Ethylenecarbodiimide-Treated Splenocytes Carrying Male CD4 Epitopes Confer Histocompatability Y Chromosome Antigen Transplant Protection by Inhibiting CD154 Upregulation

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In humans and certain strains of laboratory mice, male tissue is recognized as nonself and destroyed by the female immune system via recognition of histocompatibility Y chromosome Ag (Hya). Male tissue destruction is thought to be accomplished by CTLs in a helper-dependent manner. We show that graft protection induced with the immunodominant Hya-encoded CD4 epitope (Dby) attached to female splenic leukocytes (Dby-SPs) with the chemical cross-linker ethylenecarbodiimide significantly, and often indefinitely, prolongs the survival of male skin graft transplants in an Ag-specific manner. In contrast, treatments with the Hya CD8 epitopes (Uty-/Smcy-SPs) failed to prolong graft survival. Dby-SP–tolerized CD4$^+$ T cells fail to proliferate, secrete IFN-γ, or effectively prime a CD8 response in recipients of male grafts. Ag-coupled splenocyte treatment is associated with defective CD40–CD40L interactions as demonstrated by the observation that CD4 cells from treated animals exhibit a defect in CD40L upregulation following in vitro Ag challenge. Furthermore, treatment with an agonistic anti-CD40 Ab at the time of transplantation abrogates protection from graft rejection. Interestingly, anti-CD40 treatment completely restores the function of Dby-specific CD4 cells but not Uty- or Smcy-specific CD8 cells.   *The Journal of Immunology*, 2010, 185: 3326–3336.

C ompared to depletion of T cells, specific inactivation of only select T cell populations is favorable for the treatment of immune-mediated conditions, such as autoimmunity and transplant rejection (1). The advantages of Ag-specific therapy lie primarily in avoiding the risk of opportunistic infections, neoplasia, and toxicity associated with current-generation immunosuppressants (2, 3). We have demonstrated that peptides attached to the surface of syngeneic splenic leukocytes (SPs; Ag-coupled splenocytes [Ag-SPs]) with the chemical cross-linking agent ethylenecarbodiimide (ECDI) effectively and safely induce Ag-specific immune tolerance (4). Ag-SP tolerance has been shown to prevent and treat the symptoms of experimental autoimmune encephalomyelitis (EAE) (5), type 1 diabetes (6), and allogeneic pancreatic islet transplant rejection (7) in the absence of nonspecific immunosuppressive drugs. The involvement of clonal anergy, regulatory

T cell populations, costimulatory molecule blockade, and activation of negative costimulatory molecules, such as programmed-death–1 and CTLA-4, have been described as potential mechanisms of Ag-SP tolerance (6–9), but the precise effects on Ag-specific T cells following tolerogen encounter remain unclear.

Previous EAE studies induced tolerance using SPs coupled to a variety of CD4 T cell epitopes (10), and although exquisite specificity for inactivating pathogenic CD4$^+$ T cells was achieved, resulting in effective disease therapy, the impact of Ag-SP therapy on CD8 T cells remained unclear. Conversely, CD8$^+$ T cells play a prominent role in viral responses and allograft rejection, and Ag-SP tolerance can effectively control inflammation and/or tissue destruction in both models (7, 11). Our laboratory has suggested that the CD8 compartment can be functionally inactivated using Ag-SP for the prevention of allogeneic islet transplant rejection (7), but the complexity of that system has made it difficult to determine whether tolerance is primarily induced directly in the CD8 compartment or tolerant CD4$^+$ T cells simply fail to prime a CD8 response.

Priming of CD8$^+$ T cells can occur dependently or independently of CD4$^+$ T cell help (12–17). Helper-independent CTL responses typically occur in the context of acute inflammatory reactions associated with signals mediated by the recognition of TLR ligands found in intracellular bacterial or viral pathogens (18–20). In both helper-dependent and independent responses, a determining outcome is the upregulation of costimulatory molecules on the surfaces of APCs. In the former case, this is accomplished by interactions between activated CD4$^+$ T cells and APCs, and in the latter case, APCs upregulate these molecules secondary to TLR ligand encounter (16, 17). In the absence of potent inflammatory cues, efficient CD8 responses are critically dependent upon CD4 T cells for the acquisition of their effector function (16, 17). Several diffusible factors (IL-2 and IFN-γ) as well as surface-bound receptor–ligand pairs influence the priming of CTL responses by CD4$^+$ Th cells. Prominent among such receptor–ligand pairs within the TNF/TNFR

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Abbreviations used in this paper: Ag-SP, Ag-coupled splenocyte; B6, C57BL/6; Dby, immunodominant Hya-encoded CD4 epitope; DC, dendritic cell; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; ECDI, ethylenecarbodiimide; FMO, fluorescence minus one; GRAIL, gene related to anergy in lymphocytes; Hya, histocompatibility Y chromosome Ag; iDC, immature dendritic cell; MBP, myelin basic protein; PBS+, PBS and 0.1% Triton X-100; SP, splenic leukocyte; TMEV, Theiler's murine encephalomyelitis virus; VP2, viral protein 2.

superfamily are CD40 and its ligand CD154 (reviewed in Ref. 21). CD40 is constitutively expressed by B cells and the majority of APCs (22). CD40 ligation by CD154, expressed primarily by activated CD4$^+$ T cells, results in an increase in the expression of B7 family costimulatory molecules and proinflammatory cytokines (23, 24). Such "licensing" of APCs by Th cells enables the priming of naive CD8$^+$ T cells and induction of productive and long-lasting cytolytic immune responses (13).

Using the well-characterized histocompatibility Y chromosome Ag (Hya) model of chronic graft rejection (25), we show that Ag-SP ECDI-coupled to the immunodominant Hya-encoded CD4 epitope (Dby) significantly prolongs skin graft survival, whereas Ag-SP coupled to the immunodominant CD8 epitopes (Uty and Smcy) (26) does not confer graft protection. Ag-SPs carrying the CD4 epitope led to a failure in the priming of CTL responses to the Hya CD8 epitopes as determined by significantly suppressed CD8 activation and expansion, IFN-γ production, and cytolytic activity. Although others have previously observed the failure of Uty- and Smcy-specific lysis in Dby-tolerant animals, CD4$^+$ regulatory T cells were postulated to provide an active suppressive signal (27). In this study, we report that, secondary to encounter with ECDI-coupled cells, CD4$^+$ T cells fail to prime a CTL response. We show that tolerized CD4$^+$ T cells exhibit defective upregulation of CD154 (CD40L) and that cross-linking of CD40 in vivo with an agonistic CD40 mAb restores graft rejection in Dby-SP–treated animals. Notably, treatment of protected animals with anti-CD40 resulted in a restoration of proliferation, IFN-γ secretion, and graft infiltration by CD4$^+$ T cells but not IFN-γ secretion, lytic activity, or graft infiltration by CD8$^+$ CTLs. Collectively, these results indicate that targeting of graft-specific CD4$^+$ T cells can suppress CD8$^+$ cytolytic responses, resulting in significant protection of skin grafts. This protection was found to be dependent upon defective CD40 stimulation, and the restoration of graft rejection by anti-CD40 treatment of protected animals occurs via a CTL-independent mechanism.

Materials and Methods

Mice

Age-matched male and female C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Homozygous OT-II mice were purchased from The Jackson Laboratory and bred in-house. Mice were housed in the Center for Comparative Medicine in sterile microisolator cages with ad libitum access to water and chow.

Tolerance induction

SPs were coupled to Ags as previously described in the presence of 30 mg/ml ECDI and 1 mg/ml peptide (4). For tolerance induction to male Ags in B6 females, 10^8 Ag-SPs were administered i.v. for 7 d and again 3 h prior to engraftment. B6 females received Ag-SP coupled to either the CD4 epitope found in male Ag (Dby) or two CD8 epitopes (Uty and Smcy) added at equimolar ratios. Tolerance in OT-II mice was induced by i.v. injection of 10^8 OVA$_{323-339}$-SPs 7d prior to assay. Peptides (Dby, NAGFNSN-RANSSRSS; Uty, WMHHNMDLI; Smcy, KCSRNRQYL; OVA$_{323-339}$, ISQAVHAAHAEINEAGR) were obtained from Genemed Synthesis (San Antonio, TX).

