

**Abstract**

**Background:** The gut associated lymphoid tissue (GALT) is an early target of HIV infection and site of severe CD4+T cell depletion. Loss of CD4+T cell help negatively impacts anti-HIV CD8+T cell responses. In contrast to peripheral blood, initiation of highly active antiretroviral therapy (HAART) results in delayed and variable GALT CD4+T cell restoration. It is unknown whether a threshold level of GALT CD4+T cell help would be sufficient for inducing effective anti-HIV CD8+T cell responses that may be independent of complete GALT CD4+T cell recovery. We investigated the effect of variable GALT CD4+T cell restoration on the poly-functionality of anti-HIV CD8+T cell responses in the context of viral suppression and control of GALT immune activation in HIV infected patients on long-term HAART (>5 years).

**Methods:** We evaluated jejunal biopsies and peripheral blood of HIV infected patients on HAART (n=11), who had restored >50% or <50% CD4+T cells, comparable with uninfected controls (n=6), and HAART-naïve HIV infected patients (n=11). We evaluated the degree of CD4+T cell restoration in correlation to memory subsets (central and effector memory) and the ability of CD4+ and CD8+T cells to produce IL-2, IFN  $\gamma$ , TNF  $\alpha$ , and IL-17 in response to antigenic or mitogenic stimulation by multicolor flow cytometry. We also investigated viral burden and degree of immune activation and inflammation in GALT by immunohistochemistry and real-time RT-PCR.

**Results:** Our data demonstrate CD4+T cell restoration in GALT during long term HAART is variable. Inability to fully restore GALT CD4+T cells was attributed to incomplete suppression of GALT viral replication and maintained stability of proviral burden in GALT CD4+T cells despite long-term HAART. Increased CD4+T cell restoration correlated with accumulation and maintenance of central memory CD4+T cells, reduced immune activation and inflammation associated gene expression, and poly-functional HIV-specific CD8+T cell responses.

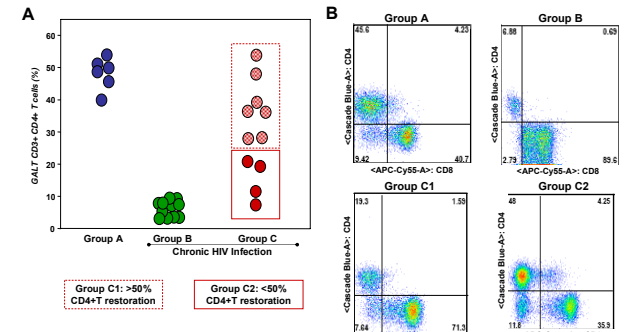
**Conclusions:** Our findings suggest >50% of GALT CD4+T cell restoration during long term HAART was sufficient to restore functional CD4+T cells and overcome CD8+T cell exhaustion, evidenced by enhanced poly-functional CD8+T cell responses. These were linked to suppression of viral replication and mucosal immune activation and were independent of stable proviral reservoirs. Our results suggest that partial restoration of GALT CD4+T cell help is sufficient for restoring mucosal immune functions.

**Sample Collection and Analysis:** Jejunal biopsies and PBMC were collected for multi-color flow cytometric analysis, immunohistochemistry and real-time PCR from 22 HIV-1 seropositive patients and 6 HIV-1 seronegative healthy patients (table 1). Long term HAART patients were divided based on whether they restored GALT CD4+T cells greater than or less than 50% as compared to ~45-52% CD4+T cells in GALT of HIV negative controls. Statistical comparisons between patient groups were performed using Kruskal-Wallis (equivalent to Mann-Whitney test). P values <0.05 considered significant. The Institutional Review Board at the University of California, Davis, CA approved the study and written informed consent was obtained from all participants in the study.

