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David M. Shepherd and Nancy I. Kerkvliet

CD154 (CD40 ligand, gp39) interaction with its receptor CD40 has been shown to be critically important for the generation of cell-mediated as well as humoral immunity. It has been proposed that ligation of CD40 on APCs, presumably by activated Th cells, leads to increased APC function as defined by up-regulation of costimulatory molecules and enhancement of IL-12 production. In this report, we directly examined the contribution of the CD154:CD40 pathway in a murine model of allograft rejection. Generation of both the CTL and alloantibody responses following injection with allogeneic P815 tumor cells was severely compromised in CD154 knockout mice and wild-type C57BL/6 mice treated with the anti-CD154 mAb, MR1. Splenic production of IL-2, IFN-γ, and TNF was significantly suppressed from CD154-deficient mice, indicating a lack of T cell priming. However, splenic cells from CD154 knockout mice induced comparable levels of CD86 expression and IL-12 production when compared with their wild-type littermates. The treatment of CD154−/− mice with the agonistic anti-CD40 mAb, FGK45, generated activated APCs yet failed to restore either the CTL or alloantibody responses to P815. Likewise, immunization with B7-transfected P815 tumor cells failed to generate expansion of the CTL effector population in CD154−/− mice. These results suggest that the generation of allograft immunity is dependent on the interaction of CD154 with CD40 but not primarily for the activation of APCs. The Journal of Immunology, 1999, 163: 2470–2477.

It is widely accepted that activation of naive T cells requires at least two signals: Ag/MHC stimulation of the TCR/CD3 complex and costimulation (1, 2). The interaction of B7 molecules on APCs with their counterreceptor, CD28, on T cells is believed to be the primary form of costimulation. However, other accessory molecules such as CD54/LFA-1 and CD2/LFA-3 also contribute significantly to T cell activation through adhesive interactions (3). Additionally, the differentiation of activated T lymphocytes into competent effector cells is dependent on cytokines (4). Both IL-12 and IL-4 have been shown to direct the development of T cells into a Th1 or Th2 phenotype, respectively. Disruption of either costimulation or differentiation of Ag-activated T cells ultimately leads to a conditional unresponsiveness termed tolerance and a compromised state of immunity (5, 6).

CD154 is a member of the TNF family (7, 8), which includes TNF-α, CD95 ligand, LT α, LT β, and the ligands for CD27, CD30,OX-40, and 4-1BB (9). Expression of CD154 is found primarily on activated CD4+ T cells (10), but has also been reported on CD8+ T cells, mast cells, and basophils (10–13). CD40, the receptor for CD154, is expressed on APCs such as B cells (14), macrophages (15), and dendritic cells (16). It is a member of the TNF receptor family (14), which includes TNF-R1 (p55), TNF-RII (p75), CD95, CD27, CD30,OX-40, and 4-1BB (9, 14).

The interaction of CD154 with CD40 has been identified as a major pathway for the activation of APC and is essential for the generation of many cell-mediated immune responses (17–19). Disruption of this pathway leads to increased susceptibility to opportunistic pathogens, illustrated by the prevalence of Pneumocystis and Cryptosporidium infections in hyper-IgM syndrome patients who fail to express functional CD154 (20, 21). Experimental animal models to investigate this immunodeficiency have been established by administering CD40-Ig fusion protein or antagonistic CD154 Abs to block CD154:CD40 interactions (17). Recently, the creation of CD154 and CD40 knockout mice have allowed further investigation of the important role of this pathway in the development of effective immunity (22, 23).

It has been shown that CD40 ligation induces accessory molecules such as CD86, CD80, CD54, and MHC II on APC populations (10, 24, 25). It has also been determined that stimulation via CD40 induces secretion of IL-12 from both dendritic cells and macrophages (26–28). Recently, several reports suggested that a primary role of activated Th cells in the generation of CD4-dependent cell-mediated immunity was to provide CD40 ligation on APC, presumably to increase B7 expression and IL-12 secretion (29–31). This activation step then empowered the APC to successfully activate CD8+ cells and drive their differentiation into CTL effectors. Therefore, we hypothesized that a lack of CD154:CD40 interaction would prevent generation of allograft immunity by failing to increase B7 expression and induce IL-12 secretion from APC. To test this hypothesis, we used the P815 tumor allograft model and followed the activation of B cells, macrophages, and dendritic cells from CD154−/− and wild-type mice over the course of the allogeneic response. However, despite a significantly reduced capacity of CD154 knockout mice to generate alloimmunity, we found no differences in the induction of costimulatory molecule expression or IL-12 secretion between CD154−/− and CD154+/− mice. These results suggest that the generation of allograft immunity is dependent on the interaction of CD154 with CD40 but not primarily for the activation of APCs.
Materials and Methods

