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TL1A-DR3 plasma levels are predictive of HIV-1 disease control, and DR3 co-stimulation boosts HIV-1-specific T-cell responses

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Abstract

Relative control of HIV-1 infection has been linked to genetic and immune host factors. Herein, we analyzed 96 plasma proteome arrays from chronic-untreated HIV-1-infected individuals using the classificatory random forest approach to discriminate between uncontrolled disease (pVL>50,000 RNA copies/ml; CD4 counts 283 cells/mm^3, n=47) and relatively controlled disease (pVL<10,000 RNA copies/ml; CD4 counts 657 cells/mm^3, n=49). Our analysis highlighted the TNF molecules relevance, in particular, TNF-like ligand 1A (TL1A, TNFSF15) and its cognate death receptor 3 (DR3, TNFSRF25), both of which increased in the relative virus control phenotype. DR3 levels (in plasma and PBMCs) were validated in unrelated cohorts (including long-term non-progressors, LTNPs), thus confirming their independence from CD4 counts and pVL. Further analysis in cART-treated individuals with a wide range of CD4 counts (137–1,835

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cells/mm^3) indicated that nor TL1A neither DR3 levels reflected recovery of CD4 counts with cART. Interestingly, in cART-treated individuals, plasma TL1A levels correlated with Tregs frequencies, while soluble DR3 was strongly associated with the abundance of effector HLA-DR +CD8+ T cells. A positive correlation was also observed between plasma DR3 levels and the HIV-1-specific T-cell responses. *In vitro*, co-stimulation of PBMC with DR3-specific mAb increased the magnitude of HIV-1-specific responses. Finally, in splenocytes of DNA.HTI–vaccinated mice, co-stimulation of HTI peptides and a DR3 agonist (4C12) intensified the magnitude of T-cell responses by 27%. Our data describes for the first time the role of the TL1A-DR3 axis in natural control of HIV-1 infection and point to the use of DR3 agonists in HIV-1 vaccine regimens.

INTRODUCTION

Combined antiretroviral treatment (cART) administered to HIV-infected subjects suppresses viral replication, leads to recovery of CD4 T-cell counts and reduces T-cell activation. While cART improves the quality of life and life expectancy of people living with HIV (PLWH), it entails substantial lifelong side effects and does not fully restore immune function (1). In addition, access and adherence to treatment are sometimes limited, especially in low-income countries. Therefore, there is an urgent need for strategies to achieve a so-called functional cure and/or interventions that can eliminate the virus from the body (2, 3).

A small group of PLWH, known as long-term non-progressors (LTNPs), do not experience disease progression, even in the absence of cART over long periods of time. Similarly, CD4 T-cell depletion occurs at a slower pace than in PLWH with standard disease progression. This relative control of HIV has been associated with several host factors, including markers of the innate and adaptive immune responses (4). Also LTNP produce more polyfunctional HIV-specific cytotoxic T lymphocyte (CTL) responses (5, 6), which has served as the rational for therapeutic CTL vaccines in HIV cure strategies.

Despite there is a considerable knowledge of the features of LTNPs, we do not fully understand the mechanisms associated with this natural control. In addition, new clinical guidelines recommend early initiation of treatment (7), thus limiting identification of these individuals and, consequently, the definition of new biomarkers to predict natural control.

Multiple mechanisms may contribute to the heterogeneous nature of LTNPs and many of these processes can be studied in plasma, an accessible biological fluid. Proteomic arrays enable the identification of key processes in plasma. The “communicome” array, a protein array detecting the levels of dozens to hundreds of plasma proteins involved in cell-to-cell communication, has recently been used to identify plasma factors predictive of plasma viral load (pVL) in untreated HIV infection (8). Herein, we extend the analysis of this multiplexed proteomic array in plasma samples from untreated PLWH using classificatory random forest analysis for discrimination between HIV progressors and individuals with relative virus control. Our analysis identified Tumor Necrosis Factor (TNF) and TNF receptors (TNFR) as crucial factors associated with HIV control. Of these, TL1A (TNFSF15) and its cognate death receptor DR3 (TNFRSF25) were of particular interest, given that validation studies in unrelated cohorts including LTNPs confirmed higher levels

of DR3 in plasma and PBMCs of individuals with relative virus control. Interestingly, neither pVL nor CD4 counts were associated with DR3 levels. Comprehensive immunological data collected from a cART-treated cohort with wide range of CD4 T cell recovery (9) indicated that, while the TL1A ligand is associated with the frequency of regulatory T cells (Tregs, CD4 and CD8), soluble DR3 is strongly associated with activated effector CD8 T cells. In addition, plasma DR3 levels are associated with HIV-specific T-cell responses, and *ex vivo* co-stimulation with DR3-specific antibody boosts these responses in untreated PLWH. An intensification of the HIV response was also detected after *ex vivo* co-stimulation of plasmid DNA-vaccinated mice splenocytes with DR3 antibody agonist (4C12). Taken together, these data indicate for the first time that TL1A/DR3 may play an important role in the immune control of HIV and highlight the potential use of DR3 co-stimulation with specific agonists in T-cell boosting regimens in HIV vaccine interventions.

