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Antigen-Induced Unresponsiveness Results in Altered T Cell Signaling

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Pretransplant exposure to allogeneic lymphocytes can result in donor-specific unresponsiveness and prolonged allograft survival. Intracellular signaling events have been described in anergic T cell clones, but the biochemical events underlying in vivo induced unresponsiveness have not been studied in detail. We employed a TCR transgenic mouse, bearing the 2C TCR, providing adequate numbers of homogenous peripheral T cells to study biochemical aspects of T cell unresponsiveness in vivo. 2C mice exposed to semiallogeneic lymphocytes (H-2b × H-2d) experienced prolonged H-2d cardiac allograft survival, and cells from these mice did not proliferate or make IL-2 in response to alloantigen (H-2d). Importantly, there were marked differences in TCR-associated tyrosine phosphorylation activation patterns. The targets for the unresponsive state appear to be diminished Lck activation and absent ZAP-70 and LAT (linker for activation of T cells) phosphorylation. Our study demonstrates that Ag-induced tolerance in vivo is accompanied by altered early TCR-mediated signaling events.


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Our study investigates the intracellular signaling events associated with alloantigen-induced T cell tolerance. For over 4 decades, human and animal studies have demonstrated that alloantigen tolerance can be induced by infusion of live donor lymphocytes (1–8). In murine studies, a single transfusion of donor cells is sufficient to inhibit a mixed lymphocyte response to donor histocompatibility Ags and decrease the frequency of donor-specific cytotoxic T lymphocyte precursors (CTLp) (9). Transfusion-induced donor-specific tolerance is dependent on the presence of viable donor T cells (10–12) and is associated, in experimental models, with lack of IL-2 synthesis (13, 14). In studies relevant to our model, transfusion-induced skin allograft tolerance can be evoked in congenic C57BL/6 mice across an H-2 class I (bm-1) and class II disparity (bm-12) (15) by transfusion of live lymphocytes.

Exposure to allogeneic lymphocytes through blood transfusions has also been successful in achieving donor-specific tolerance in clinical renal transplantation. Over 30 yr ago, it was realized that renal allograft survival rates were better among recipients of a large number of transfusions (16). In fact, nontransfused patients had inferior graft survival. More recently, transfusion of a single unit of leukocytes matched to the recipient by one DR Ag (HLA class II) has been shown to greatly improve graft survival and reduce the danger of pretransplant sensitization (17). Thus, abundant literature from both human trials and experimental animal models of transplantation demonstrates that i.v. injection of live lymphocytes leads to T cell unresponsiveness.

The biochemical events underlying in vivo induced unresponsiveness have not been studied in detail. Intracellular signaling events associated with in vitro T cell tolerance or anergy have been evaluated in several model systems, mainly using T cell clones. In T cell clones, anergy has been induced either by ligation of the TCR in the absence of costimulatory molecules or by ligation with an altered form of an immunogenic peptide (APL). Distinct alterations in TCR-mediated signals have been identified in anergic T cell clones. One example of altered TCR-mediated signaling occurs when T cell clones are stimulated in the absence of costimulatory signals, such as those of the B7 family or CD28 ligands (18). In this model, T cells are incapable of producing IL-2 and proliferating upon re-exposure to Ag and the costimulatory ligands (19). The biochemical basis for the IL-2 production defect is a selective block in signal transduction to the mitogen-activated protein kinases (MAPKs) extracellular signal-related kinase-1 (ERK-1) and ERK-2 (20, 21). Phosphorylation of ERK-1 and -2 is reduced, suggesting that the critical upstream molecule Ras is not activated (20, 21). Ras activation is essential for the transcription of cytokine genes such as IL-2. In this model of costimulatory blockade, PMA can overcome the defect in Ras in the anergic T cell clones, suggesting that the signaling defect lies either proximal or immediately distal to Ras (20, 21).

There are other examples of anergy induced in T cell clones that give evidence for defects in Ras signaling at the level of early TCR-mediated signals. Several studies induced anergy by functionally antagonist APLs. APLs have been found to stimulate a distinct pattern of TCRζ-chain phosphorylation (22) or a distinct TCRζ isoform (TCRζ pp21) (23), which was associated with absence of ZAP-70 kinase activity and IL-2 production (22, 23). These studies also substantiate that anergy in T cell clones is associated with a defect in TCR signaling that is upstream of Ras.