Animals

Five- to six-week-old male C57BL/6, CD154−/− (B6, 129-Cd40), CD154+/+ wild-type (B6, 129 F2), and female DBA/2 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in front of a laminar flow unit.

P815 mastocytoma cells

Wild-type P815 tumor cells, derived from a methylcholanthrene-induced mastocytoma, were maintained by weekly passage in DBA/2 mice. CD86-transfected P815 (clone HTR/C.B7-2) and its vector-transfected control (clone HTR/C.C) were generously provided by Dr. Thomas Gajewski (University of Chicago Medical Center, Chicago, IL). Transfected cells were maintained in vitro in RPMI 1640 medium supplemented with 10% FBS (HyClone, Logan, UT) and 1 mM gentamicin at 37°C in 5% CO2.

Reagents

HPLC-purified, antagonistic anti-CD154 mAb (MR1) was used for in vivo blocking experiments, anti-CD4 (GK1.5) used for deletion studies, and agonistic anti-CD40 mAb (FGK45) used for exogenous CD40 ligation experiments were all kindly provided by Dr. Randolph J. Noelle (Dartmouth Medical School, Lebanon, NH). Hamster IgG and rat IgG were purchased from Cappel (Organon Teknika, West Chester, PA) and used as Ig controls for the in vivo blocking and CD40 ligation experiments, respectively. Spleen cell phenotypes were determined by flow cytometric analysis using the following Abs: FITC-conjugated anti-CD34 (1E2), FITC-conjugated anti-CD11c (HL3), FITC-conjugated anti-CD62L (MEL-14), PE-conjugated anti-CD11c (HL3), PE-conjugated anti-CD86 (GL1), and PE-conjugated anti-CD54 (3E2). Peptides from P815 tumor cells alone and naive spleen cells with P815 were used to measure nonspecific production of IL-12 by adherent spleen cells. Supernatant cultures from P815 tumor cells alone and naive spleen cells with P815 were also collected for controls for each experiment. Levels of IL-2, IL-4, IL-12, and IFN-y were determined for each supernatant by using specific Ab sandwich ELISAs. Secondary biotinylated detection reagents were complexed with avidin-peroxidase and visualized with ABTS substrate. Appropriate labeled, isotype-matched Igs were used as controls for blocking experiments, anti-CD4 (GK1.5) used for depletion studies, and anti-CD11c (HL3), FITC-conjugated anti-CD62L (MEL-14), PE-conjugated anti-CD11c (HL3), PE-conjugated anti-CD54 (3E2), and FITC-conjugated anti-Mac-1 (RB-46-H4C5) were generously provided by Dr. Thomas Gajewski (University of Chicago Medical Center, Chicago, IL). Transfected cells were maintained in vitro in RPMI 1640 medium supplemented with 10% FBS (HyClone, Logan, UT) and 1 mM gentamicin at 37°C in 5% CO2.

Animal treatments

Studies to evaluate the role of CD154 in allograft rejection were conducted in CD154−/− mice and their wild-type littermates. Alternatively, C57BL/6 mice were treated i.p. with 250 μg of MR1 on day 0 relative to P815 immunization to block CD154:CD40 interactions. In vivo depletion of CD4 + T cells was performed by injecting mice with 0.25 mg GK1.5 on day −2 relative to CD86-P815 injection. Preliminary studies determined that 99% of naive cells were depleted at least 10 days using this treatment protocol. Mice were injected with either 1 × 105 tumor ascites P815 or CD86-transfected P815 cells on day 0. The protocol for providing exogenous ligation of CD40 involved i.p. injection of both CD154−/− and CD154+ mice with 5 μg of FGK45 on days 0, 2, 4, 6, and 8 relative to P815 injection. All mice were sacrificed by CO2 asphyxiation on days 5–8, or 10 relative to injection. Spleens were removed aseptically, and blood was collected by heart puncture into heparinized syringes. Plasma was separated by centrifugation and stored at −70°C.