MATERIALS AND METHODS

Participants:

Antiretroviral-naïve HIV-seropositive subjects in the chronic disease stage (n=96) recruited at Germans Trias i Pujol University Hospital, Badalona, Spain and the IMPACTA clinics in Lima, Peru were grouped in relation to their ability to control HIV disease, as previously reported (8). In brief, individuals with relative disease control were classed as “HIV-Low” (n=49) and had pVL <10,000 HIV RNA copies/ml (range 25–9,990; median 530 HIV RNA copies/ml). The median CD4 count was 657 cells/mm^3 (range 289–1,343 cells/mm^3). Individuals with uncontrolled disease were classed as “HIV-High” (n=47) and had pVL >50,000 HIV RNA copies/ml (range 50,295–1,200,000; median 105,520 HIV RNA copies/ml) and a median CD4 count of 283 cells/mm^3 (range 11–726 cells/mm^3) (Table S1).

Additional unrelated cohorts included seronegatives (n=8), chronic untreated individuals (“Untreated”, n=15), participants with one year of cART (“Treated”, n=10), and LTNPs (n=31, including viremic controllers [VC: n=13, pVL <2,000 copies/ml] and elite controllers [EC: n=18, undetectable pVL, <50 copies/ml]) (Table S1, (8)). cART-treated individuals with a wide range of CD4 counts, including immune-concordant (IC, n=12) and immune-discordant subjects (ID, n=12), were also included in the study (Table I, (9)). Finally, an additional chronically untreated HIV-infected cohort, “PLWH” (n=6, pVL range <50 – 16,041 HIV RNA copies/ml and CD4 counts range 281–1,500 cells/mm^3), was included for functional *in vitro* studies (Table S1). The study was approved by the Clinical Research Ethics Committee of Germans Trias i Pujol University Hospital (CEIC: EO-12–042), and all participants provided their written informed consent. Blood samples were processed using lymphoprep™ (STEMCELL technologies) and isolated PBMCs, PBMC dry-pellets and plasma samples were frozen until use.

“Communicome” cytokine determination in plasma samples:

Proteomic arrays were run using a custom-designed chip that enabled the detection and quantification of >600 individual proteins, as reported previously (8, 10). Raw data were background subtracted and normalized using cluster analysis and Z-scored ($z=(x-\mu)/\sigma$). Multivariate analyses were then performed with Bioconductor in R software package

(www.r-project.org) (11) using the *CMA* package R/Bioconductor with five cross-validations (12). This approach combines variable selection and model classification (random forest). The process was repeated 100 times (20 randomly repeated five-fold cross-validation processes). Finally, the AUC (area under the curve) was used to evaluate the model. The output of this multivariate analysis provides a ranking of variables according to the number of times that each variable is selected. For further analysis, we included the subset of the Top 25 scoring variables with a frequency of ≥100. Gene ontology (GO) enrichment analysis was performed using the *GOstats* package R/Bioconductor (13), and KEGG enrichment analysis was conducted with DAVID (14, 15). A hypergeometric test was applied in both cases.

Real-Time PCR:

PBMC dry cell pellet samples were used for RNA extraction (RNeasy® Plus Mini Kit, Qiagen) and retro-transcription (SuperScript®III First-Strand Synthesis SuperMix). The cDNA was used for RT-qPCR based on TaqMan gene expression assays for detection of *TL1A* (Hs00270802_s1), *DR3* (Hs00237054_m1), and *TBP* (Hs99999910_m1) in a 7500 real-time instrument (Applied Biosystems). Relative expression was calculated as $2^{-\Delta CT}$ (CT= the median of cycle threshold of 3 replicates).

Cytokine determination by ELISA:

TL1A was determined using an ELISA kit (Human TL1A/TNFSF15 DuoSet ELISA, R&D Systems). DR3 protein in plasma was determined using an ELISA kit from CUSABIO and Booster Immunoleader, following the manufacturer's instructions. Quantification of ligand and receptor (TL1A and DR3) was calculated using a four-parameter logistic nonlinear regression model.

Flow cytometry in treated HIV-infected individuals:

Data were generated as described elsewhere (9, 16–19). Spontaneous cell death was evaluated in fresh samples using 40 nM DIOC6 (Invitrogen), as described previously (16), combined with propidium iodide (Sigma-Aldrich, Madrid, Spain). For other staining panels flow cytometry was performed using a series of different antibody combinations. In brief, T-cell maturation and immunosenescence were evaluated using CD3-APC-Cy7, CD4-PerCP-Cy5.5, CD8-V500, CD57-FITC, CD27-APC, CD28-PE, CCR7-PE-Cy7, and CD45RA-V450 (BD Bioscience). Proliferation and Treg phenotype were assessed using an antibody panel comprising Ki67-FITC, FOXP3-PE, CD25- PE-Cy7, CD127-Alexa Fluor 647, CD3-APC-Cy7, CD4-V450, and CD8-V500. The activation panel included CD95-FITC, CD38-PE, HLA-DR-PerCP, CD3-APCCy7, CD4-APC, and CD8-PE-Cy7. All the stained cells were collected in LSRII flow cytometer (Becton Dickinson) and analyzed with FlowJo Software (version 7.6.5).

