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Cutting Edge: Sustained Expansion of CD8⁺ T Cells Requires CD154 Expression by Th Cells in Acute Graft Versus Host Disease

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Brief treatment with αCD154 Ab has been shown to prevent acute graft versus host disease (aGvHD). We extend these data to show that in the absence of CD154 function, donor T cells are unable to expand or generate high level anti-host CTL activity. Using transgenic (Tg) allosecretive CD8⁺ T cells adoptively transferred into allogeneic recipients, we show that short-term expansion of the CD8⁺ Tg T cells occurred in the absence of Th cells, and this short-term expansion could be facilitated with an agonistic αCD40. While CD40 agonism could enhance short-term expansion, sustained expansion of CD8⁺ Tg T cells required bona fide CD154-expressing CD4⁺ allosecretive Th cells. While CD154 was necessary for CD8⁺ Tg cell sustained expansion, IL-2 was also implicated as essential. These observations suggest αCD154 therapy in GvHD is effective because the treatment causes an abortive CD8 allosecretive activity. The data show that in the absence of CD154 function, donor T cells and the subsequent generation of anti-host CD8⁺ T cells are unable to expand or generate high levels of anti-host CTL. Furthermore, it was shown that an agonistic αCD40 mAb was able to enhance the short-term expansion of TCR transgenic (TCR Tg) CD8⁺ T cells specific for host alloantigen. However, sustained expansion of the TCR Tg T cells requires help provided by donor IL2-producing, CD154-expressing CD4⁺ T cells.

Materials and Methods

Mice

Six- to 8-wk-old (C57BL/6 × DBA/2)F₁ (B6D2F₁) and C57BL/6 (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The CD154⁻/⁻ mice (in a mixed background C57BL/6 × 129) were produced as previously described (8). 2C TCR Tg mice were kindly provided by Dr. D. Loh (University of Minnesota, Minneapolis, MN). Animals were maintained in the specific pathogen-free animal facility at Dartmouth Medical School.

Antibodies

MR1 (hamster αCD154 (9)), 1B2 (the clonotypic α-2C TCR, kindly donated by Dr. H. N. Eisen, Massachusetts Institute of Technology, Cambridge, MA), and FGK115 (rat αCD40, a kind gift of T. Rolink, Basel Institute, Basel, Switzerland) were produced as ascites and purified by HPLC. αCD8-PE mAb was obtained from PharMingen (San Diego, CA).
Induction of GvHD

Spleens from either C57BL/6, CD154+/−, or their wild-type H-2b/b littermates were aseptically removed and teased into a single-cell suspension. B6D2F1 (H-2d/b) recipients were injected i.v. with 10⁶ donor cells. Mice were injected i.p. with either αCD154 or hamster Ig, 250 μg/mouse/day, on days 0, 2, and 4 unless otherwise indicated. For studies using αCD40, mice were injected with 10 μg/mouse/day every other day starting day 0 until they were sacrificed.

Assessment of CTL activity in vitro

Spleen cells from in vivo-primed animals treated with αCD154 or untreated were rechallenged in vitro with mytomicin C-treated spleen cells from B6D2F1 mice. After 6 days the cultures were harvested, and the resulting live cells were used as effectors in the standard 4-h ⁵¹Cr release assay (6).

Adoptive transfer of L²-reactive TCR Tg 2C TCR CD8⁺ T cells

Lymphocytes from 2C TCR Tg mice were transferred i.v. (4–8 × 10⁸ Tg cells per recipient). In some experiments the IB₂⁺ Tg cells were enriched by panning on goat anti-mouse Ig-coated plates. In addition to Tg cells, some mice were given 25 × 10⁶ C57BL/6 spleen cells or 20 × 10⁶ C57BL/6 CD4⁺ T cells enriched by panning and CD8⁺ complement kill. To follow the expansion of the transferred Tg cells, lymphocytes from recipient spleens were harvested at different times and stained with 1B2-PE. Flow cytometry data was acquired using a FACScan (Becton Dickinson, Mountain View, CA).

Results and Discussion

CD154 on donor T cells is essential for GvHD-associated mortality

Previous studies from our laboratory established that treatment of mice with αCD154 blocked aGvHD (6). These observations were extended to address the question of whether CD154 expression on the donor T cells was critical for inducing aGvHD. To this end, donor T cells from CD154⁻/− mice were transferred into F₁ recipients. Results showed that animals given CD154⁺/⁺ cells died rapidly, with the average onset of death by day 21, and only 15% of the animals surviving out to day 60 (Fig. 1A). In contrast, for recipient mice given CD154⁻/− cells, the first death was not seen until day 37, and at day 60, 92% of the animals were still alive. Therefore, expression of CD154 on donor T cells is critical for the development of disease.

