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Type 2 Immune Deviation Has Differential Effects on Alloreactive CD4$^+$ and CD8$^+$ T Cells[1]

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Allograft rejection has been associated with detection of the type 1 lymphokines, IFN-γ and IL-2. The role of type 2 cytokines (IL-4 and IL-5) remains controversial, as is whether alloreactive CD4$^+$ and CD8$^+$ T cells behave similarly when exposed to type 2 cytokine-enhancing manipulations. We studied the characteristics of alloreactive CD4$^+$ and CD8$^+$ T cells before and after type 2 immune deviation induced by IL-4 plus anti-IFN-γ Ab. Alloreactive T cells from naive mice were low in frequency, produced only IL-2, and were predominantly CD4$^+$, while alloreactive T cells from allograft-primed mice were high in frequency, produced IFN-γ, IL-2, and IL-4, and were predominantly CD8$^+$. Type 2 immune deviation of allospecific CD4$^+$ T cells resulted in IL-4 and IL-5 production without IFN-γ, consistent with unipolar type 2 immunity. These T cells mediated delayed-type hypersensitivity, but not cytotoxicity. Under identical type 2 cytokine-inducing conditions, allospecific CD8$^+$ T cells were primed to become IL-4, IL-5, and IFN-γ producers, and exhibited cytotoxicity, but not classic delayed-type hypersensitivity. Adoptive transfer of either cell population into SCID recipients of allogeneic skin resulted in graft rejection, with stable allospecific type 2 cytokine production in vivo. Adoptive transfer of the IL-4/IL-5-producing CD4$^+$ T cells, but not the CD8$^+$ T cells, induced a distinct histopathology characterized by marked eosinophilic infiltration of the skin. We conclude that type 2 immune deviation has differential effects on CD4$^+$ and CD8$^+$ T cells and results in emergence of alternate effector mechanisms capable of destroying allografts. ***The Journal of Immunology,*** **1998, 161: 5236–5244.**

Allograft rejection has generally been considered to result from a type 1 immune response characterized by the preferential production of the proinflammatory lymphokines IFN-γ and IL-2 (1, 2). These cytokines contribute to graft destruction in a number of ways, including up-regulating MHC II (3), costimulatory molecule (4–6), and chemokine expression in the graft (7, 8); providing help for induction of allograft-specific cytotoxicity (1, 2); activating macrophages and macrophage-mediated effector mechanisms within the graft (9–13); and inducing Ig class switching to complement-fixing IgG2a Abs (14).

In contrast, allospecific secretion of the type 2 cytokines IL-4 and IL-5 has been associated with graft tolerance in several experimental models, including induction of neonatal allograft tolerance (15) and induction of graft tolerance using CTLA4-Ig (16) or anti-CD4 Abs (17–19). Although an allospecific immune response develops under these situations, the noninflammatory nature of the response has been hypothesized to be nondestructive as well as to prevent the emergence of pathogenic type 1 immunity (2, 16).

It is well established, however, that type 2 immunity is not truly benign and can mediate pathogenic immune responses under a variety of conditions. For example, IL-4 and IL-5 are chemoattractants and activators of eosinophils that, in turn, can mediate tissue destruction (20), in part via secretion of cationic proteins such as major basic protein (MBP)[3] (21, 22). In addition, the initial descriptions of IL-4 and IL-5 revealed that these so-called noninflammatory cytokines can prime allospecific CD8$^+$ T cells to become CTLs (23–26). These experiments, among others, provide evidence that induced allospecific type 2 immunity may indeed be destructive, not protective, to an allograft. In support of this, both IFN-γ knockout (27) and IL-2 knockout mice can reject allografts (28), and detection of type 2 cytokines has been associated occasionally with graft rejection (29). Moreover, direct attempts at type 2 immune deviation have not led to graft tolerance and in some cases have resulted in accelerated rejection (30–32).

The mechanisms of allograft destruction following induction of type 2 cytokine-mediated alloimmune responses are poorly understood, however. Furthermore, it has largely been assumed that type 2 immune deviation would have similar effects on both CD4$^+$ and CD8$^+$ T cell subsets. As transplant rejection involves a high-frequency immune response comprised of both CD4$^+$ and CD8$^+$ T cells with multiple antigenic specificities, varying activation thresholds, and differing costimulatory requirements (20, 33–36), it is not at all clear that this would be true. In addition, an inability to adequately measure and quantify cytokines produced by freshly isolated alloreactive CD4$^+$ and CD8$^+$ T cells has prevented a careful analysis of these issues.

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[3] Abbreviations used in this paper: MBP, major basic protein; DTH, delayed-type hypersensitivity; PE, phycoerythrin.

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To provide further insight into the effects of type 2 immune deviation on the cytokine profiles and functional characteristics of alloreactive T cells, our laboratory has developed a highly sensitive cytokine ELISA spot assay (33, 37). The secreted cytokines can be detected over short time intervals (<24 h in culture), thus providing an improved reflection of the in vivo immune response when compared with standard techniques (33, 37). We used this assay to evaluate cytokines secreted by alloreactive $CD4^+$ and $CD8^+$ T cells during allogeneic skin graft rejection and to determine the effects of IL-4-induced type 2 immune deviation on the cytokine profiles of these T cell subsets. We further evaluated the functional characteristics of the type 2 immune-deviated cell populations through analysis of cytotoxicity, ability to mediate delayed-type hypersensitivity (DTH), and ability to induce skin graft rejection. Our findings revealed that alloreactive $CD4^+$ and $CD8^+$ T cells respond differently to the same type 2 cytokine-inducing stimulus. The experiments also revealed that both type 2 cytokine-producing $CD4^+$ and $CD8^+$ T cells are capable of mediating allograft rejection, although the two cell types most likely function through different mechanisms. These results underscore the plasticity of the alloimmune response, and have important implications for future attempts at tolerance induction through immune deviation.

Materials and Methods

Animals

Female BALB/c (*H-2*^{d}), BALB/c SCID (*H-2*^{d}), SJL (*H-2*^{s}), C57BL/6 (B6, *H-2*^{b}), and B10.Br (*H-2*^{k}) mice, age 6 to 8 wk, were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in specific pathogen-free animal facilities at Cleveland Veteran's Affairs Medical Center and Case Western Reserve University (Cleveland, OH).

Placement of skin grafts

Full thickness trunk skin allografts were placed using standard techniques (33, 37). Skin was harvested from euthanized donor mice, the s.c. fat was removed, and the skin was cut into 0.5-cm^2 pieces and placed in sterile PBS until used for transplantation (less than 30 min). Recipient mice were anesthetized with pentobarbital (50 μg/g body weight) 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-impregnated 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 >90% necrotic. In selected animals, allograft rejection was confirmed histologically. All skin graft placements performed on SCID recipients were done under fully sterile conditions in a laminar flow hood. Adoptive transfers of T cells into SCID recipients were performed by i.v. injection into the tail vein.