Skin grafting

Orthotopic split-thickness tail skin grafting was performed as described previously (28). The grafts were scored by daily visual inspection for edema, pigment loss, and hair loss. Rejection was defined by complete hair loss and >80% pigment loss. Differences in survival times were tested for significance by log-rank tests.

T cell recall assays

Animals were sacrificed at the indicated times posttransplantation. Single-cell suspensions of spleen and draining lymph nodes were explanted into 96-well plates and challenged with Dby, Uty, Smcy, or OVA$_{323-339}$ (irrelevant Ag control) peptides (0.05–5 μM). Anti-CD3 (2C11) stimulation was included as a control for proliferation and cytokine secretion. Cultures were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 50 μM 2-ME, and 25 mM HEPES buffer for 72 h. Proliferation was measured by pulsing with 1 μCi [^{3}H]thymidine at 48 h and harvesting 24 h later. Culture supernatants were collected at 72 h, and cytokine secretion was measured by ELISA using anti–IFN-γ clones XMG1.2 (capture) and biotin-R4-6A2 (detection) (eBioscience, San Diego, CA), streptavidin-HRP, and enzyme substrate (BioFX, Owing Mills, MD). OVA$_{323-339}$ responses in naive or treated OT-II mice were measured similarly.

In vivo CTL assays

Target cells were labeled with 5 and 0.5 μM concentrations of carboxy-fluorescein diacetate (Invitrogen/Molecular Probes, Carlsbad, CA) at room temperature for 8 min and quenched in the presence of 20% heat-inactivated FBS for 5 min, allowing distinct identification by flow cytometry (11). Fluorescently labeled cells were counted and mixed at a 1:1 ratio prior to i.v. injection. Targets were loaded with Ags (5 μM) for 90 min at 37°C, and cytometric analyses of recipient spleens were performed 6 d following i.v. transfer.

Abs and flow cytometry

Allophycocyanin-conjugated H-2D^b tetramers specific for Uty and Smcy TCRs were obtained from Dr. Amy Stout (National Institutes of Health Tetramer Facility). Other primary conjugates used in this study include CD44-PE/Cy7, CD62L-allophycocyanin/Alexa Fluor 750, CD69-FITC, CD127-biotin, CD40-FITC, CD154-PE (CD40L), CD8-eFluor605, CD4-allophycocyanin, and CD3-Pacific blue or PerCP (eBioscience). Live–dead discrimination was performed using LIVE/DEAD fixable staining reagents (Invitrogen). Detection of biotinylated reagents was accomplished using streptavidin-PE/Cy7 (eBioscience). For analyses of cultured cells, dead cells were excluded from analysis using Violet Dead Cell Stain (Invitrogen/Molecular Probes). Flow cytometric analyses were carried out using a FACSCanto II (BD Biosciences, Franklin Lakes, NJ). Data were collected and analyzed using FACSDiVa software. Anti-CD40 (FGK45.5) and isotype control rIgG2a were purchased from Miltenyi Biotec (Auburn, CA).

Skin graft histology

Mouse tails were fixed in 4% paraformaldehyde in PBS overnight at 4°C followed by infiltration with 30% sucrose in PBS overnight at 4°C. Sections containing grafts were frozen in cryomolds in OCT on dry ice and sectioned at 10 μm with a cryostat. Prior to being labeled, sections were air-dried at room temperature for at least 30 min, rinsed in distilled water to remove OCT, and fixed in −20°C acetone for 10 min. Sections were dried at room temperature for 10 min, washed in PBS three times for 5 min, and blocked in 5% normal donkey serum in PBS and 0.1% Triton X-100 (PBS+) for 60 min prior to incubation with primary Abs in PBS+ overnight at 4°C. After being washed in PBS three times for 10 min, sections were incubated in secondary Abs in PBS+ for 1 h at room temperature, washed in PBS three times for 10 min, incubated in DAPI for nuclear staining for 5 min, washed three times for 5 min in PBS, and then covered with a coverslip using hard setting Vectamount (Vector Laboratories, Burlingame, CA). For biotinylated primaries, peroxidase block (DakoCytomation, Carpinteria, CA) was used for 30 min followed by streptavidin/biotin block (Vector Laboratories) prior to the PBS+ blocking step. The streptavidin/tyramide system (PerkinElmer Life Sciences, Downers Grove, IL) was used to visualize biotinylated primary Abs. Abs used include: anti-mouse CD4 (eBioscience), anti-mouse CD8, (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-mouse F4/80 (Invitrogen). Images were taken on a DM 5000B microscope (Leica Microsystems, Bannockburn, IL) using ImagePro software (MediaCybernetics, Bethesda, MD).

Results

Treatment with splenocytes ECDI-coupled with the Hya-specific MHC class II-restricted Dby epitope promotes long-term survival of B6 male skin grafts

Females of mouse strains with the $H2^b$ haplotype (e.g., B6) generate strong cellular immune responses against Hya-disparate tissue, as measured by allograft rejection kinetics and by the development of Hya-specific delayed-type hypersensitivity (DTH) and CTL responses (25). Naive B6 females were treated i.v. with either ECDI-fixed B6 male splenocytes or Dby-SPs (syngeneic female SPs ECDI-coupled with the Hya CD4 epitope) or with Uty/Smcy-SPs (female splenocytes ECDI-coupled with the Hya CD8 epitopes Uty and Smcy) on days −7 and 0 relative to engraftment with male

tail skin. Male grafts survived for significantly longer times ($p < 0.001$) on Dby-SP–treated animals (median 77 d) than those on untreated animals (median 21 d) or Uty/Smcy-SP–treated animals (median 27 d) (Fig. 1A). Ag specificity of Ag-SP therapy was tested by engrafting treated and nontreated females with skin from third-party female C57BL/10 donors. Both nonprotected and Dby-SP–treated females rejected B10 grafts with equivalent kinetics (Fig. 1E, median 17 d), indicating that Dby-SP specifically regulates Hya-expressing grafts. Furthermore, protection of male B6 grafts is not observed in animals treated with splenocytes coupled to an irrelevant Ag. OVA$_{323-339}$-SP–treated recipients rejected male grafts at a median of 22 d. Female B6 control grafts were not rejected by any treatment group and survived indefinitely (data not shown).

Dby-SP treatment suppressed Dby-specific CD4 recall responses as determined by both in vivo DTH (examined longitudinally, Fig. 1B) and in vitro proliferation (Fig. 1C) and IFN-γ production (Fig. 1D), assessed 14 d posttransplant. These observations are consistent with our previous work demonstrating that Ag-SP tolerance results in Ag-specific decreases in effector CD4 Th1/17 cell responses (1, 4). Confirming the previous work of Jenkins and Schwartz (9), addition of 25 U/ml exogenous IL-2 to recall cultures restored [^{3}H]thymidine uptake by T cells from Dby-SP–treated recipients (Fig. 1C), indicating that anergy plays a significant role in the Dby-SP–induced unresponsiveness.

IFN-γ responses to the Hya CD8 epitopes, Uty and Smcy, were suppressed in Dby-SP–tolerant animals (Fig. 1D). Collectively, these findings indicate that Ag-SP treatment targeting the immunodominant Hya CD4 epitope (Dby) is necessary and sufficient to prolong the survival of male tissue grafts and that tolerance to the CD4 epitope results in priming failure of Hya-specific CD8 T cell
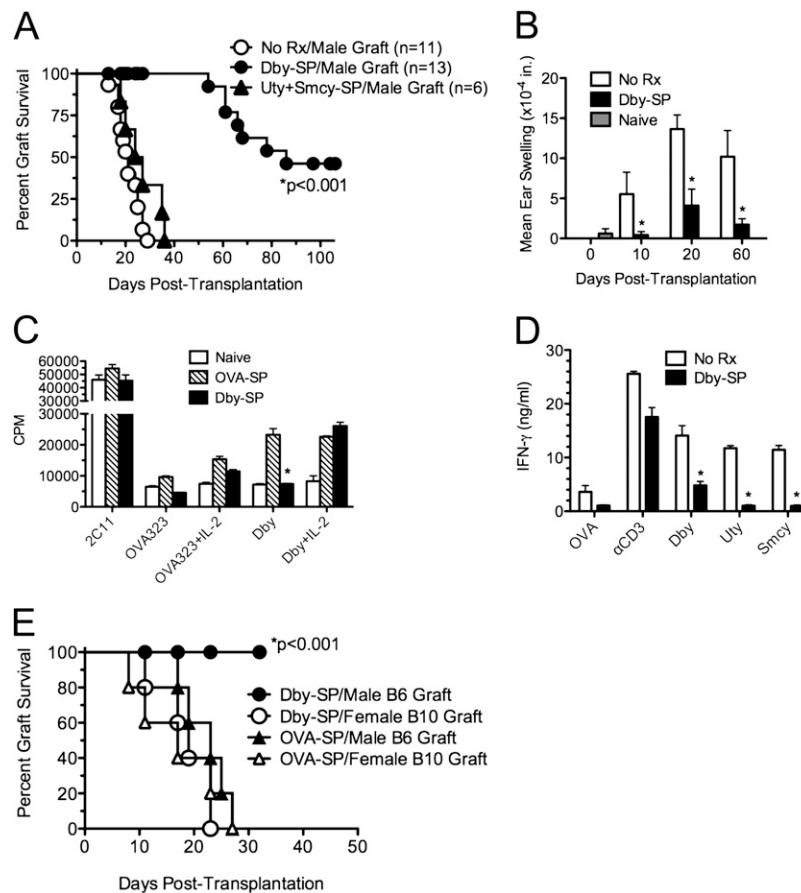

