CD103 blockade reduces CD8 T cell accumulation during GVHD, but does not attenuate GVL effects

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<u>Introduction:</u>

Every four minutes someone in America is diagnosed with a blood cancer and The Leukemia & Lymphoma Society estimates that over 50,000 people die of a blood cancer each year[1]. Many blood cancers are caused by white blood cells that undergo uncontrolled cell division. This uncontrolled replication causes the effected cells to be non-functional. Furthermore, normal white blood cells are crowded out by the malignant cells, leaving blood cancer patients highly immunosuppressed. Current therapies for blood cancers include radiation therapy and more than 50 chemotherapeutic drugs[1]. However, many of these drugs are ineffectual in treating the disease; thus, alternative therapies are desired.

Bone marrow transplants represent a curative therapy for patients with blood cancers. Immediately preceding a bone marrow transplant, the recipient is treated with drugs and/or radiation to kill the cancerous cells, thereby destroying their immune system. The recipient is then infused with bone marrow cells from a genetically similar individual. Bone marrow cells are immune cell precursors, so the bone marrow transplant recipient receives a cancer-free donor immune system.

The limiting factor to the broad use of bone marrow transplants as a curative therapy for blood cancers is graft verses host disease (GVHD). GVHD is caused by a mature subset of white blood cells (T cells) present in the bone marrow graft that recognize and destroy host tissue,

particularly epithelial cells[2-4]. Transplanted T cells that migrate to various locations in the recipient mediate GVHD. Once transplanted, T cells follow specific trafficking patterns that are determined by several factors, including cues from sites of tissue damage caused by chemotherapy and/or radiation treatment. The intestinal tract is an area of naturally dividing cells; thus, along with rapidly dividing cancerous immune cells, the anti-cancer treatment also causes significant damage to gut epithelial cells. As a result of this damage, barriers that prevent commensal bacteria in the gut from being released are dissolved causing the release of the normal flora into circulation. To combat bacteria release, newly infused and residual host immune cells infiltrate the gut causing severe intestinal damage. Immune cells in the gut release soluble inflammatory mediators called cytokines that perpetuate the inflammatory response[5].

The two subsets of T cells that are responsible for the pathogenesis of GVHD are CD4+ T cells and CD8+ T cells. Upon entry into the gut, CD4+ T cells release cytokines which recruit other inflammatory cells[6-7]. In the pathogenesis of GVHD, the primary role of CD8+ T cells is to recognize foreign host cells and destroy them. Once in the gut, donor CD8+ T cells recognize the newly infiltrated host epithelial cells as foreign and destroy them[6-7]. Although both T cell subsets play a significant role in GVHD, it is believed that CD8+ T cells are the primary culprits of GVHD pathology. It stands to reason that mature T cells should be removed from the bone marrow inoculum. However, depletion of mature T cells prior to transplant is not a viable treatment strategy because mature T cells facilitate several beneficial effects that are essential to a successful transplant. These effects include controlling opportunistic infections and preventing cancer relapse.

Integrins are molecules that facilitate adhesion between cells. Integrins present on the surface of T cells help determine where T cells are going and how long they stay in a particular location after transplant. Integrin expression on the surface of T cells promotes their accumulation at sites of inflammation, particularly in the gut in the case of GVHD. Previous work in our lab has shown that the integrin CD103 is expressed on CD8+ T cells[8]. Our work has shown that CD103 expression on CD8+ T cells promotes the association of CD8+ T cells with epithelial cells[9]. The goal of our research is to find a way to separate the T cells that cause GVHD from those that facilitate the beneficial effects post transplant. We hypothesize that CD103 is required for the retention of CD8+ T cells in the epithelium of the gut during GVHD, but will not attenuate effective clearance of a blood malignancy.

