Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

CD52 ligation induces CD4 and CD8 down modulation *in vivo* and *in vitro*

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Keywords
campath-1H, CD52, immunosuppression.

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Received: 22 December 2005
Revision requested: 16 January 2006
Accepted: 1 May 2006

doi:10.1111/j.1432-2277.2006.00350.x

Summary

To successfully induce donor-specific tolerance after immune depletion, it is essential to understand the residual and recovering immune system in the context of the depleting agent because the properties of such a recovering immune system differ based on the depleting agent used. In this study, we investigate the phenotypic and functional characteristics of T cells exposed to Campath-1H *in vivo* and *in vitro*. Recovering T cells demonstrated down modulated surface CD4 and CD8 (by flow cytometry) for up to 45 days after Campath-1H administration. Additionally, these T cells had an activated phenotype. To determine whether this CD4/8 down modulation was due to T-cell activation only or in part due to Campath-1H, whole blood from healthy volunteers was exposed to Campath-1H and the surviving lymphocytes isolated. Flow cytometry revealed a dose-dependent down modulation of CD4/8 *without* T-cell activation. Additionally, these Campath-1H-treated T cells were immunocompetent as indicated by increased surface CD69 and interleukin-2 (IL-2) production following stimulation by soluble anti-CD3 mAb. In conclusion, Campath-1H by itself down modulates surface CD4 and CD8 without activating T cells.

Introduction

Campath-1H (Alemtuzumab; Berlex Inc., Montville, NJ, USA) is a humanized monoclonal antibody against the CD52 antigen, a 12 amino acid, heavily glycosylated glycosylphosphatidylinositol-linked cell surface protein. CD52 is expressed on the surface of normal human T cells, B cells, monocytes, macrophages, eosinophils, and T/B cell lymphomas, and binds Campath-1H, resulting in long-lasting lymphocyte depletion [1–5]. It has been assumed for some time now that, after depletion by any agent, the recovering immune system has the potential to become tolerant to the transplanted organ as the emerging cells are seeing the organ in a compromised state. However, this assumption has been challenged and recent data demonstrate that such residual and recovering immune cells are resistant to tolerance induction protocols [6].

Fortunately, however, homeostatic proliferation is not a simple or uniform entity, and whereas the recovering immune system described [6] is resistant to tolerance induction, it is quite possible that immune cells recovering after depletion by another agent may very well be susceptible to tolerance induction. This assertion is supported by the fact that homeostatic proliferation is not identical in every situation of immune cell depletion. In fact, the phenotypic and functional characteristics of the recovering immune cells vary from model to model, and depend on variables such as the nature of peptide-MHC complexes (i.e. peptide that resides within the peptide binding groove) [7,8], cytokine profile [9–11], availability of space [12], and age of the host [13] among others. Thus, depending on the environment created by the depleting agent, some naïve T cells undergoing homeostatic proliferation may display a memory phenotype and function [14–22], some may undergo little or no phenotypic change at all [23,24], while others may display vigorous and clear signs of complete activation with increase in CD69 and/or CD25 [25–28]. Thus, it is

suggested that the basic immunology of homeostatic proliferation in each immune cell depletion protocol be studied in detail. Such studies will then result in sufficient understanding of the reconstituting immune system to allow timely manipulation of these homeostatically proliferating cells to induce donor-specific tolerance.

We have chosen to study the reconstituting immune system *in vivo* in transplant patients who received Campath-1H, and have also developed an *in vitro* model to study the effects of Campath-1H on T cells. We demonstrate here that T cells emerging after Campath-1H exposure demonstrate prolonged down modulation of surface CD4 and CD8 molecules *in vivo*. Additionally, our *in vitro* results demonstrate that after exposure to Campath-1H only, T cells down modulate their surface CD4 and CD8, are not activated, and remain immunocompetent.

Materials and methods

Human studies were reviewed by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in an appropriate version of the 2000 Declaration of Helsinki.

Immunosuppression in patients receiving Campath-1H

Patients receiving a renal transplant received 30 mg of Campath-1H (Berlex Inc.) (preceded by 500 mg of Solumedrol, 50 mg of Benadryl, and 650 mg of Tylenol) just before kidney implantation. Postoperatively, the patients received Cellcept 1 g twice a day, FK (Tacrolimus) to keep level around 10, and Prednisone 20 mg daily. Prednisone was weaned as tolerated (5–2.5 mg every month). Blood obtained (IRB approved) from kidney transplant recipients was immediately AC lysed (see below), and analyzed using fluorescence-activated cell sorting (FACS).

Determining the absolute number of peripheral blood mononuclear cells or lymphocytes

The cell suspension derived after ammonium chloride (AC) lysis, or Ficoll-Paque PLUS centrifugation, was mixed in 1 mL of medium. After diluting 1:4 with Trypan Blue, 10 μl was loaded onto a hemocytometer and viewed under a Micromaster Inverted Microscope (Fisher Scientific, Pittsburgh, PA, USA). The average of opposite quadrants was used in the following equation to determine cell number per milliliter – cell average × dilution factor × 10^4. Cells per milliliter were the number of peripheral blood mononuclear cells (PBMCs) or lymphocytes obtained from the original 1 mL of blood. The appropriate antibodies were then used to stain 0.5×10^6 cells in FACS tubes (Falcon, Franklin Lakes, NJ, USA).

