Technical Advance

Interferon- γ -Secreting T-Cell Populations in Rejecting Murine Cardiac Allografts

Assessment by Flow Cytometry

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Interplay between T-helper-1 (Th1) and T-helper-2 (Th2) cells is considered important in the development of acute allograft rejection and many other immune-mediated disease processes. Existing methods for evaluating expression of Th1 and Th2 cytokines, including reverse transcriptase polymerase chain reaction (RT-PCR), RNase protection assay (RPA), immunohistochemistry, and enzyme-linked immunosorbent assay (ELISA) all have limitations; alternate techniques to quantify cell populations expressing specific cytokine proteins, generate statistically analyzable data, and allow simultaneous identification of cytokine-secreting cell type are needed. To this end, we adapted a flow cytometric technique for intracellular cytokine immunofluorescence staining for use with cells isolated from solid tissue. To demonstrate the utility of the method, we determined the number of CD4⁺ and CD8⁺ cells secreting the prototypical Th1 and Th2 cytokines, interferon (IFN)- γ , and interleukin (IL)-4 in acutely rejecting murine cardiac allografts. We also measured the cytokine production via ELISA, RPA, and semiquantitative competitive RT-PCR. The number of CD4⁺ cells producing IFN- γ increased as rejection proceeded, in agreement with previous data; we detected no IL-4 production at any time, although relatively low numbers of IL-10-producing cells were identified. In addition, a high per-

centage of CD8⁺ cells, which outnumber CD4⁺ cells at day 6 after transplant, also produce IFN- γ , suggesting that cytotoxic lymphocytes contribute significantly to the local cytokine milieu. This new application of intracellular cytokine staining provides a powerful methodology for studying transplantation immunology. The method may also be easily adapted to the study of other immune-mediated processes. (*Am J Pathol* 1998, 153:1383–1392)

Acute graft rejection is a major cause of morbidity and mortality after solid organ transplantation and is thought to contribute to long-term adverse outcomes, such as the development of occlusive graft arterial disease.¹ Helper T lymphocytes are critical mediators of acute rejection; depending on the cytokines they secrete, they may either enhance or modulate rejection severity.^{2,3} Cytotoxic T lymphocytes can also contribute to the local cytokine environment.⁴

Helper T cells can differentiate into two different functional subtypes when exposed to antigen.⁵ These subtypes express and secrete different panels of cytokines and therefore are believed to play different roles in immunologically mediated processes. T-helper-1 (Th1) cells are effectors of cell-mediated immunity; they secrete primarily interleukin (IL)-2, which stimulates the growth and activation of T lymphocytes, and interferon (IFN)- γ , which, among other functions, activates macrophages.⁵ Th1 cytokines likely stimulate the development of acute rejection, and their transcript levels have been correlated with acute graft rejection severity.^{6–8} Conversely, T-helper-2 (Th2) cells express IL-4, IL-5, and IL-10 and appear to antagonize the effects of Th1 cells *in vivo*.⁵ For example, Th2 cells may modulate acute rejec-

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tion and the development of allograft tolerance.² Th2 cytokine transcripts correlate with milder rejection episodes.^{6,9} Similarly, cytotoxic T lymphocytes may differentiate into T cytotoxic (Tc)1 and Tc2 populations with cytokine profiles analogous to those seen in T helper subpopulations.⁴ The degree to which strict Th1/2 and Tc1/2 differentiation takes place in humans, and the precise roles that these cells play in rejection processes, still remains unclear.

Experimental methods used to examine the presence and contribution of discrete T cell subpopulations in acute graft rejection include reverse transcriptase polymerase chain reaction (RT-PCR), RNase protection assay (RPA), *in situ* hybridization, immunohistochemistry, radioimmunoassay (RIA), and ELISA. Each method has important limitations. For example, levels of cytokine transcript detected by RT-PCR may not always correlate with cytokine protein levels; many important cytokine transcripts contain AU sequences in the 3' untranslated region that confer mRNA instability.¹⁰ In addition, RT-PCR is at best semiquantitative and is usually performed on whole-tissue homogenates, giving no information about the source of the detected transcript. Likewise, RPA cannot distinguish the source of particular cytokines. Although *in situ* hybridization can be used to identify secreting cell types, it also detects RNA levels, not protein. It is technically difficult, and levels of cytokine transcript present *in vivo* may be insufficient for detection. Immunohistochemistry also allows identification of cytokine-secreting cell type and detects cytokine protein directly; however, it gives variable results and lacks a high degree of sensitivity, and the process of generating statistically meaningful data are tedious. *Ex vivo* methods such as RIA, ELISA, and bioassay detect the net secretions of heterogeneous, captive, and therefore artificial, culture populations that have often spent days outside the allograft environment; these methods also provide no information about secreting cell type. The need clearly exists for techniques that can quantify cytokine protein expression, generate statistically analyzable data, and allow simultaneous identification of the source of cytokine protein.

Intracellular cytokine staining (ICCS) with flow cytometric analysis was recently described as a technique for examining cytokine expression in cloned T cell lines.¹¹⁻¹³ Briefly, intracellular cytokine staining involves incubating cells with an anti-cytokine antibody in the presence of a mild detergent that permeabilizes the cell surface and allows the antibody to pass through cytoplasmic and organelle membranes and bind to intracellular cytokine protein. Cells can then be resealed and stained with monoclonal antibodies against cell-surface markers, such as CD4 or CD8, so that cytokine expression can be correlated with cell type using multicolor flow cytometry.

When applied to the study of acute graft rejection, this method offers many advantages. First, cell-surface staining may be used to identify a specific subpopulation (eg, CD4⁺ or CD8⁺) of graft-infiltrating lymphocytes from within a heterogeneous mixture, without the need for physical isolation of the cells. Using intracellular staining techniques and multicolor flow cytometry, one may define the particular cytokines produced by a specific cell type

and the number of cells producing a given cytokine. Flow cytometry permits analysis of a large number of cells and the generation of statistically significant data.