Measurement of the adaptive host immune response to HIV:

T-cell immunity to HIV was assessed in purified PBMCs using the ELISPOT assay (100,000 PBMCs/well), with an overlapping peptide (OLP) set comprising 410 OLPs, as described

elsewhere (20). The breadth (number of reactive OLPs) and magnitude (spot forming cells [SFC] per 10^6 PBMCs) were recorded.

In vitro TL1A and DR3 co-stimulation effects on HIV-infected human PBMCs:

We cultured thawed cryopreserved PBMCs from untreated PLWH and LTNPs (Table S1) (150,000 PBMCs/well) in duplicate in 96-well ELISPOT plates (MSIP 4510 plates Millipore, Mabtech) and stimulated them with a pool of selected reactive OLP peptides (each peptide, 2 μg/ml, Table S2). We also stimulated cells with a CEF (cytomegalovirus [CMV], Epstein-Barr virus [EBV], and influenza virus) peptide pool designed to stimulate T-cells (CEF-MHC Class I Control Peptide Pool Plus, C.T.L., OH, USA). Then, we co-stimulated the cells with 100 ng/ml of sTL1A (Recombinant Human TL1A/TNFSF15, R&D systems), 500 ng/ml of sDR3 (Recombinant Human DR3/TNFRSF25 Fc Chimera Protein, R&D systems), and /or 100 ng/ml of mAb anti-DR3 (Purified anti-human DR3 (TRAMP) JD3, Biolegend). Concentrations were based on the manufacturers' recommendations. Cells were incubated overnight at 37°C in 5% CO_2 and processed for the ELISPOT (see above). For evaluation of DR3 expression on T cells, cells were recovered before the ELISPOT development procedure and stained for surface markers (CD3-APC-Cy7, CD4-APC, CD8-PercP, and DR3-PE [anti-human DR3 (TRAMP) JD3] from Biolegend) acquired on an LSR Fortessa cytometer (Becton Dickinson) and analyzed using FlowJo 7.6.5 software.

Mouse immunization and splenocyte isolation:

Two groups of co-housed C57BL/6 mice (Envigo) were immunized intramuscularly with 100 μg/animal of DNA.HTI (21) in the Animal Facility of Germans Trias i Pujol University Hospital. The first group (n=3) received three doses of vaccine and the second group (n=4) four. All vaccinations were separated by three weeks, and mice were euthanized two weeks after the last vaccination. Spleens were removed and splenocytes isolated by mechanical disruption and passage through a 45-μm cell strainer (Falcon) using a 5-ml rubber syringe plunger. Following red blood cell lysis with red cell lysis buffer (17 mM TRIS and 0.14 M NH4Cl, Sigma), splenocytes were washed twice with RPMI 1640 supplemented with 10% FCS and penicillin/streptomycin (R10) and cryopreserved in N2 until use in FBS with 10% DMSO. Procedures involving mice followed EU legislation on animal experimentation and were approved by the Animal Experimentation Ethics Committee of Germans Trias i Pujol Research Institute and the Ministry of Agriculture, Livestock, Fishing and Food of the Generalitat de Catalunya (DAAM order numbers 6390, 9183).

Determination of murine T-cell responses to HTI:

IFN-g ELISPOT was used to determine vaccine–specific T-cell responses in mice. Briefly, thawed splenocytes (200,000 cells/well in RPMI 10% FCS) from the HTI-vaccinated mice were simultaneously stimulated overnight with a set of 15mer overlapping peptides covering the whole sequence of the HTI immunogen (Table S2) and increasing doses of the mouse DR3 agonist 4C12 (0.5, 1 and 2 μg/ml). The magnitude of the response (SFCs per 10^6 PBMCs) was recorded.

Statistical Analysis:

Univariate statistical analyses were performed using Prism Version 6, GraphPad. For comparisons between patient groups, the Mann-Whitney and Wilcoxon signed rank tests were applied. The Spearman test was applied for the correlation analysis. For all analyses, p-values <0.05 were considered statistically significant.

