T Cells Reactive to a Single Immunodominant Self-restricted Allopeptide Induce Skin Graft Rejection in Mice

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Abstract

Alloreactive T lymphocytes can respond to foreign MHC complexed with foreign peptides through the direct pathway of allorecognition and can additionally recognize allopeptides expressed in the context of recipient (self) MHC through the indirect pathway. To better elucidate how indirect pathway–responsive CD4$^+$ T cells mediate allograft rejection, we isolated and characterized a TH1 T cell line from BALB/c recipients of B10.A skin that responds to a defined immunodominant, self-restricted allopeptide, I-Aβ^k58–71. When transferred into BALB/c severe combined immunodeficiency recipients of B10.A skin allografts, this cell line specifically induced a form of skin graft rejection characterized by the presence of TH1 cytokines, macrophage infiltration, and extensive fibrosis. Recall immune responses and immunofluorescence of the rejecting skin revealed only the presence of the peptide-specific T cells within the recipient animals, with no evidence of a direct pathway alloresponse. These studies demonstrate that T cells reactive to a single self-restricted allopeptide can mediate a form of allogeneic skin graft rejection that exhibits characteristics of a chronic, fibrosing process. (*J. Clin. Invest.* 1998. 101:1398–1407.) Key words: T lymphocytes • transplantation immunology • cytokines • graft rejection • cellular immunity

Introduction

T lymphocytes are known to be central mediators of allograft rejection (1, 2). The majority of recipient T cells activated in response to an allograft recognize donor peptides complexed with donor MHC molecules expressed directly on the transplanted cells through the direct pathway of allorecognition (3). Recent work has also established that alloreactive T cells can respond to processed donor peptides presented in the context of recipient MHC molecules through the indirect pathway (4–9). The relevance of indirect allorecognition to the rejection process is highlighted by studies demonstrating that immunotherapy targeting peptides presented by the indirect pathway can result in allograft tolerance (10–14).

The specific role played by CD4$^+$ T cells responsive to determinants presented via indirect allorecognition is only partially understood. Elegantly designed experiments using MHC II knockout mice as skin donors have clearly shown that these T cells participate in graft rejection, and suggest that they provide help for the induction of a CD8$^+$, direct pathway–responsive effector cell population that in turn rejects the allograft (9). Additionally, there is some evidence to suggest that CD4$^+$, indirect pathway–responsive T cells can mediate graft rejection without CD8$^+$ cells (15, 16), but the rejection process under these circumstances seems to be inefficient, at best. In addition, the effector mechanisms involved are poorly understood, especially in light of the fact that without direct allorecognition, there can be no direct cytotoxicity of the transplanted cells. Furthermore, T cells responding to self-restricted allopeptides have been hypothesized to be specifically pathogenic in the development of chronic allograft rejection (14). However, despite much speculation, there is little experimental evidence to support this contention.

To provide further insight into the role of CD4$^+$ T cells responding to indirect pathway determinants as potential mediators of allograft rejection, we have been studying rejection of B10.A skin by recipient BALB/c mice (6, 7, 17). In this model, indirect alloreactivity is focused towards a defined, MHC II donor–derived, immunodominant determinant (I-Aβ^k58–71 [I-Ap])[1] expressed in the context of recipient MHC II (6, 7, 17). In these studies, we provide evidence that TH1 T cells specific for I-Ap, without concomitant reactivity to alloantigens presented by the direct pathway, can clearly mediate skin graft rejection. Moreover, the studies suggest that these T cells produce a form of rejection that exhibits features of a chronic process characterized by mononuclear infiltration and fibrosis, but only slow clinical deterioration of the graft itself. The work provides an ideal model system for isolating and studying the mechanisms of indirect pathway–responsive T cells as mediators of allograft rejection.

Methods

Animals. Female BALB/c (*H-2*^{d}), BALB/c severe combined immunodeficient (SCID) (*H-2*^{d}), C57BL/6 (B6, *H-2*^{b}), and B10.A (*H-2*^{a}; K^{k}A^{k}E^{k}D^d), age 6–8 wk, were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the specific pathogen–free animal facility at the Cleveland Veterans Affairs Medical Center.

Peptides. I-Ap (AEYWNKQYLERTRA) and hen egg white lysozyme 106–116 (HELp; NAWVAWRNRCK) were synthesized by Research Genetics (Huntsville, AL) at > 90% purity. I-Aβ^k31–49

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1. *Abbreviations used in this paper:* APC, antigen-presenting cell; Con A, concanavalin A; DTH, delayed-type hypersensitivity; HELp, hen egg lysozyme peptide 106–116; I-Ap, peptide I-Aβ^k58–71; RT, reverse transcription; SCID, severe combined immunodeficiency; TCR, T cell receptor; Vβ, variable region β chain.

and I-Aα^k49–63 were kindly supplied by Gilles Benichou, University of California, San Francisco, CA.

Placement and evaluation of skin grafts. Full-thickness trunk skin allografts were placed using standard techniques (2, 17). Skin was harvested from killed donor mice, the subcutaneous fat was removed, and the skin was cut into 0.5-cm^2 pieces and placed in sterile PBS until used for transplantation (< 30 min). Recipient mice were anesthetized with pentobarbital (50 μg/g body wt) and shaved around the chest and abdomen. The skin allograft was placed in a slightly larger graft bed prepared over the chest of the recipient and secured using Vaseline gauze and a bandage. Bandages were removed on day 7, and the grafts were then visually scored daily for evidence of rejection. The allograft was considered fully rejected when it was > 50% necrotic. In selected animals, allograft rejection was confirmed histologically. Skin grafts were placed on SCID recipients using fully sterile techniques.

Histologic evaluation of skin. Skin tissue was harvested from killed mice and prepared for histologic analysis by standard techniques. A portion of the skin tissue was frozen in OCT compound (Miles Laboratories Inc., Elkhart, IN) for immunofluorescence studies. Sections were stained with hematoxylin and eosin. Histologic evaluation of the skin was performed in a blinded fashion by a board-certified dermatopathologist (A. Gilliam).

Preparation of stimulator cells. Splenic stimulator cells were prepared by incubation with mitomycin C (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 50 μg/ml for 20 min at 37°C in PBS, followed by three washes in HBSS. The cells were counted by trypan blue exclusion and diluted for use in the various assays.

Isolation and maintenance of SH10 T cell line. SH10 T cell line was prepared using cells isolated from the draining lymph nodes of a BALB/c mouse rejecting a B10.A trunk skin allograft using previously published techniques (18). The cell line was maintained on a monthly cycle by stimulation in complete RPMI (10% FCS, 5% NCTC medium, 2-mercaptoethanol, penicillin, and streptomycin) with syngeneic mitomycin C–treated splenocytes as antigen-presenting cells (APCs) and 2 μM I-Ap for 10 d, followed by two consecutive feeding cycles with 2 U/ml recombinant human IL-2 (PharMingen, La Jolla, CA) for 10 d (without new APCs or antigen).