Preparation of spleen cells

Single-cell suspensions were prepared by pressing the spleen between the frosted ends of two microscope slides. Erythrocytes were removed by hypotonic lysis. Cells were washed once and resuspended in cold HBSS/5% FBS with 20 mM HEPEs, 50 μg/ml gentamicin, and 1.5 mM sodium pyruvate.

Flow cytometric analysis of spleen cells

Expression of the cell-surface proteins CD86, CD54, and I-Aβ was determined from viable spleen cells and then further measured on gated populations of B220+, Mac-1−, and CD11c+ cells. A distinct population of spleen cells expressing high levels of CD11c (CD11chigh) was determined to represent dendritic cells by profiles of costimulatory molecule expression. These CD11chigh cells constitutively expressed high levels of CD86, CD54, and I-Aβ similar to levels found on dendritic cells enriched over BSA−/dense gradients as previously described (32). In a typical splenic preparation, 1.5–3.3% of the cells were CD11c+ of which >88.4% were CD86+, >99.5% were CD54+, and >95.5% were I-A+. CTL effectors (CTL)3 were identified by first gating on the CD8+ T cells and then identifying the CD44+CD25+CD62Lhi population, as previously described (33). Nonspecific binding was blocked with 10 μg of rat IgG, and then the cells were stained with optimal concentrations of fluorochrome-conjugated mAb. Appropriately labeled, isotype-matched Igs were used as controls for nonspecific fluorescence. Between 20,000 and 100,000 events were collected by listmode acquisition from freshly stained cells using a Coulter XL flow cytometer (Coulter Electronics, Hialeah, FL) and analyzed using WinList (Verity Software House, Topsham, ME).

CTL assay

The cytolytic activity of spleen cells against P815 tumor (ascs) cells was measured in a standard 4-h 51Cr release assay as previously described (34). The percentage of cytotoxicity at each E:T ratio was calculated using the following equation:

\[
\%\ \text{cytotoxicity} = \frac{(E - N)(M - S)}{N} \times 100
\]

where E is the experimental release using spleen cells from P815-immunized mice, N is the nonspecific release using splenocytes from naive mice, M is the maximal release of 51Cr from cells incubated with 1% SDS, and S is the spontaneous release of 51Cr incubated in medium alone. Duplicate wells were tested at E:T ratios of 200:1 to 6:25:1.

Cytotoxic Ab assay

Cytotoxic antibody titers were determined using a complement-dependent 51Cr release assay as previously described (34). Briefly, serial 2-fold dilutions (1/10 to 1/2560) of heat-inactivated plasma were incubated with 51Cr-labeled P815 cells for 20 min at 37°C in 5% CO2. Low-Tox-M rabbit complement (1/12, Cedarlane Laboratories, Hornby, Ontario, Canada) was added for 45 min at 37°C. The amount of 51Cr released into the supernatant was measured by γ counting and specific activity was calculated. All samples were tested in duplicate on separate plates. The Ab titer was defined as the highest dilution of plasma at which a minimum of 20% specific cytotoxicity was measured.

Cytokine analysis

Spleen cells (1 × 105) were incubated in RPMI10% FBS supplemented with 1.5 mM sodium pyruvate, 20 mM HEPES, and 50 μg/ml gentamicin for 6 h with 1 × 106 P815 tumor cells at 37°C in 5% CO2 in 1.5 ml polypropylene Eppendorf tubes that were treated with silicone to prevent nonspecific production of IL-12 by adherent spleen cells. Supernatant cultures from P815 tumor cells alone and naive spleen cells with P815 were also collected as controls for each experiment. Levels of IL-2, IL-4, IL-12, and IFN-γ were determined for each supernatant by using specific Ab sandwich ELISAs. Secondary biotinylated detection reagents were complexed with avidin-peroxidase and visualized with ABTS substrate. Absorbance was measured at 405 nm using a plate reader (Bio-Tek Instruments, Winooski, VT), and cytokine values were then determined using Immunosoft software (Dynatech Labs, Alexandria, VA). TNF levels were measured by standard bioassay (34) using L929 fibroblasts.