Ammonium chloride lysis: isolation of peripheral blood mononuclear cells

10× AC lysing solution was prepared as follows: 40.1 g NH_4Cl, 4.2 g $NaHCO_3$, 1.85 g disodium EDTA, 450 ml of deionized water, pH 7.4. For lysis, 1 ml whole blood was added to 15 ml of 1× ACLS. The sample was incubated for 10 min at room temperature (RT) and centrifuged at 300 *g* for 5 min at 22 °C. Cells were washed twice in 10 ml of warm (37 °C) phosphate-buffered saline (PBS), resuspended in 1 ml of 10% fetal bovine serum in Isocove's Medium (10% FBS/IMDM), and counted using Trypan Blue (above).

Fluorescence-activated cell sorting (FACS)

All FACS staining was done at 4 °C. 5×10^5 PBMCs or lymphocytes were placed in FACS tubes (Becton Dickinson, Franklin Lakes, NJ, USA), washed twice with FACS solution (1% BSA/PBS), and stained for 40 min with different combinations of the following mouse anti-human antibodies (Caltag Laboratories, Burlingame, CA, USA): anti-CD4 APC-AlexaFluor 750, anti-CD8 PE-Cy 5.5, anti-CD69-PE, anti-CD56-FITC, and anti-CD25-APC. For detecting early apoptosis, anti-Annexin-V-FITC (Caltag Laboratories) was used. All incubations were done in the dark. Necessary single color and fluorescence minus one (FM1) controls were included. After staining, the cells were washed twice with FACS solution and resuspended in 300 μl of 3.7% formaldehyde and run on a BD LSRΠ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Flow cytometry analysis: determining the absolute number of specific T-cell populations

Percent cell survival, or percent change for any given cell population was determined by using absolute numbers of the given cell population. The absolute numbers were determined by multiplying the percent cells (FLOWJO software V4.6.2; Tree Star Inc., Ashland, OR, USA) with the total number of cells obtained from the original 1 ml of whole blood.

In vitro whole blood incubation with Campath-1H

Fresh blood from healthy human volunteers was used for *in vitro* experiments to simulate physiological conditions. The indicated concentration of Campath-1H (Berlex Inc, Montville, NJ, USA), was added to 1 ml of fresh blood,

and incubated in sterile 50 ml centrifuge tubes at 37 °C for the indicated amount of time. The blood was kept in motion continuously by a rocker to simulate blood flow. Control tubes were treated similarly, but with no Campath-1H. The blood sample was AC lysed and PBMCs isolated. Alternatively, lymphocytes were isolated using Ficoll-Paque PLUS centrifugation. Live cells were then counted using Trypan Blue and the absolute number of cells remaining determined. Experiments were done in triplicate unless otherwise indicated.

Ficoll gradient isolation of lymphocytes

Blood was diluted 1:1 with warm (37 °C) PBS. Three milliliters of Ficoll-Paque PLUS (Amersham Biosciences, Piscataway, NJ, USA) were added to 15-ml conical tubes. Four milliliter of the blood solution per tube was layered over the Ficoll-Paque Plus and the tubes centrifuged at 300 *g* (18 °C) for 40 min with slow deceleration. The upper plasma layer was then removed using a sterile Pasteur pipette, and a different sterile Pasteur pipette was used to remove the lymphocyte layer, which was then placed in 20 ml of warm PBS. Cells were spun at 400 *g* for 7 min at 18 °C, washed with warm PBS, and resuspended in 10% FBS/IMDM.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining

TUNEL kit (Roche Diagnostics, Indianapolis, IN, USA) was used. Daunomycin hydrochloride-treated cells (for positive control), untreated cells (negative control) and experimental cells were prepared by washing cells with cold PBS (4 °C) and spinning at 400 *g*. The cells were resuspended in 1% formaldehyde in PBS. After 15 min, cells were spun down and resuspended in 70% ethanol and mixed gently. After 15 min, cells were washed twice with cold PBS and 40 μl of TdT reaction mix was added. Ten microliter of Biotin-16-dUTP (B-UTP) reaction mix was added and the tubes incubated at 37 °C for 1 h. Cells were washed with cold (4 °C) PBS twice, and stained with Streptavidin-Tricolor (Roche Diagnostics).

T-cell activation assay

Whole blood from healthy human volunteers was incubated with 10 μg/ml of Campath-1H (Ilex Inc.) at 37° for 4 h. Control tubes were treated similarly but without Campath-1H. After 4 h, lymphocytes were isolated using density gradient centrifugation (see above). Following this, lymphocytes were resuspended in 10% FBS/IMDM and live cells counted (see above). After counting the cells, density was adjusted to 10^6 cells/ml. One milliliter of cell suspension was added to a 24-well tissue culture plate (in triplicate) in the absence or presence of 5 μg/ml soluble anti-human CD3 mAb (Ancell Corporation, Bayport, MN, USA), and incubated at 37 °C for 24 h. Cells were then washed with FACS solution (1% BSA/PBS) and stained with the following mouse anti-human antibodies (Caltag Laboratories): anti-CD4 APC-AlexaFluor 750, anti-CD8 PE-Cy 5.5, anti-CD25 APC, and anti-CD69 PE.