Our study uses a novel transplantation system that uniquely allows analysis of the biochemical intracellular signaling events...
associated with donor lymphocyte-induced alloantigen tolerance. We have employed a TCR transgenic mouse bearing the 2C TCR to study the cellular and biochemical aspects of induced T cell unresponsiveness. The well-characterized 2C mouse (24, 25) provides adequate numbers of a homogenous peripheral T cell population (>95% TCR+) with specificity to H-2Ld to analyze, on a biochemical level, changes in intracellular signaling associated with the unresponsive state (24).

We found that T cells from 2C mice exposed to semiallogeneic cells in vivo did not proliferate or make IL-2 in response to alloantigen (H-2d). Further, cardiac allograft survival was prolonged in the mice exposed to semiallogeneic cells. These in vitro and in vivo observations were accompanied by marked differences in TCR-associated tyrosine phosphorylation activation patterns. The targets for the unresponsive state appear to be associated with diminished Lck activation and absent ZAP-70 and LAT phosphorylation. Thus, it appears, using this TCR transgenic mouse model, that alloantigen-induced T cell unresponsiveness is associated with distinct defects in early TCR-mediated signaling molecules.

Materials and Methods

Mice

2C (H-2d) homozygous transgenic mice were provided for breeding by Drs. Lynn Dustin and Dennis Loh (Washington University School of Medicine, St. Louis, MO). The offspring were screened for inheritance of the transgenic TCR by analysis of peripheral T cells obtained from orbital eye bleed. St. Louis, MO). The offspring were screened for inheritance of the transgenic TCR by analysis of peripheral T cells obtained from orbital eye bleed. These in vitro and in vivo observations were accompanied by marked differences in intracellular signaling associated with the unresponsive state (24).

Induction of T cell unresponsiveness

To induce Ag-specific T cell unresponsiveness in 2C mice we injected, via tail vein, adult 2C mice (6–12 wk of age) with 3 × 10^6 spleen cells from C57BL/6 × BALB/cF1 mice. Donor spleens were harvested aseptically from adult F1 mice, and single cell suspensions were made by rubbing the tissue between two frosted glass slides and suspending the mixture on ice in RPMI 1640 (Life Technologies, Gaithersburg, MD). Cell counts were determined by microscopic counting using a microcytometer. The concentration that resulted in unresponsiveness was 3 × 10^5 cells/500 μl. This concentration was injected a single time only. Both donors and recipients were unmanipulated, and the spleen cells were injected in an unseparated manner.

Heterotopic heart transplantation

Vascularized, heterotopic heart allografts were performed in the manner originally described by Corry et al. (27). The aorta was anastomosed to the abdominal aorta, and the pulmonary artery was anastomosed to the abdominal wall. Rejection was defined as the cessation of all myocardial contractions, which was confirmed at laparotomy under anesthesia. BALB/c donor hearts were obtained from 6- to 12-wk-old BALB/c mice and transplanted into 6- to 8-wk-old 2C mice or C57BL/6 mice. Syngeneic controls were performed by transplantation of BALB/c hearts into 6- to 8-wk-old BALB/c donors. Cessation of cardiac pulsations within 48 h of the operative procedure was associated with operative complications, and these animals were excluded from the data analysis. These only represented a small number of animals (zero to one per group), and there was no significant difference in technical failures among the syngeneic, naive, or F1 injected animals.

Cell preparations and mixed lymphocyte reaction

Spleen and lymph nodes were removed aseptically and kept on ice. Single-cell suspensions were obtained by rubbing the tissue between two frosted glass slides and suspending on ice in RPMI 1640 and 10% FCS. Cell counts were determined by microscopic counting using a microcytometer, allowing calculations for appropriate cell dilutions.

