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Genetic Characterization of Strain Differences in the Ability to Mediate CD40/CD28-Independent Rejection of Skin Allografts

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Simultaneous blockade of the CD40 and CD28 T cell costimulatory pathways effectively promotes skin allograft survival in C3H/HeJ mice, extending median survival times (MSTs) beyond 100 days. This strategy is markedly less effective in C57BL/6 mice, with MSTs ranging between 20 and 30 days. In this study, we investigate the underlying genetic causes of these distinct phenotypes. Using H-2 congenic mice, we show that the genetic basis for the varied responses between these two strains is independent of the H-2 locus and T cell precursor frequency. C57BL/6 mice treated with costimulation blockade are able to generate allospecific CTL- and IFN-γ-producing T cells within 3–4 wk posttransplant, whereas mice with a C3H background generate neither CTL- nor IFN-γ-producing cells. Thus, differences appear to be in the generation of the immune response and not T cell homing. Strain differences in costimulation blockade-induced hyporesponsiveness persist in the absence of CD4+ T cells, implying a direct effect on CD8+ T cells. We demonstrate that genetic differences are important in cells of hemopoietic origin and that the costimulation blockade-resistant phenotype is dominant. Analysis of BXH recombinant inbred strains indicates that multiple loci contribute to the phenotype, and that the blockade resistance loci are preliminarily linked to 17 markers on four chromosomes. We conclude that strain variation in allograft MSTs following CD40/CD28 blockade results from the ability of CD8+ T cells in some strains to use alternative modes of costimulation to mount an effective alloresponse. The Journal of Immunology, 2000, 165: 6849–6857.

A ctivated T cells are required for the rejection of transplanted tissue. T cells may be directly cytotoxic to the graft or provide help for other effector cells. Because of their pivotal role in allogeic responses, T cells have long been a critical therapeutic target in attempts to induce hyporesponsiveness and, ultimately, transplantation tolerance. Much effort has gone into developing a greater understanding of the activation requirements for allospecific T cells and defining T cell subpopulations important for rejection. Several studies have indicated a crucial role for CD4+ T cells in the rejection of cardiac allografts in mice (1–3) and the rejection of skin allografts in BALB/c mice (4). Conversely, other strains have shown the ability to mediate skin graft rejection by use of the CD8+ population without the help of CD4+ T cells (5).

It is thought that optimal activation of T cells requires two types of signals. The first signal, delivered upon prolonged interaction of the MHC with the TCR, lends specificity to the immune response, whereas the second signal, mediated by costimulatory interactions between the T cell and the APC, allows for optimal T cell activation and clonal expansion. Various molecules have been shown to have costimulatory roles for T cell activation, including CD40 and CD28 (6–9). These two pathways have been shown to be particularly important in a variety of immune responses in vivo, unveiling potential therapeutic applications. For example, blockade of the CD28 pathway can ameliorate the symptoms of experimental autoimmune encephalitis, a mouse autoimmune disease that attacks the central nervous system and is mediated primarily by CD4+ T cells (10). In the transplant community, great interest has been generated by the observation that simultaneous blockade of the CD40 and CD28 costimulatory pathways significantly prolongs allograft survival in both mice and primates in the absence of further immunosuppression (11, 12). Furthermore, blockade of either or both of these pathways has been a central component of several strategies aimed at inducing donor-specific tolerance (13–15). Nevertheless, CD40/CD28 blockade alone has not been shown to induce indefinite graft survival.

Different strains of mice show greatly disparate responses when receiving skin allografts and blockade of the CD40/CD28 pathways. Our laboratory has previously observed that although C3H/HeJ mice enjoy impressive prolongation of skin allograft survival following costimulation blockade treatment, other strains do not respond as favorably. C57BL/6 (B6)3 mice are particularly refractory to the effects of CD40/CD28 blockade. In this setting, we have identified a population of CD8+ asialo GM1+ T cells that are responsible for costimulation blockade-resistant rejection (16). The disparity between the two strains is independent of the donor strain. B6 mice are similarly resistant to costimulatory blockade following receipt of either a BALB/c or C3H/HeJ skin graft, whereas C3H/HeJ mice accept both BALB/c and B6 skin allografts for >100 days following treatment (A. Bingaman, unpublished observations).

In an effort to better understand the requirements for the generation of effector T cells capable of rejecting skin allografts, we sought to explore the underlying genetic causes of the enhanced ability of B6 mice, in comparison to C3H/HeJ mice, to reject skin allografts in a CD40/CD28-independent manner. In this report we

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3 Abbreviations used in this paper: B6, C57BL/6; MST, median survival time; CD40L, CD40 ligand; GVHD, graft-versus-host disease.
show that this disparity is neither a result of MHC haplotype nor T cell precursor frequency. We demonstrate that mice with a B6 background generate allospecific CTLs or IFN-γ-producing T cells in a CD40/CD28-independent fashion, unlike mice with a C3H background. Furthermore, our data point to a direct effect on the activation of CD8+ T cells, rather than an indirect effect mediated by CD4+ T cells. By the use of irradiation chimeras, we demonstrate that the genetic differences between the two strains assert themselves in blood-derived cells. Analysis of F1 mice indicates a dominant mode of inheritance, and analysis of BXH recombinant inbred strains implicates at least two loci involved in the blockade-resistant phenotype. Furthermore, preliminary linkage analysis of the BXH strains highlights 17 markers on four chromosomes as candidates for the location of the blockade resistance loci. Taken together, our data suggest that at least two H-2-independent loci directly modulate the activation of CD8+ T cells in the absence of CD40/CD28 costimulatory signals.

