Ex vivo depletion of alloreactive cells based on CFSE dye dilution, activation antigen selection, and dendritic cell stimulation

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Eliminating alloreactive cells from T-cell populations would enable the transfer of immune function to patients who receive stem cell transplants. However, high-efficiency depletion has proved difficult to achieve. We sought to develop ex vivo approaches for the maximal depletion of alloreactive CD4+ T cells. Using a flow cytometric cell sorting approach after mixed lymphocyte reaction (MLR) culture, we have found that sorted CFSEbright (5-(and-6)-carboxyfluorescein diacetate succinimidyl ester) (nondivided) and activation antigen-negative cells are markedly depleted of alloreactivity. With HLA-mismatched peripheral blood mononuclear cell (PBMC) stimulators we have consistently attained (90%-95%) depletion of alloreactivity. Importantly, when purified mature monocyte-derived dendritic cells (DCs) are used as stimulators, a 100-fold (99%) reduction in alloreactivity was attained, resulting in abrogation of the secondary MLR. Significantly, the CFSEbright CD25+ cells recovered from these cultures retained general immunoreactivity, including responses to Candida and cytomegalovirus (CMV) antigens. In addition, a CFSE-based approach was tested and found to be sufficient for graft-versus-host disease (GVHD) prevention in vivo, in a major histocompatibility complex (MHC) class II disparate murine model. This efficient approach to selectively deplete mature allogene-specific T cells may permit enhanced immune reconstitution without GVHD. (Blood. 2004;103:1158-1165)
ENHANCED DEPLETION OF ALLOREACTIVE CELLS

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FACS and assess the function of both populations of cells by using the cell sorter to physically separate them. In this study, we evaluated the hypothesis that functional sorting of nonalloreactive cells, based on nondivision (CFSE

\textsuperscript{high}), would deplete GVHD-inducing cells. We found that CFSE sorting was sufficient to markedly decrease disease. We also found that depletion of alloreactivity as assessed by in vitro methods could be further enhanced by combining activation antigen selection and using potent purified antigen-presenting cells (APCs). Using this strategy with human CD4

\textsuperscript{+} T-cell responders, we found that small, resting, CFSE

\textsuperscript{high}, and CD25

\textsuperscript{+} cells, isolated from dendritic cell (DC)–driven MLR cultures, had no detectable secondary alloresponse in most donor combinations. Importantly, the sorted nonalloreactive cells retain third-party alloreactivity and immune responses to Candida and cytomegalovirus (CMV) antigens. In contrast, use of PBMC stimulators (even in high numbers) was found to be inefficient for alloredepletion. In addition, proof of principle for the biologic efficacy of CFSE-based elimination of GVHD-causing T cells was obtained in vivo in a murine major histocompatibility complex (MHC) class II disparate model system. In these experiments, sorting solely on CFSE

\textsuperscript{high} cells after in vitro MLR culture was able to markedly reduce GVHD incidence.

Materials and methods

Isolation of responder and stimulator cells for MLR cultures

CD4

\textsuperscript{+} T cells were isolated fromuffy coat preparations derived from the whole blood of healthy volunteer donors (Memorial Blood Centers, Minneapolis, MN). Leukocyte-rich buffy coat cells were centrifuged over Ficoll-Hypaque layers to collect PBMCs. CD4

\textsuperscript{+} T cells were isolated by positive selection with anti-CD4 magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were routinely 96% to 98% pure by FACS analysis. DCs were generated from CD14

\textsuperscript{+} monocytes, 24,25 isolated from PBMCs, by magnetic bead-based purification (Miltenyi-Biotec) and were cultured in X-vivo-15 (BioWhittaker, Walkersville, MD) media at 1 \times 10

\textsuperscript{5} cells/mL supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF; 50 ng/mL final) and IL4 (20 ng/mL final) cytokines (R&D Systems, Minneapolis, MN). Cells were cultured for 1 week and then matured with tumor necrosis factor \( \alpha \) (TNF-\( \alpha \); 25 ng/mL final) and Poly I/C (25 \( \mu \)g/mL final) (Invivogen, San Diego, CA) for 2 days. 26 Poly I/C (polyinosinic polycytidylic acid) is a synthetic molecule that acts as a double-stranded RNA (dsRNA) analog, which activates APCs through binding to TLR-3 (Toll-like receptor 3), which monocyte-derived dendritic cells have been shown to express abundantly. 26 PBMCs and DC stimulators were irradiated at 30 Gy.