RESULTS

Soluble TNF and TNF receptors in plasma discriminate between untreated HIV disease phenotypes

The “communicome” approach has served to identify plasma markers that predict plasma viral load in HIV disease (8) and to define predictive biomarkers of other diseases (10). In order to explore the soluble factors involved in natural control of HIV infection, a classificatory multivariate approach was applied to discriminate between uncontrolled and relative infection control phenotypes. Multiplexed arrays of HIV-High (n=47) and HIV-Low individuals (n=49) ((8) and Table S1) were analyzed by applying a random forest model. These analyses revealed a total of 25 soluble molecules among 600 factors that consistently (frequency of >100 of 1000 iterations) distinguished between both groups of individuals (Figure 1A). The molecules fell most noticeably into the GO categories of chemokines (CCL-8, −18, −22, and CXCL-1) and cytokines (including TGF-b family members), as well as apoptotic factors that belong to the TNF/TNF-receptor family (TNFSF15, TNFRSF10D, and TNFRSF10C) and cell adhesion categories (including SIGLEC9 and SELE and ALCAM) (Figure 1A–B). The important role of the cell death process in the pathology of HIV was further supported by KEGG analysis, which also indicated the relevance of the apoptosis pathway in the control of HIV infection (fold enrichment of 3.5, Figure 1C). TNF/TNF-receptors fall into this category and discriminate between chronic untreated HIV-infected individuals with controlled or uncontrolled disease.

Elevated plasma levels of TL1A and DR3 are associated with relative control of HIV infection

Classificatory analyses of plasma proteomic arrays indicate that TNF/TNF receptors are relevant for discriminating between the two groups of chronic untreated HIV individuals studied. Among the TNF/TNF receptors detected, the TNFSF15 (TL1A) molecule stands out, as it is the first marker when considering only the apoptosis category (Figure 1) and the fifth discriminatory factor obtained in the multivariate analyses (frequency of 531). TL1A and its cognate receptor TNFRSF25 (also known as DR3) act as T-cell costimulatory signal with pleiotropic and broad functions that have been poorly studied (22, 23). Here, higher relative plasma levels of TL1A and DR3 were detected in untreated HIV-infected subjects with lower viremia and normal CD4 counts than in those with chronic uncontrolled HIV infection (Mann-Whitney test, TL1A p=0.0003 and DR3 p=0.0445, Figure 2A–B). As the HIV-Low group includes individuals with a wide range of plasma viral loads, we compared the relative plasma levels of TL1A and DR3 in HIV-Low individuals, separating those with pVL <50 from those with pVL ranging from 50–10,000 copies/ml. No differences were observed between these two groups for either molecule (Figure S1A–B). In addition, correlation analyses in chronic untreated HIV individuals with pVL or CD4 counts indicated

that sTL1A was associated with these two parameters (pVL: Rho= −0.2475 p=0.0151; CD4 counts: Rho: 0.2355 p=0.0209; Spearman rank test, data not shown), and no significant correlation was found with sDR3 levels. In addition to DR3, TL1A ligand can also signal through the decoy receptor DcR3 (24), although the relative plasma levels of DcR3 do not differ between both groups of chronic untreated HIV-infected individuals. This observation holds when pVL differences are considered in the HIV-Low group (Figure S1C–D).

Since plasma protein concentration is a reflection of multiple cell sources not only PBMC, we decided to explore *TL1A* and *DR3* gene expression in PBMCs from chronic untreated HIV-1-infected individuals with different levels of virus control. While no differences between HIV-High and HIV-Low are observed for *TL1A* (Figure 2C), its cognate receptor *DR3* is significantly highly expressed in HIV Low individuals (p=0.0289, Mann-Whitney test, Figure 2D), suggesting that in PBMCs, the receptor DR3 plays a more important role in discriminating between the groups than the ligand. Additionally, the expression levels of ligand and receptor in PBMCs were validated in unrelated cohorts of chronic infection including individuals one year before cART treatment initiation (Untreated n=11), one year after cART initiation (Treated n=5), LTNPs (n=23) and seronegative individuals (n=6). Consistently, these analyses confirmed that *TL1A* expression was not significantly different between groups (Figure 1E) and that only the *DR3* receptor was significantly highly expressed in LTNPs than in untreated individuals (p=0.0107, Mann-Whitney test, Figure 1F). Treated individuals tend to have higher levels of *DR3* in PBMCs than untreated individuals (Figure 1F), although the difference is not statistically significant (p>0.05).

To validate the plasma levels in unrelated cohorts, absolute protein concentration of TL1A and DR3 was quantified. Absolute TL1A levels in plasma did not reach the levels of detection of the assay Human TL1A/TNFSF15 DuoSet ELISA, R&D Systems in most of the samples measured. In contrast, findings for plasma sDR3 confirmed the higher levels associated with HIV control observed in the plasma proteomic arrays and PBMC gene expression tests (LTNPs vs untreated p=0.0282; LTNPs vs treated p=0.0030; Mann-Whitney test, Figure 2G). In addition, DR3 levels in the group of HIV seronegative individuals tended to be lower compared to LTNP and more similar to chronic viremic individuals, suggesting that LTNP had particularly high levels of DR3. Interestingly, ligand and receptor gene expression did not correlate in HIV-infected patients (Figure S1E). Only the plasma levels of TL1A and DR3 correlated positively in untreated HIV individuals measured in the communicome array (Rho=0.2705 p=0.0077, Spearman rank test, Figure S1F).

These results indicate that higher sDR3 levels in plasma and DR3 expression by PBMCs are associated with control of HIV. Interestingly, plasma and PBMCs DR3 levels did not correlate with pVL or CD4 T-cell counts, pointing to mechanisms in HIV controllers that are independent of those directly associated with viral replication or its cytopathic effects.