Materials and Methods

**Mice**

Adult male 6- to 8-wk-old BALB/c, B6, C3H/HeJ, C3H.SW-H-2b, B6.AKR-H-2b, and B6.SJL mice were purchased from The Jackson Laboratory (Bar Harbor, ME). F1 mice were generated by mating B6 males to C3H.SW females.

**Skin grafting**

Full thickness skin grafts (~1 cm2) were transplanted on the dorsal thorax of recipient mice and secured with a band-aid for 7 days. Graft survival was then followed by daily visual inspection. Rejection was defined as the complete loss of viable epidermal graft tissue. Statistical analyses were performed using a Mann-Whitney U test.

**Treatment protocols**

Skin graft recipients were treated with 500 μg each of hamster anti-murine CD40 ligand (CD40L) Ab (MR1) and human CTLA4-Ig (provided by Diane Hollenbaugh, Bristol-Myers Squibb, Princeton, NJ) administered i.p. on days 0 and 2 posttransfer.

**Flow cytometry**

Analyses of splenocytes of irradiated recipients of CFSE-labeled T cells were conducted using fluorochrome-conjugated Abs (rat IgG2a-PE, rat IgG2b-APC, anti-CD4-PE, and anti-CD8-APC; PharMingen, San Diego, CA). Peripheral blood was analyzed by staining with fluorochrome-conjugated Abs (CD45.1-PE, CD45.2-FITC, CD4-APC, CD8-APC, mouse IgG2a-FTTC, and mouse IgG2a-PE) followed by RBC lysis and washing with PBS/Tween 20 (0.05%). Effector cells were incubated for 14–16 h at 37°C with or without stimulators. After the culture period, cells were removed by washing the plate in RPMI 1640 (HyClone). Effectors were preincubated with 50% CD4-depleted experimental groups received 100 μg anti-mouse CD4 (GX.1) i.p. on days -3, -2, -1, and weekly until harvest. Treated irradiated recipients of CFSE-labeled T cells received 500 μg each of hamster anti-murine CD40L and human CTLA4-Ig i.p. on days 0 and 2 posttransfer.

**CFSE labeling and adoptive transfers**

Splenic and mesenteric lymph node cells were harvested from B6 or C3H.SW mice. After RBC lysis and nylon wool passage, the cells were harvested from the recipients, the RBC were lysed, and the remaining cells were stained for flow cytometry as above.

**Determination of precursor frequencies**

Precursor frequency was determined as previously described (17). In short, peaks were numbered for the number of times the cells had divided (n). A T cell that divides n times generates 2^n daughter cells. Therefore, to obtain a number of precursors for each individual peak, the number of cells in that peak is divided by 2^n. For allogeneic transfers, the precursor numbers of peaks 4–8 were added together and divided by the total number of initial precursors to generate a precursor frequency for divisions 4–8. These divisions were chosen based on the observation that syngeneic transferred cells do not proceed detectably beyond three divisions.

**Cytotoxicity assays**

BALB/c CL.7 cells were used as targets. Target cells were suspended in saline and 5% FCS (~1 × 10^6/ml) with 750 μCi 15Cr (sp. act. 470 mCi/mg; NEN Life Science Products, Boston, MA) for 90 min at 37°C. Target cells were washed three times and plated in 96-well round-bottom plates at 1 × 10^4 targets/well in R10 medium (RPMI 1640 supplemented with penicillin, streptomycin, 2-ME, and 10% FCS (HyClone)). Effectors were preincubated with harvesting spleenocytes, lysing RBC, and passing them through nylon wool. Effectors were plated at the appropriate ratios in quadruplicate. Four wells were set aside for total lysis by addition of 2% Triton X-100 to the targets, and four wells were set aside for spontaneous lysis by the addition of R10 medium without effector cells. After 5 h, the supernatant was harvested and analyzed by gamma counting. Percent specific lysis was determined by the use of the following formula: 100 × (cpm unknown – cpm spontaneous)/(cpm total – cpm spontaneous).