T cell and T cell subset isolation

Splenic and lymph node T cells were isolated using commercially available murine T cell isolation columns from R&D Systems (Minneapolis, MN) following the instructions supplied by the manufacturer. Resultant cells were washed in HBSS medium, counted by trypan blue exclusion, and resuspended at appropriate concentrations for use in the various assays. An aliquot of cells was set aside for surface staining and FACS analysis.

Staining of cell surface molecules and FACS analysis

Phycoerythrin (PE)-conjugated anti-CD3 (2C11), PE-conjugated anti-CD8a, FITC-conjugated anti-CD4, FITC-conjugated anti-trinitrophenol (isotype-matched control), and PE-conjugated anti-trinitrophenol (isotype-matched control) were purchased from PharMingen (San Diego, CA). Splenocytes or T cells were labeled by direct (anti-CD3) staining, as previously described (38, 39). 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 Becton Dickinson FACScan and accompanying software using 5000 ungated cells.

Preparation of stimulator cells

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

Type 2 immune deviation in vitro

Freshly isolated and purified T cells or T cell subsets were mixed with stimulator cells, 500 U/ml IL-4 (Boehringer Mannheim), and XMG1.2 anti-IFN-γ Ab (10 μg/ml, produced in our laboratory from a hybridoma) in 2 ml of complete RPMI 1640 in 24-well plates for 5 days. The resultant cells were washed in HBSS and studied in recall ELISA spot assays or FACS analysis.

ELISA spot assays

Ninety-six-well ELISA spot plates (Autoimmun Diagnostika, Columbia, MD) were coated overnight with the capture Abs in sterile PBS. R46A2, 4 μg/ml (isolated and purified from hybridoma), was for IFN-γ; JES6-1A12, 3 μg/ml (PharMingen, San Diego, CA), was used for IL-2; 11B11, 2 μg/ml (isolated and purified from hybridoma), was used for IL-4; and TRFK5, 5 μg/ml (isolated and purified from hybridoma), was used for IL-5. The plates were blocked for 1 h with sterile PBS/1% BSA and washed three times with sterile PBS. Various dilutions of splenocytes, lymph node cells, or purified T cells ($0.25–8 \times 10^5$/ml) in 200 μl of HL-1 medium (BioWhittaker, Walkersville, MD) were placed in each well with 6×10^5 mitomycin C-treated stimulator cells (in duplicate) and incubated at 37°C for 24 h in 5% CO_2. Pilot studies testing $1–10 \times 10^5$ mitomycin C-treated stimulator cells demonstrated optimal detection of cytokine spots at this concentration (not shown). Further pilot studies revealed a linear relationship between the number of responder cells plated and the number of detected spots for each cytokine (not shown). After washing with PBS followed by PBS/0.025% Tween (PBST), detection Abs were added overnight. XMG1.2 horseradish peroxidase (produced in our laboratory) was used for IFN-γ, rat anti-mouse IL-4-biotin (BVD6-24G2; PharMingen) was used for IL-4, rat anti-mouse IL-2-biotin (JES6-5H4; PharMingen) was used for IL-2, and biotinylated TRFK4 (PharMingen) was used for IL-5. The plates were then washed three times in PBST. Streptavidin horseradish peroxidase (Dako, Carpenteria, CA) was added at 1/2000 dilution in PBST as a third reagent for IL-2, IL-4, and IL-5 for 2 h, followed by three washes in PBS. The plates were developed using 800 μl 3-amino-9-ethylcarbazole (Pierce, Rockford, IL; 10 mg dissolved in 1 ml dimethylformamide) mixed in 24 ml 0.1 M sodium acetate, pH 5, plus 12 μl H_20_2. The resulting spots were counted on a computer-assisted ELISA spot image analyzer (T spot Image Analyzer; Autoimmun Diagnostika), which is designed to detect ELISA spots using predetermined criteria based on size, shape, and colorimetric density.

Cytotoxicity

Cytotoxicity was performed as published (38, 40). B6 or third-party SJL target cells were made by incubating 6×10^6 splenocytes with 2 μg/ml Con A (Sigma, St. Louis, MO) in 2 ml HL-1 medium (Biowhittaker, Walkersville, MD) for 48 h. [^{3}H]Thymidine, 10 μCi, was added for the final 6 h. After three washes in HBSS medium, the target cells were counted and 10,000 cells were placed in each well of a round-bottom 96-well plate. In some experiments, B6-derived, MHC II-expressing, macrophage IC-21 cells (obtained from American Type Culture Collection, Manassas, VA) were used as targets. IC-21 cells were grown in six-well plates in DMEM/10% FCS, labeled with 10 μCi [^{3}H]thymidine overnight, washed three times in HBSS, and plated at a final concentration of 10,000 cells/well. Responder $CD4^+$ or $CD8^+$ T cells (produced as outlined above) 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 (40).

Delayed-type hypersensitivity

DTH was performed as previously described (38, 39). Briefly, $1–2 \times 10^6$ in vitro activated $CD4^+$ or $CD8^+$ T cells plus 1×10^6 mitomycin C-treated, allospecific, or third-party stimulator cells in a total volume of 25 μl of PBS were injected into the ears of three naive BALB/c mice. Ear thickness was measured at 24 and 48 h with an engineer's micrometer by an investigator blinded to the experimental groups, and the results were expressed as a difference in mean values of the thickness between the ears at time zero and 24 or 48 h.

Microscopy

Skin graft samples or ear samples were obtained at the time of sacrifice, fixed in 10% buffered Formalin, embedded in paraffin, and processed for routine histopathology. Slides were examined in a blinded fashion by a dermatopathologist (A.C.G.) and photographed.

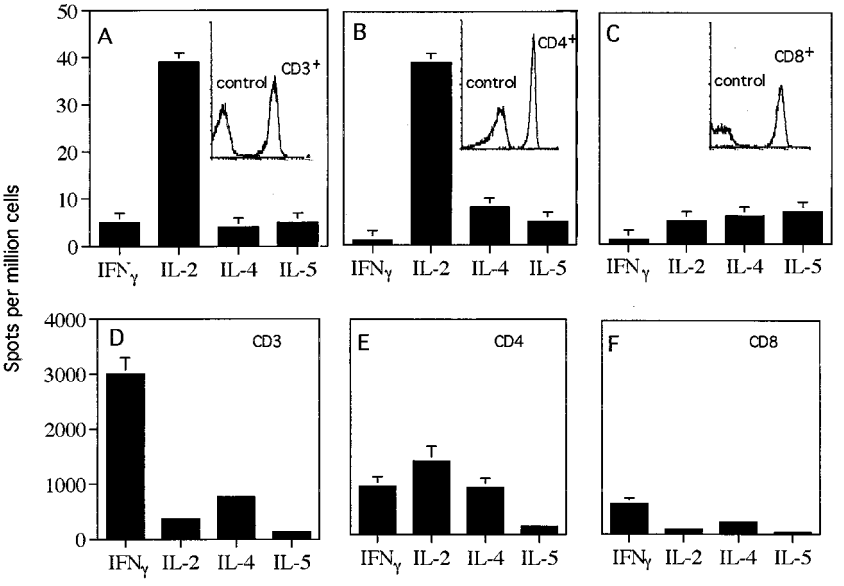

FIGURE 1. Frequency and cytokine profiles of alloreactive T cells from naive mice. Purified CD3$^+$ (*A* and *D*), CD4$^+$ (*B* and *E*), or CD8$^+$ (*C* and *F*) T cells were isolated from pooled spleen cells of 5–10 naive BALB/c mice and tested in cytokine ELISA spot assays in response to fully allogeneic B6 spleen cell stimulators. *A–C*, T cells were directly tested after isolation from naive mice. *Insets* show purity of the individual T cell fractions. *D–F*, The purified T cell subsets were first cultured in vitro with B6 spleen cell stimulators and then tested in recall responses to B6 stimulators on day 5. Third-party responses after specific priming (*D–F*) revealed <10% cross-reactivity for all cytokines tested, not shown. Spot frequencies represent the mean number of two to three individual wells counted by a computer-assisted image analyzer. The results are representative of two to four individual experiments performed on each T cell subset.