ELISA (enzyme-linked immunosorbent assay)

Purified mouse anti-human IL-2 (BD Pharmingen, San Diego, CA, USA) was coated onto a 96-well plate at 2 μg/ml in 1× coating buffer at 100 μl/well and incubated overnight at 4 °C. The plate was washed and blocked with 3% BSA (Sigma, St. Louis, MO, USA) in 1× coating buffer for 3 h at 300 μl/well at RT. The plate was incubated for 2 h at RT with standards and samples (100 μl each), washed, and biotinylated anti-human IL-2 (BD Pharmingen) introduced (1 μg/ml in 1× coating buffer at 100 μl/well) for 1 h at RT. After washing, streptavidin HRP (Roche Diagnostics) was applied at 1:20 000 in 1× coating buffer at 100 μl/well for 1 h at RT. After washing, 100 μl/well of substrate [1:1 Peroxide Solution and Peroxidase Substrate (Pierce, Rockford, IL, USA)] was added. The reaction was stopped with 100 μl/well of stop solution (2M H2SO4), and read on a universal microplate reader (Bio-Tek, Winooski, VT, USA) at 450 nm using the program KC junior (Bio-Tek).

Statistical analysis

GRAPHPAD PRISM V4.01 (GraphPad Software, San Diego, CA, USA) was used to construct graphs, calculate medians, and display SE bars. All experiments shown were done in triplicate unless otherwise stated.

Results

Campath-1H induces CD4 and CD8 down modulation *in vivo*

Three patients receiving Campath-1H as induction therapy for renal transplantation were studied. Blood was drawn before transplant (control) and at regular intervals thereafter as indicated in the figures. Blood was AC lysed, stained with indicated antibodies, and the lymphocytes analyzed using flow cytometry. The lymphocytes were identified using forward versus side scatter, and these were further narrowed down through $CD2^+$ (isolating T cells and NK cells) and $CD56^-$ gates (eliminating NK cells), thus ensuring that we were analyzing only CD4 and CD8-positive T cells. CD4 and CD8 T cell histograms were generated and the results shown in Fig. 1. Figure 1a and b are cells from