MLR cultures

CFSE-labeled responding CD4

\textsuperscript{+} T cells (20 \times 10

\textsuperscript{6} and 40 \times 10

\textsuperscript{6} PBMCs or 3.5 \times 10

\textsuperscript{5} DC stimulator APCs were cultured in T25 flasks in 20 mL media. Culture media was RPMI-1640 (Gibco-Life Technologies, Grand Island, NY) supplemented with 10% human AB serum (PelFreeze, Brown Deer, WI), l-glutamine, penicillin, and streptomycin. After 7 days cells were centrifuged over Ficoll-Hypaque to collect viable cells and were stained with anti-CD25–PE (phycoerythrin) clone M-A251 (BD Pharminett, San Diego, CA). Cells were sorted on a Vantage Flow Cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

CFSE labeling and analysis

CD4

\textsuperscript{+} T cells were labeled with 2.5 \( \mu \)mol/L CFSE (Molecular Probes, Eugene, OR) for 10 minutes at 20°C in phosphate-buffered saline (PBS; 0.1%) bovine serum albumin (BSA) buffer. Flow cytometric data were analyzed by CellQuest (Becton Dickinson) and FlowJo software. Proliferative analysis and precursor frequency quantitation was also by FlowJo software version 3.6 (Treestar, San Carlos, CA). Precursors, which give rise to proliferating T-cell blasts, can be quantitated by summing the number of cells in each peak and dividing by 2 to the power of the cell division number, ie, for cells in the eighth peak that have divided approximately 8 times (2\(^8\) = 256), 256 cells have derived from one precursor. 22,23

Secondary MLR cultures

T-cell populations after primary MLR or FACS sorting were recultured in secondary MLR. Responders (10\( ^4 \), 5 \times 10\( ^5 \), or 10\( ^5 \)) were cultured with 10\( ^5 \) frozen/thawed, centrifuged over Ficoll layers, and irradiated PBMC stimulator preparations from the same donor as the initial MLR. Third-party MLR used identical conditions with unrelated donor PBMCs. Sorted CFSE

\textsuperscript{high} or nonactivated (NA) cells alone had no counts above background. Primed cells alone had some residual proliferation, which was exhausted after 2 to 3 days, and these background counts were subtracted out of data shown. Cells were cultured in 96-well round-bottom plates and were pulsed daily for 7 days with H-thymidine on the last 16 hours of culture. Each time point had 6 replicates. Cells were harvested with 96-well plate harvester, and dried filters were counted with a direct beta counter with no liquid scintillation amplification. Counts are lower but proportionally accurate.

Antimicrobial responses

Fresh and sorted CD4

\textsuperscript{+} T cells (10\( ^5 \)) were cultured with 10\( ^5 \) frozen/thawed autologous CD4

\textsuperscript{+} PBMCs in 96-well round-bottom plates. Candida antigens were added at 10 \( \mu \)g/mL (Greer Labs, Lenoir, NC), and CMV lysate was added at 1/100 dilution (Microbix, Toronto, Canada). The no antigen controls had minimal background counts, which were subtracted away from data shown. Cultures were pulsed with H-Thymidine on day 7.

Limiting dilution analysis (LDA)

Populations of CD4

\textsuperscript{+} T cells, either fresh or sorted, were tested for their proliferative capacity at limiting numbers by plating at 3 \times 10\( ^4 \) cells per well (96-well plates) with 24 replicates per concentration and serial 1/3 dilutions. 30 All cells were cultured for 8 days with 5 \times 10\( ^5 \) PBMCs irradiated to 30 Gy. No IL2 was added to the media. On serial dilution of responding T cells, the dilution at which 37% of wells are nonreactive represents the concentration at which approximately one alloreactive progenitor/well is present (using a poisson distribution). 30 Wells with counts more than 3 standard deviations above background were scored as positive. With the DC system, X-vivo-15 medium was used, which led to slightly increased LDA frequencies.

Elisspots

Polyvinylidene difluoride (PVDF) 96-well plates, MAIPs45 (Millipore, Bedford, MA) were coated with anti–interferon \( \gamma \) (IFN-\( \gamma \)) antibody at 10 \( \mu \)g/mL overnight (Mabtech, Nacka, Sweden). Plates were washed, and 10\( ^5 \) fresh or sorted T cells were added with 10\( ^5 \) allogeneic PBMCs. Plates were then incubated for 2 hours with biotin–anti–IFN-\( \gamma \) antibody (Mabtech). Avidin-Peroxidase-Complex (Vector Labs, Burlingame, CA) was added for 1 hour at room temperature, and spots were developed with 3-amino-9-ethylcarbazole (AEC) substrate (Vector Labs). Spots from triplicate wells were counted with an Elispot reader (Zeiss).