Tissue staining for MBP

For detection of eosinophils and extracellular MBP in tissue, we utilized rabbit polyclonal antisera to murine MBP, which was prepared by Dr. Kirsten Larsen (41), and kindly provided by Dr. Gerald Gleich (Mayo Clinic, Rochester, MN). The specificity of the Ab for murine MBP and eosinophils has been previously published (41, 42). Anti-MBP was diluted 1/1000 in 1% FCS in 0.05 M Tris-buffered saline, and incubated with 5-μm paraffin sections at room temperature for 2 h. Biotinylated goat anti-rabbit Ig (Dako, Carpenteria, CA) diluted 1/200 in 1% FCS in 0.05 M Tris-buffered saline was then added for 30 min, and alkaline phosphatase-conjugated streptavidin (BioGenex, San Ramon, CA) was added for an additional 30 min. Vector Red Substrate containing 12 mg Levamisole (Sigma) was added, and sections were counterstained using modified Harris' hematoxylin (Richard-Allen, Kalamazoo, MI).

Statistical analysis

Statistical comparisons were performed using the Student's *t* test.

Results

Alloreactive T cells from naive mice produce an IL-2-dominated cytokine profile that derives from the CD4$^+$ subfraction

In initial experiments, we evaluated the cytokines produced by alloreactive splenic T cells obtained from naive BALB/c mice. The purified T cells were approximately 68% CD4$^+$ and 32% CD8$^+$ by FACS (not shown). Cytokine-producing alloreactivity was determined using an ELISA spot assay that allows detection of cytokines produced over short time intervals (≤24 h). T cells responding to fully MHC-disparate B6 stimulator cells were low in frequency (~40 per million) and produced predominantly IL-2 in this 24-h assay (Fig. 1*A*). Previous experiments have confirmed that these responding T cells lie within the L-selectin^{high} (CD62L^{high}) population, suggesting that they are naive, not memory T cells (33).

To further define the source of cytokine production, we isolated CD4$^+$ and CD8$^+$ T cell subsets using commercially available isolation columns and tested the resultant cells for cytokines produced in response to alloantigens (Fig. 1, *B* and *C*). The purity of each isolated cell fraction was >95% by FACS. As shown previously (33), the responses detected in assays using these T cell preparations represent direct recognition of allopeptides expressed in the context of allo-MHC (43). We and others have also shown that T cells responding to allopeptides expressed in the context of self (recipient)-MHC (indirect recognition) represent a significant minority of the overall alloresponse and express similar cytokine profiles to direct pathway-responsive T cells (37, 43–47). ELISA spot analysis of the purified CD4$^+$ and CD8$^+$ T cells confirmed a predominance of IL-2 producers in response to alloantigenic stimulation and further revealed that essentially all of the cytokine-producing cells derived from the CD4$^+$, and not from the CD8$^+$, subfraction (Fig. 1, *B* and *C*).

To confirm the viability of the cells and to determine the effect of in vitro priming on the alloreactive T cell population, the CD4$^+$ and CD8$^+$ T cells isolated from naive BALB/c mice were next cultured with B6 splenic stimulator cells in vitro, and recall immune responses were determined by ELISA spot analysis on day 5 (Fig. 1, *D–F*). In vitro priming of CD8$^+$ T cells yielded a predominance of IFN-γ producers (~600 spots per 10^6 cells plated) with 2- to 15-fold fewer IL-2 (~90 spots per 10^6 cells plated), IL-4 (~225 spots per 10^6 cells plated), or IL-5 producers (~35 spots per 10^6 cells plated, Fig. 1*F*). In contrast, in vitro priming of CD4$^+$ T cells yielded a higher frequency of allospecific IFN-γ (~900 spots per 10^6 cells plated), IL-2 (~1400 spots per 10^6 cells plated), IL-4 (~850 spots per 10^6 cells plated), and IL-5 producers (~150 spots per 10^6 cells plated, Fig. 1*E*). Interestingly, in vitro priming of unfractionated T cells resulted in an overwhelming predominance of IFN-γ producers (~5000 spots per 10^6 cells plated), although IL-2, IL-4, and IL-5 were detected at lower frequencies as well, suggesting that the coculture of CD4$^+$ and CD8$^+$ T cells had a synergistic effect on the priming of IFN-γ-producing cells (Fig. 1*D*). In sum, although the primary alloresponse initiates with a low frequency of IL-2-producing CD4$^+$ T cells, both alloreactive CD4$^+$ and CD8$^+$ T cells from naive mice can be primed in short-term culture to produce a mixed type 1/type 2 cytokine profile (and not a pure type 1 profile).

Alloreactive T cells from allograft-primed mice produce a mixed type 1/type 2 cytokine profile that derives from both CD4$^+$ and CD8$^+$ subfractions

To study the cytokine profiles of in vivo primed cells, we next purified T cells and T cell subsets from the draining lymph nodes of BALB/c mice at the time of rejection of B6 trunk skin allografts (day 11–12). Draining lymph nodes contained 58% CD4$^+$ and 21% CD8$^+$ T cells by FACS in this strain combination (Fig. 2*A*).

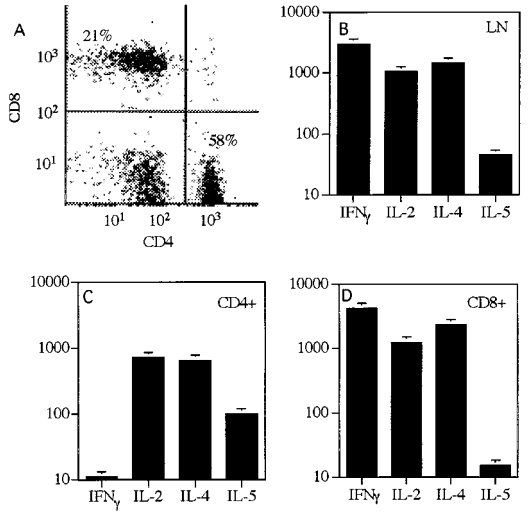

FIGURE 2. Frequency and cytokine profiles of alloreactive T cells from skin allograft-primed mice. *A*, Pooled draining lymph node cells from six BALB/c recipients of B6 skin grafts (obtained on day 12, 80% necrosis of the grafts) were stained in a two-color FACS analysis for surface expression of CD4 and CD8. *B–D*, Draining lymph node cells (*B*), enriched lymph node $CD4^+$ (*C*), or $CD8^+$ (*D*) T cells were isolated from 6–10 BALB/c recipients of B6 skin allografts (obtained on day 12, 80% necrosis of the grafts) and tested in recall ELISA spot assays for cytokine production in response to B6 spleen cell stimulators. Third-party responses revealed <10% cross-reactivity for all cytokines tested, not shown. Spot frequencies represent the mean number of two to three individual wells counted by a computer-assisted image analyzer. The results are representative of two individual experiments performed on each group.