Statistical analysis

Results are presented as the mean ± SE of six mice per group unless indicated otherwise. Most experiments were repeated at least once. ANOVA modeling was performed using Statview statistical software (Abacus Concepts, Berkeley, CA). Comparisons between means were made using the least significant difference multiple comparison t test or Dunnet’s t test for pairwise comparisons. Values of p ≤ 0.05 were considered statistically significant.

Results

Disruption of the CD154:CD40 pathway suppresses generation of allograft immunity in the P815 model

Previous studies have established the importance of CD154 in the generation of cell-mediated immunity, including models of allograft rejection (36, 37). We investigated the role of CD154 in the generation of immune responsiveness to allogeneic P815 tumor cells. Following immunization of C57BL/6 mice with P815 tumor 3 Abbreviations used in this paper: CTL E , effector cytotoxic T lymphocyte; CTL p , precursor cytotoxic T lymphocyte; MCF, mean channel fluorescence; GVHD, graft vs host disease; EAE, experimental allergic encephalomyelitis.
cells, the concomitant development of CTL and alloantibody responses effectively rejects these allogeneic cells. The allograft response peaks within 10 days of immunization and correlates with the generation of CTL activity specific for H2-D^d-bearing cells (34) and the emergence of alloantibodies in the plasma (Fig. 1, A and B). CD154 knockout mice failed to generate allo-CTL activity following immunization with P815 tumor cells (Fig. 1A). Similar results were also observed in P815-immunized CD154^+/+ mice, which had been treated with the anti-CD154 blocking Ab, MR1. Furthermore, the production of cytotoxic alloantibody was suppressed by >90% in both the CD154^−/− and MR1-treated mice when compared with their appropriate controls (Fig. 1B). Although it has already been established that IgM is the isotype primarily responsible for cytotoxicity in this alloantibody assay, we further evaluated the effect of blocking CD154:CD40 interactions on the generation of other alloantibodies isotypes. Consistent with previously published reports that illustrate the critical role of CD154 in the generation of humoral immunity (22, 23, 38), blocking CD154 ligation of CD40 resulted in significant suppression of not only IgM, but also IgG2a and IgG1 anti-P815 Abs, as measured by flow cytometry (data not shown).

Lack of T cell priming occurs in mice deficient of CD154

The inability to mount an effective CTL response to allogeneic P815 cells could be due to either lack of priming of T cells or defective effector function. Therefore, we analyzed the splenic CD8^+ cells from P815-immunized CD154^+/+ and CD154^−/− mice for the generation of cells bearing the CTL_E phenotype. Expression of the CD8^+/CD44^high/CD62L^low phenotype correlates with cytolytic activity, as shown previously by Mobley and Dailey (33). Ten days after P815 immunization, CD154^+/+ mice had generated ~3 × 10^7 CTL_E while the number of effector CTLs detected from the spleens of CD154^−/− mice did not differ significantly from that found in nonimmunized mice (Fig. 2A). These results indicate that the lack of CTL activity in CD154^−/− mice was due to an absence of CTL_E and not an inability of CTL cells to perform their effector function.

To evaluate whether T cells from immunized CD154 knockout and wild-type mice were primed, we measured cytokine production in this response. We have previously shown in the P815 model that 6-h restimulation of immunized spleen cells permits detection of IL-2, IFN-γ, and TNF on days 5–10 following immunization (34). As shown in Fig. 2, B–D, increased levels of IFN-γ and TNF were observed on days 6–8 and increased IL-2 production on days 5–8 from wild-type but not CD154-deficient mice. The reduced cytokine production from knockout mice continued through day 10 (data not shown) when both the CTL and alloantibody assays were

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**FIGURE 1.** Disruption of CD154:CD40 suppresses the generation of allograft immunity. A, Splenic CTL activity was measured from CD154^+/+ mice ( ), CD154^+/− mice ( ■ ), and C57BL/6 mice treated with 1.25 mg MR1 ( □ ) or Hamster IgG ( ● ) 10 days after injection with 1 × 10^7 allogeneic P815 tumor cells. B, Cytotoxic alloantibody titers were determined on day 10 postimmunization from mice with functional (CD154^+/+ or hyper-IgG (HIgG) treatment) or disrupted (CD154^−/− or MR1 treatment) CD154:CD40. Data represent mean ± SEM of six mice/treatment group; one representative experiment of three is shown.