Mice

B6.C-H2bm12/KhEg (termed bm12) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice were purchased from the National Institutes of Health (Bethesda, MD). C57BL/6 and bm12 (both H2\( ^b \)) mice differ at 3 amino acids because of mutations in the MHC class II IA region. Mice were used at 8 to 12 weeks of age and were housed in a specific pathogen-free facility in microisolator cages.
In vitro murine MLR cultures

CD4+ T cells were purified from peripheral lymph nodes by Cellect mouse CD4 columns (Cedarlane, Hornby, ON, Canada). The final composition of purified T cells was determined by flow cytometric analysis to be more than 94% CD4+ T cells. Responder C57BL/6 CD4+ T cells were incubated with 1 μM/L CFSE for 5 minutes at room temperature. C57BL/6 CD4+ T cells were mixed with irradiated (30 Gy) T-cell–depleted bm12 splenic stimulators.19 Responder and stimulator cells were suspended at a final concentration of 0.5 × 10^6/mL in 24-well plates (Costar, Acton, MA) containing Dulbecco modified Eagle medium (DMEM; BioWhittaker) with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 50 mM 2-mercaptoethanol (2-ME; Sigma), 10 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) buffer, 1 mM sodium pyruvate (Life Technologies, Grand Island, NY), and amino acid supplements (1.5 mM L-glutamine, L-arginine, and L-asparagine) (Sigma) and antibiotics (penicillin, 100 U/mL; streptomycin, 100 mg/mL; Sigma). After 8 days of culture, live-gated CD4+ cells and the CFSE bright nondividing cells. When high numbers of total body irradiation from a 137 Cesium source at a dose rate of 85 cGy/min. The bm12 recipients were sublethally irradiated by exposing mice to 6 Gy under these conditions, after 7 days of culture, about 33% of the cells are proliferating blasts, and 60% of the cells are CFSEbright and nondivided. Quantitative evaluation of the exponential expansion of blast number, estimates an alloreactive precursor frequency of approximately 1% in the starting CD4 T-cell population (B). DCs used at 15 000 cells/well generate the most aggressive MLR, with 67% of remaining cells blasts and less than 30% undivided. Alloreactive precursor frequency is estimated to be approximately 5%. Shown are representative examples of MLRs of each type (of 6 donor combinations each). Bars indicate population gates as percent of total cells shown. (C) PBMC-stimulated MLR culture evaluated by 2-color plot of CD25 expression versus CFSE. Quadrant gates and population statistics are shown. Also shown is FACS sorting gate, including all CFSEbright cells. (D) Contour plot of same cells as panel C, showing the nonactivated (NA cells) CFSEbright and CD25– sorting gate. Arrows indicate sort gates.

Statistics

All error bars represent one standard deviation above and below the mean. A paired, 2-tailed Student t test was used to determine the statistical significance of differences between LDA groups and proliferative responses. Survival data were analyzed by life-table methods, and actuarial survival rates are shown. Group comparisons were made by log-rank test statistics. Values of P < .05 were considered significant.

Results

CFSE-based elimination of human MLR alloreactivity

CFSE labeling of responding T-cell populations has been used to quantitate alloreactivity. By enumerating the subset of cells that have diluted out the dye because of cell division, responding cell precursor frequencies can be calculated. However, the nondividing CFSEbright T cells have not been characterized. We sought to evaluate these nondivided cells and hypothesized that they would be depleted of functionally alloreactive cells. To test this hypothesis, CFSEbright cells were isolated by cell sorting after culture in an allo-MLR and analyzed for residual alloreactivity. We found that under multiple conditions tested, a one log10 reduction in alloreactivity was readily attainable.

To evaluate reactivity in an allo-MLR culture, highly purified CD4+ responding cells were labeled with the cytoplasmic dye CFSE, cultured with HLA-mismatched allogeneic stimulators, and assessed by flow cytometric analysis. Alloreactive cells undergo activation during days 1 to 3 and a proliferative burst during days 4 to 6. The alloreactive cells undergo multiple rounds of cell division which dilute out the CFSE dye, with at least 8 cell divisions discernible. There is a wide separation of the CFSEdim proliferating cells and the CFSEbright nondividing cells. When high numbers of PBMCs are used as stimulators in this system, with a 1:2 responder cell–stimulator cell (R/S) ratio, a vigorous MLR results (Figure 1A).