To test our hypothesis, we used murine models of GVHD. Recipients were transplanted with donor bone marrow to restore immune function and donor splenocytes, a rich source of T cells, to cause GVHD. Following transplant, mice were monitored daily for clinical signs of GVHD. We found that CD8+ T cells selectively accumulate in the gut during GVHD, and optimal retention is dependent on CD103. We also found a correlation between intestinal damage and CD103 expression. Finally, we found that CD103 is not required for the effective clearance of a blood malignancy.

Research Methods:

GVHD induction:

All recipients of bone marrow transplants were irradiated with 900 Gy in 2 doses, 3 hours apart to reduce GI toxicity. Mice were irradiated in an RS2000 X-ray source. Mice were given

transplant 4-6 hours after the final radiation dose. C57Bl/6 (B6) transplant donors were sacrificed immediately before bone marrow extraction. Donor femurs and tibias were flushed with 10% FBS RPMI 1640 with media additives to remove bone marrow cells. Bone marrow cells were passed through 40 μm nylon mesh filter and centrifuged at 300 x g for 10 minutes. Cells were counted and prepared for T cell depletion with MACS Cell Separation kit (Miltenyi Biotec) according to manufacture's protocol. T cell depleted bone marrow cells (TCD BMCs) were stored in 10% FBS RPMI 1640 with media additives at 4° C until transplant. Immediately before transplant, T cell depleted bone marrow cells were washed three times in sterile PBS and resuspended at 50×10^6 cells per mL. The final injection volume was 100 μL (5×10^6 bone marrow cells). At the time of bone marrow extraction, spleens were removed and passed through 40 μm nylon mesh filter and centrifuged at 300 x g for 10 minutes. Cells were counted and either stored in 10% FBS RPMI 1640 with media additives at 4° C until time of transplant, or prepared for CD8+ T cell negative selection using MACS Cell Separation kit (Miltenyi Biotec). Immediately before transplant, whole splenocytes or purified CD8+ T cells were washed three times in sterile PBS and resuspended at appropriate volume of sterile PBS to inject desired number of cells in 100 μL. Bone marrow cells and splenocytes or CD8+ T cells were combined (total volume of 200 μL) and injected via tail vein 4-6 hours after final radiation dose.

GVHD scoring:

Mice were scored daily for clinical signs of GVHD based on the classical mouse GVHD scoring criteria. Briefly, mice are given a score of 0-2 on 5 criteria: percent weight loss, posture, activity, fur texture, and skin integrity. The scores of the 5 criteria are summed daily for a final daily score of $X/10^{10}$.

Flow cytometry:

Gut infiltrating lymphocytes were isolated by removing the small intestine and discarding the Peyer's Patches. Remaining intestinal pieces were flushed with PBS, cut longitudinally and into 1 cm sections. Small intestine pieces were incubated at 37˚C in IEL isolation solution (RPMI 1640 with 10% FBS and 0.15 mg DTE) for 20 minutes. The solution was underlayed with Lympholyte M, centrifuged at 1800 RPM for 25 minutes. The interface was collected and stained for flow cytometry. Cells used in flow cytometric analysis were resuspended at 10^6/mL in FACS staining buffer (5% FBS, 0.2% Sodium Azide in PBS). 100 μL was removed and stained with anti-CD3-APC, anti-CD8-PE, anti-CD44-PE-Cy5, anti-CD103-FITC, or isotype controls for 30 minutes at 4° C in the dark. Cells were washed three times in FACS staining buffer and fixed. Cells were analyzed using FACS Calibur (BD Biosciences) and analyzed with WinMDI 2.9 software.

Bioluminescent Imaging:

At the appropriate time post transplantation, recipient mice were anesthetized, injected with 4 mg of D-luciferin, and imaged using a Xenogen IVIS CCD camera (Caliper Life Sciences, San Francisco, CA). For imaging of the intestine and spleen, mice were given an additional 4 mg of D-luciferin, euthanized, and prepared for *ex vivo* imaging. Living image software was used to generate a pseudo-color image based on photon intensity (red = high intensity, violet = low intensity) and quantitate photon intensity.