To evaluate the applicability of this technique to the study of solid tissue processes, we developed a method for isolating and stimulating cells from acutely rejecting allografts and optimized intracellular staining conditions for the isolated lymphocyte populations. We defined the CD4⁺ and CD8⁺ lymphocyte populations within rejecting allografts by surface staining and demonstrated an increase in both populations as acute rejection proceeded. Finally, we used ICCS to determine the relative numbers of CD4⁺ and CD8⁺ cells within rejecting allografts producing IFN- γ or IL-4 at each time point.

Our results demonstrate increasing Th1-type and Tc1-type infiltrates as acute rejection proceeds, without any detectable IL-4. Moreover, we find that, at the time of graft failure, CD8⁺ T lymphocytes predominate as a source of IFN- γ .

Materials and Methods

Animals, Antibodies, and Other Reagents

C57/BL6 (B6, H-2b) and BALB/c wild-type mice were obtained from Taconic Farms (Germantown, NY) or the Jackson Laboratories (Bar Harbor, ME). All mice were maintained in the pathogen-free Harvard Medical School facility on acidified water; all experiments conformed with approved animal care protocols. The Riboquant multiprobe RNase protection assay (RPA) kit, *in vitro* transcription kit and mCK-1 template set, biotinylated anti-IFN- γ (rat IgG₁ clone XMG1.2), biotinylated anti-IL-4 (rat IgG₁ clone BVD6-24G2), biotinylated anti-IL-10 (rat IgG_{2b} clone JES5-16E3), biotinylated control antibody (anti-IgE rat IgG₁ clone R35-92 or IgG₁ isotype-matched control, clone R3-34), fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (clone RM4-5), Cy-chrome (CyC)-conjugated anti-CD4 (clone RM4-5), phycoerythrin (PE)-conjugated anti-CD8 (clone 53-6.7), Cy-C-conjugated anti-CD8 (clone 53-6.7), PE-conjugated streptavidin, unlabeled anti-IFN- γ (rat IgG₁ clone R4-6A2), unlabeled anti-IL-4 (rat IgG₁ clone 11B11), and unlabeled anti-CD16/CD32 (clone 2.4G2, Fc block) were obtained from PharMingen (San Diego, CA). Horseradish peroxidase (HRP)-conjugated ultra-avidin was from Leinco Technologies, Ballwin, MO. Ionomycin, phorbol myristate acetate (PMA), brefeldin A (BFA), collagenase (C1030, type I), saponin, bovine serum albumin (BSA), 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid), and paraformaldehyde were obtained from Sigma Chemical Co. (St. Louis, MO). IL-2 from the supernatant of X63 cell cultures was the generous gift of Dr. Andrew Lichtman, Brigham and Women's Hospital, Boston, MA. RPMI 1640 media was provided by BioWhittaker (Walkersville, MD), nonessential amino acids, L-glutamine, HEPES buffer, minimal essential medium sodium pyruvate, penicillin/streptomycin, 2-mercaptoethanol, heat-inactivated fetal calf serum, Superscript reverse transcriptase, Taq polymerase, 50 mmol/L MgCl solution, TRIzol, and 10X PCR buffer were

from Gibco BRL/Life Technologies (Grand Island, NY). Ficoll lymphocyte separation medium was from Organon Teknica (Durham, NC). IFN- γ and IL-4 primers for PCR were as published by Murray et al,¹⁴ and dNTPs were from Pharmacia (Piscataway, NJ). The competitive fragment for quantitation of cytokine mRNA was the generous gift of Dr. Jim Lederer, Brigham and Women's Hospital.

Heterotopic Murine Heart Transplant

Vascularized cardiac allografts were generated using the abdominal heterotopic murine cardiac transplantation model originally described by Corry et al¹⁵ and detailed by Nagano et al.¹⁶ Total surgical time was roughly 60 minutes, and heart ischemic time was approximately 20 minutes. At the time of graft harvest, the host was sacrificed by exsanguination under methoxyflurane anesthesia, and the graft was recovered.

BALB/c (H2^d) donor hearts were transplanted into non-immunosuppressed C57BL/6 (H2^b) recipients; using this total allogeneic mismatch strain combination, acute graft rejection is evident histologically by 4 to 6 days, and graft failure (cessation of palpable contractions) due to rejection occurs by 7 ± 1 days.¹⁷ Animals were sacrificed 3 hours after surgery to establish the baseline of cells that immediately infiltrated the graft or were already resident. Additional animals were sacrificed on days 4, 6, and 8 after transplantation. Transplanted hearts were harvested at day 7 for RNA extraction for both competitive RT-PCR and RPAs.

Extraction of Lymphocytes from Spleens or Cardiac Allografts

In most experiments, recovered cardiac tissue was minced with a sterile razor blade and placed in 10 ml of borate-buffered saline with 2% BSA and 2 mg/ml collagenase. This mixture was rocked at 37°C for 2 to 3 hours and then strained through a 70- μ m nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ). Dead lymphocytes and red blood cells were removed by centrifugation through Ficoll for 10 minutes at 2000 rpm; resulting interface lymphocytes were washed in RPMI and resuspended in C/10 media (RPMI 1640 supplemented with 1% nonessential amino acids, 1% L-glutamine, 1% HEPES buffer, 1% minimal essential medium sodium pyruvate, 1% penicillin/streptomycin, 0.1% 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum). Mechanical dissociation of heart tissue through a cytoscreen, without collagenase treatment, was also used to extract heart cells from some samples.

Spleens from transplant recipient animals were passed through a cytoscreen into 7 ml of C/10, and cells and residue were pelleted at 1200 rpm for 10 minutes and resuspended in 5 ml of ammonium chloride buffer (5 mmol/L Tris, 0.83% NH₄Cl, pH 7.2) at 37°C for 5 minutes to lyse red blood cells. Splenocytes were washed twice with RPMI medium and resuspended in C/10 for subsequent stimulation.

Figure 1. Flow chart of the method for extracting, stimulating, and staining graft-infiltrating cells from heterotopic murine allografts.