**IFN-γ ELISPOT assays**

Allospecific T cell responses were measured by IFN-γ ELISPOT assay by using nylon wool-passaged spleenocytes obtained from skin-grafted B6 or C3H.SW mice or Histopaque-separated (Sigma, St. Louis, MO) peripheral blood leukocytes obtained from B6, C3H.SW, or F1 mice. The capture Ab for this assay, rat anti-mouse IFN-γ (clone R4-6A2; PharMingen), was incubated at 4 μg/ml in PBS (100 μl/well) at 4°C overnight in esterase-bottom plates (Millipore, Bedford, MA). After washing with PBS, 3 × 10^4, 1.5 × 10^5, or 0.75 × 10^6 effector cells were added to the wells. Stimulators were either irradiated (2000 rad) donor spleenocytes added at a 1:1 stimulator-to-effector ratio or mouse dendritic cells obtained through overnight transient adherence added at a 1:10 stimulator-to-effector ratio. Effector cells were incubated for 14–16 h at 37°C with or without stimulators. After the culture period, cells were removed by washing the plate in PBS/Tween 20 (0.05%), and then biotinylated anti-mouse IFN-γ (clone XM1G1.2; PharMingen) was added at 4 μg/ml (100 μl per well). After 2–3 h at 4°C, unbound Ab was removed, and HRP-avidin D (Sigma) was added. Spots were developed with the substrate 3-amin-9-ethyl-carbazole (Sigma) with 0.015% H2O2. Each spot represents an IFN-γ-secreting cell, and the frequency of these cells can be determined by dividing the number of spots counted in each well by the total number of cells plated at that dilution. Naive splenocytes produce IFN-γ at a frequency of ~3 per 10^6 cells with or without stimulation.

**Irradiation chimeras**

C3H.SW mice (C4D.2^b) received 120 rad of irradiation to completely ablate the hematopoietic cells. One day later, irradiated mice were rescued with 1 × 10^7 B6.SJL (C4D.1^b) bone marrow cells injected i.v. Mice were immunized with 500 μg MR1 and CTLA4-Ig on days 0, 2, 4, and 6 posttransplant to prevent graft-versus-host disease (GVHD). Mice were monitored over a course of 6 wk for expression of the donor C4D alloantigen in the peripheral blood by flow cytometry. Skin grafts were given when chimerism exceeded 98%. Surprisingly, attempts to generate B6.SJL chimeras with C3H.SW bone marrow using the same protocol were not successful, precluding analysis in the opposite direction.

**Linkage analysis**

Each recombinant inbred BXH strain was assigned a phenotype based on median survival times (MSTs) of allogeneic skin grafts following treatment with CTLA4-Ig and anti-CD40L. Three strains were assigned a B6-like phenotype, and three strains were assigned a C3H-like phenotype. Linkage to 330 polymorphic markers (18) was assessed using Map Manager version 2.6.6.1 (19). Linkage was assessed within 95% confidence limits, and the software calculated logarithm of odds scores.

**Results**

Costimulation blockade resistance is independent of the H-2 locus

Previous work in our laboratory has established a significant difference in the ability of distinct strains of mice to accept skin allografts following blockade of the CD28/CD40 T cell costimulatory pathways. In particular, BALB/c skin allograft survival on C3H/HeJ recipients treated with CTLA4-Ig and anti-CD40L is
dramatically longer than the survival of these grafts on treated B6 recipients. In these experiments, the MST of BALB/c skin allografts on C3H/HeJ recipients treated with anti-CD40L and CTLA4-Ig was >100 days vs 11 days in untreated controls (Fig. 1A), whereas the MST of skin allografts on B6 mice receiving the same treatment was 22 days vs 10 days in untreated controls (Fig. 1B).

One possible interpretation of the above data is that the ability of individual strains to overcome costimulation blockade at early time points is determined by the H-2 haplotype. H-2-dependent selection of the T cell repertoire may determine the population of cells available to respond to a particular alloantigen. To understand the role of the H-2 locus in contributing to costimulation blockade-resistant rejection, we made use of H-2-congenic mice. C3H.SW (H-2b) and B6.AKR (H-2k) mice were given BALB/c skin allografts and treated with CTLA4-Ig and anti-CD40L. C3H.SW mice displayed the same phenotype as C3H/HeJ mice, with a >100 day MST following treatment with CTLA4-Ig and anti-CD40L (Fig. 1C). In contrast, B6.AKR mice acquired the more resistant phenotype of the B6 parent strain, rejecting their grafts with a MST of 20 days following treatment (Fig. 1D). These results indicate that the H-2 haplotype is not the major determinant of strain-associated allograft rejection responses after costimulation blockade.