Under these conditions, after 7 days of culture, about 33% of the responding T cells are blasts. These cells have derived from exponential expansion of the small alloreactive subset of responder T cells. The frequency of alloreactive precursors that gives rise to this cohort of blasts is estimated by quantitative evaluation of the CFSE profile. This determination indicates approximately 1% of the initial T-cell population is recruited to cell division (Figure 1A). This number has proved to be relatively consistent under these conditions, with the average (1.1%) for 8 separate experiments (range, 0.5%-1.7%). The CFSEbright cells are derived from the more than 98% of cells that have not divided.

FACS experiments were undertaken to evaluate the secondary MLR responses of the purified CFSEdim blasts and the CFSEbright nondivided cells. Sorted blasts mount extremely quick secondary responses, and their contribution to secondary MLR occurs early (Figure 2A). Although the CFSEbright cells showed much reduced alloresponses, there appeared to be some residual alloreactivity. Some or all of this alloreactivity may be due to residual blasts in the CFSEbright sorted population, note the early proliferation in the secondary MLR.

To further improve the efficiency of depletion of nonalloreactive cells we added selection against activation antigen expression. Two-color flow cytometric evaluation of CFSE-labeled MLR-activated cells revealed most all of the CFSEdim T-cell blasts to express CD25, and most all of the CFSEbright cells to lack expression of this activation antigen (Figure 1C). However, a
subset of the nondivided CFSEbright cells also expresses the activation antigen CD25 (approximately 5% of nondivided cells). It was unclear if this population represents alloreactive cells that are about to divide (impending blasts) or anergic CD25+ cells (possibly suppressor cells) that will not go on to divide.

To determine whether the sorted CFSEbright cells or CFSEbright CD25+ cells (NA, nonactivated cells) were the most allodepleted, we sorted both populations from the same 7-day MLR cultures. The sorted NA cells were a higher purity population and had less residual dividing blast cells. In addition, in secondary MLR cultures, the NA cells were the least reactive. The NA cells had undetectable responses early, and only at late time points did they mount small weak responses (Figure 2A). This result is consistent with marked depletion but also with the presence of very small numbers of alloreactive cells, which appear to have not become activated in primary culture. As noted earlier, the sorted CFSEbright cells had weak early and sustained secondary MLR responses, consistent with the presence of small numbers of primed cells or impending blasts (Figure 2A). Because of the presence of CD25− CFSEdim alloreactive cells, we did not sort solely on the basis of CD25 selection alone, reasoning that these cells would not be depleted.

To quantify the degree of alloreactivity present in the purified CD4+ T-cell populations, both before and after the sorting procedure, LDA frequencies were assessed (Figure 2B). Fresh unmanipulated CD4 T cells were determined to have LDA frequencies of approximately 1/800 (1/420-1/1600). After sorting, CFSEdim primed cells yielded increased precursor frequencies of approximately 1/110 (1/60-1/160). Sorted CFSEbright cells yielded precursor frequencies approximately 1/7500 (1/2500-1/16 500), a 70-fold reduction in alloreactivity from the cultured cells. Sorted CFSEbright and CD25− NA cells yielded even lower precursor frequencies of approximately 1/12 500 (1/6500-1/29 000), a 115-fold reduction in alloreactivity from the cultured cells. By comparison of the fresh CD4+ T cells and the sorted NA cells, there has been a 15-fold reduction in alloreactivity in the resulting cell product (P < .0002).

In addition, the CFSEbright and CD25− cells were also sorted directly and were found to have only small amounts of alloreactivity, with LDA frequencies approximately 1/1700. This frequency is much less than the primed blasts, or even fresh cells, and hence these cells do not contribute much to total residual alloreactivity of CFSEbright cells. However, they do contribute some alloreactive cells. Thus, for maximum depletion of alloreactive cells as assessed by in vitro secondary MLR assay, we favored a combination approach, using both CFSE and CD25 selection.

**DC stimulators facilitate elimination of alloreactivity**

Despite the efficient multiparameter selection approach, which resulted in highly purified small, nondivided, and activation antigen-negative cells, some residual alloreactivity was retained. This finding suggested that there was incomplete activation of all potentially alloreactive cells. Thus, we sought to determine whether DC would provide a better APC population capable of enhanced recruitment of alloreactive cells. Using the same system as described earlier only substituting DCs as APCs (versus PBMCs) and testing sorted cells in secondary MLR, we determined DCs were at least 5-fold better for depleting alloreactive cells from MLR cultures.