Proliferation assays. 4–5 × 10^4 T cells were mixed with 4 × 10^5 syngeneic BALB/c mitomycin C–treated splenocytes as APCs in triplicate in flat-bottomed 96-well plates with or without 10 μM peptide. 1 μCi [^{3}H]thymidine was added for the final 16 h of a 72-h incubation. Plates were harvested, and the incorporated label was measured by liquid scintillation counting.

Antibodies. FITC-conjugated anti-CD8α (53-6.7), phycoerythrin-conjugated anti-CD4 (RMA4-5), FITC-conjugated anti–variable region β chain (Vβ) 2 (B20.6), FITC-conjugated anti-Vβ4 (KT4), FITC-conjugated anti-CD3, and FITC-conjugated anti-CD45RB were purchased from PharMingen. FITC-conjugated anti-CD62 (L-selectin, MEL-14) was a kind gift from Paul Lehmann, Department of Pathology, Case Western Reserve University.

FACS® analysis. SH10 cells were purified by isolation through Lympholyte M (Cedarlane Labs Ltd., Hornby, Ontario, Canada) and stained in PBS/0.1% BSA for 30 min at 4°C. Single cell suspensions of spleen cells were similarly stained after red blood cell lysis. After three washes in PBS, the cells were fixed in fresh 1% paraformaldehyde and stored at 4°C in the dark until analyzed (within 24 h). Analysis was performed using a FACScan® (Becton Dickinson, San Jose, CA) and accompanying software using 5,000 ungated cells.

Direct immunofluorescence. Frozen sections of skin tissue were fixed in acetone at −20°C for 30 s and then washed three times in PBS for 5 min per wash. The tissue sections were incubated with FITC-conjugated anti-Vβ2 or anti-Vβ4 antibodies at 1:100 dilution in PBS/0.1% BSA for 30 min at room temperature and then washed three times in PBS. Slides were examined and photographed with an immunofluorescence microscope.

Preparation of RNA. Lympholyte M–purified T cells or skin tissue were homogenized in TRIzol RNA preparation reagent (GIBCO BRL, Gaithersburg, MD) and total RNA was isolated as instructed by the manufacturer.

Reverse transcription (RT) PCR for cytokine message and T cell receptor (TCR) Vβ gene expression. First-strand cDNA and RT-PCR for cytokine or TCR Vβ gene expression was performed using previously described oligonucleotide primers (18–21). Primers for β-actin, IFN-γ, IL-2, IL-4, IL-10, TNF-α, and TGF-β were purchased from Stratagene Inc. (La Jolla, CA). PCR was performed in a total volume of 50 μl as follows: 94°C denature for 5 min, 35 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min, followed by a 20-min extension at 72°C. 15 μl of each sample was run on a 1.5% agarose gel and photographed. Selected RT-PCR products were blotted onto nitrocellulose membranes and probed with an internal constant region β oligonucleotide as described previously (20).

Delayed-type hypersensitivity (DTH) assay. DTH was performed as described previously (18). Briefly, 1–2 × 10^6 SH10 cells plus 10 μM I-Ap in a total volume of 25 μl of PBS were injected into the right ear of three naive BALB/c mice, and 1–2 × 10^6 SH10 cells plus control peptide were injected into the left ear. Ear thickness was measured before immunization and at 48 h with an engineer's micrometer by an investigator blinded to the experimental groups, and the results were expressed as difference in mean values of the thickness between the ears before injection and at the 48-h time point. Ear tissue was placed in formalin, cut, and stained with hematoxylin and eosin for histologic analysis.

Cytotoxicity assays. Cytotoxicity was performed as published by Matzinger (22). BALB/c or B10.A target cells were made by incubating 2 × 10^6 splenocytes with 1 μg/ml LPS (Sigma Chemical Co., St. Louis, MO) in 2 ml of HL-1 medium (BioWhittaker, Inc., Walkersville, MD) for 48–72 h. [^{3}H]thymidine 10 μCi with or without 10 μM I-Ap (or control peptide) was added for the final 12 h. [^{3}H]thymidine-labeled A20 cells (*H-2*^{d}, B cell lymphoma obtained from American Type Culture Collection, Rockville, MD) were used as targets in some experiments. After three washes in HBSS medium, the target cells were counted, and 10,000 cells were placed in each well of a round-bottomed 96-well plate. Positive control BALB/c anti-B10.A killer cells were produced by incubating 8 × 10^6 BALB/c spleen cells with 4 × 10^6 B10.A stimulator cells in 2 ml of complete RPMI at 37°C 5% CO_2 for 5 d, and then washed and counted. SH10 cells or BALB/c anti-B10.A killer cells were added to the targets at various E/T ratios and incubated at 37°C 5% CO_2 for 5 h. The plates were harvested and counted by liquid scintillation, and the percentage of cytotoxicity was calculated as described (22).

ELISA assays. 300,000 SH10 cells were mixed with 3 × 10^6 mitomycin C–treated BALB/c stimulator cells with or without 10 mM peptide in 2 ml of complete T cell medium in a 24-well plate. The supernatants were harvested at 48 h and studied in a sandwich ELISA for cytokine production. Immulon-4 plates (PGC Scientific, Gaithersburg, MD) were coated with capture antibody in PBS overnight. R46A2 (4 μg/ml) for IFN-γ, 11B11 (1 μg/ml) for IL-4, and TRFK5 (2.5 μg/ml) for IL-5 were produced and isolated in our laboratory from hybridomas. Anti–IL-2 (3 μg/ml), anti–IL-10 (4 μg/ml), and anti–TNF-α (4 μg/ml) capture antibodies were purchased from PharMingen. After washing with PBS, cytokine standards (PharMingen) and the culture supernatants were added to wells in duplicate and incubated at 4°C overnight. After three washes in PBS/0.025% Tween (PBST), second antibodies were added for 4 h at room temperature. XMG1.2-biotin (produced in our laboratory) was used for IFN-γ. Other detection antibodies were purchased from PharMingen: rat anti–mouse IL-4–biotin (1 μg/ml) was used for IL-4, rat anti–mouse IL-2–biotin (1 μg/ml) was used for IL-2, rat anti–mouse IL-10–biotin (2 μg/ml) was used for IL-10, rat anti–mouse IL-5–biotin (2 μg/ml) was used for IL-5, and rat anti–mouse TNF-α–biotin (2 μg/ml) was used for TNF-α. The plates were again washed three times in PBST, and streptavidin-alkaline phosphatase (1:2,000 dilution; DAKO Corp., Carpinteria, CA) was added for 1 h. The plates were developed with a *p*-nitrophenyl phosphate substrate (Research Organics Inc., Cleveland, OH), and the OD was read at 405 nm.

Table I. Cytokine Production by T Cells during Rejection of B10.A Skin Grafts (Spots per 10^6 T Cells Plated)

Cytokine	I-Ap	HELp	B10.A splenocytes	Con A
IFN-γ	24	< 5	3830	> 5000
IL-2	22	< 5	990	> 5000
IL-5	< 5	< 5	< 5	> 5000

Purified T cells (> 95% $CD3^+$ by FACS®) obtained from draining lymph nodes of BALB/c mice on day 11 after placement of B10.A skin were studied in IFN-γ, IL-2, and IL-5 ELISA spot assays in response to 10 μM I-Ap or HELp, B10.A stimulator cells, or 2 μM Con A. Values are the means of duplicate wells counted by a computer-assisted image analyzer and are representative of five individual experiments. Intrawell variability was < 10%. No detectable response (< 1 per million) to I-Ap was noted in naive animals or in animals rejecting control B6 allografts (not shown).