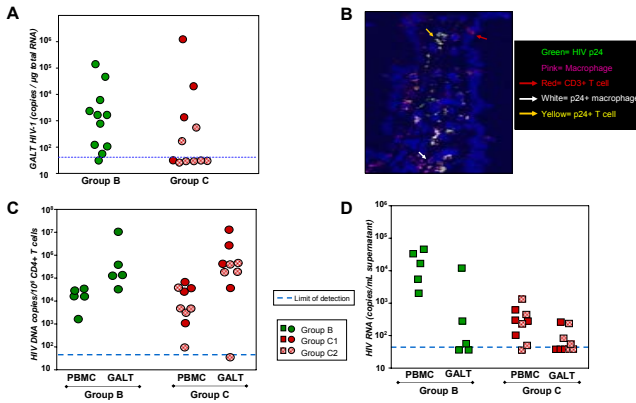
**Table 1. Patient Cohort**

| Patient Group                         | Patient ID | Gender | Age (yrs) | Time Diagnosed | Duration of HAART | HAART Regimen           | CD4+ T cells (mm <sup>3</sup> ) | Viral Load (RNA copies/mL) |
|---------------------------------------|------------|--------|-----------|----------------|-------------------|-------------------------|---------------------------------|----------------------------|
| HIV negative healthy controls         | 151        | M      | 44        | N/A            | N/A               | N/A                     | 899                             | N/A                        |
|                                       | 153        | F      | 50        | N/A            | N/A               | N/A                     | 406                             | N/A                        |
|                                       | 164        | F      | 42        | N/A            | N/A               | N/A                     | ND                              | N/A                        |
|                                       | 177        | M      | 39        | N/A            | N/A               | N/A                     | 789                             | N/A                        |
| Group A                               | 179        | M      | 21        | N/A            | N/A               | N/A                     | 601                             | N/A                        |
|                                       | 188        | M      | 28        | N/A            | N/A               | N/A                     | 520                             | N/A                        |
|                                       | 161        | F      | 32        | 10moa          | N/A               | N/A                     | 740                             | 18700                      |
|                                       | 162        | M      | 47        | 21yr           | N/A               | N/A                     | 266                             | 2410                       |
| Chronic HIV Infection HAART naïve     | 165        | M      | 51        | 2.5yr          | N/A               | N/A                     | 382                             | 89900                      |
|                                       | 154        | M      | 35        | >1yr           | N/A               | N/A                     | 581                             | 187000                     |
|                                       | 174        | M      | 37        | 6yr            | N/A               | N/A                     | 462                             | 8200                       |
|                                       | 175        | M      | 35        | 16yr           | N/A               | N/A                     | 537                             | 24400                      |
|                                       | 152        | M      | 47        | 17yr           | N/A               | N/A                     | 263                             | 14839                      |
|                                       | 156        | M      | 47        | 11yr           | N/A               | N/A                     | 193                             | 27023                      |
|                                       | 171        | F      | 43        | 11yr           | N/A               | N/A                     | 471                             | 5190                       |
| Group B                               | 170        | M      | 45        | >2yr           | N/A               | N/A                     | 250                             | 8920                       |
|                                       | 173        | M      | 29        | 4yr            | N/A               | N/A                     | 57                              | >750000                    |
|                                       | 120        | M      | 40        | >5yr           | 5yr               | episcion/norvir         | 463                             | <50                        |
| Chronic HIV Infection Long Term HAART | 155        | M      | 38        | 7yr            | 5yr               | norvir/episcion/flexiva | 558                             | <50                        |
|                                       | 167        | M      | 47        | >6yr           | 8yr               | viread/flexiva/norvir   | 434                             | 504                        |
|                                       | 178        | M      | 45        | 17yr           | >10yr             | norvir/truvada/reyataz  | 758                             | <50                        |
|                                       | 168        | M      | 55        | 21yr           | 14yr              | trizivir/sustiva        | 873                             | <50                        |
|                                       | 169        | M      | 51        | >11yr          | 10yr              | sustiva/truvada         | 384                             | <50                        |
|                                       | 168        | M      | 48        | 18yr           | 11yr              | norvir/viread/reyataz   | 1185                            | <50                        |
|                                       | 169        | M      | 55        | 20yr           | 8yr               | sustiva/viread/AZT      | 637                             | <50                        |
|                                       | 182        | M      | 27        | 7yr            | 5yr               | reyataz/truvada         | ++                              | <50                        |
|                                       | 189        | F      | 45        | >10yr          | 8yr               | combin/sustiva          | 867                             | <50                        |
|                                       | 184        | M      | 59        | 15yr           | 11yr              | episcion/reyataz        | 514                             | <50                        |

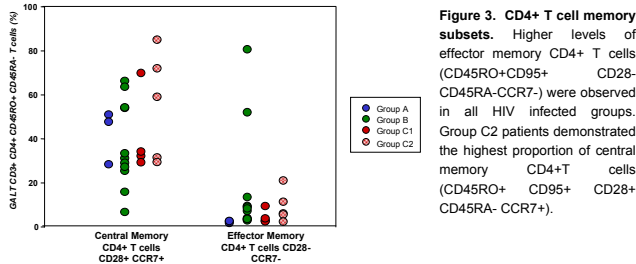
N/A= not applicable ND= none determined \*\* testing in process and results not yet available



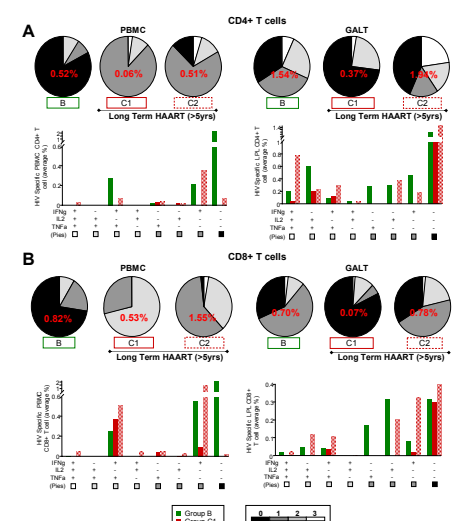
**Figure 1. Patient GALT CD4+ T cell restoration.** A. Group B patients exhibit severe depletion of CD4+T cells in GALT prior to HAART initiation. Group C patients exhibit varying levels of CD4+T cell restoration. B. Representative CD3+T cell gated flow cytometry dot plots of patients in each of the four groups.