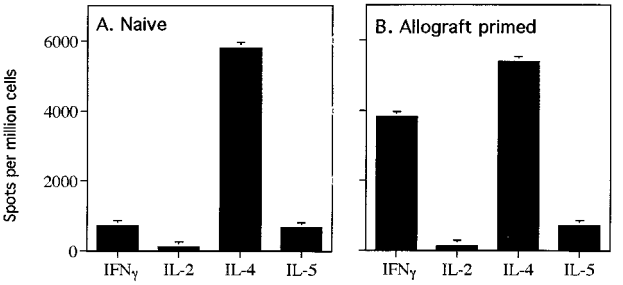

FIGURE 3. Effect of type 2 immune deviation on unfractionated T cells derived from naive and allograft-primed mice. Pooled splenic T cells from naive BALB/c mice (*A*) or pooled draining lymph node T cells from BALB/c mice primed with B6 trunk skin allografts (*B*, isolated on day 12 after graft placement) were cultured for 5 days with B6 stimulator cells, IL-4, and anti-IFN-γ Ab and then tested for cytokine production in response to B6 spleen cell stimulators in a cytokine ELISA spot assay. Third-party responses revealed <10% cross-reactivity for all cytokines tested, not shown. Spot frequencies represent the mean number of two to three individual wells counted by a computer-assisted image analyzer. The experiments were repeated twice with similar results.

Allospecific cytokine production by total lymph node cells revealed a high frequency of IFN-γ (~3000 spots per 10^6 cells plated), IL-2 (~1000 spots per 10^6 cells plated), and IL-4 producers (~1100 spots per 10^6 cells plated) with a few IL-5 producers (~50 spots per 10^6 cells plated, Fig. 2*B*). Importantly, however, the high frequency and mixed profile of cytokine-producing cells are similar to the in vitro primed alloresponse (Fig. 1*D*), but contrast markedly with the low frequency, IL-2-dominated profile found in T cells obtained from naive mice tested in a primary recall response (Fig. 1*A*).

We next tested purified $CD4^+$ and $CD8^+$ T cells from BALB/c recipients of B6 skin grafts in recall ELISA spot assays (Fig. 2, *C* and *D*). Interestingly, both the $CD4^+$ and the $CD8^+$ fractions contained IL-2 and IL-4 producers, but the $CD8^+$ subfraction was the dominant cytokine-producing population at this time point and notably contained all of the IFN-γ producers. $CD8^+$ T cells were also the dominant cytokine producers in B6 recipients of BALB/c skin grafts, confirming that this was not a strain-specific phenomenon (not shown).

Effect of type 2 immune deviation on the cytokine profile of alloreactive T cells

Because much interest has developed in understanding the role of type 2 cytokines in graft rejection, we next studied how type 2 immune deviation affected the development of allospecific T cell cytokine profiles. Previous work has implicated IL-4 as a strong inducer of type 2 immune cytokines, and IFN-γ and IL-12 as potent inducers of type 1 immunity (14). We purified T cells from naive BALB/c mice or from BALB/c recipients of B6 skin allografts and cultured them in the presence of B6 splenic stimulators with exogenous IL-4 and anti-IFN-γ Ab (XMG1.2). The resultant primed T cell populations were then studied in recall ELISA spot assays on day 5 (Fig. 3). Pilot studies revealed a maximal effect of the exogenous IL-4 at a concentration of 500 U/ml and further revealed that the addition of blocking anti-IL-12 Abs (15.1 and 15.6, kind gifts of Georgio Trinchieri, Wistar Institute, Philadelphia, PA) had no additional effects beyond that of IL-4 and anti-IFN-γ alone (data not shown). We initially postulated that the T cells obtained from naive mice would be readily polarizable toward type 2 cytokine production as the cells expressed features of undifferentiated naive T cells (Fig. 1) (33). In contrast, we postulated that allograft-primed T cells would be more difficult to deviate toward type 2 cytokine production, as many of the responding T cells were already differentiated into an IFN-γ-producing phenotype.

The results are summarized in Fig. 3. T cells obtained from naive mice and cultured in the presence of IL-4/anti-IFN-γ surprisingly produced a mixture of IFN-γ (~700 spots per 10^6 cells plated), IL-4 (~5800 spots per 10^6 cells plated), and IL-5 (~500 spots per 10^6 cells plated) in a recall assay, and were not pure type 2 cytokine producers. Furthermore, when T cells derived from allograft-primed mice were exposed to the same type 2-inducing stimulus, they also produced a high frequency, but mixed cytokine profile (Fig. 3*B*). Thus, exogenous IL-4 tended to shift the overall cytokine profile toward type 2 immunity in both naive and allograft-primed mice when compared with the naturally developing cytokine profile (Fig. 2), but additionally primed IFN-γ-producing T cells in both situations.

Type 2 immune deviation readily polarized alloreactive $CD4^+$ but not $CD8^+$ T cells from both naive and allograft-primed mice

We next tested how this immune deviation protocol affected purified $CD4^+$ and $CD8^+$ T cells from the naive (Fig. 4) and allograft-primed mice (Fig. 5). Interestingly, for T cells from naive mice, exogenous IL-4 plus anti-IFN-γ Ab induced a population of essentially pure type 2 $CD4^+$ alloreactive T cells characterized by IL-4 (~22,000 spots per 10^6 cells plated) and IL-5 producers (~6000 spots per 10^6 cells plated), and essentially no detectable IFN-γ or IL-2 producers (Fig. 4*A*). Third-party cross-reactivity was less than 5% of the allospecific response (Fig. 4*A*). In contrast, the same immune deviation protocol induced a mixed population of allospecific $CD8^+$ T cells characterized by a high frequency of IFN-γ producers (~5500 spots per 10^6 cells plated), in addition to

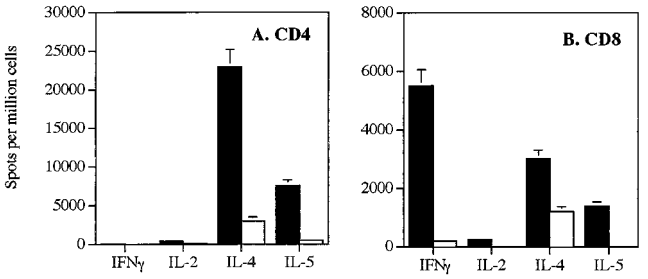

FIGURE 4. Effect of type 2 immune deviation on CD4$^+$ and CD8$^+$ T cells derived from naive mice. Pooled splenic CD4$^+$ (*A*) or CD8$^+$ (*B*) T cells from naive BALB/c mice were cultured for 5 days with B6 stimulator cells, IL-4, and anti-IFN-γ Ab and then tested for cytokine production in response to B6 spleen cell stimulators in a cytokine ELISA spot assay (black bars). Third-party responses to SJL (*H-2*^{s}) stimulators are shown in white bars. Spot frequencies represent the mean number of two individual wells counted by a computer-assisted image analyzer. The results are representative of four individual experiments.

both IL-4 (~3000 spots per 10^6 cells plated) and IL-5 producers (~1500 spots per 10^6 cells plated) (Fig. 4*B*). Thus, under identical conditions, this immune deviation protocol had markedly different effects on the two T cell subsets.