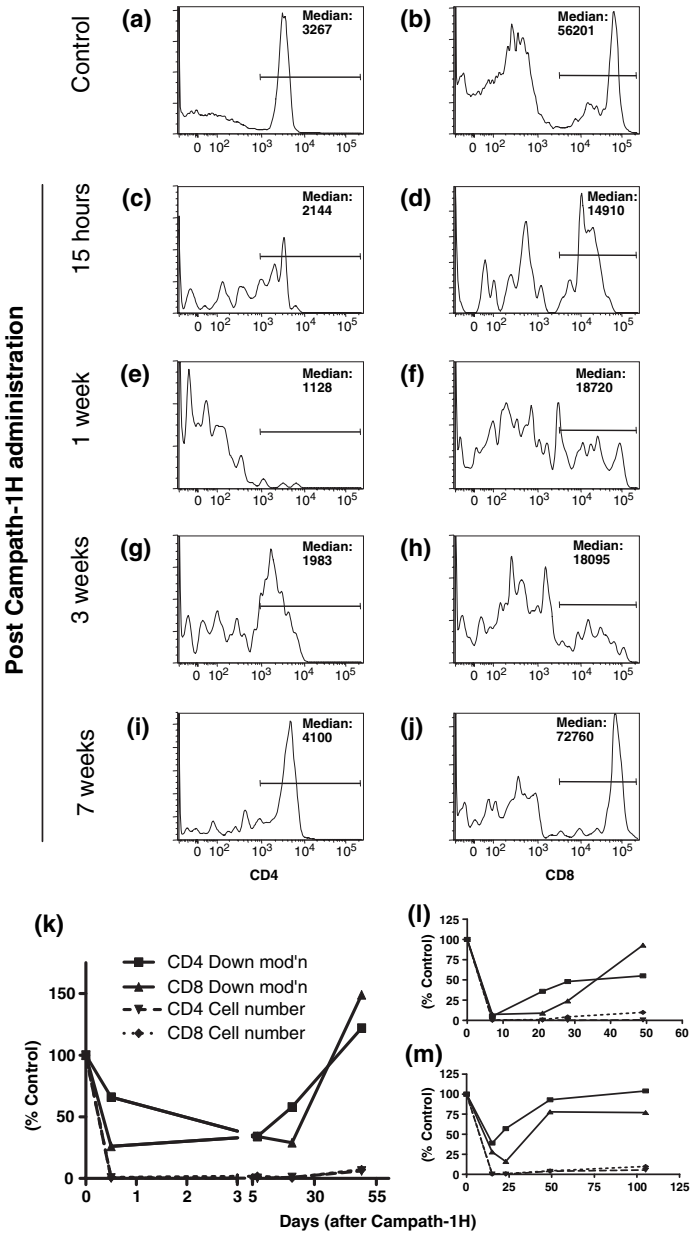

Figure 1 Campath-1H induces CD4 and CD8 down modulation *in vivo*. T cells from patients receiving 30 mg of Campath-1H as induction therapy for renal transplantation were analyzed at the indicated intervals for surface CD4 and CD8 expression. Control is the patient's T cells prior to transplantation. (a–j) Blood was drawn from one patient at the indicated time intervals, ammonium chloride (AC) lysed and lymphocytes filtered through $CD2^+$ and $CD56^-$ gates. Part figures a, c, e and g show that residual and recovering $CD4^+$ T cells have less CD4 expression than normal $CD4^+$ T cells. Part figures b, d, f and h show a similar down modulation for $CD8^+$ T cells. The MFIs for CD4 and CD8 are indicated within each histogram. (k) The indicated MFIs in a–j for CD4 and CD8 are graphed as percent control to demonstrate the degree of down modulation. In this patient, T cells recover normal surface expression of CD4 and CD8 about 40 days after Campath-1H administration. The absolute numbers of CD4 and CD8 T cells as percent control are also shown, and remain low. The absolute numbers per milliliter blood are (CD4) – 110,832 (control), 376 (15 h), 53 (7 days), 897 (21 days), and 6,659 (49 days), and (CD8) – 47,277 (control), 389 (15 h), 828 (7 days), 208 (21 days), and 3,529 (49 days). (l, m) T cells from two other patients were analyzed in a manner similar to that described in a–j, and the resulting down modulation of CD4 and CD8 shown as percent control. Recovery of normal surface expression occurs about 50 days after Campath-1H administration. Once again, the absolute numbers of CD4 and CD8 T cells, as percent control, are also shown and remain low. The absolute numbers per milliliter blood in l are (CD4) – 482,826 (control), 2,635 (16 days), 1,592 (23 days), 18,687 (54 days), 26,460 (118 days), and (CD8) – 113,303 (control), 489 (16 days), 996 (23 days), 4,902 (54 days), and 11,184 (118 days). In m (CD4) – 160,148 (control), 46 (10 days), 97 (27 days), 175 (34 days), 921 (50 days), and (CD8) – 82,630 (control), 232 (10 days), 796 (27 days), 3,265 (34 days), 1,203 (50 days).

the patient's blood prior to any Campath-1H treatment and thus serve as controls. Figure 1c and d show down modulation of surface CD4 and CD8, 15 h after Campath-1H administration. There are few T cells seen 1 week after Campath-1H administration, but those present demonstrate CD4/8 down modulation (Fig. 1e and f). At 3 weeks after transplant and Campath-1H administration (Fig. 1g and h), there is recovery of a significant number of CD4 and CD8 T cells. However, the CD4 and CD8 surface expression of these T cells remains down modulated. Nevertheless, in Fig. 1g and h, it may be possible to discern a small emerging population of CD4 and CD8 T cells, respectively, that have a normal amount of CD4 and CD8 surface expression. By 7 weeks after Campath-1H administration, most of the T cells have regained complete CD4 and CD8 surface expression (Fig. 1i and j). The degree of down modulation of CD4 and CD8 surface expression is plotted as a function of time after Campath-1H administration in Fig. 1k for this patient. Two other patients were similarly analyzed, and the data shown in Fig. 1l and m. These two patients also demonstrate that CD4 and CD8 are down modulated on recovering T cells. Additionally, Fig. 1l and m also confirm that it takes approximately 45–50 days for CD4 and CD8 expression to return to normal. The absolute number of CD4 and CD8 T cells, as percent control, are shown in Fig. 1k–m, and remain low after Campath-1H exposure. Thus, surface CD4 and CD8 molecules on CD4 and CD8 T cells that survive killing by, or are re-emerging after, Campath-1H administration are down modulated for up to 45–50 days after Campath-1H administration.

Campath-1H induces dose-dependent CD4 and CD8 down modulation *in vitro*

CD4 and CD8 down modulation could be a result of T-cell activation *in vivo*. So, we developed an *in vitro* model of T cells surviving Campath-1H treatment to determine if Campath-1H alone was able to down modulate surface CD4 and CD8. Fresh human blood was incubated with 0, 0.5, 2, and 10 μg/ml of Campath-1H for 4 h, AC lysed, and analyzed by multi-color flow cytometry. Incubating the blood with Campath-1H and then isolating the lymphocytes, rather than first isolating the lymphocytes and then exposing them to Campath-1H [29,30], is pivotal, as it faithfully mimics how immune cells are exposed to Campath-1H *in vivo*. After such exposure, there was significant depletion of CD4 and CD8 T cells (manuscript submitted), and thus only surviving T cells were analyzed as *in vivo*. Lymphocytes, identified by forward versus side scatter, were further gated through $CD2^+$ cells (T and NK cells) and $CD56^-$ cells (eliminating NK cells) to ascertain that only $CD4^+$ and $CD8^+$ T lymphocytes were being analyzed. Both CD4 and CD8 T-cell populations were then graphed as histograms at various concentrations of Campath-1H and shown in Fig. 2a. The exact median fluorescence intensities (MFI) of CD4 and CD8 expression are shown within each graph in Fig. 2a and plotted as percent control versus Campath-1H concentration in Fig. 2b. There is a dose-dependent down modulation of CD4 and CD8, where 10 μg/ml of Campath-1H results in about 50% down modulation of surface CD4 and CD8 molecules.