The treatment of mice with αCD154 (250 μg on days 0, 2, and 4) prevented GvHD mortality (100% of the animals were still alive at day 60; see Fig. 1A). Even a single dose of 250 μg of αCD154 administered the same day of parental cell transfer was adequate to protect against GvHD in >80% of mice for >70 days (Fig. 1B). Therefore, long-term blockade of CD154 is not essential for providing long-term protection.

CD40-CD154 signaling is essential for donor cell expansion and the development of CTL activity in GvHD

Although it is clear that mortality was reduced in the absence of CD154 function, other cellular aspects of GvHD were examined. To determine whether CD154⁻/− cells were capable of inducing CTL activity associated with GvHD, spleen cells from CD154⁻/− (C57BL/6 × 129) or CD154⁺/⁺ (C57BL/6 × 129) mice were transferred into B6D2F₁ recipients. On day 12, the animals were sacrificed and spleen cells were cocultured for 6 days with cells from B6D2F₁, to restimulate the alloreactive CTL generated in vivo. Secondary anti-allo-agonistic CTL activity was determined in a standard 4-h ⁵¹Cr release assay. Spleen cells from recipients given CD154⁺/⁺ cells exhibited high levels of anti-allo-agonistic CTL activity. In contrast, spleen cells from animals given CD154⁻/− cells had no detectable CTL activity (Fig. 2A). The lack of CTL activity in CD154⁻/⁻ recipients is not due to an intrinsic defect in the ability of CD154⁻/⁻ CD8⁺ T cells to generate CTL effector cells.

CD154 is essential for the expansion of alloreactive CD8⁺ T cells

To assess the possible role of CD154 on the expansion of alloreactive CD8⁺ T cells, a model employing alloreactive, Tg T cells was used. 2C TCR Tg mice express a TCR on CD8⁺ T cells which is reactive to H-2Lb (10). CD8⁺ Tg T cells (4–8 × 10⁶/mouse) were adoptively transferred into either syngeneic C57BL/6 (H-2b) or Ag-bearing F₁ (H-2bd) mice, and expansion of the Tg cells was followed using a clonotypic mAb by flow cytometry. Following adoptive transfer, mice were treated with either an agonistic αCD40 to facilitate expansion or αCD154 to block expansion. At time points after cell transfer, the recipients were sacrificed and the number of CD8⁺ Tg T cells was determined.
administration of an agonistic TCR cells/spleen) by days 10–12 after transfer (Fig. 3). Coadministic CTL activity was determined in a standard 4-h 51Cr release assay.

Ft treated with HIg. 

creased splenic cellularity is dependent on CD154. Total numbers of viable spleen cells were measured from B6D2F1 mice which received CD154+/−, or CD154−/− spleen cells. Mice receiving CD154−/+ spleen cells were treated with Hlg (●) or with anti-CD154 (●). Data in all panels are representative of three such experiments with three mice per group.

As can be seen in Fig. 3, upon adoptive transfer of CD8+ Tg T cells into an F1 recipient, there is a transient expansion of these cells. The expansion of Tg T cells is observed by day 4 posttransfer but is self-limited, diminishing to 1 × 106 cells/spleen at day 8 and reaching basal levels similar to syngeneic B6 hosts (0.1 × 106 2C TCR cells/spleen) by days 10–12 after transfer (Fig. 3). Coadministration of an agonistic αCD40 augmented the early expansion observed on days 4 and 8. However, αCD40 did not enhance the long-term expansion of the Tg T cells when measured on day 12.

Although αCD40 was ineffective at inducing sustained (12 days) expansion of the CD8+ Tg T cells, sustained expansion was observed upon the coadptive transfer of allogeneic CD154+/−CD4+ T cells. The coadptive transfer of either whole B6 spleen cells (data not shown) or purified B6 CD4+ T cells induced expansion of the CD8+ Tg T cells that could be detected at day 12 (Fig. 3). Treatment of recipient mice with αCD154 completely inhibited the helper effect of allogeneic CD4+ on the expansion of CD8+ Tg T cells. Therefore, sustained expansion of the CD8+ Tg T cells was dependent upon CD154 expression on donor-derived CD4+ T cells.

To establish that CD154 expression on the allogeneic CD4+ T cells was critical for CD8+ Tg T cell expansion, CD154+/− (129 × B6)F1 or CD154−/− (129 × B6)F1 CD4+ T cells were cotransferred with CD8+ Tg T cells. The intensity of the alloreac-

tive response by the CD154+/− CD4+ was less than that observed by CD4+ T cells on a B6 genetic background. Nonetheless, unlike CD154−/+ cells, the CD154+/− CD4+ T cells were completely ineffective at supporting CD8+ Tg T cell expansion.