DCs are the most potent APCs for activating naive T cells, as they express abundant MHC alloantigens and high levels of costimulatory molecules and have been shown to optimally activate alloreactive T cells. DCs can be readily generated from peripheral blood monocytes cultured with the cytokines GM-CSF and IL4. These cells are immature DCs and can be further matured and activated with TNF/poly I:C. The matured DCs induced strong and prolonged primary MLR and, compared with PBMC-driven MLR, induced more blast accumulation at the end of culture; approximately 66% of responding cells are blasts (Figure 1B).

CFSE-based precursor frequency analysis indicates that approximately 5% (range, 3.1%-6.5%) of the CD4+ T cells are recruited into cell division by the mature DCs. This finding contrasts with the PBMC-stimulated MLR in which approximately 1% of T cells are recruited to cell division. This has been a consistent finding from 8 donor combinations (P < .0001).

The responder frequency can vary with R/S ratio, whereby higher numbers of DCs lead to an increase in the percentage of CD4+ T cells being recruited to cell division. Titration experiments indicate a plateau in recruitment at approximately an 8:1 R/S ratio. Thus, we used a relatively high 6:1 R/S ratio for bulk culture experiments to maximize alloantigen exposure.

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**Figure 2.** Evaluation of sorted cell population alloreactivity by secondary MLR and LDA. (A) Secondary MLR evaluation of sorted cell populations. Cells (10^4) of each population were stimulated with 10^5 frozen/thawed PBMCs. Stimulator cells were derived from the same donor as the primary stimulator. Assays were done in 96-well round-bottom plates for increased sensitivity to low precursor frequencies. Blasts (or unsorted cells) mounted quick and vigorous responses. They also had short (1-2 day) background responses (500-1000 cpm) when cultured without stimulators. These have been subtracted out of the MLR data shown. Both sorted populations had no background response without stimulators. The NA cells were the least responsive, with an undetectable response for the first 4 to 5 days. The CFSEdim cells had weak stuttering responses. A representative example is shown of 4 experiments with 8 donor combinations. Day-4 comparison of CFSEdim versus NA cells was statistically significant (P < .018). Error bars indicate 1 standard deviation from the mean. (B) Limiting dilution analysis of alloreactive precursor frequencies in the sorted cell populations. Fresh and sorted cell populations were evaluated by serial dilution to determine frequency of alloreactive cells. Data are plotted on log scale. Fresh cells (approximately 1/800), primed cells (approximately 1/110), CFSEbright cells (approximately 1/7500), NA cells (approximately 1/12 500), and CFSEbright CD25− cells (approximately 1/1700). Comparison is of fresh versus NA (P < .0002) and CFSE bright versus NA (P < .04). Data are from 8 donor combinations.
For DC-driven alloelimination, CFSE-labeled CD4⁺ T cells were cultured with activated DCs for 7 days. Recovered cells were stained with CD25 and sorted by FACS for small CD25⁻ and CFSEbright cells (NA cells) or for large CD25⁺ CFSEdim cells (Figure 3A). NA cells after the sort evidence very high purity, generally more than 99.7% CFSEbright and CD25⁻ (Figure 3B).

By using this protocol, secondary MLR kinetic analysis with sorted NA cells revealed a complete abrogation of secondary MLR (Figure 4A). There were no late responses detected in 12 donor combinations. As a further assessment of magnitude of allodepletion, secondary MLR with higher numbers of responders were evaluated. In 10 of 10 donors, no response was noted for 3 × 10⁴ responder cells/well in secondary MLR, and in 6 of 8 donors no responses were noted for 10⁴ responder cells/well (Figure 5B). This degree of allodepletion was not attained with PBMC stimulator experiments.

For the DC series of experiments, we determined LDA frequencies for 4 of the donors, and they were found to be approximately 1/350 (Figure 4B). Thus, by comparison of a pretreatment MLR response detectable with less than 500 cells (Figure 5A) and a nondetectable response after treatment with more than 5 × 10⁴ cells, we have generated T-cell populations with a more than 100-fold (> 99%) reduction in alloreactivity.

As a separate indicator of allodepletion, interferon-γ ELISPOTS were determined for 2 donor MLR combinations. CD4⁺ T cells were tested on primary stimulation and found to have approximately 170 spots/10⁵ cells. After allodepletion, only a background level of spots was present (< 5/10⁵ cells).

