# INFLAMMATION AND IMMUNE ACTIVATION NOT DRIVEN BY ON-ART HIV-SPECIFIC IMMUNE RESPONSES

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## BACKGROUND

- People living with HIV (PLWH) have persistently elevated levels of inflammation and immune activation despite suppressive antiretroviral therapy (ART)
- Elevated inflammatory biomarkers and immune activation markers predict a broad array of morbidities and increased mortality (Hunt *et al*, JID, 2016; Zicari *et al*, Viruses, 2019)
- Adaptive HIV-specific immune responses also persist in PLWH on ART (Thomas *et al*, PLOS Pathogens, 2017; Keating *et al*, JAIDS, 2019; Stevenson & Ward *et al*, JCI Insight, 2021), but their role in the etiology of persistent inflammation is unknown

**Hypothesis:** Ongoing HIV-specific immune responses contribute to elevated levels of clinically-relevant inflammatory biomarkers and immune activation in PLWH on long-term ART

## METHODS

**ACTG A5321 Cohort (N=99)**

| HIV Persistence Measures | Immune Responses | Inflammation & Immune Activation |
|---|---|---|
| ○ Total HIV DNA<br>○ Cell-associated RNA<br>○ Plasma single copy assay | ○ HIV-specific T-cell responses<br>○ CMV-pp65-specific T-cell responses<br>○ HIV antibody levels | ○ Soluble biomarkers of inflammation<br>○ T-cell activation and cycling |

- PBMCs and plasma were collected and cryopreserved at a pre-ART time point and at cohort study entry (*median 7 years on suppressive ART*)
- HIV persistence measures were assessed at study entry
- T-cell responses (IFN-γ ELISPOT) to each HIV gene product, as well as to CMV-pp65, and HIV-specific antibody concentrations (Less-sensitive and Avidity-modified VITROS HIV 1+2) were measured at study entry
- Inflammatory biomarkers and immune activation markers were were measured at the pre-ART time point and at study entry

## RESULTS

### 1. Participant characteristics

| Characteristic (at A5321 study entry) | Median (range) or No. (%) |
|---|---|
| Age | 43 (23 - 74) |
| Sex, No., % | |
| Female | 25 (25.25%) |
| Male | 74 (74.75%) |
| Race/Ethnicity, No., % | |
| White non-Hispanic | 49 (49.49%) |
| Black non-Hispanic | 18 (18.18%) |
| Hispanic (regardless of race) | 30 (30.30%) |
| Asian, Pacific Islander | 0 (0.00%) |
| American Indian, Alaskan Native | 2 (2.02%) |
| Participant does not know or other/unknown | 0 (0.00%) |
| More than one race | 0 (0.00%) |
| Years on ART | 6.8 (4.2 - 14.8) |
| ARV regimen group, No., % | |
| INSTI containing | 26 (26.26%) |
| NNRTI containing | 43 (43.43%) |
| PI containing | 29 (29.29%) |
| PI + INSTI containing | 1 (1.01%) |

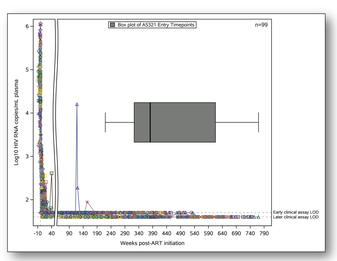



**Figure 1 (right).** ACTG A5321 cohort participants achieved viral suppression by week 48 post-ART initiation and maintained viral suppression prior to A5321 study entry

### 2. HIV-specific T-cell responses and HIV antibody levels are not associated with clinically-relevant inflammatory or immune activation markers