ELISA spot assays. ELISA spot plates (Polyfiltronics, Inc., Rockland, MA) were coated with capture antibodies in sterile PBS overnight. R46A2, produced and isolated in our laboratory from a hybridoma, was used at 4 μg/ml for IFN-γ. Anti–IL-2 capture antibody (JES6-1A12; PharMingen) was used for IL-2, and anti–IL-5 capture antibody (TRFK4; PharMingen) was used for IL-5. The plates were blocked for 1 h with sterile PBS/1% BSA and washed with sterile PBS. Various dilutions of splenocytes, lymph node cells, or SH10 T cells (0.25–8 × 10^5/ml) in 200 μl of HL-1 medium were placed in each well with or without antigen and irradiated APCs (in duplicate), and incubated at 37°C for 24 h in 5% CO_2. After washing with PBS/0.1% Tween, detection antibodies were added overnight. XMG1.2–horseradish peroxidase (produced in our laboratory from a hybridoma) was used for IFN-γ, rat anti–mouse IL-2–biotin (JES6-5H4; PharMingen) was used for IL-2, and rat anti–mouse IL-5 (TRFK5; PharMingen) was used for IL-5. Streptavidin–horseradish peroxidase (DAKO Corp; 1:2,000 in PBS/0.1% Tween for 2 h at room temperature) was used as a third reagent for IL-2, and anti–IgG2a-horseradish peroxidase (Zymed Laboratories Inc., South San Francisco, CA; 1:300 in PBS/0.1% Tween for 2 h at room temperature) was used as a third reagent for IL-5. The plates were developed using 800 μl AEC (Pierce Chemical Co., Rockford, IL; 10 mg dissolved in 1 ml dimethyl formamide) mixed in 24 ml 0.1 M sodium acetate, pH 5.0, plus 12 μl H_2O_2. The resulting spots were counted on a computer-assisted ELISA spot image analyzer (developed using software from Optimas Corp., Bothell, WA), which is designed to detect ELISA spots using predetermined criteria based on size, shape, and colorimetric density.

Results

Isolation and characterization of SH10 cells. We initially isolated draining lymph node T cells (> 95% $CD3^+$, 66% $CD4^+$,

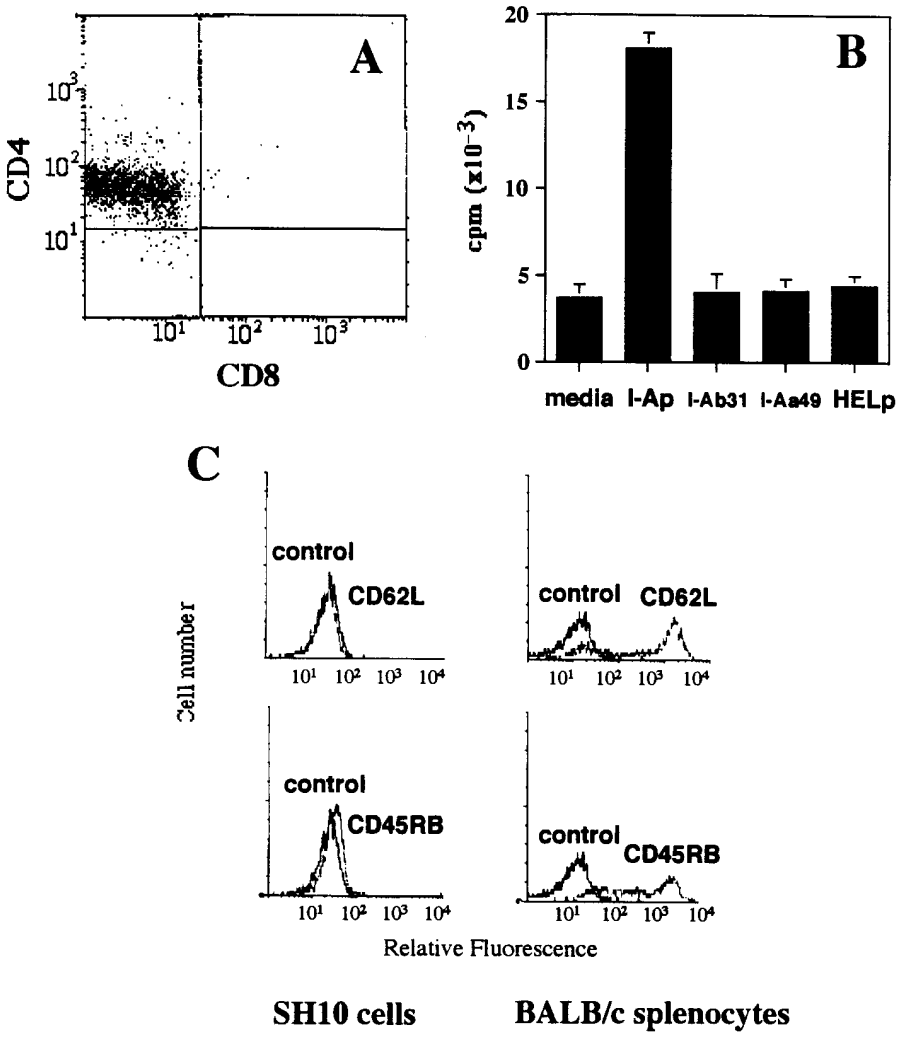

Figure 1. Surface phenotype and antigen specificity of SH10 cells. (*A*) Two-color FACS® analysis of SH10 cells stained with FITC-conjugated anti-CD8 and phycoerythrin-conjugated anti-CD4. (*B*) 72-h proliferative responses of SH10 cells to 10 μM I-Ap, or to control peptides I-Aβ^k31–49 (*I-Ab31*), I-Aα^k49–63 (*I-Aa49*), and HELp. (*C*) FACS® analysis of SH10 cells (*left*) or spleen cells from a naive BALB/c mouse (*right*) stained with FITC–anti-CD62L (*top*) or FITC–anti-CD45RB (*bottom*).

Table II. SH10 Cells Express a TH1 Cytokine Profile

Cytokine	Antigenic stimulus		
	Medium alone	I-Ap (3 μM)	HELp (3 μM)
	pg/ml	*pg/ml*	*pg/ml*
IFN-γ	9	7200	< 5
IL-2	1600	9150	1650
IL-4	< 5	< 5	< 5
IL-5	< 5	< 5	< 5
IL-10	< 5	< 5	< 5
TNF-α	< 300	400	< 300

Supernatants of 48-h cultures were studied in sandwich ELISAs as described in Methods. Limit of detection is 5 pg/ml for IFN-γ, IL-2, IL-4, IL-5, and IL-10, and 300 pg/ml for TNF-α.

