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Cutting Edge: Anti-CD154 Therapeutic Antibodies Induce Infectious Transplantation Tolerance[1]

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Nondepleting anti-CD154 (CD40 ligand) mAbs have proven effective in inducing transplantation tolerance in rodents and primates. In the induction phase, anti-CD154 Ab therapy is known to enhance apoptosis of Ag reactive T cells. However, this may not be the sole explanation for tolerance, as we show in this study that tolerance is maintained through a dominant regulatory mechanism which, like tolerance induced with CD4 Abs, manifests as infectious tolerance. Therefore, tolerance induced with anti-CD154 Abs involves not only the deletion of potentially aggressive T cells, but also a contagious spread of tolerance to new cohorts of graft-reactive T cells as they arise. ***The Journal of Immunology*****, 2000, 165: 4783–4786.**

The Holy Grail of transplantation research has been to induce tolerance by a short pulse of therapy. Long-term graft survival has been achieved in rodents through the use of nondepleting mAbs such as anti-CD4 and anti-CD154 (1–7). The potential of anti-CD154 therapy to produce prolonged graft survival even across a full MHC mismatch in nonhuman primates (8, 9) has prompted an analysis of mechanisms involved. Two elegant recent reports showed that activation-induced cell death (AICD)[4] of potentially aggressive T cells is an important feature of the induction phase of the prolonged graft survival with CD154 Abs (10, 11). We have previously shown that the CD4 T cell population in mice can indeed be tolerized by CD154 Abs in circumstances where the $CD8^+$ population have been removed by prior Ab ablation (5). In such circumstances we were able to analyze the maintenance phase of tolerance and uncover a role for a contagious process of tolerance (infectious tolerance). Infectious tolerance has been observed by us following tolerance induction with nondepleting CD4 mAb in skin and marrow transplants over multiple minor histocompatibility barriers, and heart transplants across complete MHC histocompatibility barriers (7). We show in this study that tolerance, once it has been induced by CD154 Abs, cannot be broken by the adoptive transfer of large numbers of naive nontolerant T cells. When these naive cells are allowed to coexist with the regulatory population for 6 wk, they become tolerant themselves, so exhibiting infectious tolerance. These results lead us to conclude that tolerance induced with CD154 Abs involves not just the deletion of alloreactive T cells but also maintenance through a contagious spread of tolerance to new graft-reactive T cells as they arise.

Materials and Methods

Mice

CBA/Ca ($H\text{-}2^k$), CD52-transgenic CP1-CBA/Ca ($H\text{-}2^k$), and B10.BR ($H\text{-}2^k$) mice were bred and maintained in the specific pathogen-free facilities of the Sir William Dunn School of Pathology (Oxford, U.K.). All groups were age and sex matched. All procedures were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986.

Thymectomy and skin grafting

Mice were anesthetized with a mixture of 10 mg/ml Hypnodil and 2 μg/ml Sublimaze (Janssen, Tilburg, Netherlands). A dose according to body weight (0.12 ml per 20 g) was injected i.p. Thymectomy was conducted as described by Monaco et al. (12). In short, a longitudinal incision was made on the anterior surface of the neck, and the thymus was removed as two intact lobes by the application of negative pressure through a glass tip inserted in the anterior mediastinum. Skin grafting was conducted according to a modified technique of Billingham et al. (13). Briefly, a full thickness of tail skin (1×1 cm) was grafted on the lateral flank, and rechallenge grafts were placed on the contralateral flank. Grafts were observed on alternate days after the removal of the bandage and considered rejected when no viable donor skin was present. Statistical analysis of graft survival was made by the log rank method (14).

Adoptive cell transfer

Cells were obtained from spleens of adult naive CBA/Ca mice. A single cell suspension was obtained by passing the splenocytes through a 70 μm cell strainer (Becton Dickinson, Oxford, U.K.) and the erythrocytes were depleted by water lysis. Cells were counted with a hemocytometer, diluted in PBS, and 50×10^6 were injected i.v. into the tail vein.

mAbs and cell depletion

$CD8^+$ cell depletion in athymic mice was achieved using a mixture of 1 mg YTS 156.7 (15) and 1 mg YTS 169.4 (16) injected i.p. on days 1 and 0 with respect to transplantation. For depletion of CP1-CBA T cells, 0.1 mg CAMPATH-1H (17) was injected i.p. These mAbs, as well as MR1 (18), were produced in our laboratory by culture in hollow fiber bioreactors, purified from culture supernatants by 50% ammonium sulfate precipitation, and dialyzed against PBS, and the purity was checked by native and SDS gel electrophoresis (PhastGel; Pharmacia, St. Albans, U.K.).