#### T-cell responses

| Biomarker | | IFN-γ T-cell response correlation: HIV-Gag | HIV-Env | HIV-Pol | HIV-Nef/Tat/Rev | Sum HIV | CMV-pp65 |
|---|---|---|---|---|---|---|---|
| *Inflammatory biomarkers* | | | | | | | |
| hs-CRP (ng/mL) | r | 0.06 | -0.18 | -0.05 | -0.13 | -0.01 | -0.02 |
| | p-value | 0.556 | 0.083 | 0.643 | 0.213 | 0.928 | 0.874 |
| | n | 99 | 99 | 99 | 99 | 99 | 90 |
| | Adjusted[A] r | -0.01 | -0.22 | -0.14 | -0.08 | -0.07 | -0.08 |
| | Adjusted[A] p-value | 0.961 | 0.051 | 0.230 | 0.472 | 0.518 | 0.528 |
| | Adjusted[B] r | 0.06 | -0.12 | -0.03 | -0.13 | <0.01 | - |
| | Adjusted[B] p-value | 0.580 | 0.246 | 0.759 | 0.223 | 0.991 | - |
| IL-6 (pg/mL) | r | 0.08 | -0.06 | 0.02 | 0.02 | 0.08 | 0.08 |
| | p-value | 0.433 | 0.556 | 0.820 | 0.818 | 0.444 | 0.445 |
| | n | 99 | 99 | 99 | 99 | 99 | 90 |
| | Adjusted[A] r | -0.01 | -0.09 | -0.09 | 0.03 | -0.03 | -0.03 |
| | Adjusted[A] p-value | 0.951 | 0.413 | 0.451 | 0.770 | 0.785 | 0.794 |
| | Adjusted[B] r | 0.07 | 0.01 | 0.01 | 0.03 | 0.08 | - |
| | Adjusted[B] p-value | 0.473 | 0.952 | 0.901 | 0.775 | 0.426 | - |
| IP-10 (pg/mL) | r | -0.05 | 0.03 | -0.04 | 0.02 | 0.01 | 0.10 |
| | p-value | 0.645 | 0.777 | 0.704 | 0.839 | 0.934 | 0.357 |
| | n | 99 | 99 | 99 | 99 | 99 | 90 |
| | Adjusted[A] r | NA | NA | NA | NA | NA | NA |
| | Adjusted[A] p-value | NA | NA | NA | NA | NA | NA |
| | Adjusted[B] r | -0.06 | -0.04 | -0.07 | -0.04 | -0.05 | - |
| | Adjusted[B] p-value | 0.555 | 0.699 | 0.483 | 0.730 | 0.653 | - |
| Neopterin (nMol/L) | r | 0.01 | 0.12 | -0.05 | 0.16 | 0.04 | 0.17 |
| | p-value | 0.909 | 0.240 | 0.601 | 0.120 | 0.667 | 0.105 |
| | n | 99 | 99 | 99 | 99 | 99 | 90 |
| | Adjusted[A] r | NA | NA | NA | NA | NA | NA |
| | Adjusted[A] p-value | NA | NA | NA | NA | NA | NA |
| | Adjusted[B] r | -0.06 | 0.06 | -0.12 | 0.08 | -0.06 | - |
| | Adjusted[B] p-value | 0.552 | 0.593 | 0.237 | 0.416 | 0.562 | - |
| sCD14 (ng/mL) | r | 0.04 | -0.13 | -0.11 | -0.07 | -0.06 | -0.14 |
| | p-value | 0.699 | 0.190 | 0.266 | 0.490 | 0.586 | 0.175 |
| | n | 99 | 99 | 99 | 99 | 99 | 90 |
| | Adjusted[A] r | 0.06 | -0.20 | -0.12 | -0.02 | -0.01 | -0.14 |
| | Adjusted[A] p-value | 0.595 | 0.071 | 0.310 | 0.885 | 0.898 | 0.233 |
| | Adjusted[B] r | 0.07 | -0.07 | -0.09 | -0.05 | -0.03 | - |
| | Adjusted[B] p-value | 0.496 | 0.474 | 0.394 | 0.656 | 0.764 | - |
| sCD163 (ng/mL) | r | 0.11 | -0.03 | 0.08 | -0.03 | 0.06 | 0.16 |
| | p-value | 0.259 | 0.769 | 0.407 | 0.755 | 0.564 | 0.141 |
| | n | 99 | 99 | 99 | 99 | 99 | 90 |