**FIGURE 2.** T cell priming to allogeneic P815 tumor cells requires CD154. A. The number of splenic CTL_E (CD8^+/CD62L^low/CD44^high^) cells was measured from CD154^+/+ , CD154^−/− , and nonimmunized mice on day 10 relative to P815 injection. Ex vivo cytokine production was measured from CD154^+/+ ( □ ), CD154^−/− ( ● ), or nonimmune mice ( ○ ) during the allogeneic response to P815 tumor cells. Spleen cells (1 × 10^7) were restimulated with 1 × 10^7 P815 tumor cells for 6 h, and the culture supernatants were assayed for production of IL-2 (B) and IFN-γ (C) by cytokine-specific ELISA or TNF (D) by L929 bioassay. Data points represent the mean ± SEM for duplicate samples from six mice/treatment group. P815 cells cultured alone for 6 h produced <2 pg/ml of TNF and undetectable amounts of IL-2 or IFN-γ.
nonimmune mice (B, C) over the course of the P815 response. Furthermore, mAb blocking increases their expression of CD86 on days 5–8 following immunization with P815 tumor cells (40). Although levels were higher in cultures from wild-type mice than from P815-immunized mice to produce IL-12. As shown in Fig. 3A, comparable CD86 expression was exhibited on days 5–8 from CD154+/+ and CD154−/− mice. Similarly, CD154 deficiency did not alter normal Ag-induced increases in CD54 or I-A expression on spleen cells on days 5–8 (data not shown). As it is unknown exactly which splenic APC population is responsible for the activation of allo-specific T cells in the P815 model, expression of accessory molecules on B220+, Mac-1+, and CD11c+ cells was determined. Shown in representative histograms in Fig. 3, C–E, CD154 deficiency did not alter CD86 expression on any of these APC populations following P815 immunization. Histograms are shown for day 6 data, but are representative of all days tested (days 5–8). Ag-induced increases in splenic CD86 expression were observed to be slight on the B cells (mean channel fluorescence (MCF) = 9), significant for the dendritic cells (MCF = 44), while decreased expression was exhibited on the macrophage population (MCF = 5.5). This apparent reduction in CD86 expression on Mac-1+ cells following P815 injection may be attributable to an influx of Mac-1+ cells into the spleen (2% in naive mice vs 10% in mice injected with P815). However, this increased Mac-1+ population was seen in both the wild-type as well as the knockout mice, yielding no explanation for the absence of T cell priming in mice defective for CD154. Similar patterns of CD54 and I-A expression were also detected on days 5–8 for each of the splenic APC populations examined without significant effects of CD154 deficiency (data not shown). Although these markers of APC activation are by no means exhaustive, the data taken together fail to establish any measurable differences in CD40-mediated APC activation between P815-immunized CD154-deficient or wild-type mice. The apparent lack of effect of the CD154 mutation on induction of the accessory molecules CD86, CD54, and I-A, combined with an unaltered capacity to produce IL-12 in the knockout mice, suggests that CD40 interaction with its ligand is not an essential requirement for activation of APC in this model of allograft immunity.