Retention of immune responsiveness

To determine retained potential for immunologic responsiveness, the CFSEbright NA cells were challenged with third-party allogeneic stimulators. These third-party MLRs displayed primary kinetics (peak day 7) consistent with naïve cells (Figure 6A). Responses of low numbers of cells, 10³/well, appeared intact and comparable to that of 10⁵ fresh CD4⁺ T-cell MLRs. To more accurately quantitate the retained third-party reactivity, LDA from 4 donors was assessed. These assays yielded frequencies of (approximately 1/900), similar although slightly diminished to those determined for fresh primary CD4 LDA frequencies (approximately 1/350) (Figure 4B) (P = .05).

The sorted NA cells also retained responses to infectious antigens. Proliferative responses to Candida antigens and CMV lysates were well preserved (Figure 6B). These results indicate that sorted CFSEbright NA cells are not globally hyporesponsive and they retain functional capability.

Prevention of GVHD in vivo

To determine whether CFSEbright cells were depleted of in vivo GVHD-causing cells, we tested the CFSE-based sorting
In this study we evaluated CD4+ T-cell MLR reactivity and determined factors important for activation of alloreactive cells and recruitment to cell division. We also describe an allodepletion technique based on the combined use of a CFSE parameter to aid in the functional selection of nonalloreactive cells (by nondivision) and activation antigen selection. Using this CFSE/cell sorter selection method and secondary MLR as a stringent assay, we determined factors important for activation of alloreactive cells and recruitment to cell division.

Figure 5. Sensitive detection of residual alloresponses in MLR assay. (A) Primary MLR. Serial dilution of freshly purified CD4+ T cells reveals readily detectable MLR responses with less than 500 cells. Because some wells were positive and others were completely negative at limiting dilution, there is a wide standard deviation; 5000 or less purified CD4 cells do not have auto MLR responses even in the 96-well round-bottom culture, and controls are at background. Average of 4 donors is shown from LDA experiments. (B) Secondary MLR. Sorted NA cells had no MLR reactivity at peak (day 7) in secondary MLR with 3 x 10^4 cells as responders (12 replicates); results from 8 donor combinations. In 6 of 8 of those combinations, secondary MLR with 10^5 cells as responders was also nonreactive (6 replicate wells). Error bars indicate 1 standard deviation from the mean.

Discussion

on their own unless the adoptively transferred cells cause a GVHD-mediated bone marrow aplasia with a short latency. The mouse experiments used a simplified translational protocol whereby T-cell and natural killer (NK) cell–depleted splenocytes were used as APCs, and the postculture sorting was solely selected on the basis of CFSEbright cells (Figure 7A). These are vigorous MLRs, and after 7 days about 67% of the remaining T cells are blasts. Calculation of precursor frequency yields an initial alloreactive dividing fraction of approximately 3% (4% and 1.7% in 2 separate experiments). With this protocol, and transferring 3 x 10^5 sorted CFSEbright cells, 70% of mice survived long term, whereas control-sorted CFSE-labeled cells or CFSEdim cells were uniformly lethal (Figure 7B). Interestingly, the CFSE precursor frequency correlated with in vivo outcome, in that in the first cohort 7 of 8 mice survived (frequency 4%) and in the second cohort 4 of 8 (frequency 1.7%). Importantly, the surviving mice had no signs of morbidity, as evidenced by normal weights, hematocrits, and lack of skin or gut involvement. These studies demonstrate the potential of the method to allow for the transfer of allogeneic T cells.

Figure 6. Sorted CFSEbright CD25+ NA cells retain immune responsiveness. Sorted NA cells were derived from DC-stimulated MLRs. These NA cells were also tested for retained immune reactivity, simultaneously with assessments of depletion of alloreactivity. (A) NA cells (10^5) were cultured with 10^5 irradiated MNCs from a third-party donor for 8 days. The NA cells mounted a significant MLR (NA-Third Party), roughly equivalent to a primary MLR of fresh CD4+ T cells versus same-donor PBMCs. These same NA cells did not mount a secondary MLR (NA-Restim). Results are representative of 4 experiments. (B) Proliferative responses to Candida and CMV antigens were preserved. Fresh CD4+ T cells (10^5) and 10^3 sorted NA cells were stimulated with aliquots of the same frozen/thawed APC population (autologous irradiated CD4-depleted PBMCs). Cultures were pulsed with [3H]-Thymidine on day 7. Results are compared with the alloreponses and depleted alloreponses of the same populations of cells. Results are representative of 3 experiments. Error bars indicate 1 standard deviation from the mean.