Intracellular Cytokine Staining (Figure 1)

Extracted cells were stimulated with 25 μ mol/L ionomycin and 10 ng/ml PMA for 4 hours at 37°C under a 5% CO₂ humidified atmosphere, and 10 μ g/ml of the fungal anti-metabolite BFA was added for the duration of the culture to block cytokine secretion and thereby improve cytokine detection.

After stimulation, cells were centrifuged at 1200 rpm for 5 minutes and washed with 10 μ g/ml BFA in cold phosphate-buffered saline (PBS). Cells were fixed at room temperature with 4% paraformaldehyde in PBS for 15 minutes and then washed twice with PBS. Before the initial staining, a maximum of 1×10^7 cells/tube were washed with saponin/PBS buffer (0.5% saponin, 1% bovine serum albumin, 0.1% NaN₃ in PBS) to permeabilize the plasma and intracellular membranes. Saponin permeabilization is reversible so that saponin needs to be present throughout the intracellular staining procedure.¹¹ To reduce background staining, Fc block (0.25 μ g) was applied 5 minutes before the first staining per the manufacturer's instructions. Cells in 100 μ l of saponin/PBS buffer were labeled with 10 μ g/ml of a primary biotinylated anti-cytokine antibody or isotype-matched, irrelevant control antibody for 30 minutes at room temperature, followed by two washes with saponin/PBS. The cells were incubated with PE-conjugated streptavidin (2.0 μ g/ml) for 30 minutes at room temperature. After two washes with saponin/PBS, the cells were washed with PBS to seal the membranes and then stained with FITC- and/or CyC-conjugated surface-marker antibodies (2.5 μ g/ml) for 20 minutes at room temperature. Cells were washed twice

with PBS. Flow cytometric analysis was usually performed immediately; alternatively, cells could be stored at 4°C for up to 24 hours before analysis after the addition of 200 μ l of 1% paraformaldehyde in PBS.

Flow Cytometry

Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA), equipped with a 15-mW argon laser and filter settings for FITC (530 nm), CyC (650 nm), and PE (585 nm), using CellQuest software (Macintosh). Lymphocytes were distinguished from other cardiac-infiltrating cells on the basis of light-scatter characteristics. Scatter regions for lymphocytes were established before each collection using stimulated splenocytes. Collection of cytokine staining data from allografts was restricted to this gated region (to exclude nonlymphocyte cells). Data were collected on 2,000 to 10,000 cells within the lymphocyte scatter region.

mRNA Isolation, RPAs, and Semiquantitative RT-PCR

TRIzol was used to perform mRNA isolation according to the manufacturer's protocol. The RNA pellet was dissolved in 50 μ l of diethylpyrocarbonate (DEPC)-treated water and stored at -20°C. For RPAs, the RNA was quantitated by optical densitometry, and approximately 20 μ g of RNA from fresh tissue or 5 μ g of RNA from extracted and stimulated lymphocytes was assayed using the Riboquant protocol (PharMingen). Specifically, a panel of radiolabeled probes of staggered sizes, complementary to cytokine mRNAs of interest, are hybridized to sample mRNA. Any remaining single-strand probe or RNA is enzymatically digested; resulting mRNA protected probes are electrophoresed on a sequencing gel where each cytokine migrates a specific distance based on the size of its probe. The gel is then exposed to radiographic film or a phosphor plate for detection and quantitation. Semiquantitative competitive RT-PCR was performed as described by Platzer et al.¹⁸ Briefly, cDNA was created from the mRNA samples using specific cytokine primers according to the Superscript reverse transcriptase protocol. Serial dilutions of known concentration of a competitive fragment (designed to produce products slightly larger than the native cytokine mRNA) were added to equal quantities of cDNA, and PCR was performed with specific primers to cytokines of interest. When the intensity of the authentic cytokine band is equal to the intensity of the competitive fragment band, the concentrations of the two are approximately equal.

Generation of Th2 and Th1 Control Cells *in Vitro*

To validate both our RPA and ICCS techniques, we generated Th2-like (IL-4-secreting) and Th1-like (IFN- γ -secreting) control cells *in vitro*. Th2 cells were generated by

incubating splenocytes from C57BL/6 mice with equal numbers of irradiated BALB/c splenocytes and exogenously added IL-4 (2000 U/ml) and anti-IFN- γ (40 μ g/ml) for 5 days. C57BL/6 splenocytes tend to differentiate into Th1 cells without additional cytokine stimulation, so our IFN- γ -secreting cells were generated by incubating splenocytes from C57BL/6 mice with equal numbers of irradiated BALB/c splenocytes for 5 days without additional cytokines. After washing and resting overnight in medium (with 200 U/ml IL-4 for the Th2 cells), the cells were treated like the graft-infiltrating cells, and either they were re-stimulated with PMA/ionomycin for 4 hours in the presence of BFA for subsequent ICCS or their RNA was extracted for RPA.

Quantitation of Mononuclear Cells in Allografts

Leukocyte infiltrates in rejecting allografts were quantitated by immunohistochemical staining against leukocyte common antigen (LCA) or CD45, followed by counting positively stained cells. Sections of allografts were frozen in OCT compound (Ames, Division of Miles Laboratories, Elkhart, IN) and stored at -80°C. Frozen, 4- to 5- μ m-thick sections of heart were fixed in acetone for 10 minutes and incubated with rat anti-CD45 by standard techniques. The sections were then incubated with rabbit biotinylated anti-rat IgG, developed by the avidin-biotin complex method, and counterstained with hematoxylin. Positively stained cells were counted and averaged from 10 high-power fields ($\times 100$) per allograft; two allografts were analyzed at each time point (days 0, 4, 6, and 8 after transplant).

Results

Methodology: Culture, Stimulation, and Staining Conditions

Initial reports of the technique of ICCS used stable *in vitro* lymphocyte cell lines as a source of cytokine-producing cells.^{19,20} These cells represent a homogeneous population of selected, transformed lymphocytes that produce high concentrations of cytokine protein. Primary cells are more vulnerable to cell death and may produce cytokines with lesser frequency and at lower concentrations than cultured cells. Therefore, it was necessary to re-optimize isolation and stimulation conditions for maximal lymphocyte survival and cytokine detection in cells extracted directly from cardiac allografts.

