# Memory CD4 T Cells Induce Graft Versus Host Disease

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## Abstract

In a complete MHC mismatched allogeneic model of mouse bone marrow transplantation (BMT), naïve $CD4^+$ T cells from the C57BL/6 donor spleen combined with T cell depleted bone marrow cells induce graft versus host disease (GVHD) in lethally irradiated BALB/c hosts, leading to death within 10-20 days. This is characterized by acute injury to the large bowel with severe diarrhea. It has been reported that naive $CD4^+CD62L^{hi}CD44^{lo}$ T cells induce severe GVHD, but that effector memory $CD4^+CD62L^{lo}CD44^{hi}$ T cells obtained from untreated normal donors do not induce GVHD in this model. We hypothesized that the poor GVHD-inducing capacity of effector memory cells from untreated donors may reflect their lack of previous exposure to host alloantigens. We tested this hypothesis by comparing the ability of effector memory T cells obtained from untreated donors and donors immunized to host alloantigens to induce GVHD. Donors were immunized by injecting 50 $x10^6$ host spleen cells i.p. and after one week with 10x $10^6$ cells. We sorted naïve ($CD62L^{hi}$ $CD44^{lo}$) and effector memory ($CD62L^{lo}$ $CD44^{hi}$) $CD4^+$T cell subsets from C57BL/6 donor mice four weeks after immunization, and compared their GVHD-inducing capacity to the same T cells subsets sorted from unimmunized C57BL/6 donors. We found that $CD62L^{lo}$ $CD44^{hi}$ cells from unimmunized donors failed to induce GVHD in 85% of the hosts over 100 days while $CD62L^{lo}CD44^{hi}$ cells from immunized donors caused progressive weight loss and death in 100% of hosts ($p < 0.001$). Whereas naive $CD4^+$Tcells from unimmunized donors responded vigorously to host stimulator cells in the mixed leukocyte reaction (MLR), and accumulated rapidly in the lymph nodes and spleen of irradiated hosts, effector memory $CD4^+$T cells had markedly reduced activities in these assays. We are currently comparing the activity of memory $CD4^+$T cells from immunized and unimmunized donors in these assays. In conclusion, memory $CD4^+$T cells from donors immunized to host alloantigens are able to induce lethal GVHD, but memory cells from unimmunized donors do not. We are testing whether the increased potency of memory T cells from immunized donors is due to their increased reactivity to alloantigens and increased ability to accumulate in the host target tissues of GVHD.

## Introduction

In Allogeneic transplantation, acute Graft versus Host Disease (GVHD) is mediated by mature donor T cells.

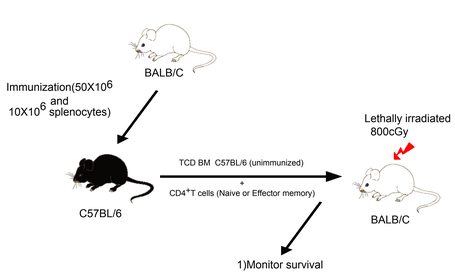
In a complete MHC mismatch model, transplantation of C57 BL/6 donor cells to lethally irradiated BALB/C hosts CD4 T cells are much more potent than CD8 T cells (Zeng *et al* , J Exp Med 1999)

The hosts die of diarrhea within 2-3 weeks of transplantation marked by characteristic colonic injury of the large bowel.

Anderson *et al* (J Clin Invest, 2003) and Chen *et al* (Blood, 2004) reported that naïve $CD4^+$T cells which are $CD62L^{hi}$ $CD44^{lo}$ cells induce acute GVHD while $CD62L^{lo}$ $CD44^{hi}$ memory cells do not.

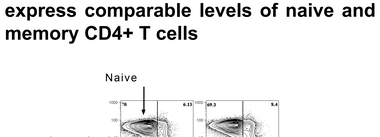
In this study we investigated whether alloantigen exposed $CD4^+CD62L^{lo}CD44^{hi}$ T cells were capable of inducing GVHD.