Figure 7. Sorted CFSEbright cells after MLR have reduced ability to mediate GVHD. Purified murine CD4+ T cells were stimulated with T-cell– and NK cell–depleted MHC class II disparate splenocytes. After 8 days of culture, viable cells were analyzed. (A) MLR cultured cells were analyzed for intensity of CFSE staining; 65% of the cells were blasts, and a precursor frequency of approximately 3% was calculated. The CFSEbright gate was the sole parameter for sorting. Postsort analysis reveals cells are markedly depleted of primed CFSEdim cells. Results were typically more than 98% purity. (B) Sorted CFSEbright T cells have much reduced GVHD-causing potential. After culture, the CFSEdim or CFSEdim cells were sorted, and 3 x 10^5 were transplanted. Results are from 2 pooled experiments. Mean survival times were as follows: sorted control, 20 days; CFSEdim cells, 21 days; and CFSEdim cells, more than 60 days (not reached). Comparison of CFSEbright versus sort control was very significant (P < .0003).
determined that the use of purified DCs was critical for high-

efficiency elimination of alloreactive cells in human MLR. Use of
this strategy has enabled a more than 2 log₁₀ reduction in
alloreactivity, as assessed by an abrogation of secondary
MLR. These alloredepleted cells retain immune responsiveness to
third-party alloantigens, *Candida* antigens, and CMV antigens. In
addition, we have been able to demonstrate markedly reduced in
vivo disease causing potential of CFSE<sup>bright</sup> sorted cells in an MHC
class II disparate murine model of GVHD.

We began these studies with the idea to use CFSE labeling as an
analytical tool to evaluate T-cell alloreactivity. We hypothesized
from these studies that sorting on the basis of the CFSE parameter
might lead to effective purification of functionally nonalloreactive
cells. The selection is based on a functional readout of cell division,
and hence the most aggressive division-prone cells should be most
efficiently eliminated. There is a wide separation of the CFSE<sup>bright</sup>
and CFSE<sup>dull</sup> alloreactive dividing cells, and this separation
allows for highly efficient sorting. In addition, the CFSE parameter
also allows for the elimination of alloreactive cells regardless of
their expression of activation antigens. We find that frequently
there are small populations of alloreactive cells that do not express
activation antigens such as CD25 (or CD69, CD71, or CD134) and,
thus, are unavailable for activation antigen-based approaches for
depletion. The only requirement for selection on the basis of the
CFSE parameter is recruitment to cell division. The fact that this
technique can be used to deplete alloreactive T cells that may be
sufficient for GVHD prevention is supported by our murine data
using spleen cell populations that proved to be potent APCs. CFSE
sorting of T cells in murine MLR cultures lead to reduced GVHD
lethality. Although we favor the explanation that the inhibition of
GVHD was due to alloreduction, we did not directly measure donor
T-cell persistence because of the small number of donor T cells
transferred and the fact that the vast predominance of T cells are of
host origin in this sublethal total body irradiation (TBI) model.
However, host T cells cannot reject donor T cells because neither
resting nor activated donor T cells are capable of expressing MHC
class II antigens.32,33

Because the CFSE parameter requires a FACS sorter, we also
took advantage of other parameters during sorting to enhance the
selection of nonalloreactive cells in the human system. Small cell
size and lack of activation antigen expression were also used to
enhance sorting efficiency and alloreduction in vitro. In theory, this
multiparameter sorting method could provide enhanced GVHD
protection in the murine system.

Of interest, we do find a small population of activation marker
(CD25<sup>+</sup>) positive CFSE<sup>bright</sup> (nondivided) cells. These cells consti-
tute about 5% of the CFSE<sup>bright</sup> cells in the human MLR. This
population appears to contain anergic CD4<sup>+</sup> CD25<sup>+</sup> cells, as well as
variable numbers of alloreactive cells. By LDA, these cells have
less alloreactivity than fresh cells, especially at later time points in
MLR (day 7-8). Thus, this population may contain some alloreactive
cells about to divide, but the majority appears to be poorly
capable of dividing. The potential suppressor function and in vivo
relevance to GVHD induction of this population are the subjects of
ongoing investigation. However, because of the small amount of
known alloreactivity, we favored adding activation antigen deple-
tion to the selection protocol.