FIGURE 1. Hya-specific MHC class II, but not MHC class I, restricted Hya epitopes ECDI-coupled to B6 female-derived splenocytes promote long-term, Ag-specific survival of male skin grafts on B6 female recipients. A, Naive B6 females were treated i.v. with syngeneic female SPs ECDI-linked to the CD4 epitope (Dby) or the CD8 Hya epitopes (Uty and Smcy) or not tolerized on days −7 and 0 relative to engraftment with male tail skin grafts (and female control grafts; data not shown). Untreated control mice were included as a baseline for rejection time. Graft survival was monitored visually for 100 d. Male skin graft survival was significantly prolonged (*$p < 0.001$) in female recipients treated with female Dby-SP (filled circles) compared with both untreated (open circles) and Uty/Smcy-SP treated (filled triangles) female recipients. B, DTH responses of female B6 mice to ear challenge with 10 μg soluble Dby were determined at 10, 20, and 60 d posttransplantation in naive, untreated and Dby-SP–treated female B6 mice. Ear swelling responses in naive B6 mice served as the baseline. Ear swelling responses in Dby-SP–treated mice were significantly (*$p < 0.05$) less than those in nontolerized controls. C and D, In vitro recall responses of splenic T cells from untreated and Dby-SP–tolerized mice were determined 10 d posttransplantation upon stimulation with anti-CD3 (clone 2C11, positive control), OVA$_{323-339}$ (negative control), and Dby peptides by [^{3}H]thymidine incorporation (C) and IFN-γ secretion (D). Proliferative and IFN-γ responses were significantly (*$p < 0.01$) suppressed in Dby-SP–tolerized animals upon challenge with either the CD4 (Dby) or CD8 (Uty and Smcy) Hya epitopes. Proliferative responses to Dby could be restored to control levels by the addition of 25 U/ml exogenous IL-2. E, Ag specificity of the induction and effector stages of the regulatory effect was tested by treating female B6 mice i.v. with syngeneic female splenocytes ECDI-coupled with either OVA$_{323-339}$ or the Dby peptide at days −7 and 0 relative to engraftment with both male B6 skin and female skin from a C57BL/10 donor. Neither Dby-SP (open circles) nor OVA$_{323-339}$-SP (open triangles) treatment significantly prolonged B10 skin graft survival. However, Dby-SPs (closed circles), but not OVA$_{323-339}$-SPs (closed triangles), significantly protected (*$p < 0.001$) male B6 grafts from rejection. Five recipients were included in each group. All of the data shown represent at least three independent experiments.

responses. CD8^{+} populations in Uty/Smcy-SP–treated animals also exhibit diminished functional responses. This is discussed in further detail below.

Hya-specific CD8 cells display a naive phenotype in Dby-SP–tolerant animals

To further investigate the effects of CD4 tolerance on the development of CD8 effector responses, we measured the activation and lytic capacity of CD8 T cells specific for Uty and Smcy in the spleens and draining lymph nodes of control and Dby-SP–tolerized male graft recipients. H-2D[b] tetramers identifying the Uty- and Smcy-specific TCRs were used to identify expression of the activation marker CD44 on Hya-specific CD8^{+} populations. Compared to naive, nonengrafted B6 females, nontolerant graft recipients contain a distinct population of activated (CD44^{+}) Hya-specific CD8^{+} T cells (Fig. 2). Dby-SP treatment significantly inhibited the expansion and activation of Hya-specific CTLs, as demonstrated by a diminished number and proportion of tetramer-positive CD44^{+} cells in both spleens and graft draining lymph nodes of tolerant animals. Fig. 2 displays data collected at day 14 posttransplantation. Similar results were observed 10, 20, or 40 d posttransplantation (data not shown), although day 14 was the peak in Uty- and Smcy-specific CD8 cells in rejecting controls. This finding also coincides with an observed diminution of in vitro proliferative responses against Uty and Smcy following

Dby-SP treatment (data not shown). To assess the functional lytic capacity of Hya-specific CD8^{+} T cells, we conducted in vivo cytolysis assays using a variety of target pairs differentially labeled with CFSE. Specific targets consisted of male splenocytes, female splenocytes pulsed with CD8 Hya epitopes (Uty and Smcy), or female splenocytes pulsed with all three Hya epitopes. These targets were paired with reference targets consisting of female splenocytes, female splenocytes pulsed with an irrelevant CD8 epitope (Theiler's murine encephalomyelitis virus [TMEV] viral protein 2 [VP2]), or female splenocytes pulsed with an irrelevant CD8 epitope (VP2) and an irrelevant CD4 epitope (OVA$_{323-339}$), respectively. Consistent with the phenotypic analyses, CD8^{+} cells in Dby-tolerant animals failed to lyse male splenocytes or female splenocytes presenting male Ag (Fig. 3). The results from both the tetramer analyses and lysis assays indicate that CD4^{+} Th cells in Dby-SP–tolerant animals fail to prime effective Hya-specific CTL responses.

Ag-SP treatment decreases CD154 upregulation by Th cells

Due to the failed priming of the Ag-specific CD8 compartment observed in animals tolerized against the immunodominant Hya-specific Dby CD4 epitope, we reasoned that there is possibly a defect in one or more of the mechanisms used by CD4^{+} Th cells to prime Hya-specific CTLs. Previous studies have demonstrated defective IL-2 secretion by Th cells secondary to Ag-SP treatment (29, 30),
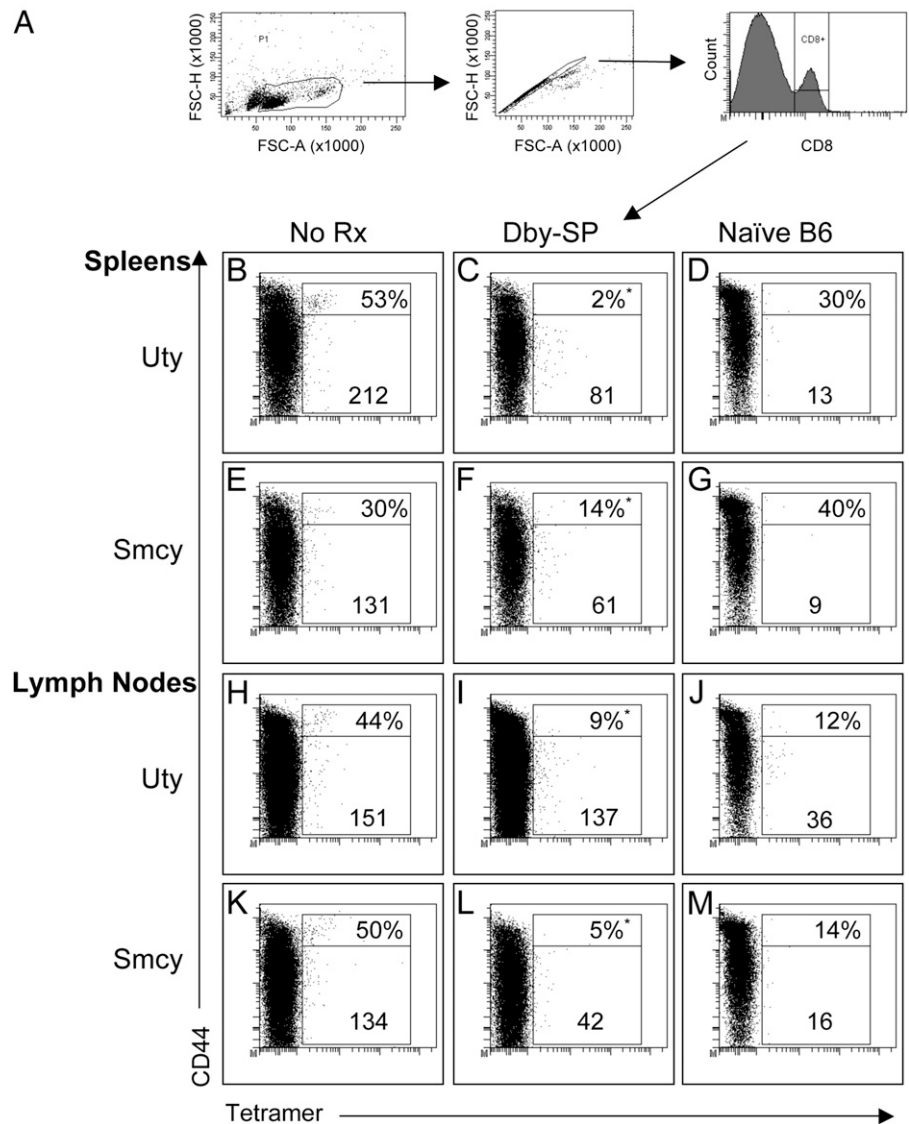