**Exogenous ligation of CD40 fails to restore allograft immunity in CD154-deficient mice**

Because CD154−/− mice are genetically incapable of expressing functional CD154, we hypothesized that exogenous ligation of CD40 would circumvent the need for its ligand and restore allograft effector functions in these mice. To test this hypothesis, both wild-type and CD154-deficient mice were immunized with P815 tumor cells and then administered FGK45, an agonistic anti-murine CD40 mAb. Treatment of mice with the 5-μg dose of FGK45 produced highly elevated expression of CD86 on splenic APC and significantly increased plasma levels of IL-12 within 48 h (Fig. 4, A and B). Surprisingly, exogenous ligation of CD40 with FGK45 did not restore CTL activity in CD154−/− mice to levels seen from wild-type mice (Fig. 4C). Although a small increase in CTL activity was observed in CD154−/− mice that had received anti-CD40 treatment, this increase was not statistically significant at any of the E:T ratios examined. It is important to note that splenic T cells from CD154−/− mice are not intrinsically incapable of generating CTL effector cells as shown in a recent report by Buhlmann et al. In this report, spleen cells from CD154−/− and wild-type mice generated comparable CTL activity after being cultured in vitro for 6 days with allogeneic stimulator cells (41). In addition to a lack of restoration of the CTL response by anti-CD40, no enhancement of cytotoxic alloantibody plasma titers was observed in anti-CD40-treated CD154−/− mice when compared with...
stimulation of alloreactive CD8+ T cells, we immunized CD154−/− mice from precursor CTL (CTLp) into CTL E possessing full cytolytic activity. To examine a possible role for CD154 in CD8+ T cell-mediated immunity, models of allogeneic immunity have also been shown to be due to compromised Th1 immunity (42–46). Additionally, models of allogeneic immunity have also been shown to depend on successful interaction of CD40 with CD154. In a model of graft-vs-host disease (GVHD), it was shown that F1 recipients that received T cells from CD154−/− mice failed to succumb to GVHD unlike recipients that were injected with wild-type T cells the appropriate controls (Fig. 4D). Interestingly, treatment of CD154−/− mice with a 5-µg dose of FGK45 partially suppressed the generation of cytotoxic alloantibodies on day 10 after P815 immunization, yet had no adverse effect on CTL activity. The dose of FGK45 administered to mice in these experiments proved to be of critical importance as higher amounts of anti-CD40 mAb (25 and 100 µg) produced moderate to severe organ-specific toxicity including thymic atrophy, hepatic coagulative necrosis and neutrophilia, and splenic lymphoproliferation within the lymphoid follicles (D.M.S. and O. R. Hedstrom, unpublished observations). Ultimately, any beneficial effects of increasing costimulatory molecule expression and systemic IL-12 production by APCs following anti-CD40 treatment failed to enhance the generation of allograft effector function.

Injection of B7-transfected P815 tumor cells fails to generate CTL E in CD154 knockout mice

We have previously reported that C57BL/6 mice depleted of CD4+ T cells failed to generate CTL activity following P815 immunization (34). However, when P815 cells transfected to express high levels of murine CD86 (Fig. 5A) were used to immunize CD4-depleted mice, increased numbers of CTL effector cells were generated when compared with CD4-depleted mice injected with vector-transfected P815 cells (Fig. 5B). The dual expression of both H2-Dd (signal one) and B7 (signal two) provides necessary stimulation of alloreactive CD8+ cells to drive their differentiation from precursor CTL (CTLp) into CTL E possessing full cytolytic activity. To examine a possible role for CD154 in CD8+ T cell activation, we immunized CD154−/− mice with B7-transfected P815 tumor cells. By providing the requisite two signals (Ag and costimulation) for T cell activation, CD154 knockout mice (similar to CD4-depleted mice) should be capable of generating CTL E unless there is a requirement for CD154 in this process. On day 10 postimmunization, the numbers of CTL E were determined for both CD154−/− and CD154+/+ mice. In contrast to the results observed in CD4-depleted mice, CD154−/− mice injected with P815-CD86 failed to generate comparable numbers of CTL E when compared with wild-type mice that had received either P815-vector or P815-CD86 tumor cells, 9 or 10 days postimmunization, respectively. Data represent the mean ± SEM of four to six mice/treatment group.

Discussion

It is well established that the CD154:CD40 pathway is important in the generation of cell-mediated immunity (17, 19). For example, the increased susceptibility of patients with hyper-IgM syndrome to intracellular pathogens is highly suggestive of defective T cell immunity in the absence of functional CD154 expression (20). Many experimental systems have also shown the critical role of this pathway in generating T cell-mediated immune responses. Increased susceptibility of CD154-deficient mice to both intra- and extracellular pathogens, as well as numerous viruses, has been shown to be due to compromised Th1 immunity (42–46). Additionally, models of allogeneic immunity have also been shown to depend on successful interaction of CD40 with CD154. In a model of graft-vs-host disease (GVHD), it was shown that F1 recipients that received T cells from CD154−/− mice failed to succumb to GVHD unlike recipients that were injected with wild-type T cells.
(47). Similarly, in a study by Parker et al. that evaluated the role of CD154:CD40 in a transplantation model, blocking CD154:CD40 led to enhanced tolerance in chemically induced diabetic mice (48). Finally, in several studies examining the role of CD154 in allograft rejection, disruption of the CD154:CD40 pathway conferred increased acceptance of cardiac allografts (36, 37, 49). Similar to the results presented in this report, generation of effector allograft immunity was critically dependent on the successful ligation of CD40 by CD154.

