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CHANGES IN MESENTERIC LYMPH NODE T CELL PHENOTYPE AND B AND T CELL HOMING PROPERTIES AFTER MURINE AIDS INFECTION

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ABSTRACT

We studied MLN (mesenteric lymph nodes) T cell phenotype and MLN T and B cells homing properties after murine AIDS (acquired immunodeficiency syndrome) infection. Our results showed an increase in the percentage of CD4$^+$ cells expressing CD44, CD54 and LPAM-1. There was also a decrease in the proportion of CD8$^+$ cells but an increase in the percentage of CD8$^+$ CD54$^+$ cells. An increased proportion of CD11b$^+$ (Mac1) cells suggested the recruitment of macrophages. Murine AIDS MLN cells labeled with ^{125}I-UDR migrated back to the MLN but did not preferentially migrate to the ILP (intestinal lamina propria). Simultaneous staining for BRDU and IgA confirmed the inability of murine AIDS MLN cells to home to the ILP. These data indicate that murine AIDS infection altered the mucosal immune system while modifying MLN T cell phenotype and MLN T and B cells migratory properties.

Murine AIDS (acquired immunodeficiency syndrome) is induced by LP-BM5 murine leukemia virus in susceptible strains of mice and resembles many aspects of human AIDS (1). Generalized lymphadenopathy and splenomegaly characterize diseased mice (2). Although LP-BM5 virus infected mice do not massively lose CD4 cells in most lymphoid organs, the remaining cells bear a different phenotype and become anergic (3,4). The final outcome is a generalized immunodeficiency with an increased susceptibility to pathogenic and opportunistic infections as in human AIDS (1,5).

In previous studies, we found that as immunodeficiency progresses mice become more susceptible to pathogens, like *Cryptosporidium parvum*, which have little impact on normal mice (6). These pathogens infect the gastrointestinal and respiratory tract of immunodeficient mice. Therefore, we decided to focus our attention on the mucosal immune system of virus infected mice (7,8). By studying the gut associated lymphoid tissues we found that while mesenteric lymph nodes (MLN) increase in size and cell number and thereby resemble other lymph nodes in a murine AIDS mouse, the opposite is true for the Peyer's patches and the intestinal lamina propria (ILP) (7). The decrease in B and T cells in the Peyer's patches that we first reported has been studied by others who found that it is associated with increased apoptosis through Fas-dependent and Fas-independent mechanisms (9).

IgA B cell precursors leaving the Peyer's patches reach the MLN through the lymphatics. In the MLN milieu, T cells secrete the cytokines that help in IgA B cell precursor maturation. Once maturation has taken place, IgA B cells can exit the MLN and seed the ILP (10-13). In our previous

work we found that although the relative percentage of IgA^+ cells in the MLN was decreased, the absolute number of cells was increased due to retrovirus induced B cell proliferation. Nevertheless, we found a decrease in the number of IgA and CD4 cells in the ILP (7). We surmised that the decrease in IgA plasma cells in murine AIDS ILP was due either to inadequate maturation of IgA precursors in the MLN or to lack of CD4 cells secreting cytokines in the ILP. Accordingly, we continued our study on the murine AIDS MLN cells, and in this paper we address the expression of T cell markers and homing receptors by MLN T cells and on the alteration of MLN cells homing properties.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice 4 weeks old were obtained from the National Cancer Institute (Frederick, MD). They were kept in the Animal Facility at the Arizona Health Sciences Center for 1 week and then were infected with LP-BM5 MuLV and sacrificed 16 weeks post-infection. Only mice showing similar level of infection–generalized lymph node enlargement—were studied. Mice were cared for in accordance with the University of Arizona College of Medicine Committee on Animal Research.

LP-BM5 murine leukemia virus infection

Five week old mice were injected intraperitoneally with 0.1 ml of an LP-BM5 MuLV inoculum, which had an ecotropic titer of 4.5 $\log_{10}$ PFU/ml, inducing the disease with a time course as previously described (14).

Lymphocyte subpopulations

Mesenteric lymph nodes were collected after mouse sacrifice using ether euthanasia. Briefly, mononuclear cells were obtained by gently pressing the tissues through a stainless steel wire mesh in RPMI 1640 medium containing 10% FBS and antibiotics. The cell suspension was washed twice with cold medium, viability was determined by trypan blue exclusion, and the cell suspension was adjusted to between 1 and 2 x 10^6 cells/0.1 ml/tube for lymphocyte surface marker determinations.