| | Adjusted[A] r | 0.16 | -0.05 | 0.16 | 0.07 | 0.12 | 0.12 |
| | Adjusted[A] p-value | 0.146 | 0.648 | 0.155 | 0.513 | 0.307 | 0.306 |
| | Adjusted[B] r | 0.08 | -0.02 | 0.04 | -0.09 | <0.01 | - |
| | Adjusted[B] p-value | 0.452 | 0.811 | 0.720 | 0.377 | 0.972 | - |
| TNF-α (pg/mL) | r | -0.07 | -0.09 | **-0.21** | -0.05 | -0.18 | -0.05 |
| | p-value | 0.477 | 0.399 | **0.040** | 0.637 | 0.081 | 0.667 |
| | n | 99 | 99 | 99 | 99 | 99 | 90 |
| | Adjusted[A] r | NA | NA | NA | NA | NA | NA |
| | Adjusted[A] p-value | NA | NA | NA | NA | NA | NA |
| | Adjusted[B] r | -0.01 | -0.05 | -0.15 | 0.02 | -0.11 | - |
| | Adjusted[B] p-value | 0.932 | 0.611 | 0.152 | 0.819 | 0.283 | - |
| %CD38$^+$HLA-DR$^+$ on CD4$^+$ cells | r | -0.03 | -0.01 | -0.06 | 0.07 | 0.02 | <0.01 |
| | p-value | 0.819 | 0.921 | 0.603 | 0.556 | 0.872 | 0.989 |
| | n | 80 | 80 | 80 | 80 | 80 | 71 |
| | Adjusted[A] r | 0.01 | <0.01 | -0.05 | 0.05 | 0.03 | -0.04 |
| | Adjusted[A] p-value | 0.903 | 0.992 | 0.692 | 0.698 | 0.816 | 0.718 |
| | Adjusted[B] r | -0.05 | -0.05 | -0.12 | -0.03 | -0.07 | - |
| | Adjusted[B] p-value | 0.692 | 0.684 | 0.306 | 0.815 | 0.555 | - |
| %CD38$^+$HLA-DR$^+$ on CD8$^+$ cells | r | 0.07 | 0.02 | 0.05 | 0.12 | 0.12 | 0.14 |
| | p-value | 0.535 | 0.875 | 0.687 | 0.302 | 0.290 | 0.241 |
| | n | 80 | 80 | 80 | 80 | 80 | 71 |
| | Adjusted[A] r | 0.08 | 0.02 | 0.04 | 0.09 | 0.10 | 0.11 |
| | Adjusted[A] p-value | 0.507 | 0.897 | 0.711 | 0.465 | 0.370 | 0.368 |
| | Adjusted[B] r | 0.06 | -0.03 | 0.03 | 0.08 | 0.10 | - |
| | Adjusted[B] p-value | 0.584 | 0.820 | 0.784 | 0.482 | 0.406 | - |
| %Ki67$^+$ on CD4$^+$ cells | r | 0.01 | -0.05 | -0.04 | -0.04 | 0.01 | -0.07 |
| | p-value | 0.963 | 0.647 | 0.726 | 0.716 | 0.950 | 0.549 |
| | n | 80 | 80 | 80 | 80 | 80 | 71 |
| | Adjusted[A] r | <-0.01 | -0.06 | -0.09 | -0.09 | -0.03 | -0.06 |
| | Adjusted[A] p-value | 0.967 | 0.593 | 0.467 | 0.465 | 0.830 | 0.622 |
| | Adjusted[B] r | 0.03 | -0.03 | -0.07 | -0.11 | -0.03 | - |
| | Adjusted[B] p-value | 0.811 | 0.786 | 0.575 | 0.329 | 0.811 | - |
| %Ki67$^+$ on CD8$^+$ cells | r | -0.04 | -0.17 | -0.04 | -0.14 | -0.04 | -0.11 |
| | p-value | 0.749 | 0.129 | 0.736 | 0.212 | 0.735 | 0.361 |
| | n | 80 | 80 | 80 | 80 | 80 | 71 |
| | Adjusted[A] r | -0.02 | -0.16 | -0.02 | -0.11 | -0.01 | -0.09 |
| | Adjusted[A] p-value | 0.872 | 0.163 | 0.835 | 0.356 | 0.960 | 0.460 |
| | Adjusted[B] r | 0.01 | -0.14 | <0.01 | -0.16 | -0.01 | - |
| | Adjusted[B] p-value | 0.936 | 0.223 | 0.981 | 0.173 | 0.923 | - |