The disparity between B6 and C3H.SW mice is not due to differences in T cell precursor frequency

One explanation of the above data is that although the H-2 locus of the B6 and C3H.SW mice is the same, other background differences may determine the precursor frequencies of alloreactive T cells, thus leading to the observed disparity in rejection time following treatment. For example, in one viral system, strain variation in the ability to mount an antiviral response is linked to expression of an endogenous mouse mammary tumor virus (MMTV) superantigen, leading to selective deletion of major portions of the T cell repertoire (20). To test this hypothesis, we used a previously described GVHD model (21). T cell-enriched splenocytes from B6 or C3H.SW mice were labeled with CFSE (Molecular Probes), a membrane-permeable ester that, when cleaved by nonspecific intracellular esterases, becomes fluorescent and remains trapped in live cells. Cellular proliferation can be monitored due to the progressive loss of fluorescence with successive cell divisions. Labeled cells were injected i.v. into lethally irradiated syngeneic (B6 or C3H.SW) or allogeneic (BALB/c) hosts. Spleens were harvested 66–72 h later, resuspended, and stained for expression of CD4 and CD8 vs CFSE fluorescence. As shown in Fig. 2B, T cells transferred into syngeneic hosts underwent only minimal replication, with no cells proceeding beyond three divisions. Conversely, allogeneic T cells transferred into BALB/c hosts divided extensively, replicating up to eight times in 3 days (Fig. 2A).

Precursor frequencies of B6 and C3H.SW donor T cells in untreated allogeneic recipients were similar between the two strains in both the CD4 and CD8 subpopulations. Six and one-half percent of naive C3H.SW CD4+ T cells divided four or more times in response to H-2d alloantigen, whereas 5.5% of B6 CD4+ T cells divided four or more times in response to the same stimulus (Fig. 2A).

FIGURE 1. The genetic basis for costimulation blockade-resistant rejection is independent of the H-2 locus. C3H/HeJ (A) and B6 (B) mice received BALB/c skin allografts. Mice either received no further treatment (open symbols) or CTLA4-Ig and anti-CD40L on days 0, 2, 4, and 6 posttransplant (closed symbols). Skin graft survival on C3H/HeJ mice was greatly prolonged following treatment with an MST of >100 days (Fig. 1A). Treated B6 mice experienced only modest prolongation of skin graft survival (MST = 19 days, n = 7). Untreated C3H/HeJ and B6 mice had MSTs of 12 and 11 days, respectively (n = 7). Two strains of H-2 congenic mice, C3H.SW (H-2b) and B6.AKR (H-2k), received BALB/c skin allografts with or without CTLA4-Ig and anti-CD40L. C, C3H.SW (H-2b) mice maintain the phenotype of the C3H/HeJ parent strain following costimulation blockade independently of the H-2 locus. C3H.SW (H-2b) (n = 6) and C3H/HeJ (●, n = 7) mice receiving CTLA4-Ig and anti-CD40L had MSTs of >100 days, compared with 11 and 12 days, respectively, in untreated controls (■, n = 7). D, B6.AKR (H-2k) mice maintain the phenotype of the B6 parent strain following costimulation blockade independently of the H-2 locus. Treated B6.AKR (H-2k) (●, n = 7) and B6 (□, n = 7) mice both rejected their skin grafts with MSTs of 19 days. Untreated B6.AKR (●, n = 3) and B6 (□, n = 3) mice rejected their grafts with MSTs of 10 and 11 days, respectively.
Among the CD8^+ subpopulation, 4.8% of donor C3H.SW and 4.1% of donor B6 T cells divided in BALB/c recipients, respectively (Fig. 2 C). CD4^+ and CD8^+ cells dividing four or more times in syngeneic hosts were undetectable above background. Precursor frequencies in either T cell subpopulation were not significantly different between strains, leading us to conclude that differences in the initial T cell precursor frequency do not account for the distinct responses to alloantigen after costimulation blockade.

Donor B6 and C3H.SW T cells were also tested for their ability to replicate in irradiated BALB/c mice in the face of costimulation blockade. Following transfer, mice were treated with anti-CD40L and CTLA4-Ig on days 0 and 2. As seen in Fig. 2C, the percentage of B6 and C3H.SW T cells able to divide in the face of costimulation blockade did not vary markedly in either the CD4^+ or the CD8^+ subpopulations. After blockade, 2.1% of CD4^+ B6 T cells and 1.7% of CD4^+ C3H.SW T cells divided four or more times in response to alloantigen, resulting in a 62 and 75% decrease from untreated mice, respectively. Following treatment, 1.8% of B6 CD8^+ T cells and 2.0% of C3H.SW CD8^+ T cells divided in response to alloantigen, decreasing from untreated mice 55 and 58%, respectively. No striking differences in the ability to proliferate in response to alloantigen in the face of costimulation blockade were observed, and we concluded that differences in the ability to mount a rejection response between the two strains could not be accounted for by differences in the frequency of T cells able to proliferate in response to alloantigen in the presence or absence of costimulation blockade.

B6 mice generate allospecific CTL and IFN-γ-producing T cells following costimulation blockade, whereas C3H.SW mice are unable to do so.

Although no early differences in proliferative responses between the two strains could be detected, we hypothesized that B6 and C3H.SW mice might vary in their ability to generate and sustain functional T cell responses following skin engraftment and treatment with CTLA4-Ig and anti-CD40L. In particular, it seemed likely that differences would be seen in the CD8^+ subpopulation, as our previous work has established a crucial role for CD8^+ T cells in costimulation blockade-resistant rejection in B6 mice (16).