FIGURE 2. Dby-SP–induced nonresponsivenss of Hya-specific CD4^{+} T cells leads to failed priming of Hya-specific CD8^{+} T cells specific for the Smcy and Uty epitopes. The activation frequency (CD44^{+}) of Hya Uty and Smcy epitope-specific CD8^{+} T cells was determined using MHC class I tetramers. A, Live, single CD8^{+} cells were identified. B–M, CD44 expression on Uty-specific CD8^{+} T cells in spleens (B–D) and draining lymph nodes (H–J) and on Smcy-specific CD8^{+} T cells in spleens (E–G) and draining lymph nodes (K–M) of naive, nontolerized (No Rx), and i.v. Dby-SP–tolerized female B6 mice receiving B6 male tail skin grafts 14 d previously are shown. The total number of tetramer-positive events is listed on each dot plot. Percentages of activated (CD44^{+}) graft-specific CD8 cells, which appear in the top region of the tetramer-positive box, are also listed. Data are representative of three independent experiments.
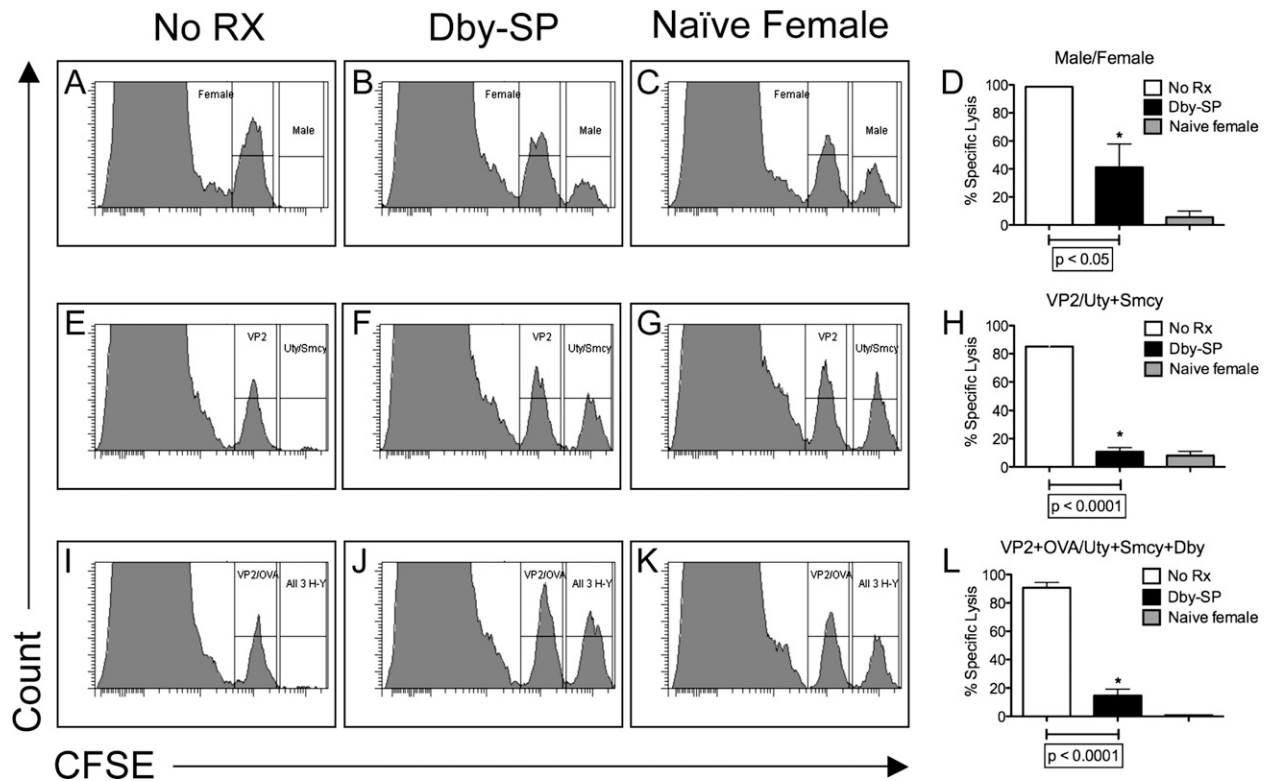
FIGURE 3. Dby-SP–induced nonresponsivenss of Hya-specific CD4^{+} T cells results in diminished cytolytic activity of Hya-specific CD8^{+} T cells specific for the Smcy and Uty epitopes. B6 females were ungrafted (naive), untreated (No Rx), or i.v. tolerized with Dby-SPs prior to engraftment with male skin. Fourteen days later, in vivo Hya-specific cytolytic activity was determined. Peptide-loaded targets were administered (one specific target and one reference target) and were discernable by differential CFSE labeling. *A–D*, Whole male splenocytes (specific) versus female splenocytes (reference) were used. *E–H*, Female splenocytes were pulsed with a combination of the Hya Uty and Smcy peptides (specific) or TMEV VP2 (reference). *I* and *K*, Female splenocytes were pulsed with a combination of the Hya Uty, Smcy, and Dby peptides (specific) or TMEV VP2 and OVA$_{323–339}$ (reference). White bars on the graphs (*D*, *H*, *L*) represent calculated lysis in nontreated graft recipients, black bars represent lysis observed in Dby-SP–treated recipients, and gray bars represent lysis observed in unmanipulated naive female controls. In vivo cytolytic responses in Dby-SP–tolerized mice were significantly (*$p < 0.01$) lower than those in nontolerized controls. Data are representative of three independent experiments.

and several studies, including the present (Fig. 1), have shown a defect in IFN-γ synthesis. Upstream of both of these cytokines is the involvement of CD40/CD154, a TNF family receptor–ligand pair that is critical for T cell costimulation, licensing of APCs, and Th-dependent activation of CD8^{+} T cells (24). CD40 ligation on APCs by CD154 expressed by activated CD4^{+} T cells increases APC expression of B7 family costimulatory molecules and proinflammatory cytokines, enabling the differentiation of naive CD8^{+} T cells to functional cytolytic effectors (reviewed in Refs. 16, 17, 24). We therefore measured the ability of CD4^{+} T cells to upregulate surface CD154 (CD40L) following treatment with Ag-SPs. OT-II TCR transgenic mice were left untreated (naive) or tolerized via the i.v. injection of 10[8] OVA$_{323–339}$-SPs or myelin basic protein (MBP)$_{84–104}$-SPs. Seven days later, spleens were removed and live CD4^{+} cells were analyzed for CD154 surface expression immediately upon explant and at serial time points (6, 12, 24, 48, and 72 h) poststimulation with OVA$_{323–339}$ in vitro. Peak expression was observed 6 h poststimulation. Upon Ag encounter, a significantly lower frequency of CD4^{+}CD154^{+} T cells was detected in cultures from mice tolerized to OVA$_{323–339}$-SPs in comparison with naive mice or mice tolerized to MBP$_{84–104}$-SPs, suggesting that Ag-SP encounter results in suboptimal activation and a decreased ability to present CD154 to other leukocytes (Fig. 4).