[A]Controlling for pre-ART biomarker level where available.
[B]Controlling for pre-ART plasma HIV-1 RNA ($log_{10}$cps/mL), pre-ART CD4$^+$ T-cell count (cells/mm$^3$), pre-ART CD4$^+$/CD8$^+$ T-cell ratio, years on ART at A5321 entry, and age at A5321 entry.

#### HIV antibody levels

| Biomarker | HIV-Env antibody signal:cutoff ratio correlation: r | p-value | n | Adjusted[A] r | Adjusted[A] p-value | Adjusted[B] r | Adjusted[B] p-value |
|---|---|---|---|---|---|---|---|
| *Inflammatory biomarkers* | | | | | | | |
| hs-CRP (ng/mL) | -0.02 | 0.891 | 80 | -0.03 | 0.779 | 0.02 | 0.859 |
| IL-6 (pg/mL) | -0.19 | 0.085 | 80 | -0.16 | 0.161 | -0.20 | 0.079 |
| IP-10 (pg/mL) | -0.04 | 0.695 | 80 | NA | NA | -0.05 | 0.686 |
| Neopterin (nMol/L) | 0.02 | 0.843 | 80 | NA | NA | 0.01 | 0.899 |
| sCD14 (ng/mL) | 0.06 | 0.572 | 80 | 0.05 | 0.660 | 0.08 | 0.481 |
| sCD163 (ng/mL) | 0.13 | 0.262 | 80 | 0.05 | 0.651 | 0.09 | 0.468 |
| TNF-α (pg/mL) | -0.07 | 0.546 | 80 | NA | NA | -0.03 | 0.766 |
| *Immune activation markers* | | | | | | | |
| %CD38$^+$HLA-DR$^+$ on CD4$^+$ cells | 0.05 | 0.641 | 80 | <0.01 | 0.976 | 0.04 | 0.751 |
| %CD38$^+$HLA-DR$^+$ on CD8$^+$ cells | 0.03 | 0.795 | 80 | -0.01 | 0.953 | 0.02 | 0.892 |
| %Ki67$^+$ on CD4$^+$ cells | 0.07 | 0.541 | 80 | 0.01 | 0.964 | 0.08 | 0.483 |
| %Ki67$^+$ on CD8$^+$ cells | 0.01 | 0.958 | 80 | 0.01 | 0.944 | 0.04 | 0.714 |

[A]Controlling for pre-ART biomarker level where available.
[B]Controlling for pre-ART plasma HIV-1 RNA ($log_{10}$cps/mL), pre-ART CD4$^+$ T-cell count (cells/mm$^3$), pre-ART CD4$^+$/CD8$^+$ T-cell ratio, years on ART at A5321 entry, and age at A5321 entry.

- Magnitudes of HIV-specific T-cell responses and HIV antibody levels, as well as CMV-pp65-specific T-cell responses, were not correlated with levels of inflammatory biomarkers or immune activation markers
  - Including after adjustment for pre-ART biomarker level, indicating that HIV-specific immune responses do not appreciably contribute to on-ART inflammation and immune activation – regardless of a participant's pre-ART immune status
  - Magnitudes of T-cell responses to HIV-Pol were inversely correlated with TNF-α levels, but this association was confounded by several factors, including: pre-ART plasma viral load, pre-ART CD4$^+$ T-cell count, pre-ART CD4$^+$/CD8$^+$ T-cell ratio, years on ART at A5321 entry, and age at A5321 entry

### 3. Plasma single copy assay levels are positively correlated with CD8$^+$ T-cell activation, but other HIV persistence parameters are not associated with inflammatory biomarkers or immune activation