Table III. SH10 Cells Are I-Ap–specific (Spots per 10^3 T Cells Plated)

Cytokine	Media	I-Ap	HELp	B10.A splenocytes	B6 splenocytes
IFN-γ	< 5	195	< 5	< 5	< 5
IL-2	< 5	159	< 5	< 5	< 5

SH10 cells (10^3) were studied in an IFN-γ ELISA spot assay with BALB/c spleen cell APCs plus or minus I-Ap (10 μM) or HELp (10 μM), or with B10.A or B6 stimulator spleen cells. Mean values of duplicate wells counted by an automated image analyzer are shown. Results are representative of three individual experiments. Positive control Con A stimulation of splenocytes consistently showed > 300 spots per well for all cytokines (not shown). Intrawell variability was < 10%.

33% $CD8^+$ by FACS®, not shown) from BALB/c mice 11 d after placement of B10.A skin grafts. Table I confirms previous studies (17) that TH1 T cells reactive to I-Ap are present during BALB/c rejection of B10.A skin at a frequency of ~ 1/20,000, ~ 1% of the response to B10.A splenocytes (direct pathway). We were unable to detect an I-Ap–specific response using splenocytes or isolated T cells from naive BALB/c mice (frequency of < 1/500,000, not shown).

To study the role of I-Ap–specific T cells as potential mediators of allograft rejection, we isolated a T cell line, SH10, from the draining lymph nodes of BALB/c mice rejecting B10.A skin. Fig. 1 shows that SH10 is $CD4^+/CD8^-$, and is both $CD62^-$ and $CD45RB^-$, consistent with a memory T cell surface phenotype. SH10 cells also express CD44 on their surface (data not shown). SH10 cells specifically proliferated in response to I-Ap but not to either of two other *I-A*^{k}–derived peptides or to an unrelated, *H-2*^{d}–restricted control peptide, HELp (Fig. 1).

The cytokine profile of SH10 cells was next determined by sandwich ELISAs performed on 48-h culture supernatants (Table II). As shown, SH10 cells produced I-Ap–specific IFN-γ and IL-2 without IL-4, IL-5, or IL-10, consistent with a TH1 phenotype. Low levels of TNF-α were additionally produced (Table II).

To further validate that SH10 cells were I-Ap–specific and did not cross-reactively respond to intact B10.A alloantigens (direct pathway), we studied SH10-induced IFN-γ and IL-2 production using a high resolution ELISA spot assay (Table III). As shown, SH10 cells responded specifically to I-Ap and not to intact B10.A stimulator cells or to control B6 splenocytes (Table III). Approximately 20% of the SH10 cells produced IFN-γ spots in this experiment. We have noted consis-

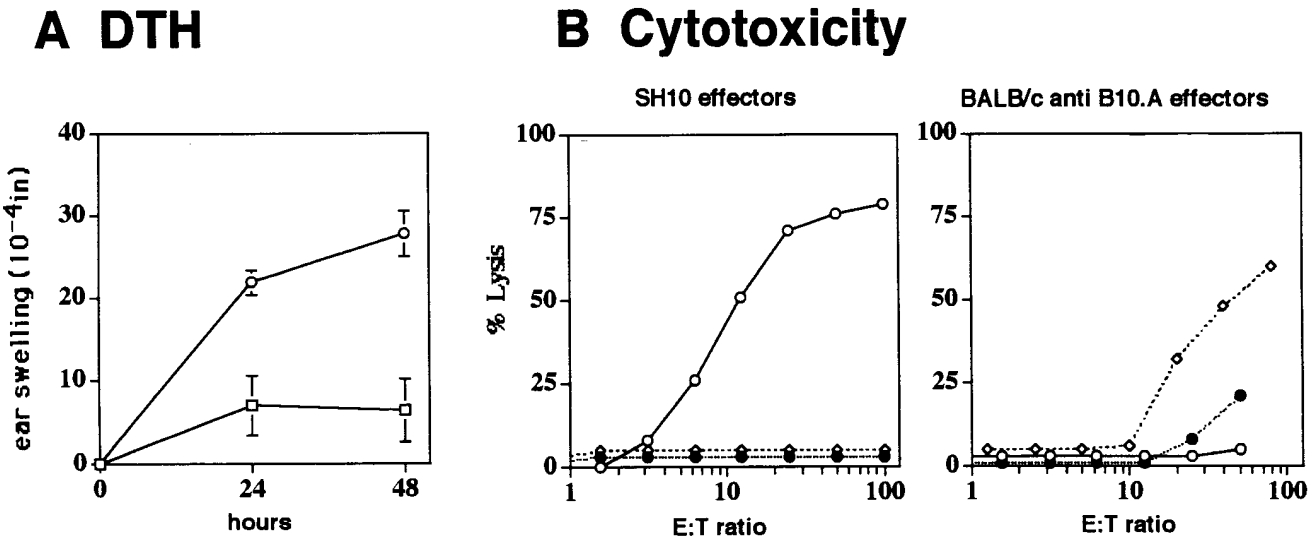

Figure 2. SH10 cells mediate DTH and exhibit cytotoxicity. (*A*) Ear swelling as a measure of DTH. Values represent the difference in ear thickness for ears injected with SH10 cells plus control HELp (□) versus ears injected with SH10 cells plus 10 μM I-Ap (○). Three animals were tested per group, and the results are representative of three independent experiments. DTH was confirmed by histologic examination of the tissue (not shown). (*B*) SH10 effector cells were tested for cytotoxicity against syngeneic A20 cells loaded with I-Ap (○) or HELp (●) or against LPS-induced B10.A spleen cell (◇) blasts. BALB/c anti-B10.A cytotoxic T lymphocytes were induced as outlined in Methods and tested for cytotoxicity against the same three targets. Results are representative of two individual experiments.

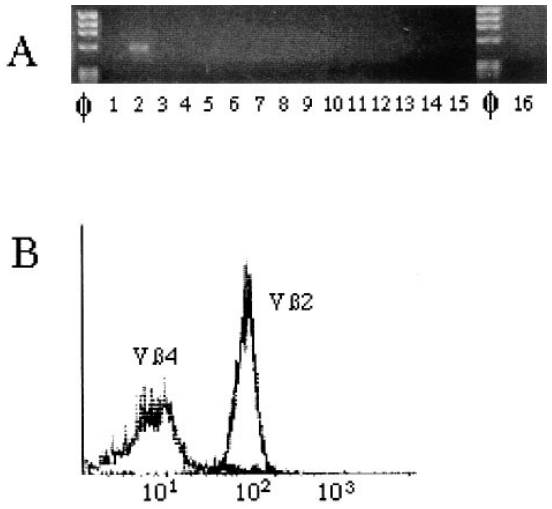

Figure 3. SH10 cells express Vβ2. (*A*) Ethidium bromide–stained agarose gel of RT-PCR products for Vβ genes expressed by SH10 cells. *Lanes*, Individual TCR Vβ gene families tested. The sample also contained a control RT-PCR product for β-actin (not shown). (*B*) FACS® analysis of SH10 cells stained with FITC-conjugated anti-Vβ2 or control anti-Vβ4 antibodies.