The frequency of T cells unresponsive in vitro to response to alloantigen was determined in a standard mixed lymphocyte reaction. Lymph node (LN) cells were prepared from unmanipulated, naive, 2C mice, 4–8 wk old. Unpurified LN cells (2 × 10^6) from 2C mice (H-2d; responder cells) were seeded into round-bottomed 96-well microtiter plates (Costar, Cambridge, MA) with 2 × 10^5 irradiated (2000 rad) BALB/c spleen cells (stimulator cells). Cultured cells were grown in 10k medium (RPMI 1640 (Life Technologies) supplemented with 10% FCS (Sigma, St. Louis, MO), penicillin, streptomycin, and 2-ME (50 μM)). Proliferation was measured by [3H]thymidine incorporation during the final 18 h in culture.

To determine whether the unresponsive state was associated with a reversible defect, T cells from 2C littermates that were either injected i.v. 3 wk earlier with (BALB/c × C57BL/6)F1 unpurified spleen cells or not injected were compared for their ability to respond to PMA and ionomycin. Cultures of 2C naive vs injected responders and BALB/c stimulators were compared with duplicate cultures treated PMA (10 ng/ml) and ionomycin (2 mM). LN cells were removed from injected mice and naive mice, but not from transplanted mice.

Cytokine analysis

Supernatants of mixed lymphocyte cultures were examined for the presence of pattern cytokine secretion. An ELISA method was used according to the manufacturer’s protocol (PharMingen, San Diego, CA). IL-2, IL-4, or IFN-γ in samples were captured by the specific primary mAb and detected by the biotin-labeled secondary mAb. The assay was developed with avidin-peroxidase and its substrate (0.03% 2,2’-azino-bis(3-ethylenbenzthiazoline-6-sulfonic acid) in 0.1 M citric acid plus 0.3% H2O2), and plates were read at 405 nm using a microplate reader. Recombinant murine IL-2, murine IL-4 (Scherer-Plough, Kenilworth, NJ), and murine IFN-γ (Agen Biologicals, Thousand Oaks, CA) were used as standards for ELISA.

Flow cytometric analysis

To analyze the expression of molecules on the surface membrane, T cells were prewashed for nonspecific binding by incubation on ice with anti-CD32 (FcγIR) Ab (clone 2.432) in PBS plus 2% FCS. Cells were then washed twice in cold PBS plus 2% FCS, and 2 × 10^5 to 1 × 10^6 cells were subsequently stained for 30 min on ice with PE- or FITC-conjugated or biotinylated (biotin) Abs in 96-well V-bottom plates. The Abs were used at 1 μg/10^6 cells, and included anti-TCRαβ (clone H57-597), anti-CD25 (clone B6.1), anti-CD69 (clone H1.2F3), anti-Vß1.8,2 (clone H75-597), anti-CD44 (clone IM7), and the 2C TCR isotype 1B2 (biotinylated). All Abs except the 1B2 Ab were washed from RIA (San Diego, CA). Biotinylated Abs were used in conjunction with streptavidin red (PE) (Life Technologies, Grand Island, NY), or purified Abs were used with affinity-isolated goat anti-mouse FITC conjugate (BioSource International, Camarillo, CA). Live cells or cells fixed in 2% paraformaldehyde (Sigma) were analyzed by flow cytometry using a FACSscan flow cytometer in conjunction with FACSscan software (Becton Dickinson, San Jose, CA).

Detection of tyrosine-phosphorylated proteins

To examine the pattern of protein tyrosine phosphorylation in T cells of 2C mice, a suspension of LN cells (10–15 × 10^6/sample) was stimulated with anti-TCR Ab (F23.1) or anti-TCR Ab and anti-CD8 for 3 min at 37°C. The cells were lysed in a buffer containing 1% Nonidet P-40, 50 mM Tris (pH 7.6), 150 mM NaCl, 1 mM NaVO4, 10 mM NaF, 10 mM sodium pyrophosphate, 10 μM/mL each of aprotinin and leupeptin, and 2 mM PMSF. Anti-phosphotyrosine Ab was added to the lysates along with protein A-Sepharose beads, and incubation proceeded for 2 h at 4°C. The beads were washed extensively, and the proteins bound to the beads were resolved by SDS-PAGE, transferred to nitrocellulose, immunoblotted with anti-phosphotyrosine, and developed with an enhanced chemiluminescence system (Amersham, Arlington Heights, IL). Identification of ZAP-70 and LAT was achieved by phosphorylation-dependent epitope tagging using anti-ZAP-70 (Transduction Laboratories, Lexington, KY) or anti-LAT (Upstate Biotechnology, Lake Placid, NY) specific Abs and Western blotting with anti-phosphotyrosine Ab as described above.