Residual lymphocytes after Campath-1H treatment are not apoptotic

In establishing the phenotypic characteristics of residual T-lymphocytes post-Campath-1H treatment *in vitro*, it is important to ascertain that these cells are not experiencing ongoing apoptosis, and that they are *bona fide* viable cells. Fresh human blood was subjected to high dose (20 μg/ml) of Campath-1H for 4 h and 8 h at 37 °C, AC lysed, and remaining T-lymphocytes analyzed in three ways. First, the number of viable T lymphocytes after 4 h of Campath-1H treatment was compared with the number of viable T lymphocytes after 8 h of Campath-1H treatment. If after 4 h of Campath-1H treatment there was still ongoing apoptosis, then the number of viable T cells should be less after 8 h of Campath-1H treatment. That, however, was not the case, and the number of viable T lymphocytes was identical in lymphocyte populations treated for 4 and 8 h with Campath-1H (data not shown). Second, the residual lymphocyte population after treatment with 4 h of Campath-1H was subjected to Annexin V staining, which also revealed no ongoing apoptosis (data not shown). Thirdly, and perhaps most rigorously, lymphocytes treated with 4 h of Campath-1H were subjected to TUNEL staining to detect any early apoptosis. These data are shown in Fig. 3, which reveals that there was no significant increase in TUNEL staining of T lymphocytes that survived 4 h of incubation with Campath-1H. Thus, lymphocytes that survive 4 h of incubation with Campath-1H are viable.

CD4 and CD8 down modulation is not an artifact of selective elimination of $CD4^{hi}$ cells

It is possible that the CD4 and CD8 down modulation seen in Fig. 2 is actually a reflection of selective elimination of $CD4^{hi}$ and $CD8^{hi}$ cells, thus increasing the *relative* number of $CD4^{lo}$ and $CD8^{lo}$ cells, and giving the illusion that there has been down modulation. To exclude this possibility, fresh human blood from healthy volunteers was incubated with 0, 0.2, 1.25, 5, and 10 μg/ml of Campath-1H and analyzed for the absolute number of cells in

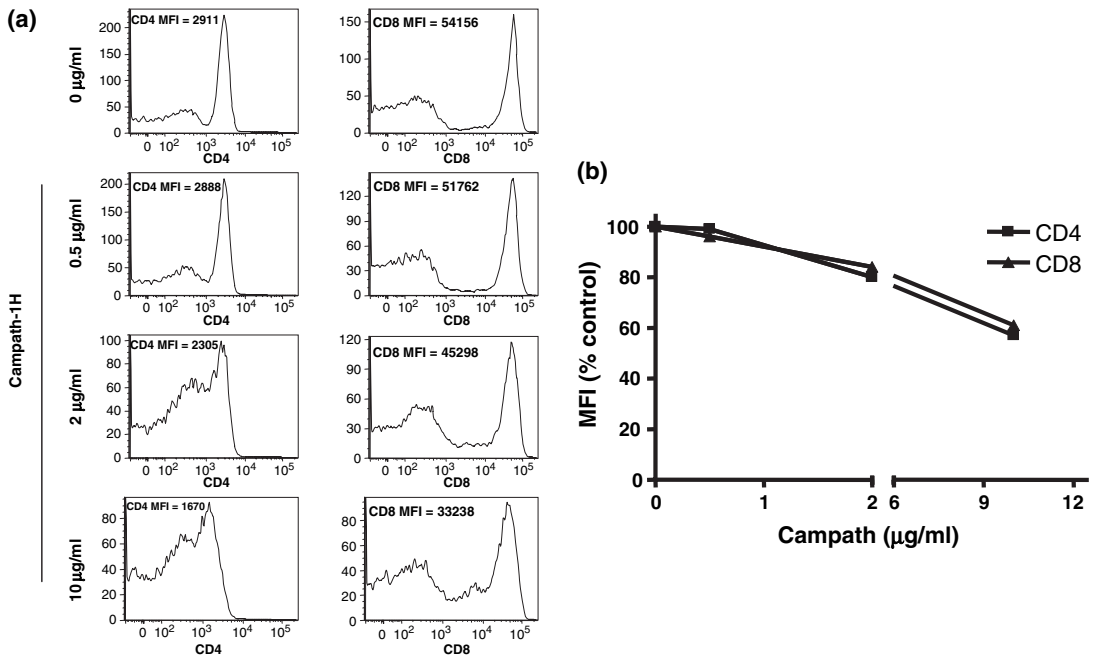

Figure 2 Campath-1H induces CD4 and CD8 down modulation *in vitro*. Whole blood from healthy volunteers was exposed to varying concentrations of Campath-1H and the residual T cells then analyzed for CD4 and CD8 down modulation in a manner similar to that described in Fig. 1. (a) The degree of down modulation of surface CD4 and CD8 is proportional to the dose of Campath-1H. The median MFIs for CD4 and CD8 are shown in the histograms. (b) The indicated MFIs are graphed as percent control versus dose of Campath-1H, and demonstrate that there is an equal degree of down modulation of surface CD4 and CD8 molecules.