#### Total HIV CA-DNA

| Biomarker | CA-DNA correlation: r | p-value | n | Adjusted[A] r | Adjusted[A] p-value | Adjusted[B] r | Adjusted[B] p-value |
|---|---|---|---|---|---|---|---|
| *Inflammatory biomarkers* | | | | | | | |
| hs-CRP (ng/mL) | -0.04 | 0.700 | 96 | 0.08 | 0.468 | -0.09 | 0.412 |
| IL-6 (pg/mL) | -0.18 | 0.087 | 96 | -0.10 | 0.386 | -0.15 | 0.153 |
| IP-10 (pg/mL) | 0.03 | 0.775 | 96 | NA | NA | -0.08 | 0.443 |
| Neopterin (nMol/L) | -0.09 | 0.393 | 96 | NA | NA | -0.20 | 0.063 |
| sCD14 (ng/mL) | -0.03 | 0.765 | 96 | -0.08 | 0.469 | <0.01 | 0.971 |
| sCD163 (ng/mL) | <-0.01 | 0.971 | 96 | -0.05 | 0.652 | -0.06 | 0.582 |
| TNF-α (pg/mL) | 0.02 | 0.831 | 96 | NA | NA | 0.04 | 0.687 |
| *Immune activation markers* | | | | | | | |
| %CD38$^+$HLA-DR$^+$ on CD4$^+$ cells | 0.12 | 0.312 | 78 | 0.10 | 0.382 | -0.01 | 0.922 |
| %CD38$^+$HLA-DR$^+$ on CD8$^+$ cells | 0.05 | 0.693 | 78 | 0.03 | 0.812 | -0.02 | 0.883 |
| %Ki67$^+$ on CD4$^+$ cells | 0.19 | 0.097 | 78 | 0.20 | 0.098 | 0.13 | 0.288 |
| %Ki67$^+$ on CD8$^+$ cells | 0.09 | 0.422 | 78 | 0.16 | 0.166 | 0.06 | 0.613 |

[A]Controlling for pre-ART biomarker level where available.
[B]Controlling for pre-ART plasma HIV-1 RNA ($log_{10}$cps/mL), pre-ART CD4$^+$ T-cell count (cells/mm$^3$), pre-ART CD4$^+$/CD8$^+$ T-cell ratio, years on ART at A5321 entry, and age at A5321 entry.

#### HIV CA-RNA

| Biomarker | CA-RNA correlation: r | p-value | n | Adjusted[A] r | Adjusted[A] p-value | Adjusted[B] r | Adjusted[B] p-value |
|---|---|---|---|---|---|---|---|
| *Inflammatory biomarkers* | | | | | | | |
| hs-CRP (ng/mL) | -0.01 | 0.955 | 95 | 0.15 | 0.199 | 0.02 | 0.849 |
| IL-6 (pg/mL) | -0.08 | 0.425 | 95 | -0.02 | 0.887 | -0.04 | 0.740 |
| IP-10 (pg/mL) | 0.06 | 0.535 | 95 | NA | NA | -0.04 | 0.738 |
| Neopterin (nMol/L) | 0.03 | 0.777 | 95 | NA | NA | <0.01 | 0.982 |
| sCD14 (ng/mL) | -0.07 | 0.523 | 95 | -0.14 | 0.219 | -0.04 | 0.681 |
| sCD163 (ng/mL) | -0.15 | 0.145 | 95 | -0.15 | 0.199 | -0.16 | 0.129 |
| TNF-α (pg/mL) | 0.09 | 0.386 | 95 | NA | NA | 0.08 | 0.466 |
| *Immune activation markers* | | | | | | | |
| %CD38$^+$HLA-DR$^+$ on CD4$^+$ cells | 0.19 | 0.101 | 77 | 0.12 | 0.314 | 0.15 | 0.199 |
| %CD38$^+$HLA-DR$^+$ on CD8$^+$ cells | 0.02 | 0.862 | 77 | -0.05 | 0.672 | -0.01 | 0.901 |
| %Ki67$^+$ on CD4$^+$ cells | 0.20 | 0.086 | 77 | 0.19 | 0.110 | 0.18 | 0.133 |
| %Ki67$^+$ on CD8$^+$ cells | 0.16 | 0.156 | 77 | 0.15 | 0.210 | 0.16 | 0.188 |

[A]Controlling for pre-ART biomarker level where available.
[B]Controlling for pre-ART plasma HIV-1 RNA ($log_{10}$cps/mL), pre-ART CD4$^+$ T-cell count (cells/mm$^3$), pre-ART CD4$^+$/CD8$^+$ T-cell ratio, years on ART at A5321 entry, and age at A5321 entry.