To test this hypothesis, splenocytes from B6 or C3H.SW mice receiving BALB/c skin allografts were assessed for direct ex vivo CTL activity and frequency of IFN-γ-producing cells at various days posttransplant. As shown in Fig. 3, untreated C3H.SW and B6 mice receiving BALB/c skin allografts mounted CTL responses in the spleen with similar kinetics. Ex vivo CTL responses were detectable by day 8, peaked at or near day 12, and remained measurable even at 26 days posttransplant. The frequency of IFN-γ-producing T cells, as measured by ELISPOT assay, was also similar between the two strains.
Substantial numbers of IFN-\(\gamma\)-producing T cells in the spleen could be measured on day 8 in both strains. The frequency of these cells peaked between days 12 and 18 and remained significant at 26 days posttransplant. These experiments showed no marked difference between the B6 and C3H.SW strains in their ability to mount allospecific IFN-\(\gamma\) and CTL responses following skin engraftment.

To assess the effects of costimulation blockade on the induction of T cell responses in vivo in these two strains, CTL activity and the frequency of IFN-\(\gamma\)-producing cells were measured in B6 and C3H.SW mice receiving CTLA4-Ig and anti-CD40L at the time of skin engraftment. Fig. 3 details the ex vivo CTL activity of T cell-enriched splenocytes at various points postengraftment. At 8 and 12 days postengraftment, no CTL activity could be detected in either B6 or C3H.SW spleens. However, by days 18 and 26, allospecific CTL activity could be detected ex vivo in B6 spleens. In contrast, C3H.SW mice showed no increase in CTL activity above naive controls at any time point, and neither strain showed an ex vivo response to syngeneic targets (data not shown). The generation of IFN-\(\gamma\)-producing cells exhibited a similar pattern. B6 mice mounted a costimulation blockade-resistant IFN-\(\gamma\) response in the spleen beginning at day 18 postengraftment, whereas C3H.SW mice failed to generate detectable numbers of IFN-\(\gamma\)-producing cells above background (Fig. 4).

These experiments demonstrate a fundamental difference in the ability of these two strains to generate effector T cells independently of the CD28/CD40 costimulatory pathways. They also imply that genetic differences defining the disparate phenotypes assert themselves in the priming and generation of the immune response and not during the effector phase, such as in T cell homing to the graft.

Inhibition of costimulation blockade-independent rejection in C3H.SW mice is at least partly a direct effect on CD8\(^+\) T cells

Although CD8\(^+\) T cells appear to be the determining factor in costimulation blockade-resistant rejection of skin allografts in B6 mice (16), CD4\(^+\) T cells have been shown to play a regulatory role in prolonging allograft survival in strategies using anti-CD40L and CTLA4-Ig (Ref. 22 and our unpublished observations). Another possible explanation for the inability of some strains to overcome costimulation blockade at early time points may include the generation of CD4\(^+\) regulatory cells. We hypothesized that perhaps...
the genetic differences between the two strains resided in the ability to generate regulatory CD4$^+$ T cells to quench the CD8$^+$ T cell response. To test this hypothesis, B6 and C3H.SW mice received BALB/c skin grafts with and without costimulation blockade. Half of the mice from each group were also depleted of CD4$^+$ T cells in vivo with the GK1.5 mAb. Responses were assessed by measuring ex vivo CTL activity and frequency of IFN-γ-producing T cells at 21 days posttransplant.

BALB/c skin allografts induced substantial direct ex vivo CTL responses in both B6 and C3H.SW mice by day 14 posttransplant (data not shown). Ex vivo CTL activity persisted at day 21 (Fig. 5A). Following treatment with CTLA4-Ig and MR1, B6 mice maintained the ability to generate a readily detectable CTL response by day 21, whereas C3H.SW mice failed to develop measurable CTL responses above background (Fig. 5A). The frequency of IFN-γ-producing cells, as measured by ELISPOT analysis, followed a similar pattern. Both mice generated high numbers of IFN-γ-producing cells at days 14 and 21. Treatment with costimulation blockade completely abrogated this response in C3H.SW mice. In contrast, B6 mice were able to generate large numbers of IFN-γ-producing cells at day 21 (Fig. 5B). Following CD4 depletion alone, both IFN-γ and CTL responses remained strong in both strains, whereas costimulation blockade combined with CD4 depletion inhibited all detectable IFN-γ or CTL responses in the C3H.SW mice. In contrast, B6 mice generated both ex vivo cytolytic activity and IFN-γ-producing cells by day 21, even in the absence of CD4$^+$ T cells (Fig. 5, A and B).

We concluded from these experiments that the disparity in early responses following blockade treatment could be at least partly explained as a direct effect on CD8$^+$ T cells, and that genetic differences between the two strains appear to affect this compartment specifically, regardless of the presence of CD4$^+$ T cells.