The CD4$^+$ and CD8$^+$ T cells isolated from allograft-primed mice behaved in a similar fashion to the cells derived from naive mice (Fig. 5, *A* and *B*). Primed CD4$^+$ T cells, which produced a mixed cytokine profile when tested in a primary recall response (Fig. 2), could still be polarized toward type 2 cytokine production with a low frequency of residual IFN-γ producers when recultured in the presence of allostimulators and IL-4/anti-IFN-γ Ab. Thus, the allograft-primed CD4$^+$ T cells were not irreversibly differentiated into type 1 cytokine-producing T cells. The CD8$^+$ T cells from allograft-primed mice (high-frequency IFN-γ producers in a primary recall response; Fig. 2) were further primed to produce a mixed cytokine profile characterized by IFN-γ, IL-4, and IL-5 producers when cultured with allostimulators and IL-4/anti-IFN-γ Ab (Fig. 5*B*). Regardless of whether the alloreactive T cells derived from naive or specifically allograft-primed mice, the CD4$^+$ T cells could be induced to overwhelmingly produce IL-4 and IL-5 with little to no IFN-γ, while the same treatment primed CD8$^+$ T cells to produce IFN-γ in addition to IL-4 and IL-5.

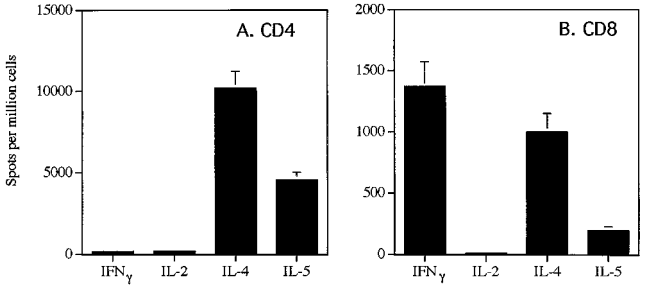

FIGURE 5. Effect of type 2 immune deviation on CD4$^+$ and CD8$^+$ T cells derived from allograft-primed mice. Pooled lymph node CD4$^+$ (*A*) or CD8$^+$ (*B*) T cells isolated on day 12 after placement of B6 trunk skin allografts onto naive BALB/c mice were cultured for 5 days with B6 stimulator cells, IL-4, and anti-IFN-γ Ab, and then tested for cytokine production in response to B6 spleen cell stimulators in a cytokine ELISA spot assay. Third-party responses revealed <10% cross-reactivity for all cytokines tested, not shown. Spot frequencies represent the mean number of two individual wells counted by a computer-assisted image analyzer. The experiment was repeated with similar results.

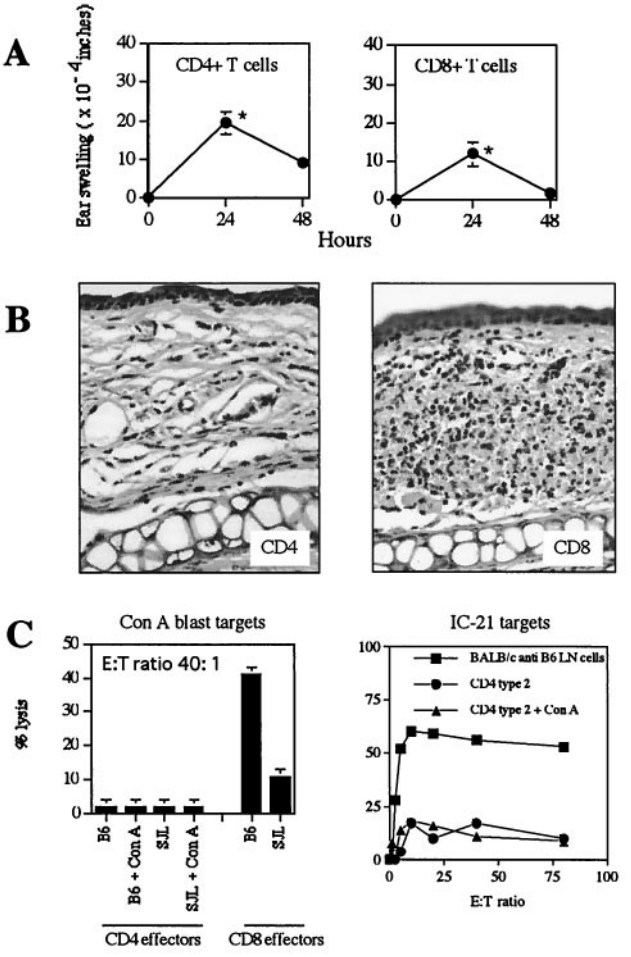

FIGURE 6. Functional analysis of type 2 cytokine-secreting allospecific CD4$^+$ and CD8$^+$ T cells. *A*, Ear swelling as a measure of DTH. Values represent the difference in ear thickness for ears injected with type 2 cytokine-secreting BALB/c anti-B6 T cells plus B6 spleen cell stimulators vs type 2 cytokine-secreting BALB/c anti-B6 T cells plus control SJL spleen cell stimulators. BALB/c splenic CD4$^+$ T cells (*left*) or enriched BALB/c splenic CD8$^+$ T cells (*right*) were primed in vitro for 5 days with B6 spleen cell stimulators, IL-4, and anti-IFN-γ Ab and then tested in a DTH assay, as outlined in *Materials and Methods*. Results are representative of three individual experiments each performed on three recipient mice. *, $p < 0.05$ vs time 0 measurements. *B*, Representative photomicrographs of the histology of the allospecific ear injection sites at 24 h (×400 magnification). *Left*, CD4$^+$ T cells, revealing typical features of DTH with perivascular and dermal edema, and mononuclear cell infiltration. *Right*, CD8$^+$ T cells revealing necrosis with a complex leukocytic infiltrate. *C*, Cytotoxicity of the type 2 cytokine-producing BALB/c anti-B6 CD4$^+$ and CD8$^+$ T cells. Target cells were Con A-stimulated spleen cells (*left*) or IC-21 macrophage cells (*right*, 50). As a positive control for killing of IC-21 targets, lymph node cells obtained from BALB/c mice on day 11 after placement of B6 skin were restimulated with B6 spleen cells for 5 days and then used in the cytotoxicity assay. Con A bridging experiments were performed with Con A added to the labeled targets plus effector cells, as described in *Materials and Methods*. The experiments were repeated twice with similar results.