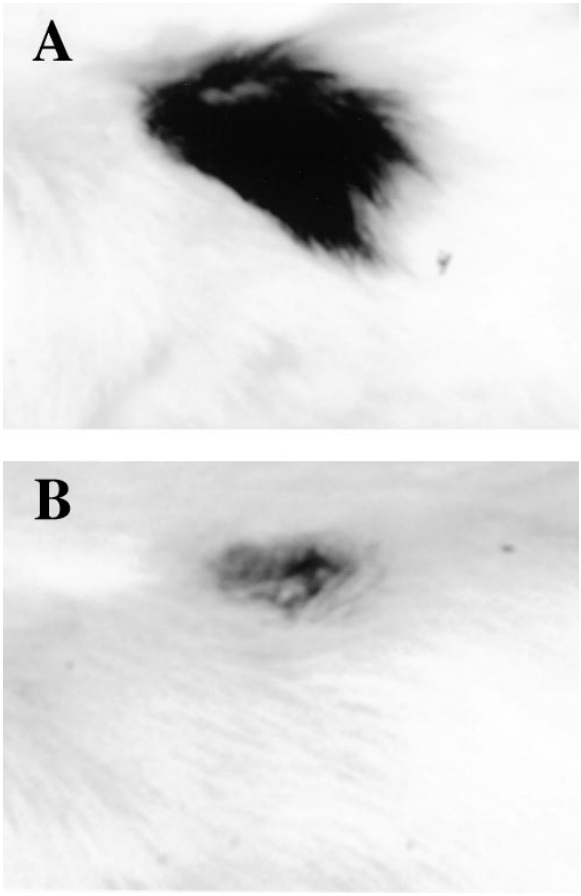

Figure 4. SH10 cells reject B10.A skin on BALB/c SCID recipient mice. (*A*) Intact B10.A skin graft on a control BALB/c SCID recipient mouse. (*B*) Rejected B10.A skin graft on a BALB/c SCID recipient mouse adoptively transferred with SH10 cells (day 18 after transfer). Representative of three individual animals.

tently that between 20 and 70% of the SH10 cells specifically responded to I-Ap in an individual experiment (this has been repeated more than five times) irrespective of the number of passages in culture (the cells have been maintained for > 20 passages), and that there has never been a detectable anti-B10.A or anti-B6–specific response. Furthermore, we found that both the cytokine profile and the detected frequency of spots were similar whether we used BALB/c splenocytes or A20 B cell lymphoma cells (*H-2*^{d}) as APCs (not shown).

Further characterization revealed that SH10 cells were capable of mediating DTH in response to I-Ap but not a control HELp (Fig. 2 *A*). Histologic evaluation revealed perivascular edema and mononuclear cell infiltration consistent with DTH (not shown). In addition, SH10 cells were capable of mediating cytotoxicity of I-Ap–loaded A20 (*H-2*^{d}) target cells (indirect recognition) but not B10.A target cells (direct recognition). Control BALB/c killer cells induced by 5 d exposure to B10.A stimulator cells in vitro provided a positive control for the assay, and lysed the B10.A targets (Fig. 2 *B*).

TCR Vβ gene expression by SH10 cells as determined by RT-PCR (Fig. 3 *A*) revealed a restriction to Vβ2. Cell surface expression of Vβ2 was then confirmed by FACS® (Fig. 3 *B*).

Table IV. Skin Graft Survival of BALB/c SCID Recipient Mice

Recipient	Donor graft	Cells transferred	Graft Survival	Comments
			d	
BALB/c SCID	B10.A	None	> 120, > 120, > 120, > 120	*n* = 4, Histology normal
BALB/c SCID	B10.A	SH10	13, 18, 18, 20, > 45, > 45, > 45*	*n* = 7, All grafts showed histologic evidence of fibrosis and macrophage infiltration
BALB/c SCID	B10.A	IID2	> 120, > 120, > 120	*n* = 3, Histology normal
BALB/c SCID	B10.A	Naive BALB/c T cells	11, 12, 12, 13, 11, 12	*n* = 6, Histology revealed necrosis and massive mononuclear cell infiltration
BALB/c SCID	B6	SH10	> 120, > 120, > 120	*n* = 3, Histology normal

5–10 × 10^6 T cells were adoptively transferred by tail vein injection into BALB/c SCID recipients of skin grafts 10–21 d after placement of the grafts. *The three grafts that did not fully reject exhibited focal areas of necrosis and hair loss. Animals in this group were killed at the time of rejection or after day 45, and the grafts were processed for histologic analysis.

SH10 cells mediate skin graft rejection in BALB/c SCID recipient mice. To test whether SH10 cells could mediate graft rejection, we first placed B10.A or control B6 trunk skin allografts on recipient BALB/c SCID mice. All skin became fully engrafted with normal hair growth (Fig. 4 *A*) and without visual evidence of rejection (selected animals followed for > 6 mo). 10–21 d after placement of B10.A skin, engrafted BALB/c SCID recipients were adoptively transferred with either 5–10 × 10^6 SH10 cells or naive unfractionated BALB/c T cells by intravenous injection into the tail vein. Transfer of SH10 cells induced B10.A skin graft rejection that was noted on days 13–20 in four of seven animals (Fig. 4 *B,* and Table IV). The three others exhibited focal evidence of hair loss and/or necrosis within the graft tissue for up to 45 d after cell transfer, at which time the animals were killed. SH10 cells did not reject control B6 skin ($n = 3$), and intravenous adoptive transfer of a $CD4^+$ *H-2*^{d}-restricted, TH1, ovalbumin–specific cell line (IID2, kindly provided by Paul Lehmann) did not result in B10.A graft rejection ($n = 3$, animals followed for > 120 d). BALB/c SCID recipients of B10.A grafts adoptively transferred with unfractionated naive BALB/c T cells rapidly rejected the allografts, by day 13 ($n = 6$, Table IV).

To circumvent the possibility that the adoptively transferred SH10 cells were unable to home reliably to the skin tissue (thus potentially accounting for the incomplete rejection in three cases), we performed additional experiments in which we directly injected SH10 cells into skin grafts placed on BALB/c SCID recipients. Three of three B10.A grafts showed focal evidence of hair loss and necrosis at the site of injection within 2 wk of the intradermal graft injection. However, injection of SH10 cells into BALB/c SCID recipients of control B6 grafts did not result in rejection ($n = 3$).

Recall immune responses in the BALB/c SCID recipients of SH10 cells. To confirm that I-Ap–specific T cells were present in the mice rejecting the skin grafts, and to rule out the possibility that the recipients developed a cross-reactive direct pathway (anti-B10.A) alloresponse in vivo, we next performed a recall ELISA spot assay using spleen cells obtained from the recipient animals. As shown in Table V, spleen cells from the SCID recipient mice rejecting B10.A grafts responded only to I-Ap, and produced IFN-γ and IL-2 (without IL-5), consistent with the original phenotype of the adoptively transferred SH10 cells. There was essentially no recall response to B10.A splenocytes, confirming a lack of direct pathway alloreactivity. Spleen cells from control graft recipients given no cells by adoptive transfer did not produce cytokines in response to I-Ap or B10.A stimulators, whereas recipients that rejected grafts after transfer of naive T cells exhibited a strong anti-B10.A response (Table V).