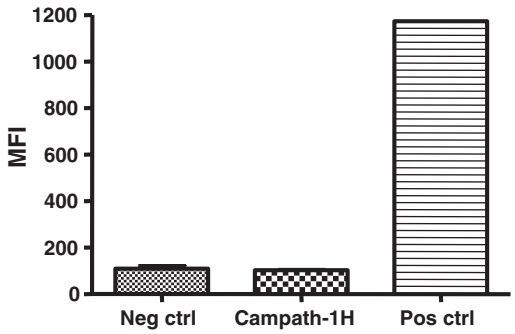

Figure 3 Residual T cells after Campath-1H treatment are not apoptotic. Whole blood from healthy volunteers was treated with 0 or 10 μg/ml of Campath-1H for 4 h, lymphocytes isolated using Ficoll gradient centrifugation, and a TUNEL assay performed. Campath-1H-treated lymphocytes did not demonstrate any TUNEL staining.

the area labeled A1 in Fig. 4a. If there is only selective elimination of CD4 high cells, then the segment labeled A1 would show no increase in the absolute number of CD4 T cells. If, however, there is *bona fide* CD4 down modulation, then the absolute number of T cells should increase in segment A1. A dot plot of CD4 versus CD25 was used to visually demonstrate the shift of CD4 T cells to the left, and this is shown in Fig. 4a. This figure once again clearly demonstrates down modulation of CD4 in a dose-dependent fashion. There are very few cells in segment A1 in the control (no Campath-1H), slightly more when treated with 0.2 μg/ml of Campath-1H, and increasingly more cells when treated with up to 20 μg/ml of Campath-1H. The changes in absolute cell numbers in segment A1 were then quantified and plotted as a function of Campath-1H concentration for CD4 T cells (Fig. 4b). There is a clear and substantial increase (eight- to ninefold) in the number of CD4 T cells seen in area A1 as Campath-1H concentration is increased. An analysis (not shown) similar to that done in Fig. 4a was also done for CD8 T cells and the absolute numbers and percent increase in the number of cells in an area identical to A1 were quantified, and the data shown in Fig. 4c. Even though there is an increase in the number of CD8 T cells in area A1, thus indicating down modulation of CD8, the increase is not as large as in CD4 T cells. Thus, CD4 and CD8 down modulation following treatment with Campath-1H is a *bona fide* down modulation.

Campath-1H by itself does not activate T cells

CD4 and CD8 down modulation could be a result of T-cell activation, and a simple explanation could be that Campath-1H was directly activating T cells *in vitro*. To determine this, fresh human whole blood was incubated with 0