The following directly conjugated rat anti-mouse monoclonal antibodies used were: FITC-Thy1.2 (CD90), PE-CD4, PE-CD8 (PharMingen, San Diego, CA). The purified monoclonal rat anti-mouse antibodies used were: CD44, LPAM-1 (integrin $\alpha4\beta7$ complex), LECAM-1 (CD62L), CD45RB, CD45RC (PharMingen), and Mac 1 (CD11b) (Boehringer, Indianapolis, IN) and the FITC conjugated goat anti-rat was used as secondary antibody (Southern Biotechnology Associates, Birmingham, AL). Monoclonal antibodies anti-mouse CD3, ICAM-1 (CD54), and $\alpha\beta$-TCR were raised in hamster (PharMingen) and an FITC goat anti-hamster (Caltag, Burlingame, CA) was used as secondary antibody. Samples were incubated with the appropriate dilution of the primary purified monoclonal antibody for 30' in an ice bath, washed with 1.5 ml of PBS, and spun down. Secondary conjugated antibody was added in the appropriate dilution, and samples were incubated for 30 minutes in an ice bath in the dark. Cell suspensions were washed again and the directly conjugated antibody was added. After the incubation and washing procedure, the samples were resuspended in 0.5 ml of 2% paraformaldehyde (pH 7.4) and stored at 4°C, in the darkness till they were analyzed. Samples were run in a FacStar flow cytometer (Becton Dickinson, Mountain View, CA) with the Consort 40 program, and analyzed using either Consort 40 or Reproman (True Facts Software, Seattle, WA). In order to draw Fig. 1 we transferred Consort 40 files through the network, converted them into Cellquest files and analyzed them using Cellquest (Becton Dickinson). FL1 and FL2 labels were replaced using Microsoft PowerPoint.

Transfer of ^{125}Iododeoxyuridine (^{125}I-UDR) labeled MLN cells

Cells were labeled with ^{125}I-UDR following a procedure described by others (10). Briefly, MLN cells synthesizing DNA were labeled by incubating 10^7cells/ml in RPMI 1640 supplemented with HEPES and 10% fetal calf serum at 37°C for 1 hour in the presence of 2μCi/ml of ^{125}I-UDR (ICN, Costa Mesa, CA). Labeled cells were washed three times and passed through nylon wool to remove clumps before transfer. Ten million cells were transferred intravenously into each recipient mouse. ^{125}I-UDR incorporation was higher in MLN cells from murine AIDS mice than in normal mice (49249 cpm vs. 3846 cpm). Recipient mice were sacrificed 24 hours after transfer. Multiple organs and feces were collected and ^{125}I radioactivity was measured in a gamma counter (LKB-Wallac clinic Gamma 1272, Baltimore, MD). Data were expressed as percentage of radioactivity injected per organ (recovery of radioactivity = cpm in the organ / cpm injected x 100).

Transfer of Bromodeoxyuridine (BRDU) labeled MLN cells

Cells were labeled with BRDU (Sigma, Saint Louis, MO) in a similar fashion as described for ^{125}I-UDR. BRDU and were used at a final concentration of 50μM. Labeled cells were washed and passed through nylon wool before transferring them. Recipient mice were killed 24 hours after transfer and their small intestines were frozen in liquid nitrogen and stored at –70°C until processed.

Tissue section staining

Cryostat sections were prepared from frozen intestines and kept at –70°C until used. Cryostat sections were fixed in cold acetone for 10 minutes and air dried. Afterwards, tissue sections were first rehydrated in PBS and then, slides were transferred to a humid chamber to proceed with the staining for IgA using goat anti-mouse IgA (Cappel, division of Organon Teknika, Durham, NC) and Texas Red rabbit anti-goat (Jackson ImmunoResearch, West Grove, PA). Then slides were fixed in 70% ethanol for 30 minutes at room temperature and air dried. Afterwards, slides were soaked in 0.07N NaOH for 2 minutes at room temperature, before passing them to 1xPBS to facilitate rehydration. Slides were then transferred into a humid chamber and a rat anti-BRDU antibody (SeraLab, distributed by Accurate Chemicals, Westbury, NY) was added followed by FITC goat anti-rat (Southern Biotechnology Associates). Slides were then mounted with glycerol:PBS (9:1) and read using an Olympus (Melville, NY) fluorescence microscope.