Lymphocytes were obtained by digesting minced heart tissue with collagenase and then pushing the fragments through a cell strainer and removing dead lymphocytes and red blood cells by centrifugation through Ficoll. Estimates based on the number of mononuclear cells in standard tissue histology sections and the number of sections per mouse heart indicate there are 5×10^6 to 40×10^6 graft-infiltrating mononuclear cells per acutely rejecting heart. Lymphocyte extraction by mechanical grinding, without collagenase treatment, yielded approximately 25,000 to 50,000 cells within the lymphocyte scat-

Figure 2. Forward scatter (FSC) and side scatter (SSC) characteristics of fresh *ex vivo* splenocytes and cells recovered from cardiac tissue after collagenase digestion. **A:** FSC and SSC characteristics of splenocytes from a graft recipient harvested on day 7. Most cells (75.2%) appear within the mononuclear inflammatory cell scatter region (boxed region, denoted R1). **B:** FSC and SSC characteristics of cells from a normal nontransplanted murine heart; the characteristic lymphocyte scatter constitutes a small percentage of the total population (6.3%). **C:** FSC and SSC characteristics of a rejecting allograft; the infiltrating lymphocytes are seen as an increase in cells in R1 (24.4%).

ter region, or approximately 0.5% to 5% of the anticipated total. Collagenase digestion of the heart tissue increased the yield of mononuclear cells to greater than 10^6 per acutely rejecting heart (data not shown).

Some existing methods for studying rejection use a separate enrichment step, such as cell sorting, to isolate lymphocytes from rejecting tissue. This additional step is likely to reduce cell yield and might alter cytokine expression. We therefore took advantage of the distinctive pattern of light scatter by lymphocytes in flow cytometry and selected a lymphocyte-enriched population from a heterogeneous group of cells on the basis of forward-scatter and side-scatter characteristics alone (Figure 2). Ficoll fractionation of collagenase-digested explants, as routinely used, resulted in decreased numbers of nonlymphocytic cells (eg, myocytes; not shown). Cells obtained after Ficoll centrifugation or after dissociation were directly cultured.

Figure 3 shows control stains with stimulated primary lymphocytes to demonstrate our ability to stain for both intracellular IL-4 (Figure 3A) and IFN- γ (Figure 3B). The staining intensity of the isotype-matched control antibody (solid dark line in both panels) was used to set the threshold for the anti-cytokine-stained samples. The threshold for positive cytokine signal was set where fewer than 5% of cells showed autofluorescence or background staining

Figure 3. Control intracellular stains for IL-4 and IFN- γ using differentiated Th2 cells for the IL-4 control and primary B6 lymphocytes for IFN- γ control. Both types of cells were stimulated with PMA and ionomycin for 4 hours in the presence of BFA. Cells stained with anti-cytokine antibody are in gray; cells stained with an isotype-matched control antibody are shown with a solid black line. **A:** Th2 cells stained with anti-IL-4 (BVD6). **B:** B6 spleen cells stained with anti-IFN- γ (XMGI.2).

with control antibody; background signal was subtracted from cytokine-positive signal.

To increase cytokine production, we stimulated cells with PMA and ionomycin for 3 to 5 hours before fixation and staining. Without this stimulation step, we could not detect any cytokine signals (Figure 4, A and B). Cell survival was judged by number, viability, and surface staining characteristics as detected by flow cytometry. In initial experiments, optimal cell survival was obtained with shorter culture times (ie, 4 to 6 hours *versus* 12 to 48 hours). PMA/ionomycin stimulation has been reported to reduce CD4 expression²¹; however, we found, in our protocol, that the PMA/ionomycin treatment did not affect the percentage of cells staining for CD4 or CD8, although it occasionally and variably decreased the signal intensity (Figure 4C). The loss of signal intensity was most pronounced with CD4 staining of highly stimulated lymphocytes (eg, from acutely rejecting hearts shortly before they stopped beating), with less effect seen on Thy1 or CD8 staining.

We also verified that the stimulation protocol did not alter the cytokine profile of the graft-infiltrating cells. This was demonstrated by using an RPA with RNA extracted from day 7 acutely rejecting hearts immediately after explantation and with RNA from extracted and PMA/ionomycin-stimulated lymphocytes (Figure 5A). IL-2, IL-10, and IFN- γ transcripts are detected in both unstimulated (lane 2) and stimulated (lane 3) extracted cells. No new cytokine message was induced by the stimulation protocol. The loss of IL-6 and IL-15 signals in the RNA from the stimulated cells is presumably due to decreased recovery of macrophages after the 4-hour culture time required for PMA/ionomycin stimulation. The ratio of IFN- γ to GAPDH housekeeping signal in the stimulated cells was 10 times greater than that seen in nonstimulated lymphocytes recovered directly from homogenized tissue. Notably, there is no IL-4 signal either before or after stimulation of lymphocytes from acutely rejecting hearts although IL-4 is detected in the control RNA (lane 1).

To increase the intracellular concentration of cytokine protein within primary *ex vivo* cells, stimulation was performed in the presence of BFA. BFA is a fungal metabolite that blocks cytokine secretion but that does not inter-

Figure 4. Effects of stimulation with PMA and ionomycin on extracted heart cells recovered 7 days after transplantation. **A** and **B**: IFN- γ staining with and without PMA/ionomycin treatment. No cytokine signal was seen without PMA/ionomycin stimulation. **A**: Dot plots of cytokine or control staining *versus* CD4. **B**: Histograms of the cytokine staining. Cells stained with anti-cytokine antibody are in gray; cells stained with an isotype-matched control antibody are shown with a solid black line. **C**: CD4 *versus* CD8 staining with and without a 4-hour stimulation with PMA and ionomycin. Percentages of positively staining cells are not altered, although signal intensity is slightly decreased by stimulation.

fere with activation and synthesis.²² Exposure to BFA thus causes the accumulation of recently synthesized cytokine protein within the cell before antibody staining. This effect greatly increases cytokine detection; we (not shown) and others^{11,20,22} have observed complete abrogation of cytokine signal when BFA is omitted. Although exposure to BFA for greater than 8 to 12 hours resulted in reduced lymphocyte viability (data not shown), the metabolite is not toxic to cells in the brief exposure time required in our experiments. Indeed, 4 to 6 hours of co-culture with BFA just before cell staining was found to be optimal for cytokine detection. The use of 0.1% sapo- nin did not adversely affect cell scatter characteristics (data not shown).