Table V. Splenic Recall Responses after SH10-mediated Rejection of B10.A Skin Grafts

Cells transferred	Cytokine	I-Ap	B10.A splenocytes
SH10	IFN-γ	1254	< 5
	IL-2	670	20
	IL-5	< 5	< 5
None	IFN-γ	< 5	< 5
	IL-2	< 5	< 5
	IL-5	< 5	< 5
Unfractionated T cells	IFN-γ	< 5	> 2000
	IL-2	< 5	> 2000
	IL-5	< 5	< 5

Spleen cells of a B10.A-grafted BALB/c SCID recipient of SH10 cells (*top*) were obtained at the time of rejection (day 18) and studied in a cytokine ELISA spot assay in response to 10 μM I-Ap or B10.A stimulator splenocytes. Spleen cells of a control B10.A-grafted BALB/c SCID recipient given no cells (skin grafts intact; *middle*) and of a recipient with a graft rejected by adoptive transfer of unfractionated syngeneic T cells (obtained on the day of rejection; *bottom*) were studied in a similar fashion. Values are the means of duplicate wells counted by a computer-assisted image analyzer and are representative of three individual experiments performed on different animals. Intrawell variability was < 10%. Con A stimulation served as a positive control and resulted in > 2,000 spots per million cells plated for all cytokines.

We further confirmed the exclusive presence of SH10 cells in recipient mice by FACS® analysis of responder splenocytes (Fig. 5). Spleen cells from SH10 recipients of B10.A grafts contained only Vβ2-expressing $CD3^+$ T cells, the TCR Vβ gene expressed on SH10 cells. In contrast, however, splenocytes from animals adoptively transferred with unfractionated T cells contained both $V\beta2^+$ and $V\beta2^-$ $CD3^+$ T cells. Age-matched control SCID mice contained no $CD3^+$ T cells in their spleens.

Histologic evaluation of the allografts. Portions of skin tissue from all animals were then studied in a blinded fashion by a dermatopathologist (A. Gilliam). Visually normal B10.A

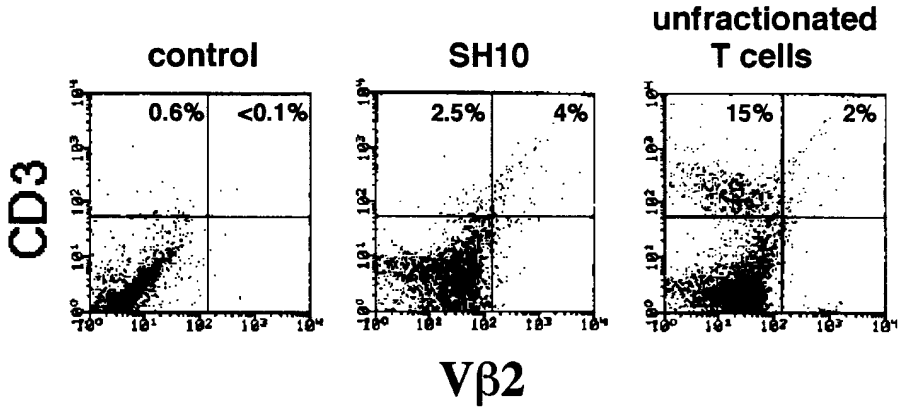

Figure 5. Only $V\beta2^+$ T cells are detectable in the graft recipients adoptively transferred with SH10 cells. Representative two-color FACS® analyses of spleen cells obtained from BALB/c SCID mice stained with phycoerythrin–anti-CD3 and FITC–anti-Vβ2. *Left*, Control naive SCID mouse; *middle*, SCID recipient of B10.A graft rejected by adoptive transfer of SH10 cells; *right*, SCID recipient of B10.A graft rejected by adoptive transfer of unfractionated T cells.

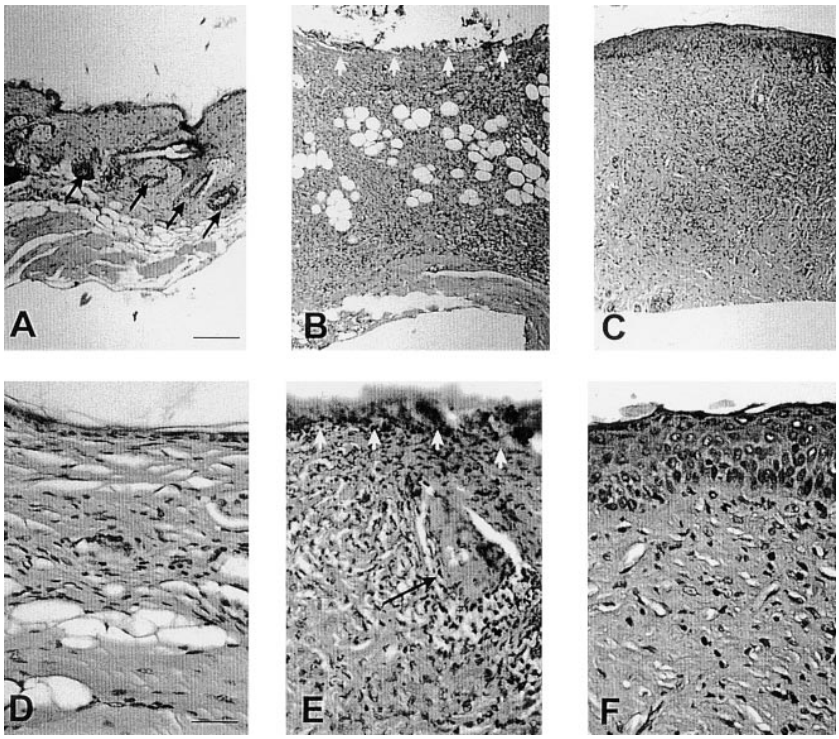

Figure 6. SH10 cell–mediated skin graft rejection exhibits distinct histopathologic features. (*A–F*) B10.A skin grafts placed on BALB/c SCID recipients. *A* and *D*, No rejection at 30 d after placement of skin grafts. *B* and *E*, Rejection at 10 d after adoptive transfer of naive BALB/c T cells. Note extensive necrosis of epidermis (*white arrows*) and hair follicle (*black arrow*). *C* and *F*, Rejection at 18 d after adoptive transfer of SH10 cells. Note extensive dermal fibrosis with infiltration by mononuclear cells and absence of hair follicles. Hematoxylin and eosin; bar for *A–C*, 180 μm, bar for *D–F*, 50 μm.

skin grafts in BALB/c SCID recipients adoptively transferred with no cells appeared histologically normal as well (Fig. 6 *A* and *D*). Hair follicles were preserved (Fig. 6 *A*, *arrows*), and the epidermis and dermis were normal in thickness and without evidence of necrosis or fibrosis. Histology of rejected B10.A grafts by naive BALB/c T cells, shown in Fig. 6 *B* and *E*, revealed a prominent mononuclear cell infiltration and necrosis of the epidermis (*white arrows*), with destruction/necrosis of a hair follicle (Fig. 6 *E*, *black arrow*). Neutrophils were concentrated in the areas of necrosis. In contrast, although B10.A skin from an animal that received SH10 cells had a markedly expanded dermal layer with a prominent mononuclear cell infiltrate (Fig. 6, *C* and *F*), the graft did not exhibit evidence of graft necrosis. Five of the seven grafts studied exhibited areas of epidermal hyperplasia. Furthermore, all grafts showed evidence of increased fibrosis compared with the grafts rejected by unfractionated T cells (including those grafts that were not rejected by visual inspection). The dermal fibrosis was seen as dense, thickened, hypercellular stroma with loss of hair follicles (Fig. 6 *C*). Dermal granulomas were noted as well (not shown). High power view (Fig. 6 *F*) suggested that the cellular infiltrate consisted predominantly of tissue histiocytes/macrophages and lymphocytes. Histologic examination of the B10.A grafts that were directly injected with SH10 cells revealed focal areas of mononuclear cell infiltration and increased fibrosis.