Statistical Analysis

Data were presented as mean ± SE. Flow cytometry data were analyzed by using the two-tailed Student's *t* test. ^{125}I-labeled and BRDU-labeled cell transfer experiments were analyzed using ANOVA followed by Student's *t* test, included in the Excel software package.

RESULTS

T cells in the MLN

MLN cells were isolated and stained with monoclonal antibodies to study the changes in cell surface phenotype. The results shown in *Fig. 1 to 4* were obtained by pooling together data from two independent experiments for each surface marker, except for CD11b, CD54, CD45RB and CD45RC that were analyzed only once. All surface markers shown were not simultaneously analyzed. The number of mice studied for each surface marker appears in the text below in brackets as normal versus murine AIDS. In *Fig. 1*, we show typical light scatter plots for normal and murine AIDS MLN cells. The

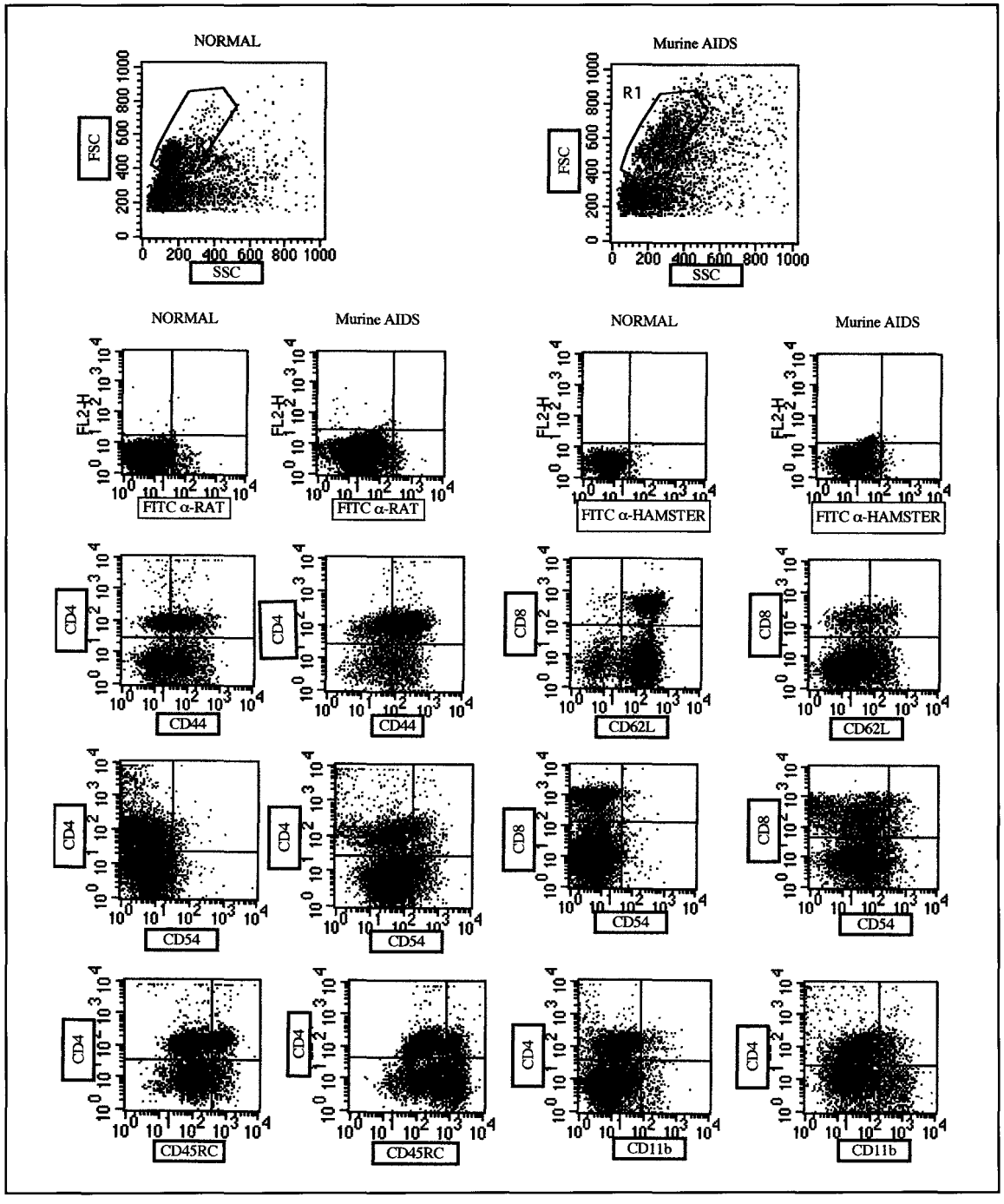