Cytokine Expression in Acute Rejection

To demonstrate the applicability of ICCS to the study of transplant immunology, we used the technique to define the cytokine production of infiltrating lymphocytes during the course of acute cardiac allograft rejection. Total allo- geneic-mismatched heterotopic allografts in nonimmuno- suppressed recipients proceed to graft failure within ap- proximately 8 days.¹⁷ Grafts were harvested from multiple animals at intervals after transplantation (days 0, 4, 6, and 8); samples were analyzed by flow cytometry for the percentage of CD4⁺ and CD8⁺ cells infiltrating the

graft, the CD4/CD8 ratio, the total cytokine production by the cells, and the percentage contributed by each pop- ulation. In addition, the recovered cells were stained with additional surface markers to determine the other cell types infiltrating the grafts. The total number of infiltrating mononuclear cells in these allografts was assessed by enumerating LCA-positive cells in high-power fields.

Figure 6 shows typical staining results from an acutely rejecting allograft harvested 7 days after transpl. We did not routinely measure the cytokine production of other cell types using ICCS because surface stains showed that the major cell population other than T lymphocytes was macrophages (Figure 6A). In general, there were only 1% to 3% natural killer cells (potential IFN- γ and IL-4 secretors) and less than 1% B cells infiltrating these grafts. As shown in Figure 6B, there are approximately 38% CD4⁺ cells, of which 58% are IFN- γ positive (with background subtracted), and none are IL-4 positive. Ap- proximately 26% of the gated cells stain positively for CD8, and 68% of these CD8⁺ cells are IFN- γ positive; none of the CD8⁺ cells stain for IL-4. In all, approximately 49% of the extracted cells are IFN- γ positive. Figure 7 demonstrates that the ICCS technique is also useful for identifying cells producing other cytokines, such as IL-10. Thus, we saw fairly faint IL-10 bands using the RPA technique (Figure 5A) compared with the IFN- γ signal; accordingly, we see a comparatively reduced number of

Figure 5. Cytokine measurements from an acutely rejecting cardiac allograft at day 7 using a RNase protection assay and semiquantitative RT-PCR. **A:** RPA cytokine measurement for unstimulated (lane 2) and stimulated (lane 3) heart cells, using the Riboquant mck1 RPA kit. More RNA from the unstimulated cells was used, as evidenced by the strength of the GAPDH bands, to ensure adequate cytokine detection. Lane 1 is control RNA from Th2 cells generated *in vitro*. After normalizing the cytokine bands to the GAPDH band, IFN- γ , IL-2, and IL-10 signals increase on stimulation; IL-6 signal decreases, probably due to adherence and subsequent loss of macrophages during 4-hour culture for PMA/ionomycin stimulation and Brefeldin A treatment. **B:** Semiquantitative RT-PCR of RNA from acutely rejecting cardiac allografts at day 7, using the protocol from Platzer et al¹⁸ as described in Materials and Methods. When the cytokine (lower band) and competitive fragment (upper band) strengths are equal, the concentration of the cytokine is approximately equal to the known concentration of competitive fragment in that lane. IFN- γ content (row 1) corresponds to approximately 0.6 ng of IFN- γ transcript per 5000 T cells. Row 2 shows no IL-4 (only competitive fragment bands), suggesting IL-4 transcript content of less than 0.2 ng/5000 T cells. Positive control for IL-4 using the I3L6 cell line show detection of 1.5 ng of IL-4 RNA per 5000 I3L6 cells (row 3).

IL-10-positive cells relative to IFN- γ -positive cells by ICCS (10% versus 49%).

To further validate our cytokine analysis method, we also performed semiquantitative RT-PCR with day 7 acute graft rejection samples (Figure 5B). We found a strong IFN- γ signal, with no detectable IL-4, consistent with the ICCS (Figure 6) and RPA data (Figure 5A). Figure 5B, lane 1, shows that there is between 1 and 0.3 ng/ μ l IFN- γ RNA in day 7 allograft tissue samples, corresponding to approximately 15 ng of IFN- γ transcript in 70 mg of wet heart tissue, or approximately 0.6 ng per 5000 T cells; lane 2 shows no IL-4 transcript in the RNA from the same heart. RNA from an IL-4-producing T cell clone (I3L6), as a positive control, showed that IL-4 could be detected at approximately 3 ng/ μ l corresponding to 1.5 ng from 5000 I3L6 cells (lane 3).

As shown in Figure 2B, before the onset of acute rejection, few lymphocytes were present within the allograft. Throughout the time-course study of acute rejection,

Figure 6. Representative staining of lymphocytes from an acutely rejecting cardiac allograft on day 7 after transplantation per the protocol in Figure 1. **A:** Surface staining showing that the majority of cells are T lymphocytes (56% CD8⁺ and 21% CD4⁺), with less than 1% B lymphocytes (B220 stain), ~3% natural killer cells (NK1.1 stain; on average we see 2% or less NK1.1-staining cells), and ~20% macrophages (CD11b stain). **B:** Intracellular staining. With an irrelevant isotype-matched control antibody, fewer than 5% of these cells stain positive, which defines background signal threshold. With the anti-IFN- γ antibody, 58% of CD4⁺ cells are defined as IFN- γ secreting, and 68% of the CD8⁺ cells are IFN- γ positive, with background staining levels subtracted. With anti-IL-4 antibody, there were no positive staining cells.