CD40 stimulation abrogates the tolerogenic effects of Dby-SP in vivo and in vitro

To assess the functional significance of the observed defect in CD40L upregulation, we determined the effect of administration of

an agonist mAb specific for CD40 (clone FGK45.5) to Dby-SP–tolerized graft recipients. This clone is known to bypass the requirement for CD4 help by stimulating the upregulation of costimulatory markers on APCs (31). B6 females were tolerized with Dby-SPs on days −7 and 0, engrafted with male tail skin on day 0, and were treated i.p. with 100 μg FGK45.5 or control rat IgG2a 24 h after engraftment. Compared to Dby-SP–treated B6 females receiving isotype control Ab, which were significantly protected from the rejection of male tail skin grafts (median 78 d), Dby-SP–treated animals receiving FGK45.5 rejected male grafts (median 28 d) similarly to nontolerized controls (median 19 d) (Fig. 5*A*), indicating that CD40 ligation overcomes the protection afforded by Ag-SP treatment. FGK45.5-induced in vivo stimulation of CD40 also reversed the suppression of Dby-specific in vitro proliferation (Fig. 5*B*) and IFN-γ secretion (Fig. 5*C*).

Cytometric analyses of Hya tetramer-positive CD8^{+} cells show that FGK45.5 administration to Dby-SP–tolerized recipients, in comparison with control IgG2a treatment, resulted in a significant enhancement of the frequency and numbers of activated graft-specific Uty- and Smcy-specific T cells (CD8^{+}tetramer^{+}CD44^{+}), reaching levels similar those observed in nontolerized controls (Fig. 6*A–G*). However, IFN-γ secretion in response to in vitro recall challenge with either the Uty or Smcy CD8 epitopes was not restored following FGK45.5 treatment of tolerized graft recipients (Fig. 6*H*), nor was in vivo CTL function restored in tolerant recipients following CD40 ligation (Fig. 6*I*). Collectively, these data indicate that CD40 ligation overcomes functional Ag-SP–induced graft protection by reversing unresponsiveness in the Hya-specific
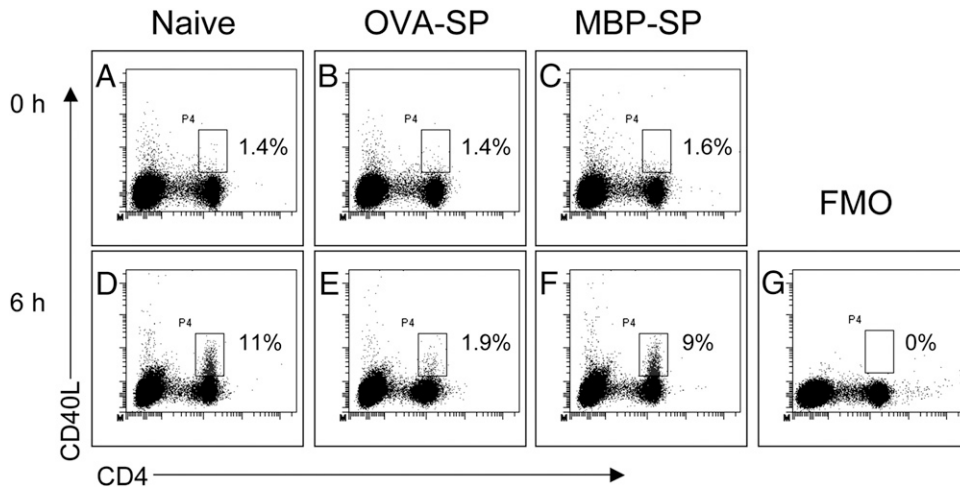
FIGURE 4. Ag-SP treatment inhibits CD154 upregulation on Ag-specific CD4$^+$ T cells upon Ag recall. *A–F*, B6 OVA$_{323–339}$-specific OT-II TCR transgenic mice were untreated (naive) (*A, D*) or injected i.v. with 10^8 OVA$_{323–339}$-SPs (*B, E*) or 10^8 MBP$_{84–104}$-SPs (*C, F*). Seven days later, splenocytes from these animals were harvested, stained, and analyzed for surface expression of CD154 immediately upon explant and again at 6 h following in vitro restimulation with 5 μM OVA$_{323–339}$. Maximal CD154 expression was observed at 6 h postculture. *G*, FMO control. Data are representative of at least 3 independent experiments. 10,000 CD4$^+$ events appear in each dot plot. FMO, fluorescence minus one.

CD4$^+$ compartment. Although anti-CD40 treatment resulted in recovery of control numbers of activated (CD44$^+$) Hya-specific CD8$^+$ T cells, the effector function of these cells as determined by IFN-γ production and lytic capacity was not restored.

In light of these findings and the observation that Uty/Smcy-SP treatment results in impaired lysis but normal graft rejection (Figs. 1, 6*I*), we reasoned that Hya-disparate graft destruction can take place despite an impaired CD8 response. This was confirmed by examining histological sections of engrafted skin for infiltrating CD4$^+$ and CD8$^+$ cells (Fig. 7). Male grafts on nontreated rejecting controls contain significant CD4$^+$ and CD8$^+$ infiltrate, whereas grafts from Dby-SP–treated animals contain greatly reduced CD4 infiltrate and virtually no infiltrating CD8$^+$ T cells, consistent with their prolonged survival. Conversely, grafts from animals receiving Dby-SPs followed by an FGK45.5 treatment contained CD4$^+$ cells but virtually no CD8$^+$ cells. A similar observation was made in grafts from Uty/Smcy-treated animals, which were found to contain significant

CD4$^+$ infiltrate but reduced CD8$^+$ infiltrate. This indicates that FGK45.5 treatment restores the ability of CD4$^+$ but not CD8$^+$ T cells to infiltrate the grafts of Dby-SP–treated mice. Together these data support the conclusion that Hya-disparate graft rejection can occur in the context of an impaired (following Uty/Smcy-SP treatment) or completely nonfunctioning (following Dby-SP and FGK45.5 treatment) CTL response.

The effects of FGK45.5 binding on the recipient APC populations were measured by cytometric analysis. Female B6 animals were transplanted with CD45.1 congenic skin 24 h prior to i.p. injection of FGK45.5 or control rat IgG. Seventy-two hours posttransplantation, spleens and lymph nodes were analyzed for APC activation, as determined by increased expression of the B7 family costimulatory molecule CD86. As expected, FGK45.5 treatment broadly activated APCs, as demonstrated by the large increase in CD86$^+$ cells (Fig. 8*D*). Lineage phenotyping of the CD86$^+$ population revealed that the primary activated APC population was