Fig. 1. Flow cytometry analysis of MLN cells. Light scatter plots showed normal and murine AIDS MLN cells. R1 was drawn to incorporate small and large cells. Anti CD4 and anti CD8 antibodies were directly conjugated. An indirect assay was used for the other surface markers. The typical secondary antibody shift to higher values is shown. The position of the cursor was uniformly always set up to consider the staining with the secondary antibody alone, and adjusted for each individual mouse. A typical plot with gated cells is shown for several surface markers that were altered after murine AIDS infection.

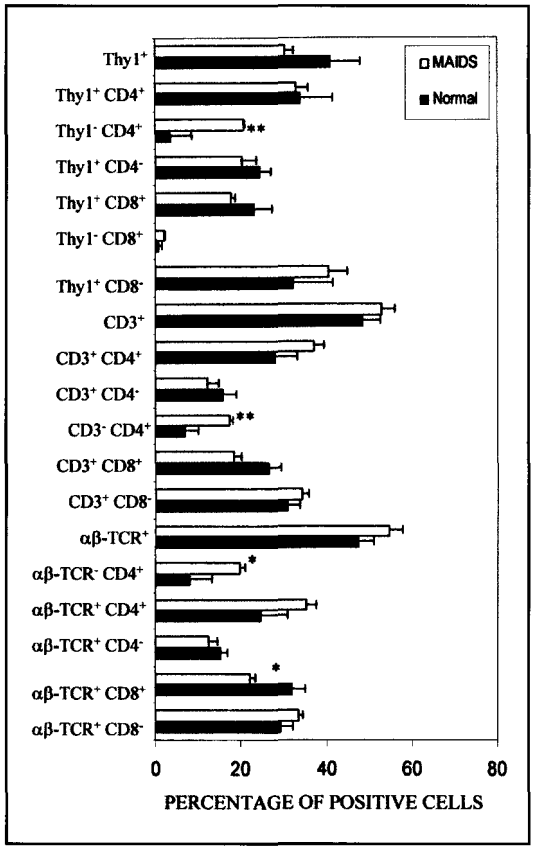

*Fig. 2. Expression of Thy1, CD3 and αβ-TCR associated with CD4 and CD8 by normal and virus infected MLN cells. MLN cells from normal and murine AIDS mice were isolated and stained and analyzed as described in Materials and Methods. Data are presented as mean ± SE, *p<0.05; **p<0.002.*

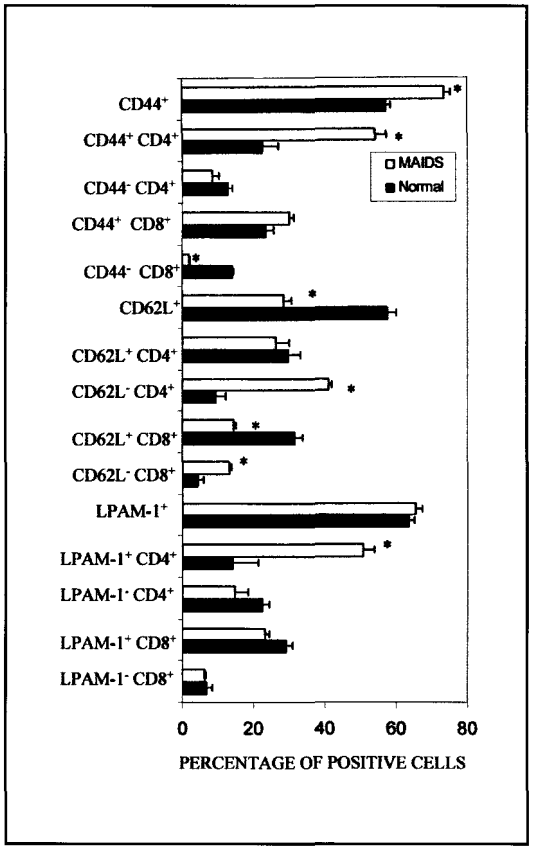

*Fig. 3. Association of CD4 and CD8 with CD44, CD62L and LPAM-1 on normal and virus infected MLN cells. MLN cells from normal and murine AIDS mice were isolated and stained and analyzed as described in Materials and Methods. Data are presented as mean ± SE, *p<0.0001.*