## Experimental Setup




## Results

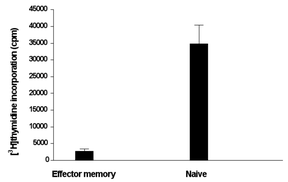
**Fig 1 Unimmunized and immunized splenocytes express comparable levels of naive and effector memory CD4+ T cells**




Flow cytometric analysis of Naive and Effector memory cells from gated $CD4^+$ T cell

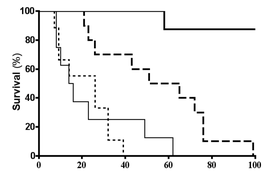
## Results

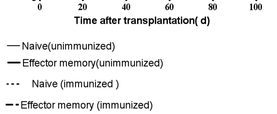
**Fig 2 Effector memory $CD4^+$ T cells from unimmunized C57BL/6 fail to proliferate in response to BALB/C stimulators**

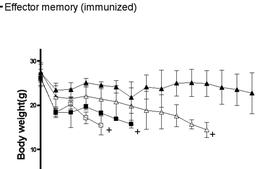


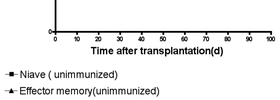

Cultures were set up with 100.000 BALB/c-derived APC and 100.000 sorted Naive and Effector memory CD4+ T cells from C57BL/6. Proliferation was assessed by labeling the cultures with $^3$H-thymidine for the final 16 h of the 5 day incubation period. Data represent mean ± SD of triplicate values.

**Fig 3 Effector memory $CD4^+$ T cells from immunized C57BL/6 donors induce GVHD**



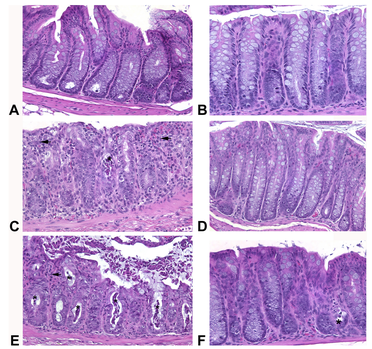



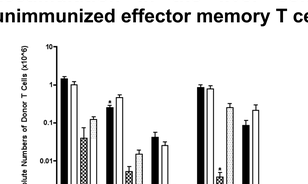

Lethally irradiated BALB/c host mice were given intravenous injections of $2x10^6$ TCD bone marrow cells from unimmunized C57BL/6 donors with Naive or Effector memory $CD4^+$T cells from immunized or unimmunized donors. There were 8-10 hosts in each group. (A) Survival of irradiated hosts given TCD bone marrow cells and $0.125x10^6$ $CD4^+$ T cells from immunized or non immunized donors. (B) Mean body weights of host mice given TCD bone marrow and $CD4^+$ T cells as in (A).Brackets show standard errors of the mean. Analysis was stopped for a given group when there were two hosts remaining (+).

**Fig 4 Histopathologic changes in the colon of lethally irradiated host seven days after transplantation of donor cells.**




All hosts received $0.125x10^6$ $CD4^+$ T cells and/or $2x10^6$ TCD bone marrow cells. (A) Colon of an irradiated control host showing normal appearing colonic mucosa and scant inflammatory infiltrate. (B) Colon of a control host given only TCD bone marrow cells showing unremarkable colon with back-to-back crypts containing numerous goblet cells. Inflammatory infiltrate is minimal.(C) Colon of a host given unimmunized Naive $CD4^+$ T cells and TCD bone marrow. Crypts are shortened and separated by a prominent inflammatory cell infiltrate composed of lymphocytes and plasma cells (arrows).Asterisk shows crypt abscess and there are decreased numbers of goblet cells lining crypts.(D)Colon of a host given unimmunized effector memory $CD4^+$ T cells.Goblet cells are retained in crypt walls with normal architecture and minimal inflammatory cell infiltrate .(E) Colon of a host given immunized Naive $CD4^+$ T cells and TCD bone marrow.Severe acute graft versus host disease is present with numerous crypt abscesses (asterisks) and erosion with exudate on surface of mucosa. Arrow highlights the accompanying mononuclear cell infiltrate.(F) Colon of a host given immunized effector memory $CD4^+$ T cells. A mild inflammatory infiltrate separates crypts which are lined by goblet cells with abundant mucin. A focal abscess (asterisk) is shown in one crypt.Tissues sections were stained with hematoxylin and eosin, with a final magnification of 200x. Each panel is representativeof each of three hosts examined. Asterisks highlight crypt abscesses with arrows showing inflammatory cell infiltrates between crypts.