Allospecific type 2 cytokine-secreting CD4$^+$ and CD8$^+$ T cells exhibit different functional characteristics

To further define the functional characteristics of our allospecific, type 2 cytokine-secreting T cells, we evaluated their ability to mediate DTH responses (Fig. 6, *A* and *B*). The type 2 cytokine-producing CD4$^+$ T cells (IL-4/IL-5 secreting) induced alloantigen-specific ear swelling. The injection site was distinguished by a

Table I. *Skin graft rejection in BALB/c SCID recipients of B6 trunk skin allografts after adoptive transfer of T cells*[a]

Cells Injected	Time to Rejection (days)
Unfractionated T cells	
Naive	12, 13, 12, 13
Primed in culture with B6 stimulators plus IL-4 and anti-IFN-γ	13, 14, 15, 21
$CD4^+$ T cells	
Naive	9, 10, 10, 12
Primed in culture with B6 plus IL-4 and anti-IFN-γ	27, 29, 29, 26, 28
$CD8^+$ T cells	
Naive	11, 12
Primed in culture with B6 stimulators plus IL-4 and anti-IFN-γ	27, 25, 24, >30[b], >30[b]

[a] Recipient mice were injected with 1–3 × 10^6 T cells by i.v. injection into the tail vein.

[b] These two grafts showed focal evidence of hair loss but were not fully rejected by visual inspection at sacrifice on day 30. Both showed histologic evidence of diffuse mononuclear cell infiltration consistent with rejection.

focal area containing increased numbers of inflammatory cells in the dermis. Histologic examination confirmed classic findings of perivascular and dermal edema, and mononuclear cell infiltration with occasional eosinophils, consistent with DTH (Fig. 6*B*). The type 2 immune-deviated $CD8^+$ T cells (IL-4, IL-5, and IFN-γ secreting) also induced a modest (although Ag-specific) ear-swelling response (Fig. 6*A*). Interestingly, however, the histology of the reaction differed significantly from the $CD4^+$ T cell-induced DTH and revealed necrosis at the site of injection, with polymorphonuclear leukocytes and nuclear dust consistent with ongoing in vivo cytotoxicity (Fig. 6*B*). These findings suggested that the type 2 cytokine-secreting $CD8^+$ T cells might have distinct functional characteristics compared with the type 2 cytokine-secreting $CD4^+$ T cells.

We further tested the function of the type 2 cytokine-secreting T cells in cytotoxicity assays. As shown in Fig. 6*C*, the $CD8^+$ T cells (which produced IFN-γ, IL-4, and IL-5) mediated allospecific killing of labeled target cells. In contrast, the immune-deviated $CD4^+$ T cells were unable to kill any target, even in an Ag-independent manner (i.e., in the presence of Con A bridging). In sum, type 2 immune deviation resulted in different functional outcomes for alloreactive $CD4^+$ versus $CD8^+$ T cells: the $CD4^+$ cells were strong mediators of DTH, but not cytotoxicity, while the $CD8^+$ cells were capable of cytotoxicity, but not histologically classic DTH.

Both allospecific type 2 cytokine-secreting $CD4^+$ and $CD8^+$ T cells mediate allogeneic skin graft rejection

To test whether the type 2 cytokine-secreting T cells could mediate allogeneic skin graft rejection, the cells were adoptively transferred into BALB/c SCID recipients of B6 skin grafts (Table I). All grafts were maintained indefinitely (>120 days, $n = 5$) when no adoptive transfers were performed. Adoptive transfer of either splenic $CD4^+$ or $CD8^+$ T cells from naive mice reproducibly led to skin graft rejection by day 12–13. Interestingly, when the $CD4^+$ T cells were primed in vitro to produce type 2 cytokines, adoptive transfer resulted in graft rejection in all animals tested, but with delayed kinetics compared with those given unprimed $CD4^+$ T cells (Table I). Adoptive transfer of $CD8^+$ T cells primed in vitro to produce type 2 cytokines also rejected their grafts (Table I), with the type 2 cytokine-producing cells exhibiting delayed kinetics vs the CD8 cells from naive mice. In additional control experiments, adoptive transfer of unfractionated T cells from naive mice and unfractionated T cells primed in vitro to produce type 2 cytokines (Fig. 3) resulted in graft rejection by day 15 (Table I).

Histologic examination of the skin grafts (Fig. 7) confirmed that adoptive transfer of both the $CD4^+$ and the $CD8^+$ T cells resulted in graft necrosis, with a complex mixed cellular infiltrate consisting of lymphocytes, tissue histiocytes, numerous degranulated mast cells, and focal areas of polymorphonuclear leukocytes associated with necrosis (Fig. 7, *A–D*). As IL-4 and IL-5 are known to induce influx of eosinophils (22, 48), we next sought to specifically determine whether eosinophils were present in the rejecting skin. Skin graft samples were stained with a polyclonal antiserum specific for eosinophil-derived MBP (21, 41). The skin grafts from all five animals adoptively transferred with type 2 cytokine-secreting $CD4^+$ T cells exhibited striking eosinophilic infiltrates, particularly in regions adjacent to hair follicles (Fig. 7, *E–F*). The rejected grafts obtained from animals transferred with type 2 cytokine-secreting $CD8^+$ T cells also showed occasional eosinophils (Fig. 7, *G–H*), but the staining was much less prominent than in the grafts obtained from the animals injected with $CD4^+$ T cells. Grafts from animals adoptively transferred with unfractionated T cells revealed typical findings of skin graft necrosis without the eosinophil infiltration (not shown).

Finally, to determine whether the adoptively transferred allospecific, type 2 cytokine-producing T cells maintained stable cytokine profiles in vivo, we performed recall ELISA spot assays using spleen cells from the recipient mice at the time of rejection. As shown in Fig. 8*A*, splenocytes of animals transferred with type 2 cytokine-secreting $CD4^+$ T cells contained only $CD4^+$ T cells that produced allospecific IL-4 and IL-5 in the absence of IFN-γ, confirming the stability of the induced cytokine profile in vivo. Similarly, recall responses performed on spleen cells from animals adoptively transferred with type 2 immune-deviated $CD8^+$ T cells contained only $CD8^+$ T cells that produced the same mixture of IFN-γ, IL-4, and IL-5, as noted in the original recall response (Fig. 8*B*). Recall responses in control animals adoptively transferred with naive $CD4^+$ and $CD8^+$ T cells revealed a mixture of IFN-γ, IL-2, and IL-4 similar to the in vivo allograft-primed response noted in Fig. 2 (not shown).

Discussion

The role of T cell cytokines as mediators of transplant rejection, and potentially as mediators of transplant tolerance, has become a confusing and controversial issue in transplantation immunology. Although some studies suggested that type 1 immunity was preferentially associated with graft rejection (1, 2), and selected animal models of graft tolerance were associated with type 2 cytokine expression (15–19), other studies have yielded conflicting results (29). Furthermore, direct attempts at inducing graft tolerance through type 2 immune deviation have generally been unsuccessful (30–32, 37), and recently published work suggests that the type 1 cytokine, IFN-γ, is in fact necessary to achieve at least one form of allograft tolerance (49).