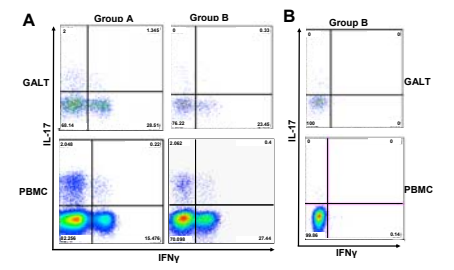
**Figure 2. Viral Burden in GALT.** A. Group C2 patients demonstrated the lowest HIV RNA levels. B. Both macrophages and CD4+ T cells are infected in GALT of patient with high viral loads. C. The level of proviral copies per million CD4+ T cells was similar in all patient groups, but the proviral burden was higher in GALT than in PBMC. D. Group B patients exhibited the highest level of HIV RNA released. Group C patients had reduced release ability of virions, indicating the HIV DNA identified in the CD4+ T cells is most likely not actively replicating virus.



**Figure 3. CD4+ T cell memory subsets.** Higher levels of effector memory CD4+ T cells (CD45RO+CD95+ CD28-CD45RA-CCR7-) were observed in all HIV infected groups. Group C2 patients demonstrated the highest proportion of central memory CD4+T cells (CD45RO+ CD95+ CD28+ CD45RA- CCR7-).



**Figure 4. HIV-Specific Responses.** Bar graphs indicate percentage of HIV-specific response based on different cytokine combinations. Pie charts depict qualitative distribution of HIV-specific T cells. Values in center of pies indicate total frequency of T cell HIV-specific responses. A. The degree of functionality in GALT CD4+T cells correlated with the level of CD4+ T cell restoration. GALT CD4+T cells in group C2 were primarily IL-2 or IFN $\gamma$  single or double producing B. Multi-functional GALT CD8+T cell responses were detectable in group C2, although GALT CD8+T cells in these patients were primarily IL-2 or IFN $\gamma$  single producing only. Media background values have been subtracted from data shown.



**Figure 5. CD4+ Th17 Populations in Chronic HIV Infection.** Lymphocytes were stimulated with PMA/ionomycin (A) or HIV gag peptides (B) for 6 hours in the presence of Brefeldin A. CD4+ Th17 cells were depleted during HIV infection.

**Table 2. Jejunal Inflammatory Gene RNA Levels Determined by Real-Time RT-PCR**

| Inflammatory Gene | Mean   | SEM    | Jejunal RNA Level (relative fold change) |          | p values for:  |                |
|-------------------|--------|--------|--|----------|----------------|----------------|
|                   |        |        | Group B                                  | Group C1 | Group B vs. C1 | Group B vs. C2 |
| TNF $\alpha$      | 2.64   | 0.64   | -2.53                                    | 2.98     | <0.0253        | NS             |
| IL-2              | -3.49  | 1.22   | -7.58                                    | -4.82    | <0.0595        | NS             |
| IL-12             | -9.71  | 0.09   | -6.90                                    | 2.54     | NS             | <0.0249        |
| IFN $\gamma$      | 8.46   | 1.88   | 11.15                                    | 8.60     | NS             | NS             |
| IL-13             | -1.50  | 1.50   | -1.50                                    | 7.93     | NS             | <0.0032        |
| IFN $\alpha$      | 797.66 | 346.30 | 345.43                                   | 395.14   | NS             | NS             |
| C1qb              | 7.24   | 1.48   | 15.67                                    | 2.60     | NS             | <0.0339        |
| Hsp70             | 127.53 | 48.91  | 74.03                                    | 24.38    | NS             | NS             |
| OAS2              | 63.29  | 43.17  | 162.13                                   | 319.27   | NS             | NS             |
| PAAP              | 1.96   | 0.20   | 4.30                                     | 2.03     | NS             | <0.0245        |
|                   |        |        | 1.31                                     | 0.19     | NS             | NS             |
|                   |        |        | -2.40                                    | -7.20    | NS             | NS             |
|                   |        |        | 0.62                                     | 3.00     | NS             | NS             |

\*Fold Change Relative to HIV negative controls. NS=non-significant