FIGURE 5. Dby-SP–induced protection of Hya skin grafts is reversed by CD40 cross-linking. *A*, Five to seven untreated (No Rx) and Dby-SP–tolerized female B6 mice received male tail skin grafts on day 0. Twenty-four hours later, separate groups of treated mice were injected i.p. with 100 μg IgG2a isotype control Ab or the agonistic anti-CD40 mAb, FGK45.5. Graft survival was monitored by visual inspection for 100 d. The prolonged survival of male skin grafts observed in Dby-SP–tolerized recipients treated with isotype control Ab was completely reversed by FGK45.5 treatment (*p < 0.001). *B* and *C*, Ten days posttransplantation and Ab treatment, recall responses of splenic T cells from the various treatment groups to in vitro stimulation with the Dby peptide were determined by [^{3}H]thymidine incorporation (*B*) and IFN-γ secretion (*C*). Suppressed proliferative and IFN-γ responses in the Dby-SP–tolerized animals were significantly reversed by treatment with FGK45.5 (*p < 0.01). Data are representative of at least three independent experiments.
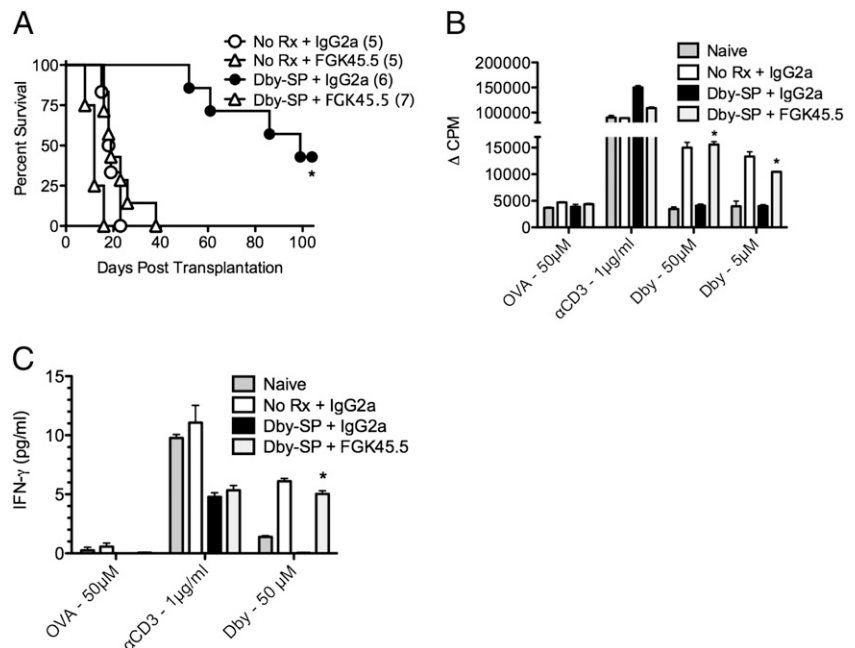
FIGURE 6. FGK45.5-mediated reversal of Dby-SP–induced protection of Hya skin grafts is not associated with restoration of Hya-specific CD8 T cell IFN-γ production or CTL activity. Untreated (No Rx) and Dby-SP–tolerized female B6 mice received male tail skin grafts on day 0. Twenty-four hours later, separate groups of Dby-SP–treated mice were injected i.p. with 100 μg IgG2a isotype control or with the agonistic anti-CD40 mAb, FGK45.5. *A–F*, Ten days posttransplantation, spleens from these treated mice were analyzed for the frequency of activated (CD44$^+$) Hya Uty (*A–C*) and Smcy epitope-specific CD8$^+$ T cells (*D–F*) using MHC class I tetramers. *G*, The total numbers of activated Hya-specific CD8$^+$ T cells are plotted. Similar results were observed upon analysis of lymph node CD8$^+$ T cells (data not shown). *H* and *I*, The in vitro IFN-γ recall response of splenocytes from the various treatment groups to recall stimulation with the Hya CD4 and CD8 epitopes was determined (*H*), as was the in vivo lytic activity of two targets pulsed with a combination of the Hya-Uty and Smcy CD8 epitopes (target) or TMEV VP2 (reference) (*I*). Interestingly, the IFN-γ response was restored to stimulation with the CD4 Dby epitope, but neither IFN-γ production nor the lytic function of CD8 T cells specific for the Uty or Smcy epitopes was restored by CD40 ligation. Data represent two independent experiments.
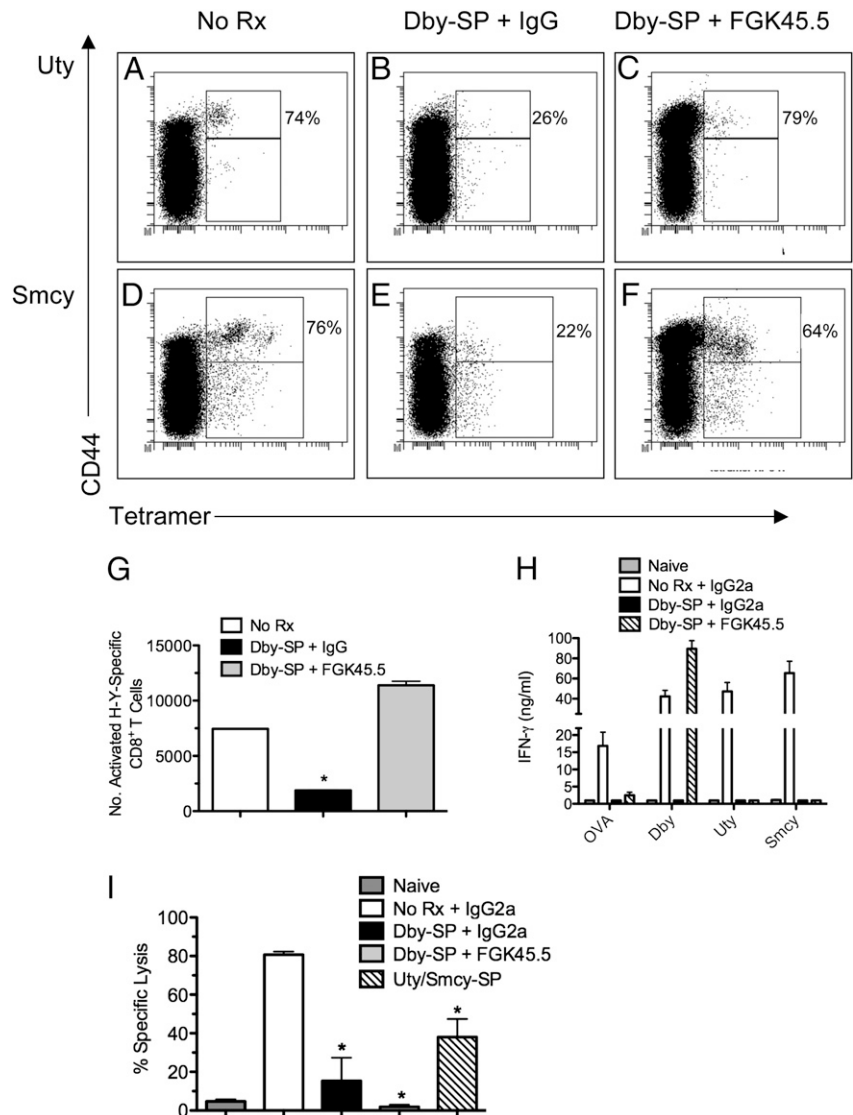
B cells (75–80% of the CD86$^+$ population, Fig. 8*E*), although a significant expansion and activation of DCs was also observed (Fig. 8*F*).

Discussion

The present data demonstrate that minor Hya alloantigenic peptides attached to syngeneic leukocytes using ECDI confer dominant Ag-specific transplant protection dependent on alteration of CD40/CD154 signaling. Induction of graft protection was dependent on the administration of cells coupled with the dominant CD4 epitope (Dby) but not CD8 epitopes (Uty and Smcy) of male Ag. These findings are consistent with a previous study by Chai et al. (27) where transplant tolerance was induced using soluble Dby peptide but not soluble Uty or Smcy. Paradoxically, a prior study from the same laboratory had reported that immature DCs (iDCs) loaded with the Uty peptide conferred tolerance to Hya-disparate skin grafts, whereas Dby-loaded iDCs sensitized female recipients and decreased male graft survival time (32). We found that Dby-coupled female splenocytes conferred significant protection to male skin grafts (Fig. 1), confirming a previous report from our laboratory that obtained a similar outcome using large numbers of ECDI-fixed male splenocytes. Although we observed some protection using an equivalent number of ECDI-fixed male splenocytes (median survival

39 d, data not shown), the protection was inferior to that conferred by Dby-SPs. As supported by titration experiments in this and in our previous study (28), this is likely due to the increased amount of Dby Ag provided to the recipient in the Dby-SP treatment versus the male splenocyte treatment.