tion, the number of leukocytes infiltrating the graft steadily increased (Figure 8A). Assuming that an average mouse heart weighs 100 μ g and that this is predominantly water, an average volume per heart is 100 mm³. Each high-power field has a diameter of 0.2 mm and a thickness of 5 μ m, giving a volume of 6.3×10^{-4} mm³. Therefore, an average of 40 to 250 cells/high-power field corresponds to 6.4×10^6 to 40×10^6 cells/heart. In addition, as the total numbers of infiltrating leukocytes increased, and the percentages of CD4⁺ and CD8⁺ cells remained approximately equal, the absolute number of both CD4⁺ and CD8⁺ lymphocytes increased as rejection proceeded. Between days 0 and 4, lymphocytes began to infiltrate the graft, with CD4⁺ cells slightly outnumbering CD8⁺ cells. During the interval between 4 and 6 days, we observed a sharp increase in the CD8⁺ infiltrate and a concomitant inversion in the CD4/CD8 ratio (Figure 8B). By day 8, when histological sections demonstrate fulminant cellular rejection,^{16,17} most graft-infiltrating cells assessed using the size-gated population from Figure 2 were T lymphocytes, with CD8⁺ cells outnumbering the CD4⁺ cells.

Figure 7. Intracellular cytokine staining for IL-10 in mononuclear cells from acutely rejecting heart allografts, harvested 6 days after transplantation. Just 9.7% of the cells stain positively for IL-10 (above background level of 4.5%), of which approximately 1% are CD11b⁺ and 5.8% are CD4⁺. The remaining IL-10-producing cells are attributed to CD8⁺ cells.

ICCS was used to enumerate the CD4⁺ and CD8⁺ cells expressing IFN- γ and IL-4 at each time point. Very few IFN- γ -producing cells were present in the cardiac allografts before the onset of rejection between days 0 and 4 (not shown). Between days 4 and 8, when histological evidence of rejection normally appears, IFN- γ production by both CD4⁺ and CD8⁺ cells increased concomitant with the increasing number of these cells present in the grafts (Figure 8C). By day 6, when CD8⁺ cells outnumber CD4⁺ cells within the rejecting allograft, IFN- γ was largely a product of CD8⁺ cells (Figure 8D). Considering both CD4⁺ and CD8⁺ lymphocytes, there is a marked production of IFN- γ at the peak of acute graft rejection.

We could not detect the production of any IL-4, with either the ICCS technique, semiquantitative RT-PCR, or RPA at any point in these acutely rejecting hearts. However, we did detect IL-4 in all three assays with control populations: RT-PCR with I3L6 cells, which constitutively secrete IL-4 (Figure 5B), and RPA and ICCS with Th2 cells generated *in vitro* from murine splenocytes (Figures 5A and 4A). This suggests that the lack of IL-4 signal in acutely rejecting grafts is due to very low or nonexistent levels of IL-4.

Discussion

Methodology

We report here the use of intracellular cytokine staining (ICCS) and flow cytometric analysis of primary *ex vivo* lymphocytes extracted from solid tissue. This is an extension of a technique initially reported for cytokine analysis in stable, long-term lymphocyte-derived cell lines, which has also been used to measure cytokine production by peripheral blood mononuclear cells, splenocytes, and

Figure 8. Surface staining and intracellular cytokine staining of lymphocytes from acutely rejecting cardiac allografts at days 4, 6, and 8. **A:** Numbers of positively stained anti-LCA (CD45) cells per high-power field, average of 10 high-power fields per allograft and 2 allografts per time point. **B:** CD4⁺/CD8⁺ ratio. **C:** Percentage of cells staining positive for IFN- γ (background levels subtracted). **D:** Contribution to total IFN- γ production by CD4 and CD8 cells.

peritoneal cells.^{23–25} However, we found that measuring the cytokine production directly from the transplanted heart tissue was necessary, as the cytokine production by splenocytes isolated from graft recipients was very low and generally not representative of the profiles from the allograft hearts. Nadeau et al²⁶ also report a lack of cytokine response in splenocytes from kidney transplant recipients. The application of ICCS to the study of solid-tissue processes required optimization of conditions for cell recovery, culture, and stimulation.

The nature of an optimal lymphocyte recovery protocol depends on the type of tissue under study. For work in cardiac tissue, the addition of a collagenase digestion step resulted in a dramatically higher yield of lymphocytes, which is especially useful when lymphocytes are scarce, ie, during very early graft rejection or during chronic rejection. On the other hand, cells from lymphoid organs are easily recovered using a cytoscreen only, without collagenase treatment; a similar mechanical procedure also allows recovery of adequate numbers of lymphocytes (with representative cytokine profiles; not shown), ie, when lymphocytes are abundant in hearts undergoing fulminant cardiac allograft rejection.

A brief episode of nonspecific stimulation immediately before cell staining was used to boost cytokine production and thus improve detection. Our results agree with previous studies that have shown that nonspecific mitogen stimulation optimizes the secretory potential of T lymphocytes but does not alter the profile of cytokine secretion from physiologically stimulated cells.^{20,22} Therefore, cells already differentiated to a Th1 phenotype continue to produce IFN- γ , whereas Th2 cells will still produce IL-4. However, the kinetics of cytokine production after mitogen stimulation are not uniform,²⁰ and it is theoretically possible that the choice of a particular culture time favors detection of a rapidly accumulated cytokine but may be too short to allow detection of a cytokine that is sluggishly expressed. Simultaneous flow cytometric detection of both IL-4 and IFN- γ has been shown to be optimal at 6 hours in Th0 (undifferentiated) lymphocytes *in vitro*, as IFN- γ expression peaks at 6 hours and then plateaus, whereas IL-4 expression peaks at 4 to 8 hours and then diminishes.²⁰ Our choice of a 4-hour stimulation duration was based on known cytokine expression kinetics in Th0 cells^{19,20} and reflected our own optimization experiments, which showed maximal lymphocyte viability and cytokine detection at the 4-hour time point.