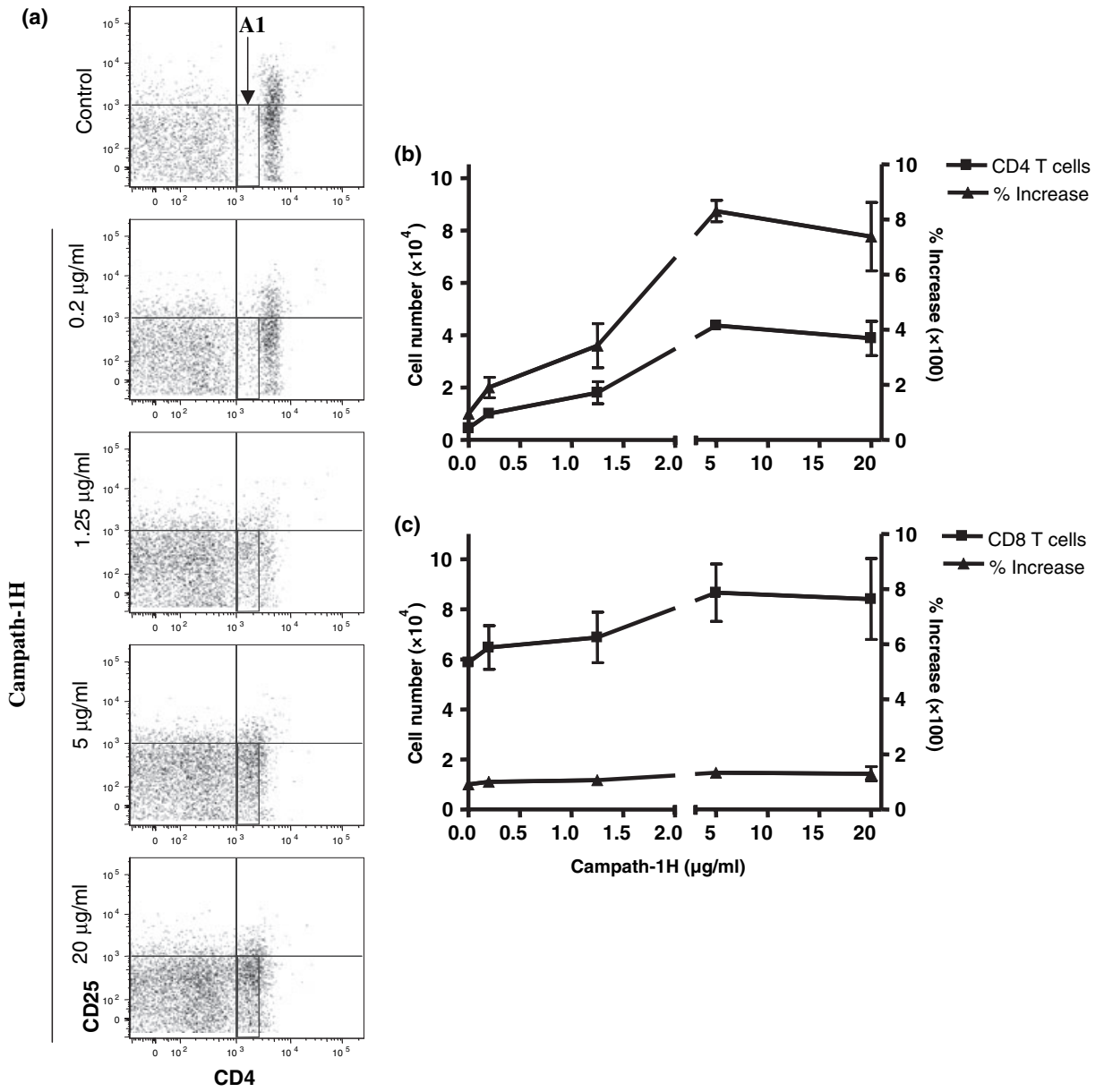

Figure 4 CD4 and CD8 down modulation *in vitro* is not a fluorescence-activated cell sorting artifact. Whole blood from healthy volunteers was exposed to increasing concentrations of Campath-1H, and lymphocytes isolated by Ficoll gradient centrifugation analyzed using flow cytometry. Lymphocytes were filtered through $CD2^+$ and $CD56^-$ gates, and then CD4 and CD8 histograms analyzed for CD4 and CD8 down-modulation. (a) An area, A1, was designated to measure the absolute number of cells and to quantify the down modulation. As the concentration of Campath-1H increases, there is an increase in the number of cells in A1. (b) The absolute number of cells in A1 are graphed on the left Y-axis and the percent increase in the number of cells is graphed on the right *y*-axis as a function of Campath-1H dose. There is an approximately eightfold increase in the number of $CD4^+$ T cells in A1 as the concentration of Campath-1H increases to 5 µg/ml and beyond. (c) An analysis (not shown) similar to that in A was done for $CD8^+$ T cells and the final results presented. There is also an increase in the number of $CD8^+$ T cells in A1, although less so than $CD4^+$ T cells.

(control) and 10 µg/ml (concentration known to induce maximal killing) of Campath-1H for 4 h. Lymphocytes were isolated using Ficoll gradient centrifugation and incubated with 0 (control) or 5 µg/ml of soluble anti-CD3, and harvested 24 h later. Using multi-color flow cytometry, CD69 histograms for CD4 and CD8 T cells were generated and the MFIs shown as bar graphs in Fig. 5a. CD4 and CD8 T cells that were exposed to Campath-1H only showed