However, the mechanism by which disruption of the CD154:CD40 pathway suppresses cell-mediated immunity varies depending on the experimental model examined. Our results show that immunization of CD154-deficient mice with allogeneic P815 cells failed to properly prime allo-specific T cells. Inadequate priming of T cells due to the absence of CD154 has been shown in several other models of T-dependent immunity. Studies by Grewal et al. demonstrated suppression of Ag-specific expansion and effector cytokine production by CD4+ T cells from mice lacking CD154 (50). Likewise, Gray and coworkers showed that T cells activated in the absence of CD40 were unable to help normal B cells undergo Ig class switching or germinal center formation (51). While data exist implicating CD40 involvement in Th cell priming, studies examining its role in priming of CD8+ cells are less clear. A limited number of studies suggest that activation of CD8+ CTLs following viral infection is unaffected in CD154−/− mice (52–54). However, a role for CD154 was indicated for the maintenance of CTL memory cells, as the anti-viral memory CTL response was defective in mice deficient for CD154 (52). Currently, while the importance of CD154 in the generation of the allograft response is accepted, the precise role is unclear. In a study by Larsen et al., the predominant effect of CD154 blockade was found to be defective effector T cell function and not the priming of these cells (36). However, in a study by Hancock et al., T cells were found to have been inappropriately primed causing them to deviate from the normal Th1 phenotype into suppressive Th2 cells that produced increased levels of IL-4 and IL-10 (37). In our model, failure to generate alloimmunity was due to defective T cell priming and not due to immune deviation (as IL-4 levels were undetectable) or compromised T cell effector function (no CTLp were generated).

Currently, a potential explanation for the suppressed cell-mediated immunity observed in CD154-deficient mice revolves around inadequate APC activation attributable to deficient CD40 ligation. CD40 ligation has been shown to induce increased APC activity by up-regulating costimulatory molecule expression and increasing the production of IL-12 as well as other proinflammatory cytokines (24, 26, 27, 55). In CD154-deficient mice infected with an adenovirus, a lack of CD40 signaling resulted in a failure to generate both CTL and Ab responses (56). Reduced expression of CD86 was noted on spleen cells from these adenovirus-infected CD154 knockout mice. Moreover, treatment of these animals with an agonistic anti-CD40 mAb restored their immune responsiveness, an outcome the authors attributed to increased CD86 expression. Likewise, in a murine model of experimental allergic encephalomyelitis (EAE), Grewal et al. showed that CD154-deficient mice that carried a transgenic TCR for myelin basic protein failed to generate the disease after antigenic immunization (57). Similar to the adenoviral model, injection of these mice with APC-expressing transgenic B7 allowed subsequent development of acute EAE, indicating that CD154 was required for induction of costimulatory activity on the APC. These reports contrast with our results that suggest that APC activation is a CD40-independent event following immunization with allogeneic P815 tumor cells. Although some evidence exists identifying the dendritic cell as the key APC in our model (58), we evaluated costimulatory profiles for all putative splenic APC populations to best identify any effect of CD154 deficiency. However, no differences in CD86, CD54, or I-A* induction were observed on the B220+ Mac-1+, or CD11c+ cells from CD154−/− and CD154+/+ mice following P815 immunization. Additionally, no effect was measured in the ability of spleen cells from both types of mice to produce IL-12 following activation. Although in contrast to the adenoviral and EAE models, these results are consistent with three reports examining the role of CD154 in a cardiac allograft model in which CD154-deficiency did not alter CD86 induction/expression (36, 37, 49). The recent study by Niimi et al. clearly demonstrated the importance of the CD40 interaction in the immune response to alloantigen and suggested that the effect of this pathway may be independent of its effect on the B7/CD28 pathway. These data taken together suggest that dependency of CD40 ligation for APC activation may be related to characteristics inherent to specific Ags. Uptake of Ags such as viruses and soluble proteins by APCs may require CD40 ligation to induce competent costimulatory activity, whereas ingestion of particulate Ags such as allogeneic cells may sufficiently activate APC and therefore have less dependence on CD40. This would provide one potential explanation for the normal expression of costimulatory molecules on APCs from CD154−/− mice in models of allograft rejection but not in other experimental systems.