The ability of ECDI-fixed Ag-SPs to induce peripheral tolerance has been documented in a number of CD4$^+$ T cell-mediated immune disorders, including EAE, type 1 diabetes, and allogeneic islet transplantation (5–7, 10, 33). As was observed in those models, we found that treatment with Dby-SPs significantly diminished DTH responses to Dby in vivo and reduced Dby-specific in vitro recall responses as assessed by proliferation and IFN-γ production, indicating profound unresponsiveness in the CD4 compartment. Further, Dby-SP–tolerized mice failed to develop CTLs specific for the immunodominant Hya CD8 T cell epitopes. Our findings indicate that Ag-SP therapy can be successfully used to specifically control the rejection of mismatched tissue grafts with the proviso that the epitopes are known. Importantly, both CD4 and CD8 responses are diminished in the absence of broad-scale immunosuppressive agents, and CD40–CD154 interactions are safely inhibited. We also find that complementing the observed defect in CD154 expression with anti-CD40 treatment restored graft rejection through CD4 reactivation but not through CD8
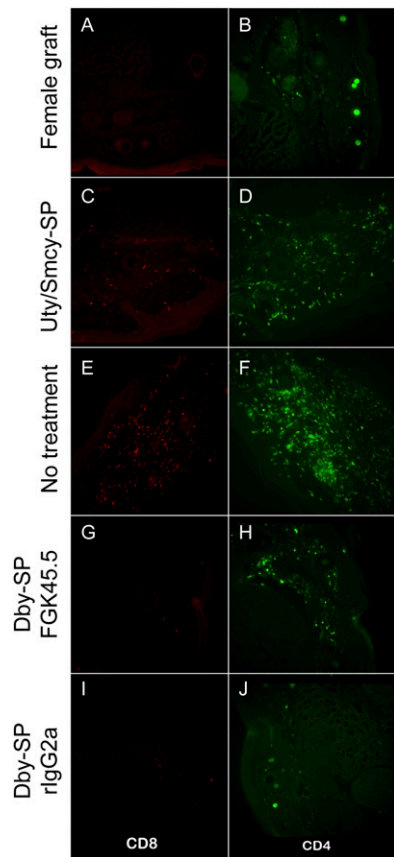
FIGURE 7. Reduced CD8 graft infiltrate does not correlate with graft survival. *A–J*, B6 female mice received female (*A, B*) and male (*C–J*) skin grafts following treatment with Uty/Smcy-SPs (*C, D*), nothing (*E, F*), Dby-SPs and rIgG2a (*G, H*), or Dby-SPs and FGK45.5 (*I, J*). Ten days after engraftment, histologic sections were prepared from graft-containing tail areas and stained for CD4 (*B, D, F, H, J*) or CD8 (*A, C, E, G, I*). Grafts from mice displaying rejection (i.e., Uty/Smcy-SP–treated, Dby-SP- and FGK45.5-treated, and untreated groups) contain variable amounts of CD8 infiltrate and considerable CD4 infiltrate. Grafts that are retained (female control grafts and male grafts on Dby-SP and rIgG2a-treated females) contain very few infiltrating T cells. Original magnification ×20.

activation. FGK45.5 has previously been reported to successfully and unsuccessfully prime CTL responses in the absence of CD4 T cells (31, 34).

We observed that FGK45 treatment of Dby-SP–treated mice restored normal levels of graft rejection as well as graft infiltration, proliferation, and IFN-γ responses in the CD4 compartment but not infiltration, lysis, or IFN-γ responses in the CD8 compartment. These findings are in agreement with pioneering work performed using FGK45, which collectively suggests that FGK45 effects on tumor and graft destruction are mediated primarily through CD4 T cells. Shepherd and Kerkvliet (31) demonstrated that CTL responses against P815 tumor cells are not initiated in CD154$^{-/-}$ mice and that neither FGK45.5 treatment nor the use of B7 over-expressing P815 cells restored CTL function, indicating that increased costimulation alone is insufficient for CTL priming in the absence of CD4 help/CD154. Likewise, FGK45 treatment only partially restores CTL function and allograft destruction in CD4$^{-/-}$ mice. CD8-mediated graft destruction was again suggested to be independent of costimulation through the use of CD28-deficient recipients, indicating that, although FGK45 treatment certainly increases B7 expression on APCs, it contributes minimally to direct priming of CTLs (34). Another study found that antitumor CTL responses could be primed in animals subjected to CD4 T cell

depletion and FGK45 treatment; however, CD4 depletion was initiated 4 d following FGK45 treatment (35). It is therefore possible that, bolstered by FGK45 treatment, sufficient CD4 activation occurred to prime protective antitumor CTL responses prior to the depletion of Th cells. Direct evidence for enhanced CD4 activation is supported by the finding that FGK45 treatment induced increases in Ag-specific CD4 T cell number, proliferation, IL-2, and IFN-γ secretion (36). Our observation of restored CD4, but not CD8, activity despite an increase in B7 family expression on recipient APCs is in agreement with the previous studies. FGK45-rescued T helper cells may fail to provide a critical determinant for CTL priming. FGK45 (administered 24 h following Ag-SP treatment and engraftment) cannot be affecting the input Ag-SPs, because ECDI-fixed splenocytes under apoptotic death are removed from the circulation and lymphoid organs within 12 h (33).

Cross-linking of peptide to the cell surfaces using ECDI rapidly induces apoptosis in Ag-SPs (33), and i.v. administration of Ag-SPs leads to the establishment of clonal anergy in T cells specific for the chemically affixed peptide (1, 6, 37). The induction of apoptosis in Ag-SPs is likely an important determinant in this therapeutic strategy, because apoptotic cells have been shown to be tolerogenic whereas cells dying by necrosis have been shown to be proinflammatory (38). More recent work exploring the mechanism of apoptotic cell tolerance determined that previously activated apoptotic expressing CD154 failed to induce tolerance (39), complimenting a previous study from the same group that showed that FGK45.5 treatment abrogated apoptotic cell-induced tolerance (40). Consistent with these findings, we found that Ag-SP encounter induces a defect in CD154 expression in the targeted T cell and that this defect is crucial to the protection of Hya-disparate grafts.

Although ECDI-catalyzed coupling of Ags to donor leukocytes has been a tool for inducing Ag-specific immune tolerance in our laboratory for nearly 30 y (4), the mechanism of suppression is not completely clear. Initial observations by Jenkins and Schwartz (9) showed that Ag-specific Th1 clones stimulated by ECDI-fixed Ag-SPs failed to produce IL-2, resulting in failure of those T cells to mount a response during secondary encounter with the cognate Ag. However, these cells remained responsive to IL-2, and anergy could be prevented or reversed by supplementing IL-2 (9). Likewise, we showed that diminished proliferative responses of T cells from Dby-SP–treated male graft recipients in response to Dby rechallenge could be rescued by the addition of exogenous IL-2 (Fig. 1C). IL-2 is also a critical factor in CD4 priming of CD8 responses. In a different model of tolerance induction to Hya-mismatched tissue, it was concluded that limited IL-2 production by Hya-specific CD4^{+} T cells was the critical helper-dependent factor for the generation of an effective and long-lasting Hya-specific CD8 T cell response (27). CD8^{+} T cells primed in the absence of IL-2 fail to mount sufficient responses during secondary encounter with Ag (41). This may account for the apparently conflicting results obtained in the paper by James et al. (32), in which Uty-pulsed iDCs conferred graft survival, but Dby-pulsed iDCs induced strong antigraft responses. Although grafts were retained in Uty-iDC recipients, Uty-specific CD8^{+} T cells displayed an activated phenotype and secreted IFN-γ in recall assays. This could indicate that Uty-iDCs may have induced Ag-specific CD8 responses but because the activation occurred in the absence of CD4-derived IL-2, a productive memory response was not induced. Despite the belief that iDCs present Ag in a tolerogenic fashion, the fact that they were administered s.c. (via the footpad) supports the idea that they actually functioned to immunize the animals in that study. In our experience with Ag-SPs, the route of administration is a critical parameter, because s.c. injection stimulates DTH responses, whereas i.v. injection induces tolerance (4).