TL1A is associated with Treg phenotypes and sDR3 with effector HLA-DR+ CD8 T cells in treated HIV infected individuals

The TL1A/DR3 axis is a key pathway for the development of effective T-cell immunity (25). Thus, we investigated whether absolute TL1A and DR3 plasma levels were reflections of differential T-cell immunity and were associated with CD4 T-cell repopulation upon cART

in HIV infection. To do so, we studied cART-treated individuals from a cohort of immune concordant and discordant participants. These groups show a wide range of CD4 T-cell counts, which differ significantly between the immune-concordant phenotype (IC, median CD4 T-cell counts= 829 cells/mm^3) and the immune–discordant phenotype (ID, median CD4 counts=214 cells/mm^3) (Table I, (9)). No differences in TL1A and DR3 plasma levels were detected between the two groups (TL1A, IC mean=45.82 pg/ml *vs.* ID mean=50.06 pg/ml, p=0.064; DR3, IC mean=0.56 ng/ml *vs* ID mean=0.84 ng/ml, p=0.42; unpaired *t* test, data not shown), suggesting that CD4 T-cell repopulation mechanisms are independent of the TL1A/DR3 signaling axis.

Since the TLA1-DR3 axis is involved in several T-cell immune processes (25), the plasma levels of TL1A and DR3 in treated HIV-infected participants were correlated with immune-phenotypic T-cell markers measured by flow cytometry (Table I). While sTL1A levels positively correlated with transitional memory phenotype CD4 T cells (Rho=0.488 p=0.011, Spearman rank test, Table I), sDR3 levels were positively correlated with the frequency of effector CD8 T cells (Rho=0.6616, p=0.0002, Spearman rank test, Table I and Figure 3A) and inversely correlated with the naïve CD8 T-cell subpopulation (Rho= −0.512 p=0.0008, Spearman rank test, Table I). These data indicated that the TL1A and DR3 plasma levels are associated with different CD4 and CD8 T-cell populations and their maturation status, suggesting that the ligand and its receptor may serve variable functions that have been reported for different cell types (23).

Given that DR3 is a multifaceted receptor involved in cell death and proliferation processes (23, 26) and that IC and ID treated subjects clearly differ in terms of cell death, proliferation (Ki67+), and senescence processes (CD57+) (9), we performed an association analysis of these T-cell markers and plasma levels of TL1A and DR3. Only TL1A plasma levels correlated with naïve CD57+ CD4 T cells (Rho=0.407 p=0.039, Spearman rank test, data not shown), suggesting a potential relationship between TL1A and the proportion of senescent naïve CD4 T-cells in peripheral blood.

As this ligand-receptor axis has been shown to play a key role in the activation of T cells and Tregs in several disorders (22, 27, 28), further studies of the association between TL1A and DR3 plasma levels and activation markers and Treg percentages were undertaken. Interestingly, while sDR3 was clearly associated with effector CD8 T cells with an activated cell phenotype (HLA-DR^+ $CD45RO^+$ CD8 T cells, Rho=0.457 p=0.019, Spearman rank test, Table I and Figure 3B), sTL1A correlated with non-proliferating T-cells (CD4 and CD8) with regulatory phenotypes ($CD25^{High}$ $Foxp3^+$ CD4 T-cells, Rho=0.479 p=0.013; $CD25^{High}$ $Foxp3^+$ CD8 T-cells, Rho=0.545 p=0.004, Spearman rank test, Table I and Figure 3C).

As sTL1A is closely associated with CD4 and CD8 Treg subsets, and sDR3 is strongly associated with CD8 T cells with an effector and activated phenotype, these data suggest a dichotomous mode of action for the TL1A ligand and sDR3 in HIV infection, as previously reported for other diseases (22, 28). Accordingly, the disruption of the TL1A/DR3 axis favors tolerance processes while its stimulation might benefit antitumor and antiviral T-cell–mediated responses (25, 29), in line with the associations between higher levels of DR3 and TLA1 and improved HIV control (see above).

DR3 as a new marker and co-stimulator of HIV-specific T-cell responses

Given that the TL1A/DR3 axis is a key pathway for the development of effective CD4 and CD8 T-cell antiviral responses (25), we evaluated the association between plasma TL1A and DR3 levels in chronic untreated HIV-infected individuals and the magnitude and breadth of HIV-specific T-cell responses. While sTL1A did not correlate with the magnitude or the number of responses, plasma sDR3 levels were associated with broader and stronger HIV-specific T-cell responses (breadth: Rho=0.2589 p=0.0155; and magnitude: Rho: 0.2214 p=0.0393; Spearman rank test, Figure 4A–B). These results reinforce the aforementioned association between DR3 and activated effector CD8 T cells (Table I and Figure 3A–B). Interestingly, a stronger correlation between sDR3 and T-cell breadth was observed in HIV-Low individuals (Rho: 0.3175 p=0.0380; Spearman rank test, Figure 4A), suggesting a potential role for this receptor in the generation of broad HIV T-cell responses in the setting of more effective virus control.