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[4] Abbreviations used in this paper: AICD, activation-induced cell death; ATx, adult-thymectomized; CyCr, CyChrome; MST, median survival time.

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Flow cytometric analysis

Peripheral blood samples were depleted of erythrocytes by water lysis, washed, and resuspended in PBS, 1% w/v BSA, 5% v/v heat-inactivated normal rabbit serum, and 0.1% w/v sodium azide. Cells were incubated for 45 min at 4°C with directly conjugated CAMPATH-1H-FITC, CD8α-PE (Sigma, Poole, U.K.), and CD4-CyChrome (CyCr; PharMingen, San Diego, CA). The cells were washed, resuspended in PBS, 1% w/v BSA, 0.1% w/v sodium azide, and fixed in 2% v/v formaldehyde solution. Tricolor FACScan analysis (Becton Dickinson) was performed using CellQuest (Becton Dickinson) software.

Results and Discussion

We used CD8-depleted mice treated with CD154 Abs to produce tolerance to B10.BR skin grafts which differ over multiple minor histocompatibility Ags. This model has proven useful in previous descriptions of infectious tolerance with CD4 Abs, and many parameters of cell dose and potency in adoptive transfer studies have been well characterized (19). Previous studies have demonstrated that regulatory cells involved in CD4 Ab-induced transplantation tolerance are themselves $CD4^+$ (20). We wished to be able to distinguish any such $CD4^+$ regulatory T cells from naive CD4 T cells that could reject grafts. Therefore, we used CBA/Ca mice, transgenic for the human CD52 gene expressed under the control of the CD2 promoter, as the tolerized host, and normal CBA/Ca mice as a source of naive nontolerant T cells for adoptive transfer. It was thus possible to identify and specifically deplete host T cells using the CD52 specific CAMPATH-1H mAb (21). These transgenic mice, named CP1-CBA, are histocompatible with CBA/Ca mice (confirmed by acceptance of reciprocal skin grafts (data not shown)). Furthermore, when grafted with B10.BR skin (differing by multiple minor histocompatibility Ags), both transgenic and CBA/Ca strains rejected at a comparable rate (Fig. 1*A*). To study infectious tolerance we needed to be sure that once host T cells had been ablated by the CAMPATH-1H Ab, they would not replenish from the thymus. For that reason we used adult-thymectomized (ATx) CP1-CBA mice. Such ATx mice depleted of T cells with 0.1 mg of CAMPATH-1H mAb accept B10.BR skin grafts indefinitely (Fig. 1*A*). Analysis of PBLs from CAMPATH-1H treated mice by flow cytometry confirms that T cells are depleted to $<1\%$ (Fig. 1, *B* and *C*). This enabled us to use the CAMPATH-1H mAb to ablate T cells of the tolerant transgenic host whenever we wished, allowing us to determine the impact of their prolonged coexistence with naive CBA/Ca T cells.

In this study, ATx, $CD8^+$ cell-depleted CP1-CBA were tolerized to B10.BR skin under the cover of three doses of the nondepleting anti-CD154 mAb MR1, administered on days 0, 2, and 4 with respect to time of transplantation (5).

We investigated whether anti-CD154-induced tolerance was dominant by testing whether the CP1-CBA mice tolerized to B10.BR skin as described above could resist the adoptive transfer of spleen cells from naive CBA/Ca mice (22). Ninety days after tolerance induction, CP1-CBA-tolerant mice were injected i.v. with 50×10^6 spleen cells from naive CBA/Ca mice, and received a fresh B10.BR skin graft the following day. The naive cells did not break tolerance, as both the new and old skin grafts were accepted indefinitely (Fig. 2*A*). However, in control groups in which mice received MR1 treatment in the absence of a first skin graft, or where at the time of cell transfer, the host T cells had been depleted with CAMPATH-1H Ab, B10.BR skin grafts were readily rejected (Fig. 2*A*). The levels of donor T cell chimerism and host T cell depletion were analyzed by flow cytometry of PBLs (Fig. 2*B*). These observations indicate that although naive T cells did engraft, they were prevented from rejecting transplanted B10.BR skin by tolerized host T cells.