FIGURE 8. FGK45.5 treatment results in increased numbers of activated B cells and DCs. B6 females were engrafted with skin from a CD45.1 congenic donor female. Twenty-four hours posttransplantation, recipient animals received 100 μg FGK45.5 or rat IgG2a via i.p. injection. Forty-eight hours after Ab treatment (and 72 h posttransplantation), spleens and lymph nodes were analyzed for the expression of the B7 family costimulatory molecule CD86 on various lineages of APCs. *A–D*, CD86 expression was upregulated on the cells of graft recipients treated with FGK45.5 (*D*) but not those treated with isotype control (*C*) nor on naive control animals (*B*). *E* and *F*, Enumeration and phenotyping revealed that the majority of CD86 expressers are B cells (*E*) and DCs (*F*). Three mice were included in each group.
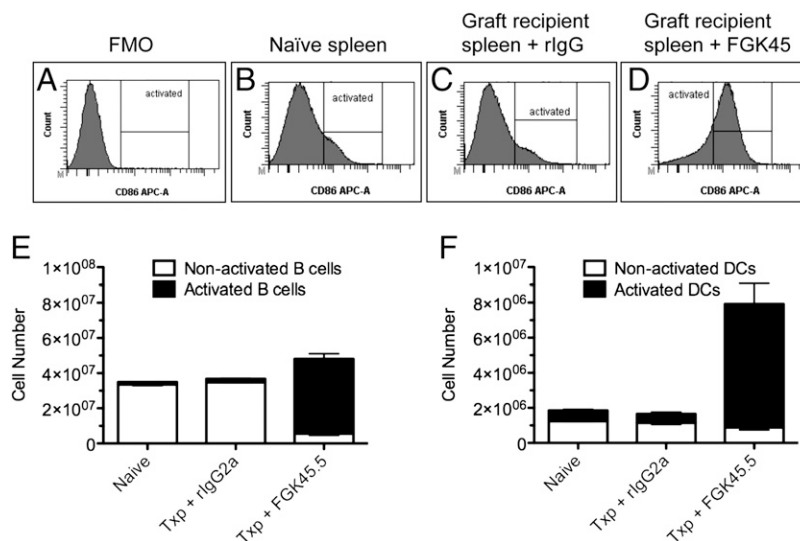
It has been proposed that clonal anergy is the major mechanism by which Ag-SPs confer immune tolerance. The induction of clonal anergy is thought to occur when T cells receive "signal 1" (via the TCR) but not "signal 2" (via CD28) (reviewed in Ref. 42). This control mechanism probably evolved to avoid inappropriate activation of T cells in the absence of a "danger signals" (in the form of a TLR ligand, for example), which induce APCs to upregulate expression of B7 family costimulatory molecules and secretion of proinflammatory cytokines. Ag-SP administration clearly does not provide a danger signal to APCs, as demonstrated by the fact that LPS-induced APC activation abrogated the protective effect of Ag-SP therapy (8, 43). Induction and maintenance of T cell anergy following encounter with ECDI-fixed splenocytes is critically dependent upon a delicate balance of positive and negative costimulatory signals consistent with the demonstrated involvement of both CTLA-4–B7 and programmed-death-1–programmed-death ligand-1 interactions (6–8). In addition to TLR ligands, CD40–CD154 interactions are critical regulators of costimulatory molecule expression, cytokine secretion, and APC survival. Both mechanisms are known to regulate CD4 priming of CTL responses, and both abrogate Ag-SP–induced immune tolerance. The involvement of other TNFR family molecular pairs has not been determined in Ag-SP–induced immune tolerance. We expect perturbations to molecular pairs, such as 41-BB/41-BBL and OX40/OX40L, may also contribute to Ag-SP–induced hyporesponsiveness.

The role of CD8+ T cells in the rejection of Hya-disparate tissue is not completely clear. Male skin grafts are accepted by both β2-microglobulin–deficient and CD8 T cell-depleted B6 females (44–46); however, attempts to specifically inactivate Hya-specific CD8+ populations suggest that graft survival and CTL activity often do not correlate. For instance, Uty-pulsed iDCs given s.c. were shown to prevent rejection of male grafts but did not prevent Uty-specific CD8 cells from becoming activated and secreting IFN-γ (32). Our results (Fig. 1) and others (27) noted that Uty/Smcy-tolerized mice rejected Hya-disparate grafts (Fig. 1A) despite the fact that in our study these cells had diminished ability to produce IFN-γ and mediate lysis in response to recall with the CD8 epitopes (data not shown). In addition, increased male graft survival observed following the i.v. injection of large numbers of viable male splenocytes correlated with diminished Hya-specific DTH, but not CTL, responses (28). These studies collectively indicate that CD8 effector functions are insufficient for male graft rejection or can be complimented by other CD4-mediated effector mechanisms to

reject Hya-disparate grafts in the absence of CTL activity. Consistent with the notion of a disconnect between graft rejection and CTL activity, we found that graft rejection observed in Dby-tolerized, FGK45.5-treated females is associated with a restoration of the Hya-specific CD4+ response (proliferation or IFN-γ secretion) but not the CD8+ response (lysis or IFN-γ secretion). We also show that tetramer-positive CD8 T cells express CD44 following anti-CD40 treatment, indicating that surface phenotype is not necessarily indicative of effector function, in agreement with previous observations (47). It is also possible that Ag-SP–induced regulatory T cells (7) allow expansion and partial activation of CD8 populations but regulate their effector functions (27, 47).

That CD4 T cells are necessary and sufficient for the rejection of allogeneic tissue has been well described in models of transplantation across MHC class II-disparate barriers. Waldmann and colleagues showed that CD4 T cells expressing a transgenic TCR specific for Hya on a RAG1 knockout background was sufficient for rejection of Hya-mismatched skin grafts (48), although they were unable to determine the mechanism of graft rejection. Effector molecules, such as IFN-γ, Fas/Fas ligand, and granzyme B, have all been implicated in CD4 T cell-mediated tissue rejection (49), of which IFN-γ may have the most significant role due to its ability to mobilize immune responses against the target tissue and modify the local milieu. The pleiotropic effects of IFN-γ include the inducible expression of MHC class II and costimulatory molecules on endothelium and tissue parenchyma, increased vascular permeability, the downstream recruitment of effector cell types, and enhanced activation of macrophages, monocytes, and NK cells (50). Recently, IFN-γ production by CD4 T cells was shown to be critical for the recruitment of cytotoxic CD8 T cells to target tissues via its ability to enhance local tissue expression of CXCL9 and CXCL10 (51). Interestingly, in our model, we did not find a role for IFN-γ production in the recruitment of CD8 T cells, because FGK45.5 restored IFN-γ production in Dby-SP–tolerized CD4 T cells, and yet we failed to see any significant presence of CD8 T cells in the rejecting grafts of Dby-SP–tolerized recipients receiving FGK45.5. These results suggest that the impairment of the Hya-specific CD8 T cell response goes beyond a defect in their ability to traffic to the graft site and that rejection is a CD4 T cell-mediated event. Because IFN-γ can induce tissue expression of MHC class II, allowing for direct recognition of Hya-expressing tissue by CD4s, it is possible that Hya-specific T cells are mediating graft rejection by direct cytotoxicity, as was recently observed in a model of CD4 T cell-mediated tumor immunity (52). Alternatively, as favored by the correlation between

DTH responsiveness and chronic graft rejection, it is likely that the Hya-specific CD4s may mediate graft rejection via an indirect pathway, through the activation of Hya-bearing, tissue-resident APCs. Bone marrow transplantation across an MHC class II-disparate barrier was capable of inducing 100% lethal graft-versus-host disease in a CD4 T cell-dependent manner, even when chimeric recipients (MHC class II knockouts reconstituted with wild-type APCs) were used (53). Thus, CD4 T cells were able to mediate graft-versus-host disease with wild-type kinetics when only the tissue-resident APCs expressed the alloantigens. Propagation of tissue destruction and inflammation by macrophages and microglia has also been described in models of autoimmunity (54, 55).

Aside from a defect in IL-2 production, anergy is associated with the expression of several E3 ubiquitin ligases that function to dampen activation signals in T cells by targeting the relevant signaling molecules for proteasomal degradation. One such E3 ligase is gene related to anergy in lymphocytes (GRAIL), which was found to mediate the ubiquitination of CD154 in experimentally anergized T cells (56). Because T cells encountering Ag-SPs exhibit defective CD154 upregulation after subsequent antigenic challenge, it is possible that Ag-SP tolerance may induce GRAIL expression leading to CD154 degradation. Conversely, Ag-SP treatment may induce a GRAIL-independent defect in CD154 expression associated with anergy induction. In support of the latter hypothesis, CD154 expression has been shown to be the critical factor in determining the diabetogenic potential of pancreas-specific CD4$^+$ T cells; NOD/4.1/RAG2$^{-/-}$/CD154$^{+/+}$ mice develop type 1 diabetes, whereas NOD/4.1/RAG2$^{-/-}$/CD154$^{-/-}$ mice fail to develop disease even after treatment with agonistic anti-CD40 (57). Ongoing experiments are determining the involvement of GRAIL in regulating CD154 expression in Ag-SP–induced tolerance.

Acknowledgments

Disclosures

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