R1 region was drawn to exclude dead cells. We set up a big R1 region including all living cells in murine AIDS infected mice taking into account the increase in forward scatter. As previously described these mice presented cells with increased forward scatter values (4). Murine AIDS mouse cells showed a shift in fluorescence staining toward higher values when only the conjugated secondary antibody was added. This shift was taken into account when setting up the quadrant position for each individual mouse. Murine AIDS infection favored the appearance of a Thy1$^-$ CD4$^+$ (7 vs. 7; p<0.002) population in the MLN as shown previously in the spleen *(Fig. 2)* (11). There was also an increase in the percentage of CD3$^-$ CD4$^+$ and αβ-TCR$^-$ CD4$^+$ cells (7 vs. 4; p<0.002, 9 vs. 5; p<0.05). We found an increase in the percentage of CD11b$^+$ CD4$^+$ cells (3 vs. 5; p<0.0005), suggesting that some CD4 expressing cells could be macrophages *(Fig. 4)*. Moreover, we found an increase in the percentage of CD11b$^+$ CD4$^-$ cells (p<0.05), confirming the increase in the macrophage population in murine AIDS MLN. There was a slight decrease in the percentage of αβ-TCR$^+$ CD8$^+$ cells (8 vs. 9; p<0.05) in the MLN *(Fig. 2)*.

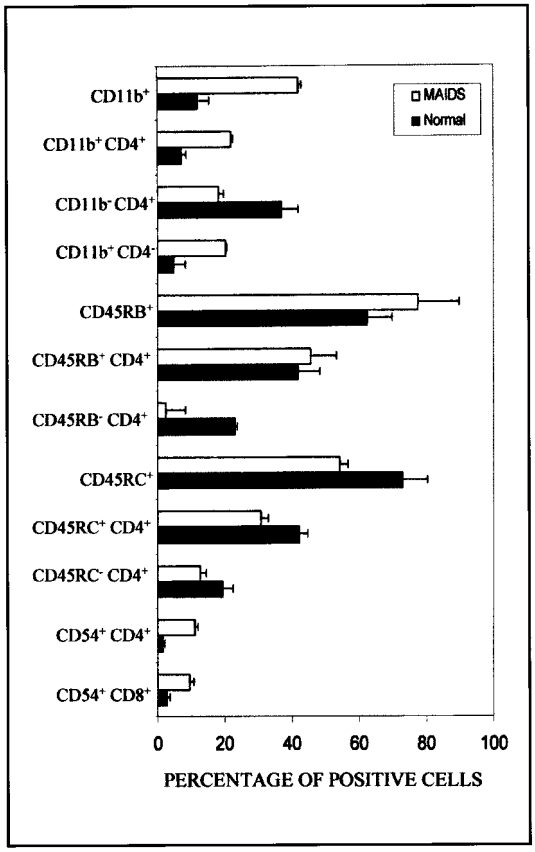

*Fig. 4. Association of CD4 and CD8 with CD45RB, CD45RC, CD54 and CD11b on normal and virus infected MLN cells. MLN cells from normal and murine AIDS mice were isolated and stained and analyzed as described in Materials and Methods. Data are presented as mean ± SE, *p<0.05, **p<0.005, ***p<0.0005.*

The total percentage of cells expressing CD44 (9 vs. 9; p<0.0001) was also increased in murine AIDS mice MLN *(Fig. 3)*. This increase may relate to an increase in the percentage of $CD4^+$ $CD44^+$ cells (9 vs. 7; p<0.0001). On the other hand, there was a decrease in the percentage of cells expressing CD62L (9 vs. 9; p<0.0001) a homing receptor associated with migration to Peyer's patches and peripheral lymphoid organs (15,16). Interestingly, the data showed a highly significant decrease in the percentage of $CD62L^+$ $CD8^+$ cells (9 vs. 9; p<0.0001) in the MLN, suggesting that this decrease could be the population responsible for the decrease in the total percentage of CD62L cells *(Fig. 3)*. There were no changes in the proportion of LPAM-1^+ cells (9 vs. 9). Nevertheless, there was an increase in the percentage of LPAM-1^+ $CD4^+$ cells (9 vs. 6; p<0.0001) *(Fig. 3)*.