We have compared the sorting method with an immunotoxin
(DT-IL2)–mediated depletion in the PBMC-stimulated system. In 4
of 4 donor combinations, the sorting approach yielded cell
populations with 2- to 3-fold less secondary alloreactivity and
better retention of third-party reactivity (W.R.G. and M.R.K.,
unpublished data, October 2001). In addition, we have compared
the sorting approach directly with an anti-CD25 magnetic mi-
crobead-based depletion in the DC system. In 3 of 3 donor
combinations, the bead-based approach did not eliminate second-
ary MLR reactivity with 10<sup>5</sup> responder cells. Exhaustive depletion
with anti-CD25 microbeads decreased anti-microbial responses,
possibly because of removal of CD25<sup>+</sup> memory cells. The sorting
method appears to result in cell populations that are consistently
more depleted of primed alloreactive blast cells by FACS analysis
and lower secondary MLR reactivity. Importantly however, we
found that independent of the method of selection, a critical factor
for elimination of alloreactivity is getting all the potentially
alloreactive cells to become activated.

This study demonstrates that matured activated DCs facilitate
recruitment to the MLR and enhance the elimination of alloreactivity.
Given the primary importance of recruitment to cell division for
the CFSE-based approach (and activation antigen expression for
the combination sorting approach), we reasoned that the use of high
relative numbers of potent APCs would be important for maximiz-
ing recruitment of alloreactive cells. Thus, we used high relative
numbers of matured DCs for activating the T cells.34 Although
PBMC stimulators recruit approximately 1% of CD4<sup>+</sup> T cells
to divide in an MLR, activated DCs recruit approximately 5%. More
important, the sorted CFSE<sup>bright</sup> NA cells derived from DC-
stimulated cultures were depleted of secondary MLR reactivity.
Our results indicate that PBMC-stimulated MLRs are not com-
pletely efficient and that a number of alloreactive cells do not join
the proliferative reaction (up to 10% of the potentially alloreactive
pool), even with an efficient purified CD4<sup>+</sup> MLR and high numbers
of stimulators. Most studies of alloreduction have used PBMCs as
MLR stimulators, including the only reported clinical trial of
alloreduction.21 Because residual alloreactivity correlated with
GVHD,21 we believe the extra efficiency afforded by matured
DC-stimulated cultures may improve the results.

One group has used adherent cell-derived DCs, adherent cells
treated with GM-CSF and IL4 and reported an 80% decrease in
secondary MLR reactivity and (helper T-cell precursor) HTLp
frequency.14 We have found that adherent cell-derived DCs,
although generating a robust MLR, do not as efficiently recruit all
the alloreactive cells as CD14-purified derived DCs. We speculate
that this occurs because of the co-puriﬁcation of B cells and other
cells in the adherent population. Other studies have suggested
lymphoblastoid cell lines (LCLs) are better MLR stimulators for
alloreduction.15 A recent report demonstrated reduced residual
proliferation in primary MLR (after immunotoxin treatment) with
the use of LCL (R/S ratio of 40:1) versus PBMC stimulators.35 By
CFSE analysis, we found that the use of high numbers of LCL
stimulators can lead to very high calculated precursor frequencies,
possibly indicative of bystander proliferation, which we have not
seen with DC-stimulated cultures.

Our work has concerned CD4<sup>+</sup> T-cell responses, both in human
and mouse systems. We focused on CD4<sup>+</sup> T cells to have a more
robust, consistent, and sensitive experimental system. It has proved
difficult to eliminate secondary MLR reactivity, and it required the
combined use of an efficient multiparameter selection strategy and
purified potent APCs in high numbers to generate effective
alloreduction. The resulting populations can respond proliferatively
to *Candida* and CMV antigens and retain some ability to produce
interferon-γ in short-term Elispot assays. Transfer of alloredepleted
CD4 cells could provide immune protection, as CD4<sup>+</sup> T cells alone
can provide direct antileukemic36 and anti-infectious activity.37 Of
importance, CD4+ T cells orchestrate immune responses by providing proinflammatory factors for activating monocytes, dendritic cells, and B cells and providing accessory factors for CD8+ T-cell survival and effector differentiation.

In haploidentical BM transplantation only small numbers of allogeneic cells may need to be transfused to facilitate immune reconstitution. With the sorting approach approximately 200 million sorted CFSEhigh NA cells could easily be prepared from small apheresis products (2-4 billion MNCs), as routine recovery is about 10% NA cells from total input CD4+ T-cell number. Thus, a CFSE-based strategy is technically feasible in humans. Even a small number of functional T cells could help prevent Epstein-Barr virus (EBV) or CMV reactivation38 and may provide for broad-based immune protection as well as some antitumor reactivity.17,18

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Ex vivo depletion of alloreactive cells based on CFSE dye dilution, activation antigen selection, and dendritic cell stimulation

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