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CTLA4Ig PROMOTES THE INDUCTION OF HEMATOPOIETIC CHIMERISM AND TOLERANCE INDEPENDENTLY OF INDOLEAMINE-2,3-DIOXYGENASE (IDO)

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Abstract

Bone marrow transplantation (BMT) under costimulation blockade induces mixed chimerism and tolerance in rodent models. Recent data, predominantly from *in vitro* studies, suggest that in addition to blocking the CD28 costimulation pathway CTLA4Ig also acts through upregulating the tryptophan-catabolizing enzyme indoleamine-2,3-dioxygenase (IDO). Here we demonstrate that even though CTLA4Ig is critically required for the induction of chimerism and tolerance in a murine model of non-myeloablative BMT, IDO activity is not. No significant differences were detectable in the kynurenine to tryptophan ratios (indicative of IDO activity) in sera of BMT recipients treated with CTLA4Ig (tolerant group) versus BMT recipients treated without CTLA4Ig (non-tolerant group) versus naïve controls. *In vivo* inhibition of IDO immediately after BMT with CTLA4Ig or several months thereafter did not block achievement of chimerism and tolerance. Thus, IDO does not play a critical role in the induction or maintenance of chimerism and tolerance in a CTLA4Ig-based BMT model.

Keywords

tolerance; mixed chimerism; costimulation blockade; CTLA4Ig; IDO

The dimeric fusion protein CTLA4Ig (abatacept) has been rationally designed with the intent to prevent T cell activation by blocking the CD28 T cell costimulation pathway (through saturating binding to CD28's only known ligands CD80 and CD86) (1). Indeed, CTLA4Ig has been shown to effectively modulate T cell responses in numerous *in vivo*

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transplantation and autoimmune disease models (1-5) and has recently been approved by the FDA for the treatment of rheumatoid arthritis (6).

It has been suggested that CTLA4Ig and CTLA4-expressing T regulatory cells (T regs) have an additional mode of action by inducing the immuno-regulatory enzyme indoleamine-2,3-dioxygenase (IDO) in antigen-presenting cells (APC) by triggering a signal through CD80/CD86 leading to IFN γ -dependent induction of IDO (7;8). IDO is a tryptophan-catabolizing enzyme impairing T cell proliferation by tryptophan starvation and by the emergence of toxic downstream products (e.g. kynurenine) (9). The IDO inhibitor 1-methyltryptophan (1-MT) prevented maternal tolerance towards fetal alloantigen (10), and abrogated the graft-prolonging effect of CTLA4Ig in a pancreatic islet transplantation model (11) which is to date the only *in vivo* allotransplant model where the benefit of CTLA4Ig was shown to critically depend on IDO. Overall, the biologic relevance of IDO-mediated immunoregulation, however, remains a matter of debate (12;13).

A promising application of costimulation blockade is its use as a component of various BMT protocols for the induction of mixed chimerism and donor-specific skin graft tolerance (14-19). The immunological mechanisms underlying induction and maintenance of tolerance in such protocols have not been fully delineated yet. In particular, the role of IDO in mixed chimerism models has not been investigated so far. Here we demonstrate that induction of chimerism and tolerance in such a murine BMT model occurs independent of IDO, even though the regimen critically depends on CTLA4Ig.

First, in order to determine whether the success of this tolerance model depends on CTLA4Ig, groups of C57BL/6 (H2^b) mice received non-myeloablative total body irradiation (TBI, 3 Gy, d-1), approximately 20×10^6 unseparated, fully mismatched Balb/c (H2^d) bone marrow cells (d0) and costimulation blockade consisting of an anti-CD154 mAb (MR1; 1mg d0) with or without CTLA4Ig (0.5mg d+2) as previously described (20). The majority of mice treated with CTLA4Ig developed high levels of chimerism which remained stable in all tested leukocyte lineages for the length of follow-up (up to 46 weeks), whereas chimerism was not successfully induced in most recipients treated without CTLA4Ig (8/10 with vs. 3/10 without CTLA4Ig at week 12; $p < 0.05$; Figure 1A-B, data from one representative experiment). Also, permanent donor skin graft survival was observed in the majority of CTLA4Ig-treated BMT recipients (7/10, Figure 1C), while almost all donor grafts were rejected in the group treated without CTLA4Ig (1/10 acceptors, $p < 0.01$). Third party grafts were promptly rejected in all groups (Figure 1C) (pooled data of several similar experiments are shown in Table 1). Hence, CTLA4Ig plays a critical role in inducing chimerism and tolerance in this fully mismatched BMT model.

