because we aimed at the analysis of CTLA-4 function on Treg cells for the suppression of conventional T cells, we performed a Treg-suppression assay. Conventional CD25- T cells were co-cultured with CD25+ Treg cells and the proliferation was assayed routinely by CFSE dilution. As shown in Figure 4B, the addition of CD25+ Treg cells led to a drastic reduction in the proliferation of conventional CD25- Th cells. These results show the validity of our antagonistic CTLA-4 blockade as well as that of the assay system used to examine the functionality of Treg cells.

Next, we analysed the effect of CTLA-4 blockade for the production of cytokines by Treg cells. We examined the production of typical early pro-inflammatory cytokines GM-CSF, TNF-α, IL-1β and IL-2, and effector cytokines of Th cells such as IL-4, IL-5, IL-10 and IFN-γ. In conventional CD25- Th cells the blockade of CTLA-4 signalling led to a significant or at least numerical increase in the production of the pro-inflammatory cytokines IL-2 ($p<0.01$) and IFN-γ ($p<0.05$) but also of the Th2-associated cytokines IL-4 and IL-10 (Figure 5). In addition, the production of TNF-α, IL-1β ($p<0.001$) and GM-CSF ($p<0.01$) was enhanced after the inhibition of CTLA-4 in CD25- conventional T cells. In contrast, the production of the Th2 cytokine IL-5 was unaffected by anti-CTLA-4 Fab-fragment mediated blockade of CTLA-4. These results obtained in CD25- cells were in marked contrast to the effect of CTLA-4 blockade in CD25+ Treg cells alone. Here, none of the cytokines investigated was affected by the inhibition of CTLA-4 signalling. This result was also recapitulated in the co-culture of CD25- T cells and CD25+ Treg cells during a suppression-assay. The secretions of IL-1β, IL-10 and IFN-γ were only marginally and non-significantly increased upon CTLA-4 blockade. All other cytokines analysed were not influenced. Thus, the blockade of CTLA-4 during the inhibition-assay in the co-culture of CD25- T cells and CD25+ Treg cells did not lead to any significant up-regulation of cytokine production indicating that the repressive capacity of CD25+ Treg cells on the cytokine production of CD25- responder cells was not significantly abrogated by CTLA-4 inhibition. Interestingly, the cytokine secretion of all cytokines analysed was strongly suppressed in the co-cultures of CD25- T cells and CD25+ Treg cells when compared to CD25- T cells cultured alone with the exception of IL-5. CD25+ Treg cells showed only little or no effect on the production of IL-5.

Discussion

In this study we have examined the role of CTLA-4 for the production of cytokines in CD4+ Th cells with a special focus on CD25+ regulatory T cells. We could show that CTLA-4 is important for the homeostasis of CD4+ Th cells in several ways. Besides being responsible for the cell-intrinsic inhibition of effector T cells, a major reason for the phenotype of CTLA-4 deficient mice is the absence of CTLA-4 in regulatory T cells leading to altered homeostasis of Treg cells (1, 31, 32).

Here we demonstrate that CTLA-4 is not involved in the regulation of the production of either pro- or anti-inflammatory cytokines by CD25+ Treg cells indicating that the altered homeostasis observed in the absence of CTLA-4 is not caused by changes in cytokine production. Our observation using CD25- effector T cells in which CTLA-4 blockade does not equally regulate all cytokines analysed in the same way points towards a specific regulation of cytokine secretion by CTLA-4. The observed effect of enhanced cytokine production is not a mere cause of changes in the activation status of the effector T cells. How this specificity of CTLA-4-signalling is brought about remains a matter of further research, since the intracellular signal transduction induced by CTLA-4 is to our knowledge not yet fully understood (33). One mechanism is the activation of the ubiquitin ligase Itch, which has been shown to be responsible for the down-regulation of IFN-γ and IL-4 but not that of IL-2 (34).

Another important finding was that IL-10 seems to be dispensable for the suppression of conventional T cells since the blockade of CTLA-4 led to an up-regulation of IL-10 in CD25- T cells; however, these cells display an activated phenotype in CTLA-4 deficient mice. In addition, although regulatory T cells from CTLA-4 deficient mice are less effective in the suppression of other T cells, a lack of CTLA-4 mediated signalling in these cells does not lead to a conversion of these cells into pathogenic, pro-inflammatory T cells. This effect of Treg conversion has been described recently and the underlying mechanism is not known so far