Leukemia induction:

Transgenic mice that develop a human-like cancer (henceforth referred to as Eu-TCL1 mice) were euthanized and 10^7 splenocytes were adoptively transferred into Balb.SCID recipients. 10^7 wild type (WT) or CD103-/- Balb/C splenocytes were co-injected with Eu-TCL1 splenocytes. Recipients were bled weekly and flow cytometric analysis was done to detect the presence of CD5+CD19+ cells.

Results and Discussion:

Donor specific T cells accumulate in the gut during GVHD

To confirm that donor specific CD8+ T cells accumulate in the host gut during GVHD, we transplanted donor 2C+ B6 CD8+ T cells along with WT splenocytes and TCD BMCs. 2C+ B6 CD8+ T cells are genetically modified to only recognize a molecule expressed on the host cells. Our 2C CD8+ T cells also express luciferase. We transferred $2x10^6$ 2C.LUC+ CD8+ T cells and imaged cohorts of recipient mice on days 7, 14, and 21 post-transfer. To eliminate the confounding effects of CD4+ T cells and prolong survival, we depleted CD4+ T cells on days 2, 3, and 4 post-transfer. CD4+ T cell depleted recipients survive past day 25 post transplant, while untreated recipients succumb to lethal GVHD by day 10 post-transfer (Figure 1). On days 7, 14, and 21 post-transplant the majority of CD8+ T cell accumulation occurs in the gut (Figure 2A). The bioluminescent signal decreases dramatically from day 7 to day 14, indicating that the number of CD8+ T cells decreases during that time. However, the signal does not decrease significantly from day 14 to day 21, suggesting that CD8+ T cells are being retained in the gut (Figure 2B). It is to be expected that the bioluminescent signal drastically decreases from day 7 to day 14 as the acute infiltration of T cells subsides. However, the stabilization of CD8+ T cell

populations in the gut from day 14 to day 21, suggests that CD8+ T cells are actively

accumulating in the gut.

CD103 is upregulated concurrent with progressive intestinal damage

To investigate CD8+ T cell-mediated pathology over time, we euthanized a cohort of GVHD

recipient mice on day 7 or 22 post-transplant. We isolated and performed flow cytometric

analysis on immune cells infiltrating the gut. Eleven percent of CD8+ T cells expressed CD103

on day 7, and 53% of CD8+ T cells expressed CD103 on day 22 post-transplant (Figure 3A).

Concurrent with CD103 upregulation, we found increased intestinal pathology as compared to

naïve control tissue (Figure 3B). Taken together, our data suggest that CD103 promotes the

accumulation of CD8+ T cells, allowing progressive intestinal damage.

CD103 promotes optimal retention of CD8+ T cells in the gut during GVHD

To test the hypothesis that CD103 is required for retention of CD8+ T cells in the recipient gut

during GVHD, we adoptively transferred WT bone marrow cells along with WT splenocytes and

either WT or CD103-/- luciferase positive CD8+ T cells. Recipients were CD4+ T cell depleted

and imaged on day 9 following transplant. CD8+ T cells demonstrate optimal accumulation in

the host gut only when they express CD103 (Figure 4A). To ensure that this finding is due to a

deficiency in CD103 and not an overall reduction in CD8+ T cell numbers, we analyzed the

number of CD8+ T cells in the spleen of recipient mice. We found equivalent bioluminescent

signals in the spleens of both groups, suggesting there are equal numbers of CD8+ T cells

present (Figure 4B). These data suggest that if CD8+ T cells are unable to express CD103 they

will not accumulate in the gut as efficiently as CD8+ T cells that express functional CD103.