While the TL1A ligand is produced mostly by endothelial and antigen-presenting cells, the DR3 receptor is expressed mainly in antigen-experienced T cells (22). To evaluate the protein levels of DR3 in CD4 and CD8 T cells from HIV-infected subjects with a controller (n=3) or non-controller phenotype (n=3) we used flow cytometry. Figure S2 shows that upon *in vitro* HIV peptide stimulation, controllers upregulate DR3 expression in CD4 and CD8 T cells, while non-controllers are unable to upregulate this receptor after 24 hours of peptide stimulation.

As DR3 is a co-stimulator of CD8 T-cell responses (25, 29) and has been proposed as a potential adjuvant for vaccination regimens (30), *ex vivo* intensification of HIV-specific T-cell responses were evaluated in the presence of recombinant sTL1A, sDR3, and anti-DR3–specific antibody in untreated PLWH (Table S1, Figure 4C). After 24 hours, the stimulation of HIV-specific peptides combined with DR3-specific antibody significantly enhanced the HIV-specific T-cell responses (12%, p=0.0210, Wilcoxon matched pairs test, Figure 4C) compared with peptide stimulation alone. Similarly, the CEF-specific T-cell response was also boosted upon co-stimulation with DR3 antibody (20% of increased responses, p=0.0781, Wilcoxon matched pairs test, Figure 4D).

Since no data on agonistic or blocking capacity for human DR3-specific antibodies has been reported, we further evaluated the use of DR3 antibody agonist (4C12) *in vitro* in splenocytes from DNA.HTI-vaccinated mice. In line with the effect of DR3 specific co-stimulation seen in human cells, when splenocytes from DNA.HTI-vaccinated mice were cultured and stimulated with HTI peptides and 4C12, the HTI-specific responses were boosted by 27% (p=0.0156, Wilcoxon signed rank test, Figure 4E). These data are in line with the effect of DR3 specific co-stimulation seen in human cells and further support the important role of the DR3 receptor in driving cellular immunity against HIV.

DISCUSSION

LTNPs maintain low levels of HIV virus for long periods of time without the need for cART, indicating that some host mechanisms are able to control viral infection without total eradication of the virus. This group of individuals with special features, including highly

polyfunctional CTL responses against HIV (5), has been extensively studied with the aim of identifying key mechanisms involved in relative control of HIV infection and guiding new therapeutic interventions.

Here, we focused on the search for traits that could discriminate between phenotypes of controlled and uncontrolled HIV infection using a previously reported “communicome” approach (8, 10). The main plasma factors that distinguish between untreated HIV-infected individuals with relative control or absence of control fell into the GO and KEGG categories related to the cell death process. It is known that uncontrolled HIV infection causes direct CD4 cell death, as well as indirect immune cell depletion and dysfunction. Thus, although the enrichment of cell death processes in these plasma samples may be expected, the mechanisms involved in CD4 T-cell loss remain unclear and could involve cell death and proliferative pathways (31).

Among all the proteins in the cell death category, TL1A (TNFSF15) emerged as the most relevant factor for differentiating between individuals with controlled and uncontrolled HIV infection. In addition, high plasma levels of its cognate receptor DR3 (TNFRSF25) are associated with control of HIV infection. These elevated plasma levels are supported by higher gene expression levels of DR3 in PBMCs in individuals with superior control of HIV. No such association was observed for TL1A plasma protein levels and PBMC gene expression; this observation is consistent with the major source of TL1A being endothelial/epithelial cells and, to a lesser extent, lymphoid and myeloid immune cells, while its cognate receptor DR3 is expressed mainly in activated T cells (CD4 and CD8) present in PBMCs (22).

Twenty years ago, the DR3 receptor was classified as a “death receptor” because of its capacity to activate caspase-dependent and -independent apoptosis processes (32, 33). However, it was subsequently reported that in lymphocytes, DR3 signaling predominantly induces the MAPK and NF-κB pathways associated with inflammation, survival, and proliferation (22, 24). While the specific trigger of the different functions of DR3 receptor remains unclear, the increase in DR3 expression in TCR-activated T-cells and the enhancement of T-cell proliferation as effector functions through the TL1A-DR3 axis are well documented (22, 25). Therefore, levels of DR3 in plasma or PBMCs are not necessarily expected to correlate with CD4 T-cell counts or cell death processes in our study. Moreover, the higher plasma levels detected in elite controllers are not recovered with cART or CD4 T-cell repopulation. Therefore, in this report, we point to a key role of the TL1A-DR3 axis in T cell function for the control of HIV infection.