Costimulation-based mixed chimerism models critically require anti-CD154 mAb, and fail if this component is eliminated from an otherwise successful regimen (15;16;19). In contrast, costimulation blockade-based BMT models not requiring CTLA4Ig have been described (using anti-CD154 alone), but usually involve additional interventions such as donor-specific transfusion or CD8 depletion (21;22). Since the CTLA4Ig-based protocol used in the present studies crosses full major and minor antigen barriers it is a very stringent model with clinical relevance. The rate of successful chimeras achieved with the described protocol in the present study is consistent with previous experience (14;20). Likewise, we have previously observed that not all chimeras accept donor skin grafts permanently, the reason for which is currently under investigation, and might be related to tissue-specific antigens that are disparate between donor and recipient in this fully mismatched model.

Two approaches were employed to determine whether the effect of CTLA4Ig in this chimerism-tolerance model depends on IDO: 1) assessment of IDO activity by determining

the ratio of kynurenine to tryptophan in serum (denoted as kyn/trp in this study) (23); and 2) *in vivo* inhibition of IDO by implantation of pellets releasing 1-methyltryptophan (1-MT) (10;11).

Kyn/trp of plasma or serum samples taken at several time points remained low and were similar in BMT recipients treated with CTLA4Ig (tolerant group) or without CTLA4Ig (non-tolerant group) and in naïve controls, arguing against a systemic CTLA4Ig-induced tryptophan catabolism *in vivo* (day 3 post-BMT: Kyn/trp ratio 11.8 ± 2.7 with CTLA4Ig vs. 12.1 ± 3.3 without CTLA4Ig vs. 18.3 naïve control, day 4: 10.4 ± 3.4 vs. 13.9 ± 4.6 vs. 17.5, day 11: 16.2 ± 4.3 vs. 17.4 ± 6.3 vs. 15.4; $p = \text{n.s.}$ for each time-point; $n = 8-10$ per group) (Figure 2D). Furthermore, 1-MT pellets (see below) did not cause a change in kyn/trp during pellet release time (measured on day 10 after implantation), neither early nor late after BMT (Figure 2E). Studies investigating CTLA4Ig-mediated IDO mechanisms measured kynurenine levels in supernatants from *in vitro* or *ex vivo* cultured splenic DCs but not in the serum from experimental mice (8;11;24). In a Cyclosporine A-based murine cardiac allograft model kyn/trp in plasma was only elevated in untreated, rejecting mice but not in 1-MT treated mice (25). In our CTLA4Ig-based mixed chimerism model kynurenine levels remained low, close to the baseline kynurenine production which is predominantly maintained by the hepatic enzyme tryptophan-pyrrolase (26). These data reveal that CTLA4Ig has no detectable systemic influence on tryptophan catabolism in this model.

While the above described results suggest that IDO does not play an important role in this model, the effect of more localized IDO activity could theoretically be missed by serum measurements of tryptophan and kynurenine. To directly investigate whether IDO activity is necessary for chimerism and tolerance, we inhibited IDO activity with subcutaneously implanted pellets continuously releasing 1-MT, a competitive inhibitor of IDO (10) (7 day release time, 200 mg/pellet; purchased from Innovative Research of America, Sarasota, FL, USA (10))(11;27). An additional group of mice was implanted with placebo pellets. 1-MT pellet implantation (d-1, 7-day release) did not negatively affect chimerism induction, as the rate of successful chimeras (6/8 with 1-MT, Figure 1D) was similar to that of controls without pellets and placebo controls in the same experiment (7/12, not shown; $p = \text{n.s.}$). To rule out an insufficient duration of IDO inhibition, we implanted an additional pellet on day 6 in a separate experiment achieving a 14-day-release period that covers the main phase of tolerance induction during which CTLA4Ig and T regs are active (20). Again, chimerism was comparable to those in untreated mice in the same experiment (Figure 1E). Chimerism among all lineages even tended to be somewhat higher in 1-MT-treated animals (1-MT for 7 days or 14 days) compared to controls without pellets (from the same experiment) throughout follow-up, reaching statistical significance two weeks post-BMT for the myeloid lineage (10.1% vs. 35.8% MAC1⁺ donor cells; chimeras with CTLA4Ig vs. chimeras plus 1-MT for 14 days, $p < 0.05$). Moreover, although more subtle effects of inhibiting IDO cannot be completely ruled out, there was no significant negative influence on the median survival time (MST) or the rate of donor skin graft acceptance between mice treated with 1-MT and the controls from the same experiments (pooled data shown in Table 1). Thus, in this BMT model the chimerism- and tolerance-promoting effect of CTLA4Ig is not reversed by inhibition of IDO.