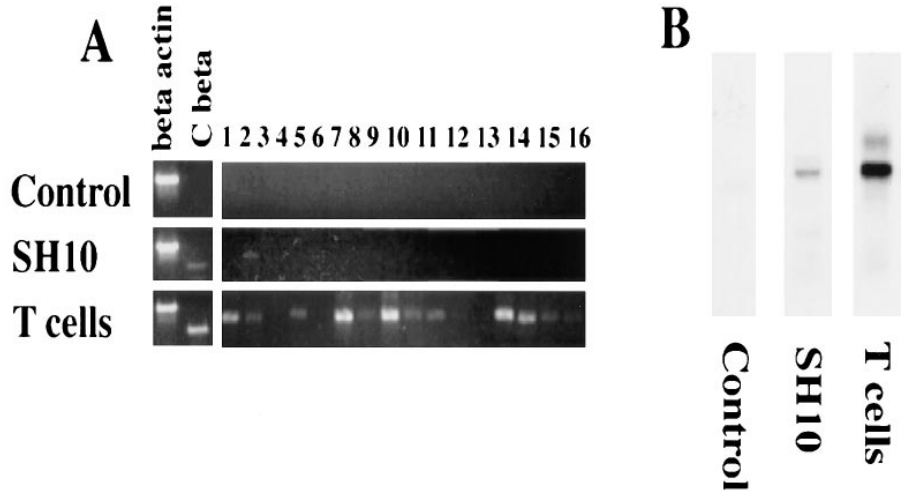

Figure 7. TCR Vβ2 RT-PCR product is present in the skin of animals adoptively transferred with SH10 cells. (*A*) Ethidium bromide–stained agarose gels of Vβ gene RT-PCR are shown. Representative gels are shown for control nonrejected B10.A skin (*top*), rejected B10.A skin on a BALB/c SCID recipient after adoptive transfer of SH10 cells (*middle*), and rejected B10.A skin on a BALB/c SCID recipient after adoptive transfer of naive BALB/c T cells (*bottom*). *Lanes*, Individual TCR Vβ gene families tested. *C beta*, RT-PCR product for constant region of the TCR β chain. Results are representative of two to four animals tested per group. (*B*) Southern blot of Vβ2 RT-PCR products from *A* probed with an internal constant region oligonucleotide. The detected band is at the predicted size of 310 bp.

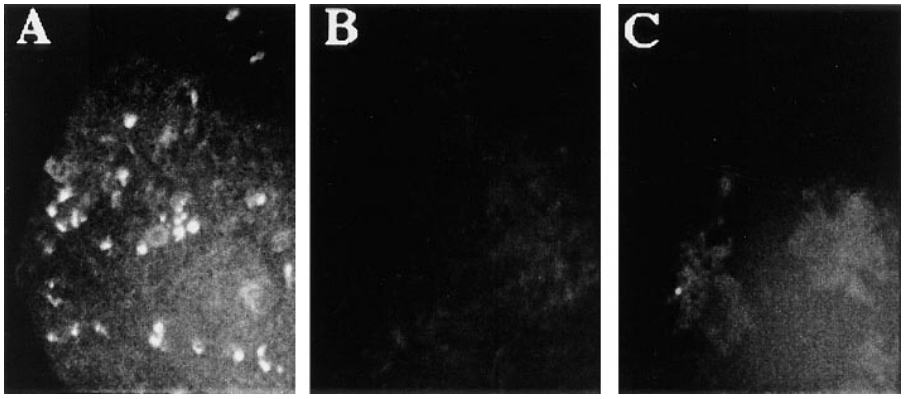

Figure 8. Immunofluorescence of rejected skin reveals the presence of TCR Vβ2–expressing cells. (*A*) Rejected B10.A skin after adoptive transfer of SH10 cells stained with FITC-conjugated anti-Vβ2. (*B*) Rejected B10.A skin after adoptive transfer of SH10 cells stained with FITC-conjugated anti-Vβ4. (*C*) Unrejected control skin stained with FITC-conjugated anti-Vβ2. Results are representative of studies performed on two individual animals; ×400.

SH10 cells were detectable in the rejected skin grafts. The skin grafts of recipients adoptively transferred with SH10 cells were then studied for TCR Vβ gene expression. The skin contained a weak RT-PCR product for Vβ2 (Fig. 7 *B*) without other Vβ genes. No Vβ gene expression could be detected in the control grafts (Fig. 7 *A*), whereas multiple Vβ genes, including Vβ2, were detected in the grafts rejected by purified naive BALB/c T cells (Fig. 7 *C*). Specificity of the Vβ2 PCR product was confirmed by Southern hybridization with an internal oligonucleotide probe as described previously (Fig. 7 *C*) (20).

The presence of SH10 cells within the rejected skin was further confirmed by immunofluorescence using specific anti–TCR Vβ antibodies (Fig. 8). The rejected B10.A grafts contained Vβ2-expressing T cells (Fig. 8 *A*), but did not stain with the control anti–Vβ4 antibody. Unrejected, histologically normal skin did not stain with the anti-Vβ2 antibody (Fig. 8 *C*).

Finally, cytokine gene expression in the skin grafts at the time of rejection was determined by RT-PCR (Fig. 9). Unrejected control skin contained a detectable PCR product for TGF-β and a weak band for TNF-α. In contrast, rejected B10.A skin grafts obtained from animals adoptively transferred with SH10 cells expressed a strong RT-PCR product for the TH1 cytokine, IFN-γ, in addition to TNF-α and TGF-β. Control rejected skin obtained from BALB/c recipients of B10.A skin and adoptively transferred with naive BALB/c T cells contained RT-PCR messages for TNF-α, TGF-β, IFN-γ, and IL-2, and a weak product for IL-10 (message for some type 2 cytokines have been found by others in allografts undergoing rejection) (for a review, see reference 23).