There was also an increase in the percentage of CD4 and CD8 cells expressing CD54 ($CD4^+$ $CD54^+$: 4 vs. 4; p<0.0005, $CD8^+$ $CD54^+$: 3 vs. 3; p<0.05) *(Fig. 4)*. There was no significant change in the percentage of cells expressing CD45RB or CD45RC (3 vs. 5 for both markers). Nonetheless, there was a decrease in the percentage of $CD4^+$ $CD45RC^+$ cells (3 vs. 5; p<0.05) *(Fig. 4)*. Although the percentage of $CD4^+$ $CD45RB^+$ cells (3 vs. 5) was not modified by murine AIDS infection, the percentage of $CD4^+$ $CD45RB^-$ cells (3 vs. 5; p<0.005) decreased significantly.

MLN T and B cell homing properties

Studies describing MLN cell migratory patterns were performed in the early 1970s (10,17), which formed the bases for this investigation. In our studies we took cells either from normal or murine AIDS infected mice, labeled them with ^{125}I-UDR, and transferred them into normal or murine AIDS infected recipients. These experiments were performed three times. Data from one representative experiment were shown. ^{125}I-UDR incorporation per cell suspension basis was higher in MLN cells from murine AIDS mice than in normal mice (49249 cpm vs. 3846 cpm). This was due to an increase in the percentage of cells in S phase in murine AIDS mice. We have previously reported changes in the cell cycle in murine AIDS mice (8). In several experiments we determined that the percentage of cells in S phase in murine AIDS MLN was in the range of 3.7 to 12.7, whereas in normal mice, it was from 0.56 to 1.18. We only presented findings from those organs where the ANOVA analysis showed statistical differences. Our data was in agreement with earlier studies

TABLE 1
Abnormal Migratory Pattern of Murine AIDS MLN Cells

Donor	Recipient	Number of Mice	Organ	Recovery of Radioactivity (%/Organ)
Normal	Normal	5	MLN	0.454±0.097
			L. Int.	0.443±0.059
			S. Int.	2.854±0.630
			Spleen	1.212±0.126
			PLN	0.214±0.167
Normal	Virus	4	MLN	1.440±0.321[a)]
			L. Int.	0.328±0.139
			S. Int.	1.700±0.584[a)]
			Spleen	1.027±0.381
			PLN	0.470±0.211
Virus	Normal	5	MLN	0.026±0.008[c)]
			L. Int.	0.214±0.010[b)]
			S. Int.	1.630±0.132[a)]
			Spleen	0.523±0.047[c)]
			SPN	0.024±0.003
Virus	Virus	5	MLN	0.778±0.324
			L. Int.	0.089±0.039[c)]
			S. Int.	0.381±0.121[b)]
			Spleen	0.753±0.293[a)]
			PLN	0.336±0.205

MLN cells from normal and virus infected mice were labeled with ^{125}I-UDR and transferred into normal or virus infected recipients. The experiment was repeated three times. Data from one representative experiment were shown. ^{125}I-UDR incorporation in $1x10^7$ cells was 3846cpm for normal and 49249 cpm for murine AIDS virus infected MLN cells. Several lymphoid organs were collected and radioactivity was measured as described in Materials and Methods. Only those organs showing an ANOVA $p<0.05$ were shown. Student's *t* test was further used to compare different donor-recipient combinations with the experimental control (normal into normal transfer). Data were presented as mean±SE, [a)] $p<0.05$, [b)] $p<0.002$, [c)] $p<0.0009$.

showing that MLN cells from normal mice preferentially home to the small intestine of normal recipients *(Table 1)*. This migratory pattern was not clearly defined as to whether normal MLN cells were transferred into murine AIDS mice or murine AIDS MLN cells were transferred into murine AIDS mice. A similar picture was observed when the migration into large intestine was studied *(Table 1)*. ^{125}I-UDR labeled all cells that synthesized DNA; therefore, it labeled T and B cells.