We also found that *in vitro* stimulation was necessary to detect cytokine. Although PMA and ionomycin stimulation has been shown to decrease CD4 expression,²¹ this was not a major problem in our hands. We also found that CD8 expression was less affected than CD4 by the PMA and ionomycin treatment and that Thy1, which is expressed by all T cells, was unaffected. Therefore, it may also be possible to obtain a reasonably accurate assessment of CD4 cell numbers by subtracting the CD8 signal from the Thy1 signal. Other protocols for ICCS have used anti-CD3 antibody to stimulate cells (eg, the PharMingen protocol); we observed comparable results using either PMA/ionomycin or anti-CD3 stimulation. We chose not to use the anti-CD3 method because CD3 down-regulation on T cell activation has also been reported.²⁷

The choice of an appropriate anti-cytokine antibody is critical to the success of the intracellular staining technique. As a fixation step is necessary to immobilize the cytokines within the cell before permeabilization, the anti-cytokine antibody must be able to recognize epitopes on paraformaldehyde-fixed cells. Although chromophore-labeled antibodies are now commercially available for use with ICCS, we and others^{28,29} have also had greater success with biotinylated primary antibodies followed by chromophore-labeled avidin.

Cells stained with irrelevant fluorescent antibody show higher signal intensity, as detected by flow cytometry, than unstained cells, perhaps due to nonspecific interactions between cells and labeled antibody. Such an effect necessitates that a threshold for positive cytokine signal intensity be established using isotype-matched, irrelevant control antibody. We have routinely set our threshold for specificity at the signal intensity where fewer than 5% of cells stained with isotype-matched irrelevant antibody would be considered positive.^{19,20}

IFN- γ - and IL-4-Secreting Cells in Rejecting Allografts

Because IFN- γ is a potent macrophage activator and mediator of cellular immunity, Th1-differentiated cells have been thought to drive the rejection process.³⁰ High levels of Th1 cytokines correlate with moderate to severe rejection,³⁰ whereas high levels of Th2 cytokines correlate with milder rejection episodes.^{6,9} In addition, Th2 cells have been identified as modulators of cellular immunity, and investigators have proposed that Th2 cells are involved in antagonizing rejection and in the development of tolerance.² Although some investigators have been able to detect significant Th2 cytokine mRNA in graft-infiltrating cells from acutely rejecting allografts,² our results agree with other investigators who report very little to no detectable IL-4 in acute grafts.^{7,8} We did, however, reproducibly detect relatively low-level IL-10 production, which has been correlated with acute rejection in human renal allografts.^{1,32} The differences in detection in IL-4 between this report and earlier investigators may reflect differences in assay sensitivity or particular cytokine responses in selected mouse strains.

As Th1 and Th2 cells have been proposed to play such critical roles in acute allograft rejection, interest in identifying IFN- γ and IL-4 within allografts has grown. Intracellular cytokine staining should be a valuable addition to current methods for studying the roles of IFN- γ , IL-4, and other cytokines in acute allograft rejection because of the ability to detect the source of the cytokine proteins.

With increasing time, allografts show increasing numbers of infiltrating mononuclear cells. Using the intracellular cytokine staining method, we found that the number of graft-infiltrating CD4⁺ cells producing IFN- γ increased with time as rejection proceeded, in agreement with previous data.² We also identified CD8⁺ cells as a major cellular source of IFN- γ . These cytotoxic T lymphocytes undoubtedly influence the local cytokine environment within the allograft; however, it is unclear whether IFN- γ production by CD8⁺ cells is a cause or an effect of Th1-type cell proliferation. By immunohistochemical staining, the majority of the mononuclear cells in rejecting allografts are macrophages.^{16,17} The predominance of T lymphocytes over macrophages in our analyses results from the initial size gating of the infiltrating cells, which will tend to increase the relative percentages of lymphocytes.

In any event, emerging Th1 dominance is clearly not accomplished by CD4⁺ cells alone. Our observations suggest that CD8⁺ cells should be viewed as important contributors to the cytokine milieu in rejecting allografts and that disruption of CD8⁺ cell cytokine should also be considered when developing immunosuppression regimens. In agreement with our data, Chan et al⁸ found that unmodified allografts did not produce Th2 cytokines; however *in vivo* depletion of CD8⁺ T cells resulted in allograft Th2 cytokine production. They suggest that donor-reactive CD8⁺ T cells inhibit intragraft production of Th2 cytokines.⁸

The information made available by cell-surface staining for CD4 and CD8 highlights one great advantage of the ICCS method, that is, the ability to clearly identify the source of cytokine protein production. Using directly chromophore-conjugated anti-cytokine antibodies, it is possible to stain simultaneously for two intracellular products, such as IFN- γ and IL-4, as well as identify the cytokine-secreting cell using a surface marker of a third color. This technique could be used to explore the role of Th0 cells in acute rejection and the kinetics of Th0 differentiation. This technique might also serve to define the cytokine contributions of every cell type present in a rejecting allograft and could be adapted to examine other intracellular products of interest, such as inducible nitric oxide synthase production by macrophages.

The present experiments establish flow cytometric analysis of intracellular cytokine staining as a useful tool in the evaluation of solid organ rejection; this approach allows quantification of cellular cytokine protein production and concurrent identification of the secreting cell type. This technique should be of great utility in the study of transplantation immunology. The methodology could also be used to examine cytokine production in other immunologically mediated or inflammatory processes, such as response to pathogens, autoimmune disease, atherosclerosis, or tumors. As the roles of Th1 and Th2 cytokines in allograft rejection are clarified, intracellular cytokine staining with flow cytometric analysis could conceivably be performed on tissue from human biopsy samples to monitor the success of immunosuppression and for diagnostic and prognostic purposes in the clinical pathology laboratory.