**The genetic disparities that lead to costimulation blockade-resistant rejection are not relevant in the parenchyma**

At least two possibilities could explain the differing ability of the two strains to generate costimulation blockade-resistant immune responses. One was that the disparate phenotypes are an intrinsic property of hemopoietic cells, and that genetic differences between the two strains manifested themselves specifically in immune cells. Alternatively, we considered the possibility that strain-associated differences in parenchymal tissue may determine the generation of costimulation blockade-resistant effector T cells. Irradiated chimeras were used to better define the respective roles of parenchymal and blood-derived immune cells. C3H.SW mice, which express the CD45.2 alloantigen, were lethally irradiated, then rescued with bone marrow cells from B6.SJL mice expressing the CD45.1 alloantigen. Mice were treated with CTLA4-Ig and anti-CD40L at the time of bone marrow infusion to facilitate engraftment and prevent GVHD. Chimerism was measured by tracking the CD45 alloantigen in the peripheral blood by flow cytometry. At 6 wk posttransplant, peripheral blood leukocytes were >98% CD45.1$^+$. Chimeric mice then received a BALB/c skin allograft with or without costimulation blockade and were assessed for allograft survival.

Untreated chimeric C3H.SW mice rejected BALB/c skin allografts in normal control time (MST = 10 days, data not shown). When treated with CTLA4-Ig and anti-CD40L at the time of engraftment, these mice acquired the more aggressive costimulation blockade-resistant phenotype displayed by B6.SJL mice receiving the same treatment (Fig. 6). Treated chimeric C3H.SW mice had a MST of 17 days, whereas treated B6.SJL and C3H.SW controls had MST values of 17 and >70 days, respectively. Unfortunately, we were unable to generate the reciprocal B6.SJL chimeras. Nonetheless, these results strongly support a pivotal role for cells of hemopoietic origin in determining the strain-associated response to allogeneic skin grafts following costimulation blockade.

Costimulation blockade resistance is a dominant phenotype in F1 mice

We next sought to further characterize inheritance of the costimulation blockade resistance phenotype by determining its expression in F1 mice. B6 mice were mated to C3H.SW mice to generate F1 hybrids that were of a single H-2 haplotype. F1 mice received BALB/c skin grafts with or without costimulation blockade at 6–10 wk of age and were assessed for allograft survival. As seen in Fig. 7A, F1 mice readily rejected their skin grafts following costimulation blockade (MST = 20 days). This is similar to the rate of rejection of skin grafts following costimulation blockade in
The costimulation blockade-resistant phenotype is dominant. B6 mice were mated to C3H.SW mice. The F1 progeny were assessed for their ability to mount IFN-γ responses and reject BALB/c skin grafts following costimulation blockade. Because the genomes of these strains represent a patchwork of inheritance from the B6 and C3H/HeJ parent strains, we hypothesized that analysis of their rejection phenotypes would allow us to make use of available linkage maps to identify areas of the genome that potentially contribute to costimulation blockade-resistant rejection. BXH, B6, and C3H/HeJ mice were tested for significant differences in survival of BALB/c skin grafts after costimulation blockade.

BXH recombinant inbred strains segregated into at least three distinct phenotypes. BALB/c skin graft survival in three strains, BXH-10, -11, and -14, was not significantly different from that in B6 mice. Graft survival in three other strains, BXH-3, -9, and -19, was not significantly different from that in C3H/HeJ mice. The remaining strains (BXH-2, -4, -6, -7, -8, -9, and -12) showed an intermediate phenotype, as graft survival was significantly distinct from that in both the B6 and C3H/HeJ mice. It remains unclear whether the intermediate phenotypes can be further grouped and distinguished, as no significant differences between them were evident with the numbers of mice tested. Survival scores and MST values are outlined in Table I.

These results suggest that multiple loci contribute to the costimulation blockade resistance phenotype and that their effect is cumulative. Furthermore, based on the frequency distribution of recombinant inbred strains to the respective phenotypes, we propose that these data are consistent with a two-gene hypothesis.

Analysis of BXH recombinant inbred strains implies that at least two loci contribute to costimulation blockade resistance

To further investigate the genetics of this phenotype, we assessed the ability of 12 BXH recombinant inbred strains to reject BALB/c skin grafts following costimulation blockade. Because the genomes of these strains represent a patchwork of inheritance from the B6 and C3H/HeJ parent strains, we hypothesized that analysis of their rejection phenotypes would allow us to make use of available linkage maps to identify areas of the genome that potentially contribute to costimulation blockade-resistant rejection. BXH, B6, and C3H/HeJ mice were tested for significant differences in survival of BALB/c skin grafts after costimulation blockade.

BXH recombinant inbred strains segregated into at least three distinct phenotypes. BALB/c skin graft survival in three strains, BXH-10, -11, and -14, was not significantly different from that in B6 mice. Graft survival in three other strains, BXH-3, -9, and -19, was not significantly different from that in C3H/HeJ mice. The remaining strains (BXH-2, -4, -6, -7, -8, -9, and -12) showed an intermediate phenotype, as graft survival was significantly distinct from that in both the B6 and C3H/HeJ mice. It remains unclear whether the intermediate phenotypes can be further grouped and distinguished, as no significant differences between them were evident with the numbers of mice tested. Survival scores and MST values are outlined in Table I.