To further confirm that the paucity of IgA plasma cells in the ILP was due to inability of their precursors to migrate, we performed a single experiment where we labeled cells with BRDU and transferred them as explained before. In this experiment

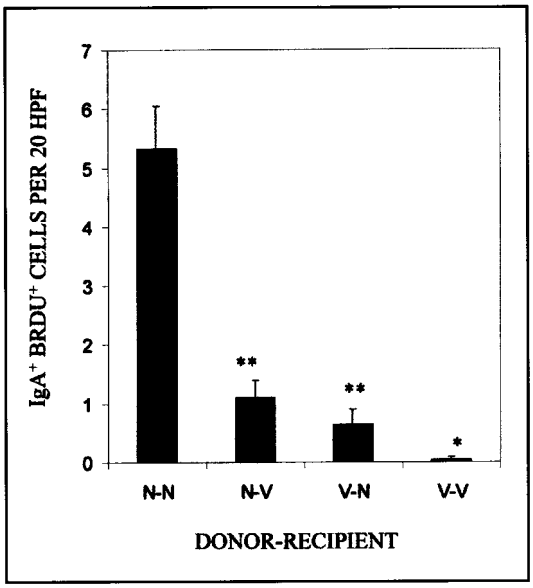

*Fig. 5. Abnormal migratory pattern of IgA B cell precursors in murine AIDS. Normal and murine AIDS MLN cells were labeled with BRDU and transferred into normal or murine AIDS recipients. Small intestines were collected, frozen and cryostat sections were prepared in order to study the presence of BRDU+ and IgA+ cells. ANOVA analysis showed a statistical difference at $p<0.05$. Experimental groups were compared with normal into normal migration using a Student's t test. Results were presented as mean number of double positive (IgA+ BRDU+) cells per 20 high power fields (100X objective) ± SE, *$p<0.04$, **$p<0.02$. N-N: MLN cells from normal mice transferred into normal mice (number of mice: 3). N-V: MLN cells from normal mice transferred into virus infected mice (2). V-N: MLN cells from virus infected mice transferred into normal mice (3). V-V: MLN cells from virus infected mice transferred into virus infected mice (2).*

we stained frozen small intestine sections with antibodies specific for IgA and BRDU. The data confirmed the experiments done using radioactivity, suggesting that IgA precursors from normal mice were unable to home to murine AIDS infected small intestine; murine AIDS IgA precursors also were unable to migrate into murine AIDS infected intestine *(Fig. 5)*.

As we studied the ability of ^{125}I-UDR labeled MLN cells to migrate back to MLN in the recipient mice, we observed the following:

Cells from normal mice preferentially migrated back to viral infected MLN *(Table 1)*, whereas viral infected cells were unable to migrate into normal mice MLN, but were able to migrate back to viral infected mice MLN.

DISCUSSION

Murine AIDS is a life-threatening disease resembling many features of human AIDS. Opportunistic pathogens can easily enter the body through either the respiratory or the gastrointestinal mucosa. Therefore, understanding the changes induced by LP-BM5 virus infection at the mucosal sites can help us to understand the mechanisms used by opportunistic pathogens.

Our previous work demonstrated a significant decrease in the number of IgA plasma cells in the ILP of murine AIDS mice (7). We suspected that IgA plasma cell precursors in the MLN were not maturing, as in normal mice; therefore, they could not seed the ILP. We also considered the possibility that the lack of CD4 T cells in the murine AIDS ILP was impeding B cell retention. We opted to further extend the analysis of MLN T cell phenotypic characteristics. MLN have a critical role in IgA B cell differentiation (11). The differentiation step that IgA B cells undergo in the MLN has been shown to depend strictly on helper T cells (13).

In the present study, we found phenotypical changes in $CD4^+$ and $CD8^+$ T cells in the murine AIDS infected MLN. The percentage of $CD8^+$ $CD62L^+$ and of $\alpha\beta$-TCR^+ $CD8^+$ decreased; whereas the percentage of $CD8^+$ $CD54^+$ and of $CD8^+$ $CD62L^-$ cells increased. These data indicated that in murine AIDS MLN, not only a decrease in the proportion of $CD8^+$ cells occurred but also a change had taken place in their activation level. Activated cells that migrate into inflammatory sites express CD54 that mediates their adhesion and trans-endothelial migration (18). Others have demonstrated that an increased expression of CD54 on CD4 and B cells in murine AIDS is a requirement

for successful virus infection. Virus infected mice treated with antibodies, anti-CD54 and anti-CD11a did not develop murine AIDS while the treatment lasted (19). All these results suggested environmental changes in the MLN after murine AIDS infection. Moreover, an increase in CD54 expression may be associated with the presence of inflammatory cytokines, like IFN-γ released in the virus modified MLN milieu (20). In a previous study, culturing MLN whole cell population promoted an increase in the secretion of IFN-γ, IL-5 and IL-6 in murine AIDS mice (21).