**Fig 5 Immunized effector memory $CD4^+$T cells show significantly increased accumulation in the liver compared to unimmunized effector memory T cells**




Absolute number of donor T cells in the spleen, mesenteric lymph nodes, peripheral lymph nodes, liver, and colon of irradiated hosts day 6 after the injection of $0.5x10^6$ Naive or Effector memory $CD4^+$ T cells (day 6). Bars show the means of the absolute number of donor T cells, and brackets show standard errors of groups of mice given $CD4^+$T cells from immunized or immunized donors. There were three separate experiments, with 5 to 6 mice in each experiment. #, indicates the absolute number of donor T cells was less than $0.001x10^6$ *, indicates statistically significant difference between immunized and unimmunized group ($p < 0.05$). All hosts received $2x10^6$ WT TCD bone marrow cells.

## Conclusions

We found that $CD62L^{lo}$ $CD44^{hi}$ effector memory $CD4^+$ T cells from unimmunized donors failed to induce GVHD in almost all of the hosts over 100 days while the effector memory $CD4^+$ T cells from immunized donors caused progressive weight loss and death in 100% of hosts.

The naive $CD4^+$ T cells home significantly better to mesenteric lymph nodes and colon while the effector memory $CD4^+$T cells from immunized donors show significantly increased accumulation in the liver compared to the effector memory $CD4^+$T cells from unimmunized donors.

### Acknowledgment

Diane Tseng would like to thank the Howard Hughes Medical Institute for their support with a Stanford University Undergraduate Research Opportunity Major Grant.

## Materials and Methods

**Animals** Wild type C57BL/6 (H-2Kb) male mice, 6 to 8 weeks old and male BALB/c (H-2Kd) mice 8 to 10 weeks old were purchased from the breeding facility of the Department of Comparative Medicine, Stanford University. All mice were housed in a specific pathogen free facility. Care of all experimental animals was in accordance with institutional guidelines.

**Antibodies and Flow Cytometric Analysis (FACS)** The following reagents were used for flow cytometric analysis: unconjugated anti-CD16/32 (2.4G2), anti-CD4 FITC (RM4-5), anti-TCRb APC (H57-597), anti-CD62L APC, (Mel-14), anti-CD44 PE (IM7), anti-H-2Kb FITC (AF6-88.5) mAbs were purchased from BD Pharmingen (San Diego, CA). All stainings were performed in PBS/1% calf serum in the presence of purified anti-CD16/32 at saturation to block unspecific staining via FcRI/III. Propidium iodide (Sigma, St. Louis, MO) was added prior to analysis to exclude dead cells. All analyses were done on a modified dual laser FACS® Vantage (Becton-Dickinson, Mountain View, CA) in the Shared FACS Facility, Center for Molecular and Genetic Medicine at Stanford University, using FlowJo, software (TreeStar, Ashland, OR) for data analysis.

**Cell preparations and Sorting** Single cell suspensions were prepared from spleens, washed twice and filtered through a fine nitex membrane. The samples were then enriched for CD4+ cells with anti-CD4 magnetic microbeads using the MidiMACS® system (Miltenyi Biotech, Auburn, CA. After staining with anti-CD4 FITC, anti-CD62LAPC and anti CD44 PE, cells were sorted into $CD4^+CD62Lhi$ $CD44lo$ and $CD4^+CD62Llo$ $CD44hi$ populations on a modified dual laser FACS® Vantage (Becton-Dickinson, Mountain View, CA). For preparation of T cell-depleted bone marrow (TCD BM), bone marrow cells were obtained from the femur and tibia, single cell suspensions were prepared and filtered through nitex membrane and were stained with anti-Thy1.2 biotin (5a-8; Caltag, Burlingame, CA) and streptavidin-magnetic beads (Miltenyi Biotech, Auburn, CA) and passed over two consecutive MACS LS-separation columns (Miltenyi Biotech, Auburn, CA). TCD BM contained less than 0.01% T cells, as determined by staining with anti-TCRb APC . Thy1.2-depleted splenocytes were used as allogeneic stimulator cells.