These results suggest that multiple loci contribute to the costimulation blockade resistance phenotype and that their effect is cumulative. Furthermore, based on the frequency distribution of recombinant inbred strains to the respective phenotypes, we propose that these data are consistent with a two-gene hypothesis.

**Analysis of BXH recombinant inbred strains shows preliminary linkage to 17 markers on four chromosomes**

Recombinant inbred strains bearing the distinctive B6 or C3H/HeJ phenotypes were analyzed using Map Manager software (19). The blockade resistance phenotype was tested for linkage to 330 known polymorphic markers (18) in each of the RI strains. Preliminary linkage was demonstrated to 17 markers on four different chromosomes within 95% confidence limits. All linked markers had logarithm of odds scores of 1.8 and are as follows: chromosome 5, En2, Emv1, D5H4S43, Pmv5, Qdpr; chromosome 9, D9 Mii8, D9Nds2, D9Nds1, Gtpc, Kfo1, Ltw3; chromosome 13, Pmv9; chromosome 15, Xitr5, L6y, Ly6d, lapls1–22, Pmv17. This low-resolution linkage analysis is limited by the relatively small number of recombinant inbred strains available. However, it provides an important starting point for further studies using large numbers of these strains represent a patchwork of inheritance from the B6 and C3H/HeJ parent strains, we hypothesized that analysis of their rejection phenotypes would allow us to make use of available linkage maps to identify areas of the genome that potentially contribute to costimulation blockade-resistant rejection. BXH, B6, and C3H/HeJ mice were tested for significant differences in survival of BALB/c skin grafts after costimulation blockade.

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**Analysis of BXH recombinant inbred strains implies that at least two loci contribute to costimulation blockade resistance**

To further investigate the genetics of this phenotype, we assessed the ability of 12 BXH recombinant inbred strains to reject BALB/c skin grafts following costimulation blockade. Because the genomes of these strains represent a patchwork of inheritance from the B6 and C3H/HeJ parent strains, we hypothesized that analysis of their rejection phenotypes would allow us to make use of available linkage maps to identify areas of the genome that potentially contribute to costimulation blockade-resistant rejection. BXH, B6, and C3H/HeJ mice were tested for significant differences in survival of BALB/c skin grafts after costimulation blockade.

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The role of CD40 and CD28 in the costimulation of CD8$^+$ T cells is not clearly understood. CD40 and CD28 themselves do not appear to be crucial elements of CD8$^+$ T cell activation in B6 mice in allosresponses to skin. Our lab has characterized a population of CD8$^+$ T cells in these mice able to overcome blockade of both the CD40 and CD28 pathways (16). Newell et al. have also reported the rejection of small bowel grafts by CD8$^+$ T cells independently of CD28 (30). In addition, antiviral responses to LCMV and viral clearance are impaired only minimally following disruption of the CD40 and CD28 pathways (31–33). However, other antiviral responses, such as to vesicular stomatitis virus (VSV), seem to have a much greater requirement for CD8$^+$ T cell costimulation than CD40 and CD28 costimulation in the activation and expansion of CD8$^+$ antiviral T cells (34). In various experiments, CD40L has been shown to play a central role in conditioning APCs for the priming of CD8$^+$ T cells (35–37). Still other groups, using tetramerized MHC molecules in vitro, have shown that CD8$^+$ T cells can potentially be activated without any second signal whatsoever, requiring only signaling via the TCR (38). Clearly, the conditions that dictate the requirements for CD8$^+$ T cell activation in vivo in these widely varied models are poorly understood. The identification of alternative and relevant means of CD8$^+$ T cell costimulation in vivo is essential to a proper understanding of their biology, activation requirements, and interactions with other cell types. We propose that during allosresponses to skin grafts in some strains, including B6, CD8$^+$ T cells have distinct costimulatory requirements from that of CD4$^+$ T cells and are able to mediate skin graft rejection through alternative routes of T cell activation. The results in this paper provide new insights into CD28/CD40-independent rejection of skin allografts by CD8$^+$ T cells and represent a major step toward identifying the precise pathways used in vivo by B6 mice in overcoming costimulation blockade that are unavailable to C3H/HeJ mice.