CD103 is not required for graft versus leukemia effects

If the primary aim of GVHD research is to identify ways to prevent GVHD, the secondary, but equally important, aim is to identify ways to maintain graft versus leukemia (GVL) effects. Therefore, it is important to confirm that blockade of CD103 will not impact the donor immune system's ability to fight off residual cancer cells. To test our hypothesis, we utilized a transgenic mouse that develops a form of cancer that is very similar to the human chronic lymphocytic leukemia (CLL). We transferred cancer cells into a mouse lacking an immune system to allow cancer cells to proliferate and invade the host lymphoid tissue. Mice receiving cancer cells alone died by day 80 post-transfer (Figure 5A). However, if WT splenocytes are co-injected to fight the transferred cancer cells, cancer-mediated mortality is completely prevented. Interestingly, if we co-inject splenocytes from a CD103-/- mouse, we also prevent cancer-mediated mortality (Figure 5A). Furthermore, on day 35 post-transfer, recipients of both WT and CD103-/- splenocytes had completely cancer-free peripheral blood as noted by the lack of CD5+CD19+ cells compared to recipients of cancer cells alone (Figure 5B). Taken together, our results demonstrate that CD103 is not required for GVL effects.

Implications:

We have shown that CD103 promotes the accumulation of GVHD-causing CD8+ T cells in the gut and is dispensable for GVL effects. Thus, we propose CD103 is an attractive therapeutic target, as it separates GVHD from GVL. Targeting CD103 in blood cancer patients receiving bone marrow transplants could not only spare patients the devastating effects of intestinal

GVHD, but also allow the maintenance of the beneficial, cancer-fighting and host defense

properties of T cells post-transplant.
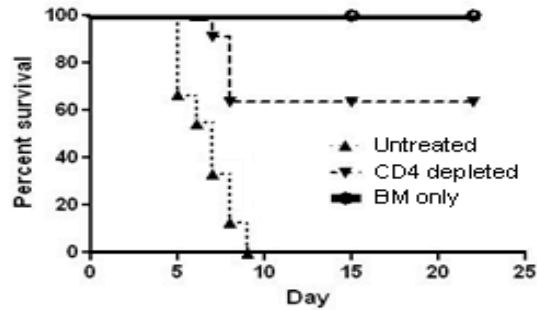
Figures:

Figure 1

Figure 1: Lethally irradiated Balb/C mice were adoptively transferred with either B6 TCD-BMC or TCD-BMC and splenocytes from WT B6 mice. Survival data is combined data from three separate experiments.
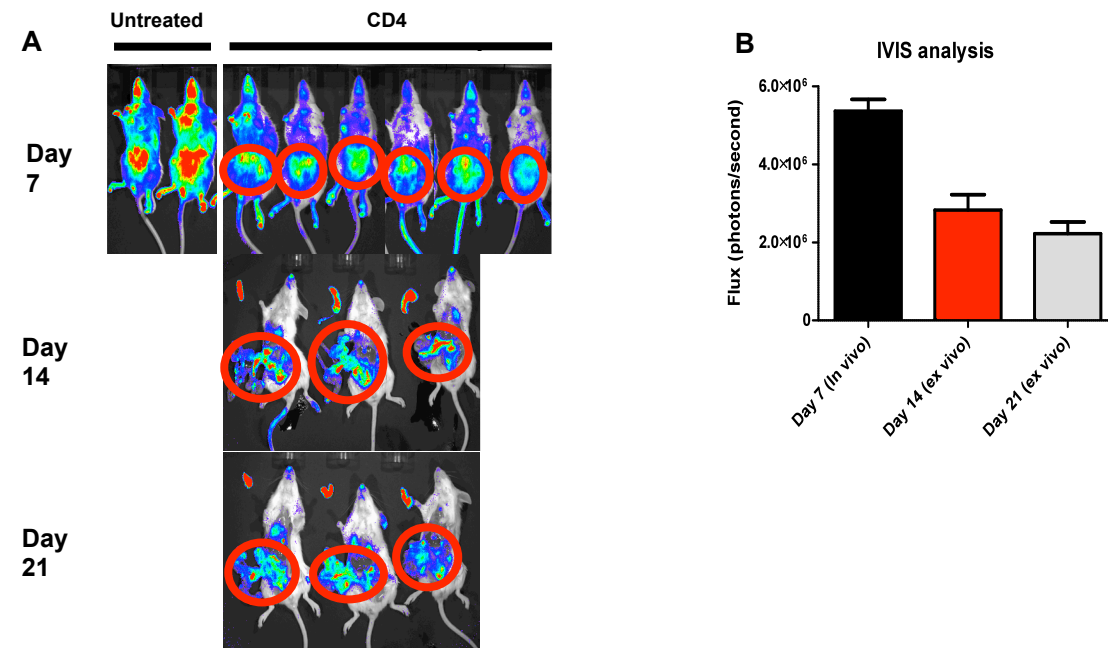
Figure 2