The failure to restore allograft immunity in CD154−/− mice immunized with P815 tumor cells and treated with an agonistic anti-CD40 mAb is an interesting result. If the only contribution of CD154 toward the generation of this alloimmune response was to provide ligation of CD40, then it should follow that providing that stimulus would restore allograft effector functions. This result did not occur in our studies, possibly because ligation of CD40 with agonistic Abs did not reproduce the same physiological effects as CD154. Also, it is possible that signaling could be occurring directly into the T cells via CD154 following ligation of CD40. Interestingly, many potentially therapeutic effects were observed in our mice receiving anti-CD40 treatment. These included induction of high levels of costimulatory molecule expression on splenic APC combined with an increased production of IL-12. Recently, it has been shown by Bennett et al. that similar anti-CD40 treatment of CD4-depleted mice provided necessary signals to induce competent cross-priming of Ag-exposed APC (30). It is conceivable then that exogenous ligation of CD40 in our system also enhanced cross-priming in our APC populations. However, no restoration of CTL activity was seen in the P815-immunized CD154-deficient mice that received anti-CD40 treatment, suggesting that the defect may not reside in the APC but in the T cell. Our results differ from those reported by Yang and Wilson (56) and several recent reports in which anti-CD40 mAb treatment of CD154−/− mice restored CTL activity (29–31, 56). However, all of these studies evaluated CTL activity from anti-CD40-treated mice only after driving potential CTLp in vitro for ~5–7 days with Ag, APC, and even added growth factors, making it difficult to properly compare their results to those generated in our study. In addition to the lack of restorative effects of anti-CD40 treatment on CTL activity, we also observed no beneficial effects of this treatment on cytotoxic alloantibody production in CD154-deficient mice. Furthermore, CD154−/− mice treated with similar doses of FK545 displayed partially suppressed titers of alloantibodies when compared with the rat IgG controls. This negative effect of anti-CD40 mAb treatment is likely due to excessive signaling of B cells through this receptor. Although ligation of CD40 has been shown to be a critical step in the generation of humoral immunity, hyperstimulation can lead to a lack of Ab production due to an arrest of B cell terminal differentiation (59). Therefore, caution should accompany any attempt to boost cell-mediated immunity in human hyper-IgM.
syndrome or AIDS patients with anti-CD40 Ab (or any other reagent capable of ligating CD40) as it may be at the expense of the other arm of acquired immunity, the Ab response.

We have previously reported that generation of CTL activity in the P815 tumor allograft model is CD4- dependent (34). However, this dependency can be circumvented by immunizing CD4-depleted mice with B7-transfected P815 cells as shown in Fig. 5B. We assume that these tumor transfectants are capable of driving CD8+ CTLp directly to become CDLp because they possess both Ag (H-2D) and costimulatory molecules (CD86) on their cell surface. Therefore, it was totally unexpected to find that CD86-P815-immunized CD154-deficient mice, in contrast to CD4-depleted mice, did not generate comparable numbers of CDLp to those observed in CD154+/+ mice. The only difference in the CD8+ cells from each of these mice was the inability of the CD154 knockout mice to express functional CD154. However, this singular genetic defect negated the ability of the P815-CD86 to generate competent CTL activity from these mice or properly prime their T cells as reflected in the suppressed production of Th1 cytokines. The data reflect that mice deficient for CD154 are clearly not equivalent to CD4-depleted mice, possibly explaining the differences in our inability to generate cell-mediated immunity in CD154+/− mice treated with anti-CD40 when compared with previous reports performed in CD4-depleted mice (29–31).

The possible function of CD154 to transduce intracellular signals into the T cell remains to be proven. Several studies have provided some evidence that CD154 may serve as a receptor capable of transducing a costimulatory signal into the T cell. In these reports, it has been suggested that CD154 is a viable signal-transducing molecule in T cells, which, when ligated, is capable of activating a sphingomyelinase and releasing ceramide (60) and/or p40 messenger RNA expression and IL-12 production of macrophages via CD40-activation. Alternatively, the expression of CD154 on the cell surface of activated T cells may be a temporal bridge between a CD4+ T cell and APC activation. Moreover, CD154 ligand-positive CD8+ T cells allow B cell growth and differentiation. Eur. J. Immunol. 25:2972.

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References