The divergent results between islet allograft studies (11) and this BMT model regarding IDO dependency might be due to the different tolerance mechanisms playing a role in these protocols (28). Importantly, tolerance induction in a related costimulation-blockade based mixed chimerism protocol has previously been shown not to depend on IFN- γ (17), the suggested key regulator of the IDO pathway (7). Moreover, we used the recently FDA-approved version of CTLA4Ig (abatacept) which is a human CTLA4-IgG1 construct, which may have different binding and signaling properties than the murine CTLA4-IgG3 which

showed IDO-dependency in the pancreatic islet allo-transplantation model. Murine IgG3 is known to self-aggregate via Fc-portions, which could potentially enhance signaling through B7 (29). Notably, we have previously shown that tolerance induction with the protocol used herein requires regulation by CD4 T regs (20), which the present data seem to suggest is not prevented by IDO inhibition.

In order to investigate whether IDO activity has a critical role in the maintenance of tolerance, we implanted 1-MT inhibitor pellets (14-day-treatment; n=5) and placebo pellets (n=3) in long-term chimeras bearing healed-in donor skin grafts. All mice maintained chimerism and only one mouse in the 1-MT group rejected its donor graft (Figure 2 A-C), suggesting that IDO inhibition does not abrogate established tolerance in mixed chimeras. Plasma samples of mice having received 1-MT pellets, placebo pellets or no pellets were taken on day 10, during the 14-day-release time and were analyzed for 1-MT concentrations by HPLC (Figure 2F). All tested mice implanted with 1-MT pellets showed 1-MT levels well above the concentration shown to be effective in inhibiting IDO (11), demonstrating that the 1-MT pellets used for IDO inhibition were active (Figure 2F).

Taken together, these data demonstrate that IDO plays no critical role in the induction and maintenance of chimerism and tolerance in this *in vivo* model, even though the regimen critically depends on CTLA4Ig. Thus, CTLA4Ig presumably acts by virtue of its costimulation-blocking effect.

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ABBREVIATIONS

BMT	bone marrow transplantation
IDO	indoleamine-2,3-dioxygenase
kyn	kynurenine
mAb	monoclonal antibody
TBI	total body irradiation
T regs	regulatory T cells
trp	tryptophan
1-MT	1-methyltryptophan

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Figure 1. Induction of chimerism and tolerance depends on CTLA4Ig but not on IDO activity (A-C) 8/10 C57BL/6 mice that received 3 Gy TBI, BMT (20×10^6 Balb/c bone marrow cells), anti-CD154 with CTLA4Ig, but only 3/10 mice receiving the same regimen without CTLA4Ig showed high-levels of multi-lineage mixed chimerism 12 weeks post BMT. In the group receiving CTLA4Ig the two non-chimeras and one chimera died before the end of follow-up, resulting in 7/7 long-term chimeras. Of those animals all accepted donor skin grafts for more than 200 days. In the group without CTLA4Ig, one chimera lost chimerism at week 32 and another chimera died before week 46, leaving only 1/7 with a donor graft. Third party grafts were promptly rejected in all groups. Data shown are from one representative experiment. **(D-E)** In a separate set of experiments mice were again treated with the CTLA4Ig protocol. Inhibition of IDO activity through the competitive inhibitor 1-MT was associated with long-term chimerism in 6/8 animals (subcutaneously implanted with 1-MT pellets on d-1, 7-day release time) and in 5/5 animals when implanted with an additional pellet on d 6 (14-day-treatment) (all mice received 3 Gy TBI, BMT, anti-CD154 with CTLA4Ig). Chimerism among all lineages tended to be even somewhat higher in 1-MT-treated animals compared to controls without pellets (from the same experiment) throughout follow-up, reaching statistical significance two weeks post-BMT in the myeloid lineage (e.g. 10.1% vs. 35.8% MAC1⁺ donor cells at two weeks post-BMT; chimeras with CTLA4Ig vs. chimeras plus 1-MT for 14 days, $p < 0.05$; *note*: chimerism levels in the groups with CTLA4Ig show some variability from experiment to experiment, as has been observed previously). Mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Anti-CD154 mAb was purchased from Bioexpress Inc. (New Hampshire, USA); hCTLA4Ig was generously provided by Bristol-Myers, Squibb Pharmaceuticals (Princeton, New Jersey). Experiments were approved by the local review board of the Medical University of Vienna. Skin grafting and flow cytometric analysis of chimerism were performed as previously described (20). A two-tailed Student's T test was used to compare chimerism levels between groups. The chi-square test was used for comparing rates of chimerism and rates of skin graft acceptance between groups. Skin graft survival was calculated according to the Kaplan-Meier product limit method and compared between groups by using the log-rank test. A P value less than 0.05 was considered to be statistically significant.