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Therefore, injection of naive 2C mice with Ag alone (F1 cells) trocellulose membrane, and developed by autoradiography. Time points, and proteins were resolved by SDS-PAGE, transferred to ni-

mM DTT, 50

and F1 injected groups. There was no difference in the percentage of technical failures among syngeneic, naive, and these animals were excluded from the data analysis. There was no difference in the percentage of technical failures among syngeneic, naive, and F1 injected groups.

FIGURE 1. In vitro unresponsiveness correlates with prolonged allograft survival. Vascularized heterotopic BALB/c heart allografts from 6- to 12-wk-old donors were transplanted into BALB/c recipients (n = 5; syngeneic; □), naive, unmanipulated 2C recipients (n = 7; naive; ○), or 2C mice that had been injected 3–4 wk earlier with F1 cells (n = 6; F1 injected; ●). All recipients were between 6 and 8 wk old. Function of the transplanted heart was assessed by daily palpation of ventricular contractions through the abdominal wall. Rejection was defined as the cessation of all myocardial contractions, which was confirmed at laparotomy under anes-

thetica. Cessation of cardiac pulsations within 48 h of the operative proce-

dure (zero to one per group) was associated with operative complications, and these animals were excluded from the data analysis. There was no difference in the percentage of technical failures among syngeneic, naive, and F1 injected groups.

FIGURE 2. Injection with semiallogeneic cells results in a hyporespon-
sive mixed lymphocyte response and defective IL-2 and IFN-γ production. 2C mice were not manipulated (A; naive; ■), were injected i.v. with 3 × 107 syngeneic spleen cells (A; syngeneic; ●), or were injected i.v. with 3 × 107 unmanipulated spleen cells from adult (B6 × BALB/c)F1 mice (A; F1 injected; ○). Treated mice were sacrificed 3 wk after injection, LN cells were harvested, and a MLR was performed using irradiated BALB/c spleen cells as stimulators. To assess cytokine production, supernatants of dupli-

cate mixed lymphocyte cultures were collected at the time of harvest from cultures of naive (B; naive; ■) and F1 injected mice (B; F1 injected; ○). One representative experiment of five is shown using one mouse per group. Multiple other experiments using pooled LN cells from two to four mice have shown no difference in results. SDs are shown from triplicate samples of each determination.

Functional activity of Lck

After identification by immunoprecipitating Ab, the functional activity of Lck was assayed in vitro using standard immune complex kinase assays. For immune complex assays, whole-cell extracts in 20 mM HEPES, pH 7.5, containing 150 mM NaCl, 2 mM MnCl2 or MgCl2, 150 μM EDTA, 1 mM DTT, 50 μM Na3VO4, 10 μM NaF, 2 μg/ml leupeptin, aprotilin, pepstatin, 1 mM PMSF, and 0.075% Triton were mixed with the immu-

noprecipitating Ab and protein-A Sepharose beads and rotated at 4°C for 2 h in a microfuge tube. The immunoprecipitate was washed twice in wash buffer and resuspended in kinase buffer, 10 μCi [32P]ATP, and substrates for the proteins of interest. The reaction was terminated at predetermined time points, and proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and developed by autoradiography.

Results

Prolonged allograft survival in 2C TCR transgenic mouse

Many studies have shown that injection of semiallogeneic cells can induce unresponsiveness and prolong allograft survival (6, 9, 11, 16, 17). Therefore, we wanted to determine whether in 2C mice exposure to semiallogeneic cells could also result in prolonged graft survival. To induce unresponsiveness, naive 2C (H-2d) mice were injected i.v. with 3 × 107 semiallogeneic (H-2b × H-2d)F1 spleen cells. Heterotopic cardiac grafts were placed in naive and injected 2C mice. Fig. 1 demonstrates that unmanipulated 2C mice rejected their BALB/c cardiac grafts by day 8, as shown by others (28) (Fig. 1, naive). Mice injected with semiallogeneic cells had greatly prolonged graft survival, with the majority of cardiac grafts in injected mice surviving longer than 20 days (Fig. 1, F1 injected). Therefore, injection of naive 2C mice with Ag alone (F1 cells) causes prolonged cardiac allograft survival (Fig. 1).