Figure 2: Donor-specific CD8+ T cells preferentially accumulate in the gut of recipients. 2C B6 CD8+ T cells were adoptively transferred along with WT splenocytes and TCD-BMC. A) *In vivo* and *ex vivo* bioluminescent images from days 7, 14, 21 post-transfer. B) Light

quantification in the gut of recipients at day 7 (*in vivo*), day 14 (*ex vivo*), and day 21 (*ex vivo*). P > 0.05 between day 14 and day 21.

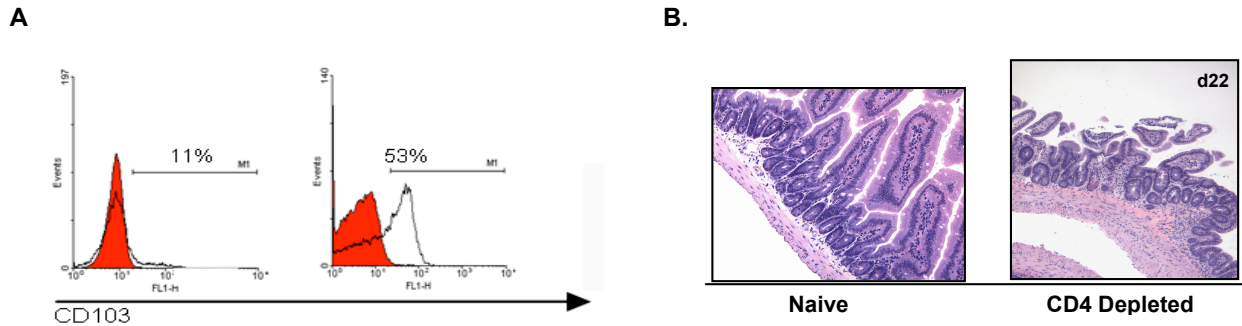**Figure 3**

A

B.

| Naive | CD4 Depleted |

Figure 3: CD103 upregulation on gut-infiltrating CD8+ T cells is concurrent with progressive intestinal damage. Lymphocytes were isolated from the gut of CD4+ T cell-depleted recipients on days 7 and 22. A) Flow cytometric analysis of gut-infiltrating lymphocytes. A representative histogram shows CD103 expression gated on CD8+ T cells. B) H&E-stained duodenal sections of naïve or CD4+ T cell-depleted recipients taken on day 22 post-transfer.
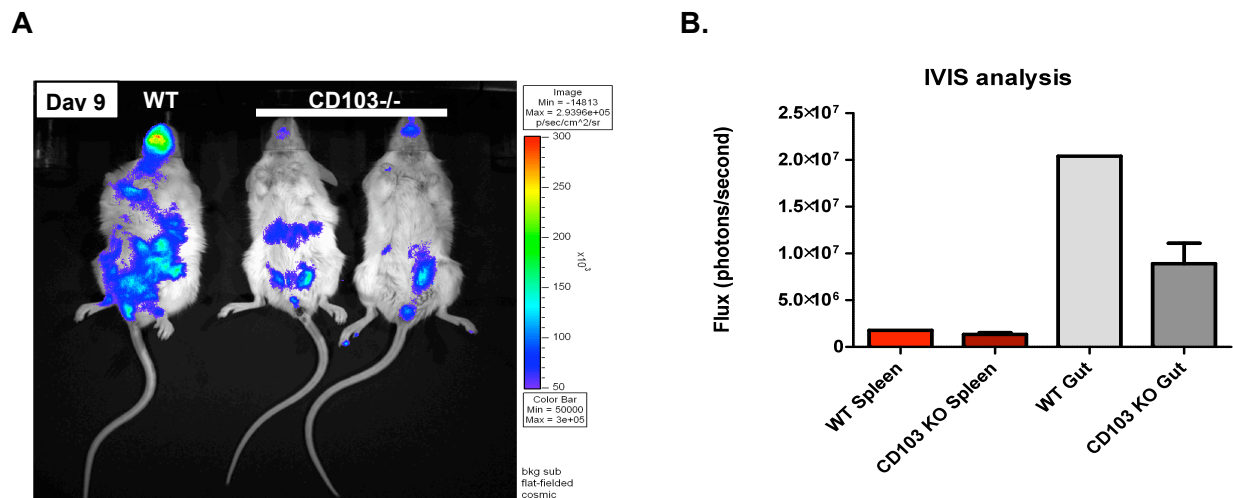
Figure 4