The studies described in this work provide evidence that allospecific IL-4- and IL-5-producing T cells responding to alloantigens via the direct recognition pathway do not induce allograft tolerance, but instead mediate graft rejection. Although these experiments do not address the role of type 2 cytokine-secreting cells responding to the indirect pathway, we noted similar results upon induction of type 2 immunity toward a single immunodominant, self-restricted allopeptide in another model system (indirect recognition) (37). Our findings also provide some insight into the mechanisms of rejection after type 2 immune deviation, and show that type 2 immune deviation leads to distinctly different effects on alloreactive $CD4^+$ and $CD8^+$ T cells.

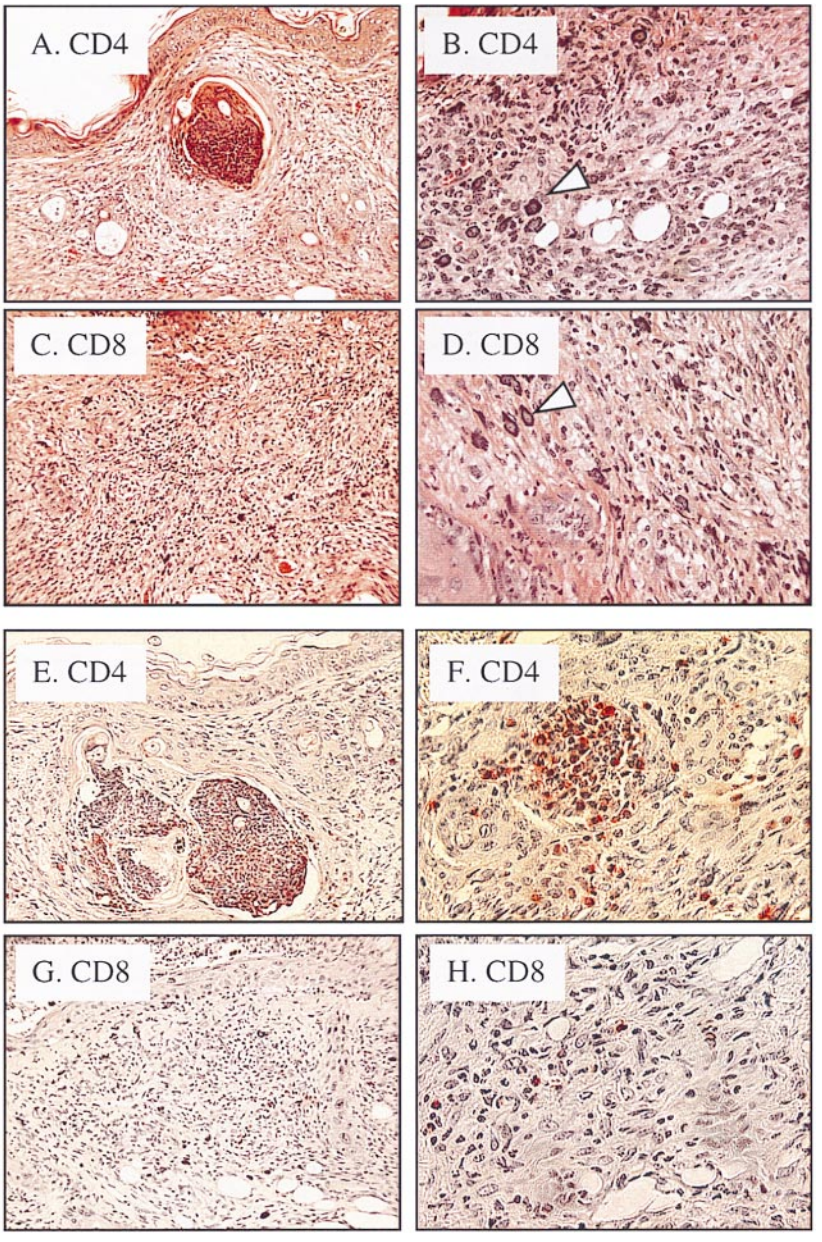

FIGURE 7. Histology of skin graft rejection in BALB/c SCID recipients of B6 skin grafts adoptively transferred with type 2 cytokine-secreting CD4$^+$ and CD8$^+$ T cells. *A–D*, Hematoxylin and eosin stain. *A* and *B*, Skin grafts in animals adoptively transferred with type 2 cytokine-secreting CD4$^+$ T cells showing folliculitis (*A*) and a complex mixed cellular infiltrate (*B*). Arrowhead points to mast cells in the graft. *C* and *D*, Skin grafts in animals given CD8$^+$ T cells showing a predominantly mononuclear cell infiltrate (*B*). Arrowhead points to mast cells in the graft. Magnification, ×200 for *A* and *C*, ×400 for *B* and *D*. *E–H*, MBP stain (red). *E* and *F*, Skin grafts in animals adoptively transferred with type 2 cytokine-secreting CD4$^+$ T cells showing prominent follicular and dermal infiltration by eosinophils (*E*, ×200 magnification; *F*, ×400 magnification). *G* and *H*, Skin grafts in animals adoptively transferred with type 2 cytokine-secreting CD8$^+$ T cells showing occasional dermal eosinophils (*G*, ×200 magnification; *H*, ×400 magnification).

In particular, IL-4 plus anti-IFN-γ Ab induced essentially unipolar, allospecific type 2 cytokine-secreting CD4$^+$ T cells (Fig. 3) that were capable of mediating DTH, but not cytotoxicity (Fig. 6). In contrast, allospecific CD8$^+$ T cells primed in the presence of anti-IFN-γ Ab and IL-4 produced IFN-γ as well as IL-4 and IL-5 (Fig. 3) and were capable of mediating cytotoxicity, but not typical DTH (Fig. 6). The finding that IL-4 can prime CD8$^+$ T cells to both produce IFN-γ and induce CTLs is not unprecedented, and is consistent with previously reported effects of IL-4 in other experimental systems (23–26). In fact, IL-4 was initially described as an inducer of CTL activity (26).

Our studies further demonstrated that both the allospecific IL-4- and IL-5-secreting CD4$^+$ and CD8$^+$ T cells were capable of rejecting allografts. The in vitro functional data (Fig. 6) suggested, however, that the two T cell subsets may have reached the same endpoint through different effector mechanisms. T cell-mediated cytotoxicity was most likely the dominant mechanism of the type 2 cytokine-secreting CD8$^+$ cells. In contrast, the lack of cytotoxicity and the striking eosinophilic infiltrate found in the IL-4/IL-5-secreting, CD4$^-$-mediated graft rejection (Fig. 7) raise the possibility that infiltrating eosinophils themselves participated in the graft destruction.