T cell unresponsiveness in vitro in 2C TCR transgenic mouse

To assess the effects on cellular responses in these unresponsive mice, LN cells were harvested from naive vs injected 2C mice (3 wk after injection of semiallogeneic (H-2b × H-2d)F1 spleen cells), and in vitro proliferative responses to irradiated BALB/c stimulator cells were compared (Fig. 2A). Naive 2C LN cells proliferated vigorously in response to BALB/c stimulators (Fig. 2A, naive), as did LN cells from 2C mice injected with 2C spleen cells (Fig. 2A, syngeneic). In marked contrast, LN cells from 2C mice that had
been injected with semiallogeneic cells (Fig. 2A; F1 injected) did not proliferate in response to a wide dose range of BALB/c stimulators. The proliferative response in the F1 injected mice was diminished by 3 wk after the injection, but was not diminished at earlier time points (data not shown), suggesting that acquisition of T cell unresponsiveness requires a period of time after Ag exposure. In addition, 2C mice that were injected with BALB/c spleen cells (3 x 10^7 cells/injection) were noted to be hyper-responsive rather than unresponsive. Therefore, it is unlikely that the unresponsive state is associated with a graft-vs-host response eliminating the potentially responsive cells.

LN cells from naive 2C mice produced the characteristic CD8 T cell cytokines (IL-2 and IFN-γ) (29) during their proliferative response to BALB/c (Fig. 2B, naive). In marked contrast, LN cells from 2C mice injected with the semiallogeneic cells (3 x 10^7 cells/injection) were noted to be hyper-responsive rather than unresponsive. Therefore, it is unlikely that the unresponsive state is associated with a graft-vs-host response eliminating the potentially responsive cells.

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To determine whether T cells from mice rendered unresponsive could, in turn, induce unresponsiveness in T cells from naive mice, we performed a MLR, mixing LN cells from naive and injected mice. We found no evidence for infectious tolerance (30) when mixing LN cells from injected 2C mice with those from naive 2C mice (Fig. 3). The diminished response seen was simply due to the dilutional effect of the nonresponsive LN cells.

We also found the transient appearance of CD25 and CD69, suggesting transient activation of T cells from the semiallogeneic injected mice (Fig. 4). The time points shown in Fig. 4 are 12 h, 1 and 3 days, and 3 wk after injection with the F1 spleen cells. We found that by day 1 after injection, there was a small increase in CD25 and CD69 cell surface expression. By day 3, CD44 expression was enhanced and persisted for >3 wk after the injection. By day 5, CD25 and CD69 expression was no longer evident, and CD44 levels were elevated (data not shown). We did not find at any time down-modulation of TCR or CD8 surface molecules (data not shown). In addition, we could not detect a difference in the total number of 2C, TCR^+ (determined by positive staining with the clonotypic mAb 1B2), CD8 T cells in LN, spleen, or thymus among age-matched, naive, and injected 2C mice at any of these time points (data not shown). These data suggest that the
MAPK (ERK-1 and ERK-2) activation is defective in anergic signaling pathway. Li et al. (21) and Fields et al. (20) have shown that T cell anergy in vitro has been linked to a block in the Ras-mediated signaling pathway.

Peripheral T cell unresponsiveness is overcome by bypassing a block in Ras-mediated signaling pathway.

T cell anergy in vitro has been linked to a block in the Ras signaling pathway. Li et al. (21) and Fields et al. (20) have shown that MAPK (ERK-1 and ERK-2) activation is defective in anergic CD4+ T cells. The MAPK block can be overcome by treating the anergic cells with the protein kinase C-activating phorbol ester, PMA, and the calcium ionophore, ionomycin, suggesting that the block is proximal to Ras activation (20, 21).

To examine whether the lack of alloresponsiveness in our system is associated with a reversible defect in the Ras signaling pathway, we treated LN cells from unresponsive mice with PMA and ionomycin. Naive cells proliferated vigorously in response to BALB/c stimulators (Fig. 5, naive + BALB/c), and their proliferation was enhanced with the addition of PMA and ionomycin (Fig. 5, naive + P/I). LN cells from unresponsive mice did not proliferate in response to BALB/c stimulator cells (Fig. 5, F1 injected + BALB/c), yet proliferated vigorously in the presence of PMA and ionomycin (Fig. 5, F1 injected + P/I). This reversal in unresponsiveness suggested that the signaling defects in the unresponsive cells reside upstream of Ras.