As dominant tolerance induced with CD4 Abs has been shown to involve infectiousness (tolerant cells imposing tolerance on naive cells (7, 20, 23)), we investigated whether infectious tolerance had been induced by anti-CD154 therapy. Indicator CP1-CBA mice that had been adult thymectomized and depleted of $CD8^+$ T cells were tolerized to B10.BR skin grafts as above. After tolerance had been confirmed by graft maintenance for 90 days, 50×10^6 spleen cells from naive CBA/Ca mice were transferred i.v. into these tolerant mice, which then received a second B10.BR skin graft the following day. This dose of spleen cells is well in excess of the dose needed to get rapid graft rejection in this model (19). When host cells were depleted with the CAMPATH-1H mAb on the day following the adoptive transfer, the naive CBA/Ca cells were fully competent to reject the new skin graft, as well as the original one (Fig. 3*A*). However, if the naive CBA/Ca cells were allowed to coexist with the tolerant cells for 6 wk, before depletion

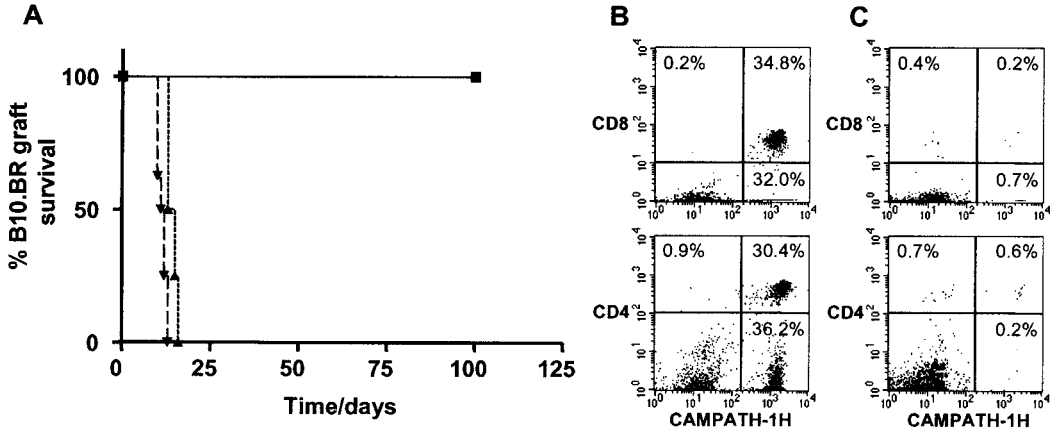

FIGURE 1. CP1-CBA reject B10.BR skin grafts at a similar rate to CBA/Ca, but not after T cell depletion with CAMPATH-1H mAb. *A*, Adult CP1-CBA (▲, $n = 4$) mice reject B10.BR skin grafts at a rate comparable with CBA/Ca (▼, $n = 8$) recipients. Adult ATx CP1-CBA donors treated with 0.1 mg CAMPATH-1H mAb before B10.BR skin grafting permanently accept these grafts (■, $n = 5$, median survival time (MST) > 100 days). *B*, PBLs were analyzed by flow cytometry following staining with CAMPATH-1H-FITC, CD8-PE, and CD4-CyCr. CP1-CBA mice T cells are double positive for either CD4 or CD8 and CAMPATH-1H. It should be noted that the $CD8^-$CAMPATH-$1H^+$ cells in the upper panel are superimposed close to the horizontal axis and can be seen as the $CD4^+$ cells in the accompanying lower plot. *C*, Euthymic CP1-CBA mice treated with 0.1 mg CAMPATH-1H show depletion of both $CD4^+$ and $CD8^+$ T cells 7 days after treatment.