Figure 2. Maintenance of chimerism and tolerance are not dependent on IDO activity
 (A-C) 1-MT pellets and placebo pellets were implanted into stable, skin graft tolerant chimeras 8-11 months post-BMT (BMT performed with 3 Gy TBI, anti-CD154 and CTLA4Ig). 3/3 mice in the placebo group (A) and 5/5 mice treated with the IDO inhibitor 1-MT for 14 days (B) retained multilineage chimerism for the length of follow-up (13 weeks after pellet implantation) with no significant decline in chimerism levels. (C) Only one mouse in the 1-MT-treated group (n=5) and none of the placebo-treated mice (n=3) rejected healed-in Balb/c skin grafts. (D) At the indicated time points, tryptophan, kynurenine and 1-methyltryptophan serum concentrations were measured by HPLC (rp-18, acetate buffer, flow-rate 1 ml/min) via fluorescence and UV absorption detection, as described previously (23). Serum kynurenine/tryptophan ratios (kyn/trp, depicted as ratio multiplied by a factor of 1000) indicative of IDO activity were determined at several time points early after BMT (3 Gy TBI, BMT, anti-CD154 with and without CTLA4Ig) (p=n.s. for each time point, groups with CTLA4Ig vs. w/o CTLA4Ig vs. a naïve control; n= 8-10 per group). (E) Kynurenine and tryptophan levels were also measured in animals that received the BMT protocol with CTLA4Ig plus 1-MT pellets (measured during the 14-day-release time, day 10 post implantation). Kyn/trp ratios were similar between recipients of 1-MT pellets, placebo pellets or no pellets. When implanted into stable skin graft tolerant chimeras late after BMT, there was also no significant difference between mice receiving placebo pellets and 1-MT treated mice (again measured 10 days post implantation during 14-day-release time). A two-tailed Student's T test was used to compare ratios between groups. (F) HPLC results show that 1-MT (measured during the 14-day-1-MT release time, on day 10) is present at high levels in plasma of mice implanted with 1-MT-pellets (one day before BMT) or implanted into tolerant chimeras (8-11 months post-BMT), whereas 1-MT is undetectable in mice receiving no pellets or placebo pellets. n.d. indicates not detectable. Error bars indicate standard deviations.

Table 1

Pooled data of chimerism rates and skin graft survival with or without CTLA4Ig and with 1-MT treatment at the time of BMT

3 Gy, BMT, anti-CD154	rates of chimeras	donor skin acceptance >60 d	MST donor grafts (days)	MST 3 rd party grafts (days)
a. with CTLA4Ig (5 exp.)	32/46 [°]	23/41 [°]	64.5	11
b. w/o CTLA4Ig (2 exp.)	6/18 ^{°°}	1/17 ^{°°}	15.5	13
c. plus 1-MT for 7 days	6/8	3/8	26.5	12
d. plus 1-MT for 14 days (2 exp.)	13/14	6/12	59	13

Pooled data show that rates of chimeras and of donor skin graft acceptors were significantly different between BMT recipients treated with or without CTLA4Ig (32/46 vs. 6/18 chimeras and 23/41 vs. 1/17 tolerant; $p<0.01$ and $p<0.001$) with a median survival time (MST) for donor skin of 64.5 vs. 15.5 days. When mice received the IDO inhibitor 1-MT for 7 or 14 days at the time of BMT there was no significant effect on chimerism rates and skin graft survival (MST 26.5 and 59 days, respectively). Rates of chimeras and of tolerant mice were significantly higher after 1-MT treatment when compared to BMT recipients treated without CTLA4Ig (13/14 chimeras with 1-MT for 14 days vs. 6/18 without CTLA4Ig, $p<0.001$; 6/12 vs. 1/17 tolerant mice, $p<0.01$). Not all mice were successfully grafted due to a few isolated technical failures (two chimeras and one non-chimera in group a and one non-chimera in b). Two chimeras in d developed ulcers and rejected at day 53. 3rd party grafts were promptly rejected in all groups with a MST between 11 and 13 days. Exp. denotes experiments.

[°] a vs. b $p<0.01$, a vs. c $p=n.s.$, a vs. d $p=n.s.$

^{°°} b vs. c $p<0.05$, b vs. d $p<0.01$