Among the phenotypic changes found in virus infected mouse MLN T cells, there was an increase in the percentage of Thy1$^-$ CD4$^+$ cells as previously described for spleen and peripheral lymph nodes (22,23). The function of this unique subset is presumed infiltration of the lymphoid follicles that trigger their destruction (24). In the MLN, an increased percentage of CD3$^-$ CD4$^+$ and $\alpha\beta$-TCR$^-$ CD4$^+$ cells, suggest that at least some of these CD4$^+$ cells are CD11b$^+$ cells, since macrophages can bear CD4 and, in fact, there was an increase in the percentage of CD4$^+$ CD11b$^+$ cells, and an increase in the overall macrophage subpopulation in virus infected MLN. Taken together these results suggested an important change in the phenotypic eukarotic profile because normal MLN harbor only low numbers of macrophages. The increase in the percentage of CD4$^+$ CD44$^+$ and CD4$^+$ LPAM-1$^+$ and CD4$^+$ CD54$^+$ cells was profound, and the increase in the CD4$^+$ CD44$^+$ cell population agreed with findings in the spleen, where cells displayed a memory-activated phenotype (4).

In the migration studies using ^{125}I-UDR, MLN cells synthesizing DNA were labeled including T and B cells and macrophages. MLN cells from normal mice showed a greater tendency to migrate back to MLN in virus infected recipients, whereas MLN cells from virus infected mice were unable to preferentially home to virus infected small intestine. These data point to the importance of phenotypic changes in MLN cells after murine AIDS infection to interfere with their further retention. Moreover, murine AIDS MLN cells migrated back to virus infected MLN instead of homing to virus infected ILP *(Table 1)*. Migration studies using *in vivo* activated T cells have shown that these cells can recirculate through the entire body; however only after reentering the tissue of origin were they able to proliferate and bypass apoptotic signals (25). It seems reasonable, therefore, that once virus infected MLN cells were not retained in the ILP, they could readily reenter the MLN and still survive.

We found several changes in MLN CD4$^+$ T cell phenotype but did not detect similar changes in the expression of IgA and CD11b, CD11a, CD62L, CD44 and LPAM-1 surface markers (data not shown). By labeling MLN cells with BRDU and studying the simultaneous expression of BRDU and IgA in the recipient's ILP, we determined again that MLN IgA cells from virus infected donors were unable to repopulate the normal or the virus infected ILP, and that normal MLN IgA cells were not retained in the murine AIDS ILP. We cannot entirely rule out abnormalities in the virus infected MLN B cell population since there is an increased proportion of virus infected MLN cells in S phase, as previously reported (8).

In conclusion, murine AIDS infection alters the mucosal immune system by modifying MLN T cell phenotype and MLN T and B cell migratory patterns. In a similar way, human studies have shown that CD4$^+$ T lymphocyte numbers in blood decrease while lymphadenopathy develops (26-28). Studies using resting human lymphocytes in vitro exposed to HIV showed upregulation of lymph node-homing-receptors that, after transfer into SCID mice, facilitated lymphocyte migration into lymph nodes (29). Moreover, studies in SIV-infected macaques have shown increased homing to lymph nodes (30,31). Therefore, re-directing lymphocyte trafficking may be a common strategy used

by several retroviruses. On one side, this strategy might be useful to prevent the destruction of lymph-node-sequestered-retrovirus-infected CD4 cells by CD8 cytotoxic cells. On the other side, the lack of effector lymphoid cells at peripheral sites clearly favors opportunistic infections like *Cryptosporidium parvum*. These findings reinforce previous observations suggesting similarities between the mouse model and HIV infection in humans. Furthermore, they validate the use of an experimental model to understand how retroviruses manipulate the host immune system.

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