TL1A-DR3 signaling is associated with several T-cell–dependent autoimmune diseases, as well as with the induction and maintenance of chronic inflammation (23, 24, 28, 34), although its involvement in human infectious disease is unknown. Here, we found that TL1A was associated with regulatory and exhausted phenotypes in CD4 T-cell populations from cART-treated individuals, while DR3 was strongly correlated with activated effector CD8 T cells, possibly because of the pleiotropic functions of the TL1A-DR3 axis. In this regard, DR3 is constitutively expressed in Treg cells, where the TL1A-DR3 axis is involved in Treg expansion. In fact, evidence from murine models has shown that treatment with

TL1A-Ig limits graft-versus-host disease (35–37). In contrast, DR3 is only expressed in conventional CD4 and CD8 T cells and after stimulation of TCRs with foreign cognate antigens, it enhances effector functions. It has been proposed that in infectious disease, TLA1-DR3 signaling in conventional T cells induces killing of infected cells, while its signaling in Treg might prevent exacerbated immune responses that damage host tissues (22). Therefore, while blocking of the TL1A-DR3 axis has been suggested when this system is disrupted, such as in autoimmune or chronic inflammatory diseases (24, 34, 38), its stimulation might benefit antitumor and antiviral T-cell–mediated responses (25, 29).

Interestingly, we show for the first time that in individuals with uncontrolled HIV infection and reduced plasma levels of TL1A, *in vitro* stimulation of their PBMCs with HIV T-cell epitopes is not sufficient to upregulate DR3 expression in CD8 T cells (Figure S2, Figure 5A). In contrast, in a situation of HIV natural control, HIV-1 antigen presentation to CD8 T cells upregulates DR3 on cell membrane of T cells and together with high levels of sTL1A favors TL1A-DR3 axis signaling (Figure 5B). Actually, in the general untreated HIV population, stimulation of the HIV peptide pool combined with DR3 co-stimulation by a DR3-specific antibody increased HIV-specific T-cell responses by 12%. Therefore, we propose that the agonistic stimulation of TL1A/DR3 axis would allow a more effective killing of HIV infected cells (Figure 5B). This finding may also hold for other pathogens, since the addition of DR3 antibody could increase CEF-induced responses by 20%. In the murine cytomegalovirus (MCMV) model, the essential role of DR3 for an efficient antiviral T cell response has been documented and the absence of DR3 been shown to results in dysregulated virus control (25). While the increases observed upon CEF pool stimulation are modest, stimulation of the TL1A/DR3 axis at the time of induction of *de novo* responses *in vivo* may increase their magnitude.

Co-stimulation with the TL1A and DR3 axis is essential for effective CD4 and CD8 T-cell responses against murine cytomegalovirus infection (25). Accordingly, TL1A protein or DR3 agonistic antibodies have been proposed as adjuvants in antigen-specific vaccinations (23, 39). We observed that the use of antibody agonists of DR3 in responder DNA.HTI-vaccinated mice intensified CD8-specific antigen responses by >25% in *ex vivo* stimulation experiments. These data clearly support the future use of DR3 stimulation *in vivo* together with vaccine candidates designed for the induction of cellular immunity, as proven in murine cancer models (29), and which could possibly be extended to cure of HIV infection.

The TL1A-DR3 axis can be modulated by other host soluble factors such as the decoy receptor-3 (DcR3), which binds and neutralizes the signaling function of TL1A, as well as other ligands, such as FasL and light protein. However, while its blockade might be interesting in a cancer setting with increased DcR3 levels (40, 41), we did not detect any differences between HIV-infected controllers and non-controllers. Inhibition of ADAM17 (a member of the ADAM metallopeptidase family, also known as tumor necrosis factor-α–converting enzyme) has also been proposed as a therapeutic target in inflammatory diseases, since it increases levels of sTL1A (42). However, given the pleiotropic effects of this metalloprotease, including supporting HIV virus infection (43–45), it may not offer a therapeutic target in the HIV antiviral response. However, in the “communicome” array, increased plasma levels of TIMP3, an ADAM17 inhibitor, were detected in HIV controllers,

thus further highlighting the potential of targeting the TL1A-DR3 axis in strategies aimed at boosting specific CD8 T-cell responses in HIV infection and, by inference, the immune response to other pathogens.

On the whole, this is the first study to identify the TL1A-DR3 axis in plasma as a new marker for HIV control and to show that this axis is associated with virus-specific T-cell responses. Therefore, our results show that immune intensification involving stimulation of the TL1A-DR3 axis could be considered in future vaccination strategies, not only for HIV, but also for other viruses and emerging pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ADAM17	ADAM metallopeptidase domain
AUC	Area under the curve
ALCAM	activated leukocyte cell adhesion molecule
cART	combined antiretroviral treatment
CT	cycle threshold
DAVID	Database for Annotation, Visualization and Integrated Discovery
DcR3	decoy receptor-3
DR3	death Receptor 3
EC	elite controllers
FasL	Fas Ligand
GO	Gene Ontology
HTI	HIV T cell immunogen
IC	immune-concordant
ID	immune-discordant

KEGG	Kyoto Encyclopedia of Genes and Genomes
LTNP	long-term non-progressors
OLP	overlapping peptide
PLWH	people living with HIV
pVL	plasma viral load
R10	RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin
sDR3	soluble DR3
SELE	selectin E
SFC	spot forming cells
SIGLEC9	sialic acid–binding Ig-like lectin 9
SN	Seronegatives
sTL1A	soluble TL1A
TIMP3	TIMP metallopeptidase inhibitor 3
TL1A	TNF-like ligand 1A
TNFSF15	TNF superfamily member
TNFRSF10C	TNF receptor superfamily member 10C
TNFRSF10D	TNF receptor superfamily member 10D
TNFRSF25	TNF receptor superfamily member 25
Treg	regulatory T cell

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KEY POINTS

- DR3 levels are associated with spontaneous *in vivo* control of HIV.
- Upon treatment, TL1A is associated with Tregs, and sDR3 with effector CD8 T cells.
- DR3 agonists can be used to boost HIV-specific T cell responses *in vitro*.