T cell unresponsiveness is associated with altered TCR-mediated protein-tyrosine phosphorylation.

To isolate Ras signaling events from any potential signals evoked from other costimulatory molecules, we activated T cells from naive and unresponsive mice by Ab-mediated cross-linking of the TCR and CD8 molecules. We compared the overall pattern of tyrosine-phosphorylated proteins in naive and unresponsive T cells. Fig. 6 shows that there is a marked difference in the TCR/CD8-mediated tyrosine phosphorylation cascade between T cells from naive and unresponsive 2C mice. Stimulation with anti-TCR and anti-CD8 Abs for 3 min at 37°C caused enhanced tyrosine phosphorylation, especially of molecules in the range of known critical early Ras signaling proteins (Fig. 6, naive +). T cells from
unresponsive mice also showed enhanced protein tyrosine phosphorylation upon Ab-mediated cross-linking. The pattern of phosphorylated proteins in T cells from unresponsive mice was quite distinct from that in naive mice (Fig. 6, unresponsive +). Several differences were particularly notable in molecules in the M$_r$ range of ZAP-70 (70 kDa), Lck (55–56 kDa), LAT (36 kDa), and the TCR-$\zeta$-chain (21–25 kDa).

We examined the activation state of the well-characterized early TCR-mediated protein tyrosine kinases, Lck and ZAP-70, and the important linker protein, LAT (33). To examine the kinase activity of Lck, T cells from naive vs injected mice were compared for enhanced Lck autophosphorylation upon Ab-mediated cross-linking of the TCR and CD8 molecules for 3 min at 37°C (Fig. 7, Lck). In T cells from naive mice, Lck autophosphorylation was greatly enhanced upon TCR stimulation. This was in marked contrast to T cells from unresponsive mice, in which only minimal Lck autophosphorylation was observed. There was no difference between naive and nonresponsive T cells in total cellular Lck, Lck associated with CD8, or cell surface expression of TCR or CD8 (data not shown).

As Lck activity is requisite for the recruitment and activation of ZAP-70, we also examined the phosphorylation state of ZAP-70. ZAP-70 phosphorylation correlates with its activation and is required for subsequent downstream Ras pathway signals (34). We found that ZAP-70 was expressed, but not phosphorylated, in freshly isolated T cells of both naive and unresponsive mice (Fig. 7, ZAP-70;anti-ptyr). Upon stimulation of the TCR and CD8 molecules, enhanced ZAP-70 phosphorylation was noted in naive mice, but ZAP-70 phosphorylation was absent in unresponsive mice. Equivalent amounts of ZAP-70 protein were detected in both naive and unresponsive mice (Fig. 7, ZAP-70;anti-ZAP-70), suggesting that the induced unresponsive state is associated with an alteration in activation of ZAP-70.

We also noted a distinct difference in tyrosine phosphorylation in a tyrosine-phosphorylated protein, of 36–38 kDa. This 36- to 38-kDa band with diminished tyrosine phosphorylation in unresponsive T cells was identified as LAT by immunoprecipitation with anti-LAT Ab and Western blotting with an anti-phosphotyrosine Ab (Fig. 7, LAT). LAT was markedly phosphorylated following TCR/coreceptor engagement in T cells from naive mice (Fig. 7, LAT; anti-ptyr), but was not tyrosine phosphorylated in T cells of unresponsive mice despite equivalent levels of LAT in both groups (Fig. 7, LAT; anti-LAT). LAT is believed to be downstream of Lck and to be phosphorylated by ZAP-70, and its phosphorylation leads to recruitment of multiple signaling molecules and activation of Ras (33). Diminished Lck activity and absence of ZAP-70 phosphorylation may be induced by prior Ag exposure and result in diminished LAT phosphorylation, contributing to diminished proliferation, cytokine production, and prolongation of heart graft survival.