The data show that allogeneic, CD154-expressing T cells are necessary, but CD40 triggering alone is not sufficient for sustained expansion of CD8+ Tg T cells. Additional support for this conclusion is provided by studies showing that T cells from IL-2−/+ mice, but not from IL-2−/− mice will support CD8+ Tg T cell expansion. Thus, in addition to CD154, IL-2 appears to be another factor critical for the sustained expansion of the CD8+ Tg T cell population (Fig. 3).

The stimulation of allogeneic CD8+ T cells in the absence of CD4+ cell help appeared to result in long-term CD8 anergy. A previous study (11) showed that when 2C TCR Tg T cells were adoptively transferred into an Ag-bearing host, a large percentage of them apoptosed after a transient, abortive expansion. Our own studies show that the abortive expansion of the Tg T cells observed on day 4 was not dependent on CD154 (data not shown); however, αCD40 potentiated the short-term expansion. Thus, short-term expansion of the CD8+ Tg T cells is CD154-independent but can be potentiated by CD40 triggering.

Recently, a number of reports have shown that αCD40 replaced T cell help in the development of CD8+ T cell responses. The generation of cytotoxic T cell responses specific to OVA (12), adenovirus E1B peptide (13), or H-Y (14) was enhanced by αCD40. We (15) and others (12, 14), have suggested that CD154-induced maturation of dendritic cells may be a critical event inducing CTL responses. Interestingly, blocking B7.1 and B7.2 signaling (16–18) or neutralizing IL-12 (19) has been shown to ameliorate GvHD. While we support the view that CD40 signaling of APCs is likely an essential element in driving CD8+ T cell expansion, αCD40 agonism cannot completely mimic the physical presence of CD4+ cells. In the model presented, we show that the

FIGURE 2. CD154−/− cells do not generate alloreactive CTLs in aGvHD. A, B6D2F1 recipients were given the equivalent of 1 spleen i.v. of either CD154−/− cells or CD154+/+ cells, and on day 12 the animals were sacrificed. After 1 wk of in vitro culture with B6D2F1 spleen cells, allogeneic CTL activity was determined in a standard 4-h 51Cr release assay using cells obtained from CD154+/+ recipients (●), CD154−/− recipients (▲), naive CD154+/+ cells (○) and naive CD154−/− cells (○). B, Decreased splenic cellularity is dependent on CD154. Total numbers of viable spleen cells were measured from B6D2F1 mice which received CD154+/+, or CD154−/− (▲) spleen cells. Mice receiving CD154−/− spleen cells were treated with Hlg (●) or with anti-CD154 (●). Data in all panels are representative of three such experiments with three mice per group.

FIGURE 3. Kinetics of the expansion of anti-Ld specific 2C TCR Tg CD8+ cells after adoptive transfer to Ag-bearing H-2bH F1 hybrids. Each recipient mouse was injected i.v. with 4–8 × 106 2C TCR Tg cells. Mice from some groups received an additional 20 × 106 purified CD4+ from wild-type, CD154−/−, or IL-2−/− C57BL/6 donors. Additionally, groups as indicated were treated with either αCD154 or αCD40 as indicated in Materials and Methods. At different points after cell transfer, lymphocytes were collected from the spleen and the number of Tg cells calculated by multiplying the total number of viable cells by the percentage of CD8+ IB2+ determined by flow cytometry.

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expansion in vivo of alloreactive Tg T cells is enhanced for only a short period of time by αCD40, with the number of Tg cells falling to basal levels by day 10–12 after injection. In contrast, a large number of CD8+ Tg cells persists by day 10–12 in the mice that had received bona fide alloreactive CD154+ CD4+ T cells as a source of help. Furthermore, we show that IL-2 production by alloreactive CD4+ T cells is also critical for CD8 expansion. Taken together, these observations suggest that the maturation of dentritic cells by CD154 (or αCD40) is essential for CD8+ T cell expansion but Th cells provide stimuli in addition to CD154 which are necessary to sustain the expansion of CD8+ T cells in vivo.

It can be suggested that in the absence of CD154 function, factors required for the survival of alloreactive CD8 T cells are not produced and these cells die and/or become anergic. Such a conclusion is consistent with the data presented and studies in another GvHD model whereby perforin mRNA-expressing T cells were undetectable in αCD154-treated recipients (7). With regard to the impact of CD154 blockade on the CD4 compartment, αCD154 treatment also has been shown to impair the expansion of the alloreactive CD4 compartment and reduce the frequency of inflammatory T cells expressing mRNA for IL-2, IL-12 p40, and IFN-γ mRNA (7). Therefore, because of the loss of alloreactive CD8 T cells and the skewing and reduced expansion of alloreactive CD4+ T cells, long-term tolerance is observed when CD154 function is impaired. Such observations provide a rationale basis for the use of αCD154 in the management of GvHD in humans.

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