A

B.

Figure 4: CD103 is required for optimal accumulation of CD8+ T cells in the gut. Recipients were adoptively transferred with WT TCD-BMC and LUC+ or LUC+CD103-/- B6 splenocytes.

A) Bioluminescent representation of accumulation patterns of WT or CD103-/- CD8+ T cells on day 9 post-transfer. B) Quantification of CD8+ T cell accumulation in the gut and the spleen.

Figure 5

A

CLL (n=5)
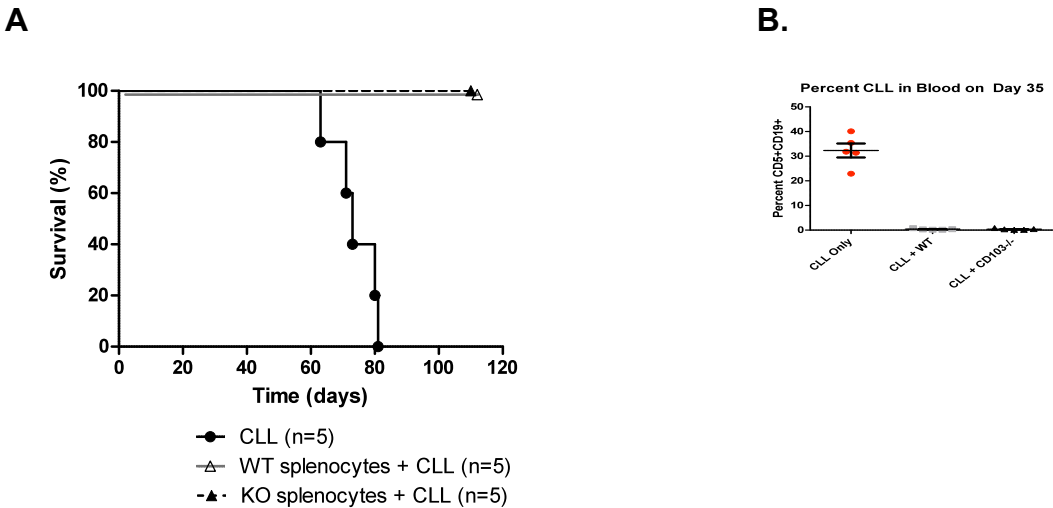WT splenocytes + CLL (n=5)
KO splenocytes + CLL (n=5)

B.

Figure 5: CD103 does not attenuate clearance of malignant B cells. Balb.SCID mice were adoptively transferred with Eu-TCL1 TCD splenocytes alone or with either WT or CD103-/- Balb/C splenocytes. A) Survival curves of recipients of Eu-TCL1 cells alone, Eu-TCL1 cells and WT splenocytes, or Eu-TCL1 cells and CD103-/- splenocytes. B) Flow cytometric analysis of CD5+CD19+ cells in each group on day 35 post-transfer.

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