**Mixed lymphocyte reaction**. Cultures were set up in 96 well round bottom plates (BD Biosciences, Franklin Lakes, NJ) in a total volume of 200 µl. Cells were cultured in RPMI-C, i.e. RPMI 1640 (Bio Whittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum, 10 mM HEPES, 1% non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml Penicillin + 100 µg/ml Streptomycin, 2 mM L-Glutamine (all Gibco BRL, Gaithersburg, MD), and 50 µM 2-Mercaptoethanol (Sigma, St. Louis, MO). Naive or effector memory CD4+T cells (responder cells ) were mixed with irradiated (3000 cGy) Thy1.2 depleted BALB/splenocytes as stimulator cells (100,000 cells each). Proliferation was assessed after 5 days by pulsing the cells with 1µCi/well [3H]-thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) for the last 16 h. Cells were harvested onto filter membranes using a Wallac harvester (PerkinElmer Life Sciences, Gaithersburg, MD), and the amount of incorporated [3H]-thymidine was measured with a Wallac Betaplate counter (PerkinElmer Life Sciences, Gaithersburg, MD).

**Immunization** Five week old male C57BL/6 mice were immunized intraperitoneally with $50X10^6$ splenocytes from age matched male BALB/C mice followed by a dose of 10 $X10^6$ splenocytes one week later. Four weeks after immunization, splenocytes from the immunized C57BL/6 mice were used for further studies.

**GVHD model** In brief, BALB/c hosts were lethally irradiated (800 cGy) from a 200 Kv X-ray source and injected with donor cells via tail vein within 24 hours. All mice received $2x\ 10^6$ TCD BM cells for reconstitution with or without CD4 +T cells as indicated in the text and figures. Mice were kept on antibiotic water (25µg/ml neomycin /0.3 U/ml polymyxin B; Sigma Aldrich, St Louis MO) for the first 28 days. Survival and the signs of GVHD, hair loss, hunched back, swollen faces, and diarrhea were monitored daily and body weight was measured weekly.

**Histopathology** Histopathological specimens from the small and large intestines of hosts were obtained at 7 days after transplantation and fixed in formalin before embedding into paraffin blocks. Tissue sections of 4-5mm thickness were stained with hematoxylin and eosin using standard protocols. Microscopic images were obtained using an Eclipse E1000M microscope (Nikon, Melville, NY) with SPOT RT digital camera and acquisition software (Diagnostic Instruments, Sterling Heights, MI) with a final magnification of 300x magnification for all images. Image processing was performed with Photoshop CS (Adobe, San Jose, CA) with standard adjustments of brightness, contrast and color balance to the entire image.

**Cell distribution studies** Cell preparation and acute GVHD induction were performed as described above. For day 6 analysis lymphocytes from tissues of individual mice for flow cytometric analysis. Single cells suspensions from mesenteric lymph nodes, peripheral lymph nodes (bilateral axillary and inguinal nodes were pooled) and spleen were filtered through fine nitex membrane to remove aggregates. Mononuclear cells from the liver were isolated by lympholyte M gradient. Lamina propria (LP) lymphocytes were purified from the interphase betwen a 67% and 44% Percoll gradient.

**Statistical Analysis** Kaplan-Meier survival curves were made using Prism (GraphPad Software, San Diego, CA). Statistical differences in animal survival were analyzed by log-rank test. Differences in donor type T cell recovery in tissues of hosts were analyzed using the two tailed Student t test. For all tests, $p < 0.05$ was considered significant.