#### Plasma single copy assay

| Biomarker | iSCA correlation: r | p-value | n | Adjusted[A] r | Adjusted[A] p-value | Adjusted[B] r | Adjusted[B] p-value |
|---|---|---|---|---|---|---|---|
| *Inflammatory biomarkers* | | | | | | | |
| hs-CRP (ng/mL) | 0.04 | 0.722 | 98 | 0.08 | 0.471 | 0.06 | 0.580 |
| IL-6 (pg/mL) | -0.07 | 0.493 | 98 | -0.06 | 0.590 | <-0.01 | 0.969 |
| IP-10 (pg/mL) | 0.08 | 0.453 | 98 | NA | NA | 0.02 | 0.875 |
| Neopterin (nMol/L) | -0.01 | 0.885 | 98 | NA | NA | -0.07 | 0.477 |
| sCD14 (ng/mL) | -0.01 | 0.933 | 98 | 0.03 | 0.820 | 0.03 | 0.790 |
| sCD163 (ng/mL) | 0.02 | 0.882 | 98 | <0.01 | 0.966 | 0.03 | 0.766 |
| TNF-α (pg/mL) | -0.07 | 0.477 | 98 | NA | NA | -0.10 | 0.354 |
| *Immune activation markers* | | | | | | | |
| %CD38$^+$HLA-DR$^+$ on CD4$^+$ cells | 0.10 | 0.396 | 79 | 0.08 | 0.521 | 0.03 | 0.770 |
| %CD38$^+$HLA-DR$^+$ on CD8$^+$ cells | **0.25** | **0.027** | 79 | **0.26** | **0.024** | **0.24** | **0.037** |
| %Ki67$^+$ on CD4$^+$ cells | -0.08 | 0.495 | 79 | -0.02 | 0.854 | -0.18 | 0.126 |
| %Ki67$^+$ on CD8$^+$ cells | -0.07 | 0.554 | 79 | <0.01 | 0.969 | -0.13 | 0.280 |

[A]Controlling for pre-ART biomarker level where available.
[B]Controlling for pre-ART plasma HIV-1 RNA ($log_{10}$cps/mL), pre-ART CD4$^+$ T-cell count (cells/mm$^3$), pre-ART CD4$^+$/CD8$^+$ T-cell ratio, years on ART at A5321 entry, and age at A5321 entry.

- Plasma HIV RNA levels by single copy assay were weakly but significantly positively correlated with CD8$^+$ T-cell activation in participants on long-term suppressive ART
  - This association remained significant after controlling for potential confounders
- Total HIV CA-DNA levels and CA-RNA were not correlated with inflammatory or immune activation markers
  - Including when associations were adjusted for pre-ART biomarker level

### 4. HIV-specific immune responses do not mediate associations between HIV persistence measures with levels of inflammation or immune activation

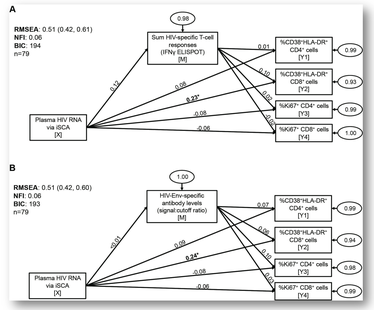



**Figure 2 (left).** No significant statistical mediation by HIV-specific immune responses between plasma single copy assay levels and immune activation markers.

**Figures 3 & 4 (below).** No significant statistical mediation by HIV-specific immune responses between total HIV DNA levels and inflammatory (*left*) or immune activation (*right*) markers.







Structural equation modeling was used to test for statistical mediation. Path model diagrams are depicted, with path coefficients representing standardized effect estimates using rank-transformed data; statistically significant path coefficients are bolded. Circled numbers represent unexplained (error) variances. X indicates the independent variable, M the mediating variable, and Y the outcome variable(s). Values for goodness-of-fit statistics including RMSEA (with 95% confidence interval), NFI, and BIC are shown. RMSEA, root mean square error of approximation; NFI, Bentler-Bonnet Normed Fit Index; BIC, Bayesian information criterion. * p<0.05, ** p<0.01, *** p<0.001.

## CONCLUSIONS

- Contrary to our hypothesis, neither HIV-specific T-cell responses nor HIV antibody levels – as measured in peripheral blood – appear to contribute to on-ART levels of inflammation and immune activation
- Significant association between on-ART plasma HIV RNA levels by single copy assay with CD8$^+$ T-cell activation differs from lack of association reported at year 4 on-ART in the A5321 Cohort (Gandhi *et al*, PLOS Pathogens, 2017)
  - Possible that very low-level viremia and/or viral blips may become increasingly influential drivers of CD8$^+$ T-cell activation over longer durations of ART – confirmatory studies needed
- Results add evidence to the "die is cast" theory proposing that pathogenic mechanisms that occur before therapy is initiated are the predominate drivers of long-lasting immune dysregulation

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