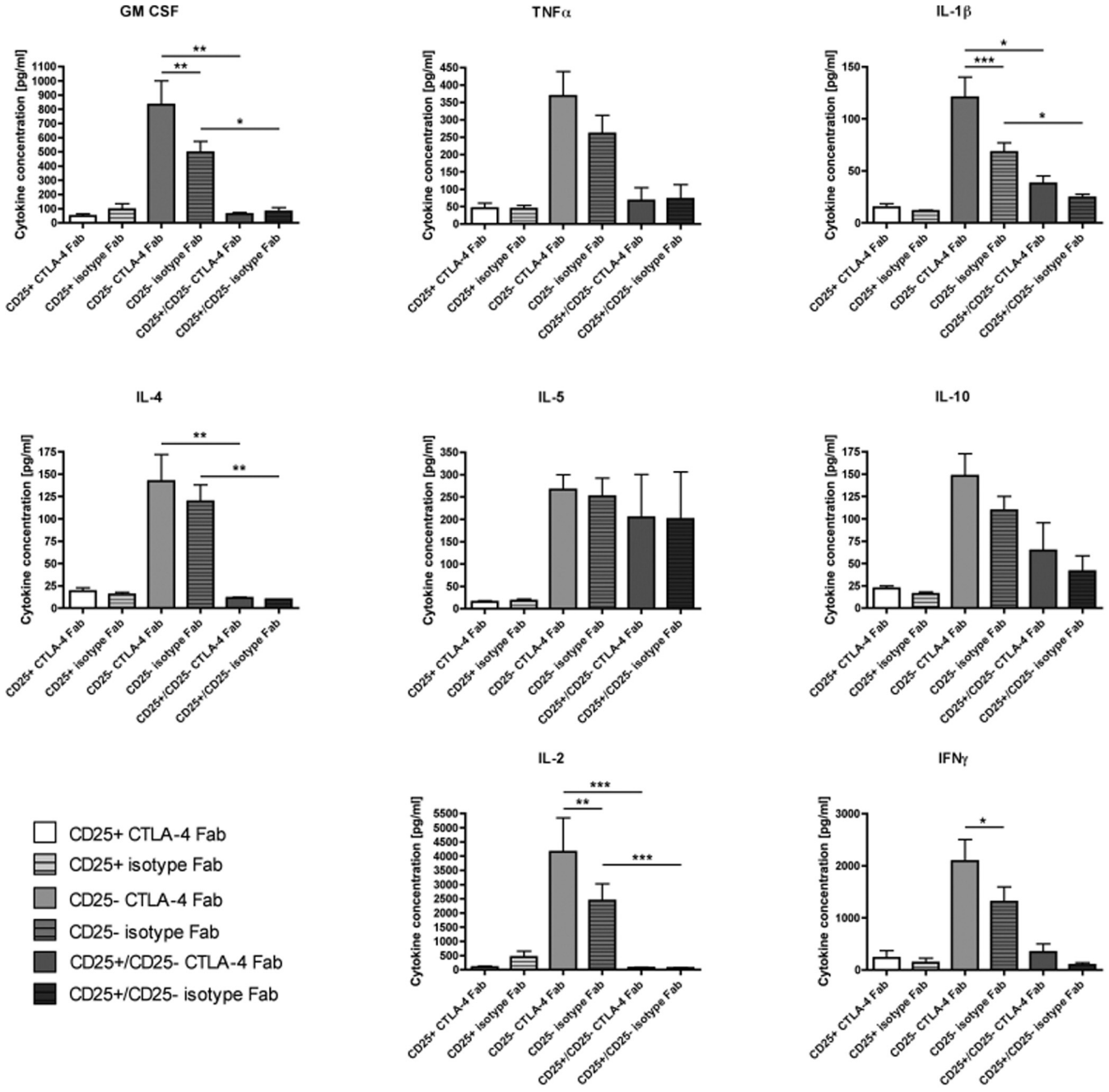


Fig. 5. CTLA-4 blocking does not cause a pro-inflammatory cytokine profile in $CD4^+CD25^+$ Treg cells. **(A)** $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ T cells were isolated and stimulated by anti-CD3 and APC either alone, or $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ T cells were co-cultured ($CD25^+/CD25^-$; 1:1). Blocking anti-CTLA-4 Fab fragments (CTLA-4 Fab) or control Fab fragments (isotype Fab) were added to the cultures. On day 3, the supernatants of the cultures were harvested and followed by cytokine analysis in the multiplex suspension array, n=6, Wilcoxon signed rank test (dependent samples), and Mann Whitney test (independent samples); $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

(30). Our results now show clearly that CTLA-4 is not involved in this process. Instead, cell-extrinsic factors must be responsible for the conversion of $Fop3^+$ Treg cells into pathogenic, cytokine secreting cells. It is very likely that it is a pro-inflammatory cytokine milieu present at sites of ongoing inflammation which leads to the instable phenotype of $Foxp3^+$ Treg cells. Moreover, this also has important implications for CTLA-4 targeting therapies such as CTLA4-Ig for autoimmune diseases or the blockade of CTLA-4 by anti-CTLA-4 antibodies, which is used in cancer therapy (33). Our results clearly point towards the safety of these therapies, as the induction of potentially dangerous converted ex-Treg cells is not mediated by the inhibition of CTLA-4.

In summary, our study highlights the role of CTLA-4 as a major and specific regulator of the production of pro- and anti-inflammatory cytokines, however, to a different extent in effector and regulatory T cells. While it plays an important role in the regulation of cytokines in conventional T helper cells, it has none or only a minor influence on the production of cytokines by

regulatory T cells. The stability of the regulatory phenotype in $CD25^+$ cells in the presence of antagonistic CTLA-4 agents suggests the safety of CTLA-4 targeting therapies in many clinical settings such as autoimmune diseases or cancer.

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