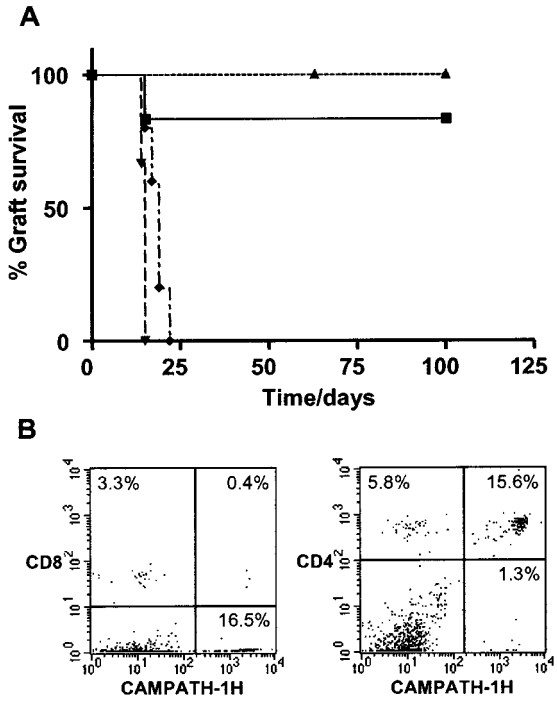

FIGURE 2. Anti-CD154 treatment of CD8-depleted mice induces dominant tolerance that is not broken by adoptive transfer of naive spleen cells. *A*, Adult ATx and CD8 cell depleted CP1-CBA transgenic mice received a B10.BR skin graft (multiple minor histocompatibility differences) at day 90, with three doses of 0.67 mg of the nondepleting anti-CD154 mAb MR1 i.p. at days 90, 88 and 86 to induce tolerance. At day 0, mice from groups designated ▲, ▼, and ♦ received 50×10^6 spleen cells i.v. from naive CBA donors. All mice received a fresh B10.BR skin graft the following day and were monitored for graft rejection. Tolerant mice that received naive spleen cells accepted the grafts indefinitely (▲, $n = 7$, MST > 100), as did tolerant mice receiving no naive cells (■, $n = 6$, MST > 100 days, $p < 0.28$). The absence of rejection was not due to the persistence of MR1 mAb as all animals in the control group that received the tolerizing MR1 treatment in the absence of an initial skin graft rejected the graft (▼, $n = 6$, MST = 15 days). The transferred cells were competent to reject the B10.BR skin, as mice depleted of their own T cells with 0.1 mg CAMPATH-1H i.p. before cell transfer and skin grafting also rejected (♦, $n = 5$, MST = 19 days). *B*, PBLs were analyzed by flow cytometry following staining with CAMPATH-1H-FITC, CD8-PE, and CD4-CyCr. Host lymphocytes, from CP1-CBA transgenic mice, are CAMPATH-1H$^+$. The CBA/Ca T cells that were adoptively transferred were CAMPATH-1H$^-$. Therefore, it was possible to monitor the efficiency of cell transfer by flow cytometry of the CD4 and CD8 populations of CAMPATH-1H$^-$ cells. An example of a mouse from group ▲ is shown where ~25% of CD4$^+$ and almost all of CD8$^+$ T cells are from the donor (the host had been CD8 depleted).