We do not believe these results are unique to these particular strains, as differences in skin graft survival following costimulation blockade are also seen in other strain types. For example, DBA/2 mice enjoy impressive prolongation of skin graft survival following costimulation blockade, much like the C3H/HeJ mice, whereas BALB/c mice reject their grafts in a similar time course to

Table I. BXH RI strains segregate into at least three distinct phenotypes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Survival</th>
<th>MST</th>
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<tr>
<td>C57BL/6</td>
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<td>C3H/HeJ</td>
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<tr>
<td>BXH-10</td>
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<td>BXH-11</td>
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<tr>
<td>BXH-14</td>
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<tr>
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<td>BXH-8</td>
<td>28, 32, 36, 50, &gt;65</td>
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</tr>
<tr>
<td>BXH-12</td>
<td>23, 59, 59, 98</td>
<td>59</td>
</tr>
</tbody>
</table>

All mice were treated on postoperative days 0, 2, 4, and 6 with 500 mg MR1 and CTLA4-Ig. Groups were tested for significant differences from either the B6 or the C3H/HeJ groups using a Mann-Whitney test. Significance was defined as $p < 0.05$.

Discussion

These results demonstrate a clear difference in the ability of CD8$^+$ T cells in mice with a C3H genetic background and mice with a B6 genetic background to functionally respond to alloantigens and reject skin grafts in a CD40/CD28-independent manner. As shown by the skin graft survival data of C3H.SW-H-2b and B6.AKR-H-2k mice, the disparity is independent of the H-2 haplotype. Furthermore, differences in T cell precursor frequency do not explain the strain-associated differences in CD40/CD28-independent CD8$^+$ T cell responses. B6 and C3H.SW mice had similar precursor frequencies responding to the H-2$^b$ haplotype in both the CD4 and CD8 subpopulations. Also, no differences could be distinguished in either subpopulation in early proliferative events following costimulation blockade. Our data demonstrate a functional difference between the two strains in their ability to mount IFN-γ and CTL responses following receipt of a BALB/c skin allograft, anti-CD40L, and CTLA4-Ig. Although B6 mice are able to mount delayed but substantial IFN-γ and CTL responses to alloantigen, C3H.SW responses are almost completely suppressed. Furthermore, we have shown that costimulation blockade directly blunts CD8$^+$ T cell activation and expansion in C3H.SW but not B6 mice, regardless of the presence of CD4$^+$ T cells. Our analysis of allogeneic skin graft survival on BXH recombinant inbred strains following costimulation blockade implies that multiple loci contribute to the phenotype, and we propose that the phenotype distribution among the 12 strains is consistent with a two-gene hypothesis. Preliminary linkage mapping has been performed using data available from the BXH strains to identify candidate chromosomal segments for the location of the blockade resistance loci.

Using this type of analysis, we have excluded the genes encoding CD40, CD154, CD28, CD80, and CD86, showing that resistance to Ab therapy is not simply due to polymorphisms in the target molecules themselves. Furthermore, we are able to exclude numerous other molecules that have been shown to play a costimulatory role in T cell activation or as growth factors for activated T cells, including ICAM-1, 4–1BB, 4–1BB ligand, Fas, Fas ligand, TRANCE, IL-2, IL-15, CD25, IL-15R, etc. However, it should be noted that we cannot exclude the possibility of differences in downstream events in one or more of these pathways. These results set the stage for further analysis of large numbers of experimental backcrosses or intercrosses to establish definitive linkage and provide a high resolution map.

Eventual identification of the loci encoding this phenotype will provide important information regarding the activation and costimulatory requirements of alloreactive CD8$^+$ T cells. Several molecules have previously been implicated as important in the costimulation of CD8$^+$ T cells. Among these is 4-1BB, a member of the TNF superfamily that is important in CD28-independent costimulation of CD8$^+$ T cells during antiviral responses (23). However, disruption of the 4-1BB costimulatory pathway alongside CD28/CD40 blockade in B6 mice does not significantly prolong skin allograft survival (our unpublished observations). However, it should be noted that at least one other report has observed delayed skin allograft rejection upon simultaneous disruption of the CD28 and 4-1BB pathways (24). Likewise, the Fas-Fas ligand pathway has been proposed as a CD8$^+$ T cell costimulatory factor (25). Recent data from our laboratory using B6.gld mice has implicated this pathway in the costimulation blockade-resistant CD8$^+$ T cell response in mice with a B6 genetic background. Other molecules may play a role in CD8$^+$ T cell costimulation, including IL-6 and TNF-α (26), ICAM-1, LFA-3 (27), Ly-6C (28), and NO generated by inducible NO synthase (29).

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4 J. Trambley, A. Lin, E. Elwood, A. W. Bingaman, F. Lakkis, T. C. Pearson, and C. P. Larsen. FasL is important in costimulation blockade-resistant skin graft rejection. Submitted for publication.
36 mice following treatment (our unpublished observations). These results further reinforce our conclusion that these distinct phenotypes are controlled by H-2-independent loci, as well as raise important questions about the use of costimulation blockade in humans. It is likely that in dealing with an outbred population such as humans, various forms of resistance to costimulation blockade therapy might occur. Strategies using costimulation blockade alone may well prove ineffective due to genetic differences in patients’ abilities to mount CD40/CD28-independent immune responses. The identification of costimulation blockade resistance loci in mice may provide clues to understanding modes of genetic resistance in humans. It may also provide targets for further therapeutic intervention to block alternative costimulatory pathways.

Acknowledgments

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References