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References

- Schoen FJ, Libby P: Cardiac transplant graft arteriosclerosis. *Trends Cardiovasc Med* 1991, 1:216–223
- Takeuchi T, Lowry RP, Konieczny B: Heart allografts in murine systems: differential activation of Th2 like effector cells in peripheral tolerance. *Transplantation* 1992, 53:1281–1294
- Duquesnoy R, Demetris A: Immunopathology of cardiac transplant rejection. *Curr Opin Cardiol* 1995, 10:193–206
- Mosmann TR, Li L, Hengartner H, Kagi D, Fu W, Sad S: Differentiation and function of T cell subsets. *Ciba Found Symp* 1997, 204:148–154
- Abbas AK, Murphy KM, Sher A: Functional diversity of helper T lymphocytes. *Nature* 1996, 383:787–793
- Cunningham DA, Dunn MJ, Yacoub MH, Rose ML: Local production of cytokines in the human cardiac allograft: a sequential study. *Transplantation* 1995, 57:1333–1337
- Piccotti JR, Chan SY, Goodman RE, Magram J, Eichwald EJ, Bishop DK: IL-12 antagonism induces T helper 2 responses, yet exacerbates cardiac allograft rejection. *J Immunol* 1996, 157:1951–1957
- Chan SY, DeBruyne LA, Goodman RE, Eichwald EJ, Bishop DK: In vivo depletion of CD8⁺ T cells results in Th2 cytokine production and alternate mechanisms of allograft rejection. *Transplantation* 1995, 59:1155–1161
- Salom R, Maguire J, Esmore D, Hanock W: Endothelial cell activation and cytokine expression during acute graft rejection. *Transplant Proc* 1995, 27:2164–2165
- Shaw G, Kamen R: Conserved AU sequence from a 3'-untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* 1986, 46:659–667
- Sander B, Andersson J, Andersson U: Assessment of cytokines by immunofluorescence and the paraformaldehyde-saponin procedure. *Immunol Rev* 1991, 119:65–93
- Jung T, Schauer U, Heusser C, Neumann C, Reiger C: Detection of intracellular cytokines by flow cytometry. *J Immunol Methods* 1993, 159:197–207
- Carter LL, Swain SL: Single cell analyses of cytokine production. *Curr Opin Immunol* 1997, 9:177–182
- Murray LJ, Lee R, Martens C: In vivo cytokine gene expression in T cell subsets of the autoimmune MRL/Mp-lpr/lpr mouse. *Eur J Immunol* 1990, 20:163–170
- Corry RJ, Winn HJ, Russell P: Primary vascularized allografts of hearts in mice. *Transplantation* 1973, 16:343–350
- Nagano H, Mitchell RN, Taylor MK, Hasegawa S, Tilney NL, Libby PL: Interferon- γ deficiency prevents coronary arteriosclerosis but not myocardial rejection in transplanted mouse hearts. *J Clin Invest* 1997, 100:550–557
- Nagano H, Libby P, Taylor MK, Hasegawa S, Stinn JL, Becker G, Tilney NL, Mitchell RN: Coronary arteriosclerosis following T cell-mediated injury in murine cardiac allografts: role of interferon- γ . *Am J Pathol* 1998, 152:1187–1197
- Platzer C, Richter G, Uberla K, Muller W, Blocker H, Diamantstein T, Blankenstein T: Analysis of cytokine mRNA levels in interleukin-4 transgenic mice by quantitative polymerase chain reaction. *Eur J Immunol* 1992, 22:1179–1184
- O'Garra A, Murphy K: Role of cytokines in determining T cell function (mouse). *Handbook of Experimental Immunology*. Edited by Weir DM. Cambridge, UK, Blackwell Science, 1996, pp 221–226
- Openshaw P, Murphy EE, Hosken NA, Maino V, Davis K, Murphy K, O'Garra A: Heterogeneity of intracellular cytokine synthesis at the single cell level in polarized T helper 1 and T helper 2 populations. *J Exp Med* 1995, 182:1357–1367
- Anderson SJ, Coleclough C: Regulation of CD4 and CD8 expression on mouse T cells. *J Immunol* 1993, 151:5123–5134
- Pickar LJ, Singh MK, Zdravski Z, Treer JR, Waldrop SL, Bergstresser PR, Maino VC: Direct demonstration of cytokine heterogeneity among human memory/effector cells by flow cytometry. *Blood* 1995, 1408–1419
- Sander B, Hoiden I, Andersson U, Moller E, Abrams J: Similar frequencies and kinetics of cytokine producing cells in murine peripheral blood and spleen. *J Immunol Methods* 1993, 166:201–214
- Hsieh B, Schrenzel MD, Mulvania T, Lepper HD, DiMolfetto-Landon L, Ferrick DA: In vivo cytokine production in murine listeriosis: evidence for immunoregulation by $\gamma\delta$ ⁺ T cells. *J Immunol* 1996, 156:232–237
- Ferrick DA, Schrenzel MD, Mulvania T, Hsieh B, Ferlin WG, Lepper H: Differential production of interferon- γ and interleukin-4 in response to Th1- and Th2-stimulating pathogens by $\gamma\delta$ T cells in vivo. *Nature* 1995, 373:255–257
- Nadeau KC, Azuma H, Tilney NL: Sequential cytokine dynamics in chronic rejection of rat renal allografts: roles for cytokines RANTES and MCP-1. *Proc Natl Acad Sci USA* 1995, 92:8729–8733
- Salio M, Valitutti S, Lanzavecchia A: Agonist-induced T cell receptor down-regulation: molecular requirements and dissociation from T cell activation. *Eur J Immunol* 1997, 27:1769–1773
- Elson LH, Nutman TB, Metcalfe DD, Prussin C: Flow cytometric analysis for cytokine production identifies T helper 1, T helper 2, and T helper 0 cells within the human CD4⁺CD27⁺ lymphocyte subpopulation. *J Immunol* 1995, 154:4294–4301
- Meyaard L, Hovenkamp E, Keet IPM, Hooibrink B, Jong IHD, Otto SA, Miedema F: Single-cell analysis of IL-4 and IFN- γ production by T cells from HIV-infected individuals. *J Immunol* 1996, 157:2712–2718
- Dallman MJ, Larsen CP, Morris PJ: Cytokine gene transcription in vascularized organ grafts: analysis using semiquantitative polymerase chain reaction. *J Exp Med* 1991, 174:493–496
- Suthanthiran M, Strom TB: Mechanisms and management of acute renal allograft rejection. *Surg Clin North Am* 1998, 78:77–94
- Suthanthiran M: Molecular analyses of human renal allografts: differential intragraft gene expression during rejection. *Kidney Int Suppl* 1997, 58:S15–S21