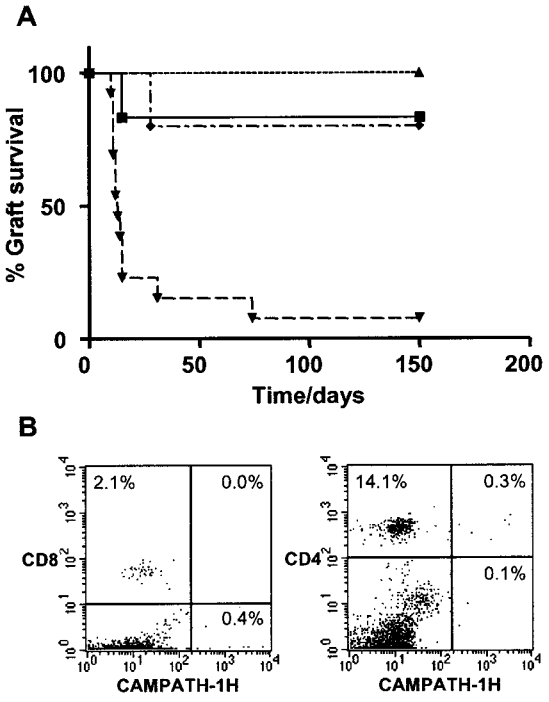

FIGURE 3. Anti-CD154 treatment induces infectious transplantation tolerance. *A*, Transplantation tolerance to B10.BR skin was induced in ATx CP1-CBA transgenic mice with MR1 mAb as described before. Mice from the group designated ■ ($n = 6$) did not receive naive cells. Spleen cells (50×10^6) from naive CBA donors were adoptively transferred into the animals of all other groups at day 0 (90 days after tolerance induction), followed by a fresh B10.BR skin graft at day 1. In group ▼ ($n = 13$), where host transgenic T cells were depleted at day 1 with 0.1 mg CAMPATH-1H mAb i.p., both fresh and old skin grafts were rejected (MST = 13 days). In group ♦ ($n = 5$), the CBA lymphocytes were allowed to coexist with the CP1-CBA cells for 6 wk, after which host T cells were depleted with CAMPATH-1H and a further B10.BR skin was grafted (MST > 150). There was no statistically significant difference in graft survival for this group when compared with the groups that did not receive naive CBA spleen cells (■, $n = 6$), or in which the CP1-CBA cells were not depleted (▲, $n = 7$). There is a significant difference between the groups depleted at day 1 (▼) and day 45 (♦) after transfer ($p < 0.006$). In animals receiving multiple grafts, the most recent one was always the first to be lost and the one considered in the analysis of data. *B*, PBLs were stained with CAMPATH-1H-FITC, CD8-PE, and CD4-CyCr and analyzed by flow cytometry, as before, to confirm the efficiency of depletion and cell transfers. An example of a mouse from group ♦, 45 days after CAMPATH-1H depletion is shown, where the presence of CAMPATH-1H$^-$ T cells of donor origin can be seen while the host T cells are <1%.

of host cells with CAMPATH-1H mAb, and challenged with a third B10.BR skin graft on the day following depletion, all three B10.BR skin grafts were accepted indefinitely (Fig. 3*A*). Flow cytometry confirmed donor T cell chimerism, as well as effective host T cell depletion (Fig. 3*B*).

Taken together with our previous findings of linked suppression in this model of transplantation tolerance (5), these results indicate that therapy with anti-CD154 in this context has a more profound impact than can be explained just by deletion of potentially aggressive T cells by AICD (10, 11). The notion of the need for AICD arose from transplants across MHC barriers, and it is conceivable that AICD may not be essential in tolerance across multiple minor differences (24), although this remains to be established. Equally, although infectious tolerance can be shown to operate across MHC barriers when tolerance is induced with CD4 Abs, it has not been formally demonstrated with CD154 Abs. Whether or not AICD is operational in tolerance achieved across multiple minor differences, we must conclude that a population of CD4$^+$ regulatory T cells emerges from among the Ag-reactive T cells, and that these are responsible for the maintenance phase of tolerance. They do so not just by actively suppressing rejection, but also by imposing tolerance on naive cells through infectious tolerance.

There are two possible mechanisms by which these regulatory cells may arise. One (two-population model) is that these regulatory cells are already present in the T cell pool as a distinct subpopulation. If they were less susceptible to AICD than potentially aggressive cells they would persist after anti-CD154 therapy and thus the ratio between regulators and aggressors would be altered to favor tolerance. A variant of this first model might require that

AICD of the potentially aggressive T cells permits the graft to survive long enough, for a regulatory $CD4^+$ cell population to expand and then dominate. The other possible mechanism (single-population model) is that potentially aggressive T cells which failed to die from AICD might have changed function to become regulators as a consequence of the Ag recognition in a tolerogenic environment. We cannot, at present, distinguish between these two possibilities.

To exert infectious tolerance, such regulatory T cells would need to influence naive T cells. This could be either by influencing the local microenvironment of Ag presentation where both types of cells (regulatory and naive) were in close proximity, or alternatively by decommissioning APCs so that they presented to naive T cells for tolerance rather than immunity.

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