Eosinophils are a hallmark feature of helminth infection and exposure to environmental allergens (48) and have been shown to be capable of cytolysis in a number of experimental models (21, 41, 48). The cytotoxic properties of eosinophils are derived primarily from their granules, which contain several preformed cytotoxic proteins, including eosinophil MBP, eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase (22, 48). Eosinophil MBP, found extensively in the grafts rejected by type 2 cytokine-secreting CD4$^+$ T cells (Fig. 7), is a highly cationic protein that forms the crystalline core of the eosinophil granule (22, 48). Its likely mode of cytotoxicity is through a nonspecific interaction with the anionically charged lipid membrane, causing disruption of the cell membrane and subsequent lysis (21). In addition to direct cellular cytotoxicity, MBP induces platelet and mast cell degranulation, thereby exacerbating the inflammatory response (49). Interestingly, degranulated mast cells were a prominent feature of the graft destruction induced by both the type 2 cytokine-secreting CD4$^+$ and CD8$^+$ T cells (Fig. 7).

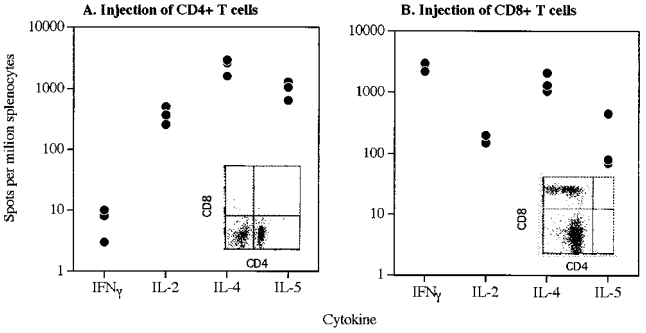

FIGURE 8. Stability of the adoptively transferred T cells in vivo. Spleen cells were isolated at the time of sacrifice from BALB/c SCID recipients of B6 trunk skin allografts after adoptive transfer of type 2 cytokine-secreting $CD4^+$ (*A*) or $CD8^+$ (*B*) T cells. The cells were tested for cytokine production in response to B6 stimulators in ELISA spot assays. Each dot represents the response from a single animal ($n = 3$ per group). Third-party responses were less than 5% of the detected allospecific frequencies (not shown). Spot frequencies represent the mean number of two individual wells counted by a computer-assisted image analyzer (less than 10% variability between wells). Aliquots of the spleen cells were studied in two-color FACS analysis for CD4 and CD8 expression (*insets*).

The data reported herein are consistent with several studies evaluating the role of type 2 cytokines as mediators of graft rejection. Bishop and colleagues, for example, showed that type 2 immune deviation, through inhibition of IL-12 in vivo, led to accelerated graft rejection in a murine heterotopic cardiac rejection model in which $CD8^+$ T cells were the dominant effectors (30). Interestingly, the anti-IL-12 intervention led to an increase in IL-4, IL-5, and IFN-γ message in the graft, but did not affect the number of CTL precursors (30). These findings could be readily explained by our data: type 2 immune deviation primes $CD8^+$ T cells to become IFN-γ, IL-4, and IL-5 producers (Fig. 3), and primes CTL effector function (Fig. 6). Further studies by this group revealed that CD8-depleted animals exhibited marked eosinophilic infiltration of cardiac allograft tissue (30, 31), suggesting that the remaining $CD4^+$ alloreactive T cells functioned similarly to those induced in our studies. In experiments performed by VanBuskirk et al., adoptive transfer of alloantigen-specific, $CD4^+$ T cells that produced IL-4, but not IFN-γ, led to accelerated heterotopic cardiac rejection (32), further establishing that type 2 cytokines can mediate the rejection process under certain conditions.

How can these findings, that IL-4- and IL-5-producing alloreactive T cells mediate rejection, be reconciled with the many studies showing an association between type 2 cytokines and the presence of allograft tolerance (15–19)? Importantly, the majority of these previous studies have demonstrated that type 2 cytokines, in particular IL-4, were detectable in tolerant animals, but the source of the detected cytokines and their role as mediators of tolerance were as not clearly defined. As many cell types other than T lymphocytes can produce these cytokines (14), detection of RNA message may reflect APC secretion and not an Ag-specific type 2 alloimmune response. It remains possible that the IL-4 detected in these other experimental models was derived from non-T cells in a bystander response and that the alloreactive T cells in the tolerized animals were not expanding or producing cytokines at all. Alternatively, our studies showed a prominence of IL-5 production within the population of allospecific, type 2-deviated T cells. The presence or absence of IL-5 in the previously published tolerogenic protocols (15–19) has not been well established, however. It therefore remains possible that the IL-5, a potent mediator of eosinophil influx and activation (14, 41), may play a critical role in triggering rejection, while preferential production of IL-4 (and/or IL-10) in the absence of IL-5 may lead to tolerance. Analogous studies of allograft rejection using type 2 immune-deviated $CD4^+$ T cells produced from IL-5 knockout mice may be required to fully address these issues.

Our studies also reveal that alloreactivity in naive mice initially derives from a relatively low frequency of IL-2-producing $CD4^+$ T cells. This finding, in conjunction with previous work by our laboratory showing that the alloresponse derives from the L-selectin^{high} population, confirms that alloreactivity initiates from T cells with a naive, and not an environmentally preprimed, phenotype (33). Both the $CD4^+$, and the $CD8^+$, T cells were capable of producing a mixture of type 1 and type 2 cytokines when primed in vitro (Fig. 2) or in vivo (Fig. 3), but only the $CD4^+$ T cell population responded initially. These findings are consistent with the hypothesis that in fully allogeneic graft rejection, the T cell response initiates in the $CD4^+$ subset, but then rapidly differentiates and spreads to include a mixed population of $CD4^+$ and $CD8^+$ T cells (36). The data are further consistent with the suggestion that $CD4^+$ T cells are needed to provide help for differentiation of alloreactive $CD8^+$ T cells (36).

These experiments also demonstrate that alloreactive IFN-γ production was derived from $CD8^+$ T cells, and not from $CD4^+$ T cells, during allograft rejection. This unanticipated result was noted in two fully MHC-disparate mouse strains (BALB/c, Fig. 2, and C57BL/6, not shown). Similar findings were noted during rejection of cardiac allografts (30).

Although the primary function of $CD8^+$ T cells has largely been assumed to be direct cytotoxicity of the transplanted tissue, the production of IFN-γ by these cells suggests they may have other functional characteristics in vivo as well. The IFN-γ could contribute to up-regulation of MHC II and costimulatory molecule expression on the transplant, thus reducing activation thresholds for infiltrating T cells, with resultant spreading of the alloreactive T cell repertoire. In addition, the secreted IFN-γ may activate macrophages, and thus provide alternative effector mechanisms of graft destruction, including induction of $CD4^+$ T cell-mediated DTH and macrophage release of TNF.

In conclusion, our studies definitively show that treatment with IL-4 and anti-IFN-γ Abs has different effects on alloreactive $CD4^+$ and $CD8^+$ T cells, and further demonstrates that allospecific IL-4/IL-5-producing T cells are not tolerogenic. Instead, both $CD4^+$ and $CD8^+$ T cells secreting IL-4 and IL-5 reject allografts and may do so by employing different effector mechanisms. This documented plasticity of the alloimmune response implies that approaches other than cytokine immune deviation will be required to achieve the evasive goal of inducing allograft tolerance.

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