Discussion

This work clearly demonstrated that SH10, a T cell line specific for a single MHC II–restricted epitope presented by the indirect pathway of allorecognition, was capable of mediating allogeneic skin graft rejection. SH10 cells responded to I-Ap without cross-reactivity to direct pathway antigens expressed on B10.A spleen cells, and induced rejection of B10.A skin grafts (but not control grafts) on BALB/c SCID recipient animals. Recall responses revealed only I-Ap–specific TH1 immunity without a response to the direct pathway. Only Vβ2-expressing T cells were found in the recipient animals, and both Vβ2 and IFN-γ were found within the graft itself, consistent with infiltration of SH10 cells.

Our findings confirm and extend previous work showing that reactivity to peptides presented by the indirect pathway is important for allograft rejection (4, 5, 8, 9, 11, 16). Published experiments from two laboratories explored the role of the indirect pathway in allograft rejection by placing MHC II–deficient skin on MHC I disparate SCID or athymic nude recipients reconstituted with $CD4^+$ T cells (15, 16). Both sets of experiments were designed such that T cell–mediated rejection could only occur if the transferred $CD4^+$ T cells recognized donor MHC I peptides expressed in the context of recipient class II on recipient APCs (indirect recognition). Both sets of experiments concluded that allograft rejection could occur under these circumstances, but that it was delayed markedly (and sometimes did not occur visually) compared with rejection mediated by $CD4^+$ or $CD8^+$ T cells responding to alloantigens presented by the direct pathway (15, 16). Importantly, the specific peptides being recognized could not be defined in these previous experiments. Additionally, there was no direct evidence that peptides derived from donor MHC I (or other,

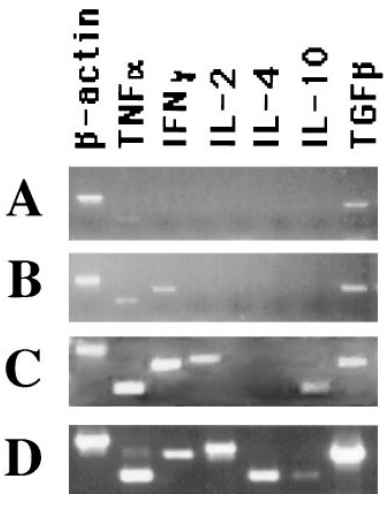

Figure 9. SH10-mediated rejection is associated with IFN-γ message in the skin. Ethidium bromide–stained agarose gels of RT-PCR products for cytokines are shown. (*A*) Unrejected normal B10.A skin on a BALB/c SCID recipient. (*B*) Rejected B10.A skin on a BALB/c SCID recipient after adoptive transfer of SH10 cells. (*C*) Rejected B10.A skin on a BALB/c SCID recipient after adoptive transfer of naive BALB/c T cells. Skin samples were obtained 3 wk after graft placement for group in *A* and at the time of rejection for *B* and *C*. (*D*) Positive control RT-PCR products derived from 48-h Con A–stimulated BALB/c splenocytes. Results are representative of experiments performed on two to four animals per group.

minor antigens) presented in the context of recipient MHC II were relevant to the natural rejection process in these model systems. On the other hand, it has been well established that reactivity to I-Ap is immunodominant during naturally occurring rejection of B10.A skin in recipient BALB/c mice (6, 7, 17). Thus, the finding that T cells responsive to this immunodominant determinant alone can mediate skin graft rejection represents an important step forward.

Interestingly, and similar to the studies noted above (15, 16), our SH10 adoptive transfer experiments did not result in full necrosis of all of the allogeneic skin grafts. Four of the skin grafts became devoid of hair growth, decreased significantly in diameter, and exhibited focal areas of necrosis within 18 d, but three others remained viable for up to 45 d after cell transfer. However, histologic analysis consistently showed evidence of a mononuclear cell inflammatory infiltrate as well as significant fibrosis, even when the graft was not fully rejected by visual inspection. Thus, subclinical rejection of the skin may be present in a manner analogous to findings noted in several studies of vascularized organ transplants (24–27). Overall, these studies confirm that skin grafts should be evaluated histologically even if the graft appears relatively normal visually.

The specific effector mechanisms involved in SH10-mediated skin allograft rejection remain undefined, but our studies implicate the induction of a DTH response as potentially relevant in this regard. This suggestion is consistent with recently published studies which demonstrated that a panel of T cell clones reactive to peptides presented by indirect allorecognition could mediate DTH, although their ability to induce/modulate allograft rejection has not yet been reported (28). Activation of SH10 cells may occur within the draining lymph nodes of the allografted animal, as processed B10.A antigens may be presented by host APCs under these circumstances. Alternatively, graft-invading APCs of host origin may allow activation of the SH10 cells within the graft itself. Subsequent production and release of IFN-γ by the activated T cells may then induce a proinflammatory TH1 environment, with consequent upregulation of MHC II (29) and costimulatory molecules on the surface of APCs allowing further activation of the invading cells. In addition, the TH1 cytokines may induce the expression and release of chemokines, particularly inflammatory protein 10 and monocyte chemotactic protein 1, which would then attract macrophages and potentially natural killer cells into the graft site (30). Upon exposure to the proinflammatory environment, the invading macrophages may become activated, forming granulomas and producing TNF-α, which can be directly cytotoxic to the donor cells (15). The proinflammatory environment may additionally induce TGF-β expression and subsequent fibrogenesis (31). The presence of messages for IFN-γ, TNF-α, and TGF-β in SH10-mediated skin graft rejection (Fig. 9) support this hypothesis. Nitric oxide production and subsequent bystander cytotoxicity may additionally occur (32), leading to necrosis of the foreign cells and subsequent uptake and degradation by the activated macrophages themselves (25).

We cannot entirely rule out cytotoxicity as a mechanism of rejection, as SH10 cells can mediate killing of I-Ap–loaded target cells in vitro. However, preliminary experiments showed that neither Fas ligand nor perforin message was detectable in the SH10-rejected grafts but were found in grafts rejected by unfractionated T cells (not shown), suggesting that cytotoxicity is not a dominant mechanism of rejection under these circumstances. Furthermore, it remains unclear how cytotoxic lysis of I-Ap–expressing self APCs would result in destruction of allogeneic graft tissue in vivo, even if it is demonstrable in vitro. Delineation of the role of these potential effector mechanisms is ongoing in our laboratory.

Our studies are also consistent with the interpretation that SH10 cells induce a form of chronic skin graft rejection in the SCID recipients. The relatively slow rejection of the skin (compared with that mediated by unfractionated T cells) and the extensive fibrosis are consistent with a chronic form of skin rejection. Further studies of the effect of SH10 cells in a vascularized organ model of allograft rejection will be required to better elucidate the role of indirect allorecognition and chronic rejection. Importantly, indirect alloreactivity has been postulated to play an immunopathogenic role in the induction of chronic rejection for several years (14), but an inability to measure immune responses to low frequency peptide determinants has technically limited the testing of this hypothesis. The ELISA spot assay and this model system now provide a reliable means for specifically addressing these issues.

In conclusion, these studies reveal that indirect alloreactivity focused towards a single immunodominant determinant is sufficient to induce allogeneic skin graft rejection in mice. This work provides an ideal model system for studying the mechanisms of acute and, potentially, chronic allograft rejection as they apply to the indirect pathway of alloreactivity.

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