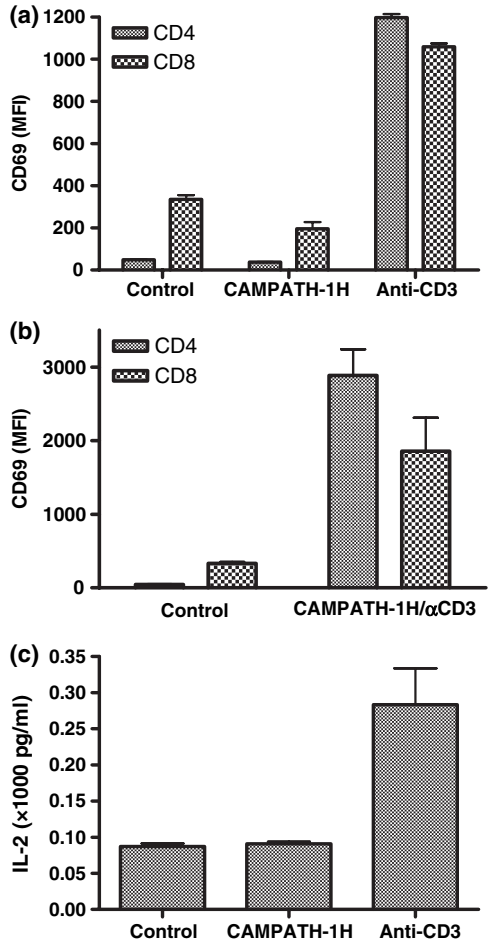

Figure 5 Campath-1H does not activate CD4 or CD8 T cells. (a) Whole blood from healthy volunteers was exposed to 10 μg/ml of Campath-1H (a dose known to elicit maximal killing) for 4 h and lymphocytes isolated by Ficoll gradient centrifugation. A negative control group (no Campath-1H) was exposed to no soluble anti-CD3 and the positive control group (no Campath-1H) was exposed to 5 μg/ml of soluble anti-CD3 for 24 h. CD4 and CD8 T cells were then analyzed by flow cytometry for CD69 expression as a measure of T-cell activation. Campath-1H does not by itself activate CD4 or CD8 T cells. (b) Whole blood was treated as in A, and Campath-1H-treated T cells were further stimulated by 5 μg/ml of soluble anti-CD3. Campath-1H exposure to T cells does not compromise their ability to activate as measured by surface expression of CD69. (c) Supernatants from lymphocytes isolated and treated as in A above were analyzed by ELISA for the production of interleukin-2 (IL-2). Campath-1H incubation with lymphocytes leads to no IL-2 production.

no increased expression of CD69, indicating that they were not activated. Cells treated with anti-CD3 only showed normal activation (positive control). T cells treated with Campath-1H and subsequently with soluble anti-CD3 mAb also showed activation (Fig. 5b), indicating that Campath-1H did not render the T cells immuno-incompetent. Additionally, there was no increase in IL-2 production when treated with Campath-1H alone (Fig. 5c). Thus, Campath-1H alone is not able to fully activate CD4 and CD8 T cells. Further evidence supporting this conclusion can be found in Fig. 4a where, despite being treated with high-dose Campath-1H (20 μg/mL), there is no increase in the number of $CD25^+$ T cells. Thus, T cells exposed to Campath-1H remain immunocompetent and are not activated (as measured by CD69, CD25 expression and IL-2 production, there may be some tyrosine phosphorylation, see Discussion).

Discussion

We have established here that CD4 and CD8 surface expression remains down modulated in surviving and emerging T cells for approximately 40 days after Campath-1H administration and that such down modulation can be induced by Campath-1H alone.

CD4 and CD8 down modulation *in vivo* could also be due to T-cell activation, which itself could be elicited by an antigen (infection, transplanted organ) or homeostatic proliferation [25–28]. In fact, our data (not shown) demonstrate that there is an increase in the percentage of $CD25^+$ T cells during homeostatic proliferation after Campath-1H administration. Thus, it would be difficult to determine how much of the CD4 and CD8 down modulation is attributable to Campath-1H, if at all, and how much to T-cell activation. These T cells, in conjunction with the rest of the immune system, do not appear to be functionally compromised as there is no increase in mortality and morbidity, especially infections, in patients treated with Campath-1H [31–33]. Whereas there is some suggestion that these T cells may be somewhat compromised [4], clinical studies showing no increase in infection [31–33], and our studies (Fig. 5, data not shown), and the literature [22] would suggest that these residual T cells are in fact immunocompetent. The one study that demonstrated decreased immunocompetence [4] probably did not see any T-cell division after anti-CD3 stimulation because the T cells were already activated *in vivo* and stimulation may have led to some activation-induced cell death. Thus, the preponderance of evidence currently would suggest that T cells remaining or T cells recovering after exposure to Campath-1H are immunocompetent.

Whereas these T cells may be immunocompetent *in vivo* after Campath-1H treatment, it is difficult to isolate the effects of Campath-1H alone as the phenotypic and functional characteristics of recovering T cells are influenced by the presence of homeostatic proliferation. Thus, we recreated the model *in vitro* and demonstrated that the same CD4 and CD8 down modulation can be seen after only 4 h of Campath-1H exposure. Additionally, and perhaps more importantly, these T cells *in vitro* did not display an activated phenotype as there was no

increase in the surface expression of CD25 (Fig. 4), CD69, or in the production of IL-2 (Fig. 5), as is the case *in vivo* [4]. Thus, the CD4 and CD8 down modulation observed is not a consequence of T-cell activation and there are two possible explanations. First, that ligation of CD52 activates a novel pathway that results in down modulation of CD4 and CD8 independent of T-cell activation. Alternatively, it is possible that ligation of CD52 creates a state of partial T-cell activation that results in CD4 and CD8 down modulation, but does not lead to full T-cell activation, as recognized by increased surface expression of CD25, CD69, and by increased production of IL-2. In fact, there is some evidence to suggest that T cells might be partially activated through CD52 ligation, as there is some tyrosine phosphorylation, but only if Campath-1H is cross-linked [29,34]. However, even after cross-linking, there was no IL-2 production (CD25 and CD69 expression was not studied), indicating incomplete activation at best. Our studies avoided the use of facilitating molecules such as cross-linking antibodies or Phorbol esters, and exposed T cells to Campath-1H only, and then only in human whole blood, so that the actual physiological and *in vivo* effect on T cells could be studied. In these conditions, there was no T-cell activation as measured by CD69 and CD25 expression and IL-2 production.

In conclusion, T cells down modulate surface expression of CD4 and CD8 up to 45 days post-Campath-1H administration *in vivo*, and Campath-1H by itself can induce CD4 and CD8 down modulation without fully activating T cells. The significance of such CD52-mediated signaling and down modulation is currently under study.

Acknowledgements

This study was funded by Department of Surgery, University of Vermont.

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