**Discussion**

2C TCR transgenic mice can be rendered unresponsive to a class I MHC alloantigen (H-2$^d$) by in vivo Ag exposure. The requirement for induction of this unresponsive state appears to be antecedent i.v. administration of live semiallogeneic cells. The unresponsive state is characterized in vivo by prolonged allograft survival and in vitro by diminished proliferation and diminished IL-2 and IFN-$\gamma$ production. Biochemically, upon stimulation, the unresponsive T cells have a unique pattern of tyrosine-phosphorylated intracytoplasmic proteins associated with several defects in early TCR-mediated signaling molecules.

Prolonged BALB/c allograft survival and diminished proliferation mark the acquired state of unresponsiveness in this system. The T cells do not appear to be unresponsive until about 3 wk after exposure of 2C mice to the semiallogeneic cells. This suggests that a period of time may be required to develop either an anergic T cell phenotype or a regulatory population of T cells. The unresponsive T cells appear to have a characteristic phenotype, CD25 and CD69, and are enhanced by 1 day after injection. By 3 days the peripheral T cells in the injected mice express high amounts of CD44, distinguishing T cells of F1 injected mice from T cells of naive mice.

Although the unresponsive T cells express the CD44 Ag, the intracellular signaling pattern of the unresponsive cells is clearly distinct from the signaling pattern seen in memory T cells. Specifically, memory T cells possess phosphorylated TCR-$\zeta$-chains (35), phosphorylated ZAP-70 (35), and phosphorylated proteins at 36–38 kDa (36). In fact, they may even possess phosphorylated proteins that are distinct to the memory phenotype (36). The unresponsive cells in our study clearly lack phosphorylation of TCR-$\zeta$, ZAP-70, or proteins in the range seen in memory cells.

Another characteristic of the unresponsive cells in this system is diminished secretion of two cytokines that characterize the 2C CD8$^+$ T cell population, IL-2 and IFN-$\gamma$ (29). The absence of IL-2 secretion is characteristic of anergy in many other systems. Further, there is no in vitro evidence for infectious tolerance (30) based on in vitro experiments in which the unresponsive T cells do not confer unresponsiveness upon naive T cells in a mixed lymphocyte reaction. Taken together, the in vitro findings suggest that anergy is the primary mechanism for the unresponsive state induced by exposure to semiallogeneic cells in this system.

The well-characterized 2C TCR transgenic mouse provides the opportunity to analyze intracellular signaling events when unresponsiveness is induced in vivo. The biochemical analysis cannot be performed in a nontransgenic system, where only 1–2% of the peripheral T cell population is clonally expanded after alloantigen recognition (37–39). In the 2C mouse >95% of the T cells bear the same TCR and coreceptor molecules. In our colony only 1–5% of the peripheral T cells are CD4+. Although the CD4$^+$ T cells represent a small number, they may play a role in the induction of the unresponsive state. However, analysis of the role of the CD4$^+$ T cell population is beyond the scope of this report.

The TCR-mediated intracellular tyrosine phosphorylation patterns between naive and unresponsive mice demonstrated several striking similarities to anergy induced in clones (18, 19), including a distinct pattern of protein tyrosine phosphorylation in proteins at 70, 55, and 36–38 kDa. We identified defects in activation of Lck, ZAP-70, and LAT following exposure to semiallogeneic cells. These early TCR signaling defects were associated with distinct in vitro and in vivo correlates.

Our study demonstrates that TCR-associated signaling is altered in vivo in T cells made unresponsive to alloantigen. This unresponsiveness can result in the prolonged survival of heterotopic cardiac allografts. We induce unresponsiveness by Ag exposure, avoiding any confounding affect on signaling molecules by immunosuppressive drugs. Alterations in signaling molecules proximal to Ras occurred in the unresponsive T cells and include diminished Lck activation and absent phosphorylation of ZAP-70 and LAT. Diminished Lck activation is currently thought to be the proximal source of absent ZAP-70 and LAT activity. As ZAP-70 and LAT may link the early TCR signals to both the Ras and PKC pathways,
their lack of activity would explain unresponsiveness in terms of absent cytokine production and lack of proliferation. Identification of the precise targets in the TCR signaling cascade causing the block in Ras activation may prove useful in therapeutic strategies aimed at in vivo induction of nonresponsiveness that can result in prolonged allograft survival.

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References