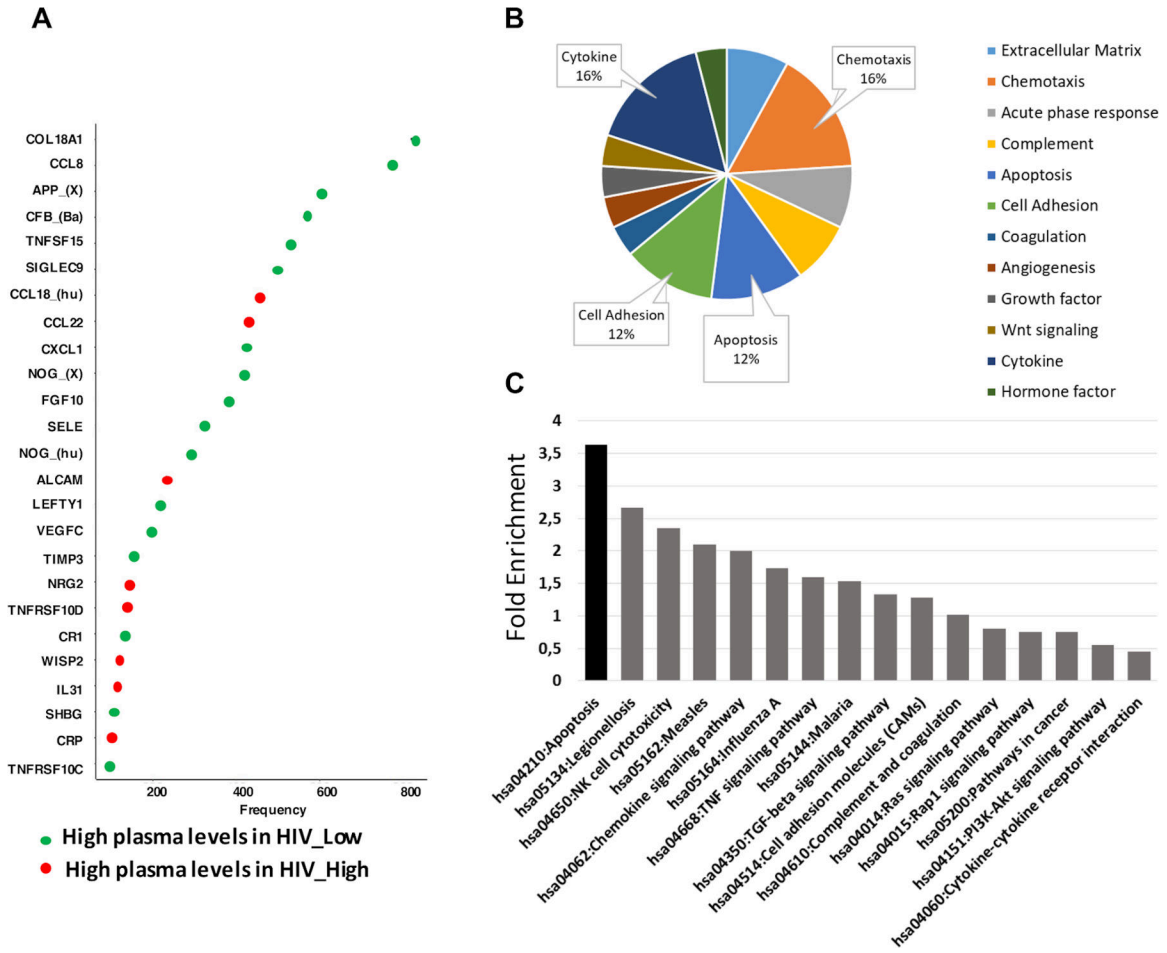

Figure 1: Plasma soluble factors in chronic untreated HIV groups.

"Communicome" arrays performed in 96 HIV-infected individuals with HIV-High (n=47) and HIV-Low (n=49) ((8), Table S1) were analyzed by applying a random forest model. A) Frequency plot indicating the relevance of the top 25 scoring soluble factors with frequency ≥100 obtained after CART analysis. B) Pie chart representing the gene ontology (GO) biological processes gene enrichment analysis percentages for each category represented among the top 25 candidates. C) KEGG enrichment analysis showing the fold enrichment value of each pathway among the top 25 selected factors.

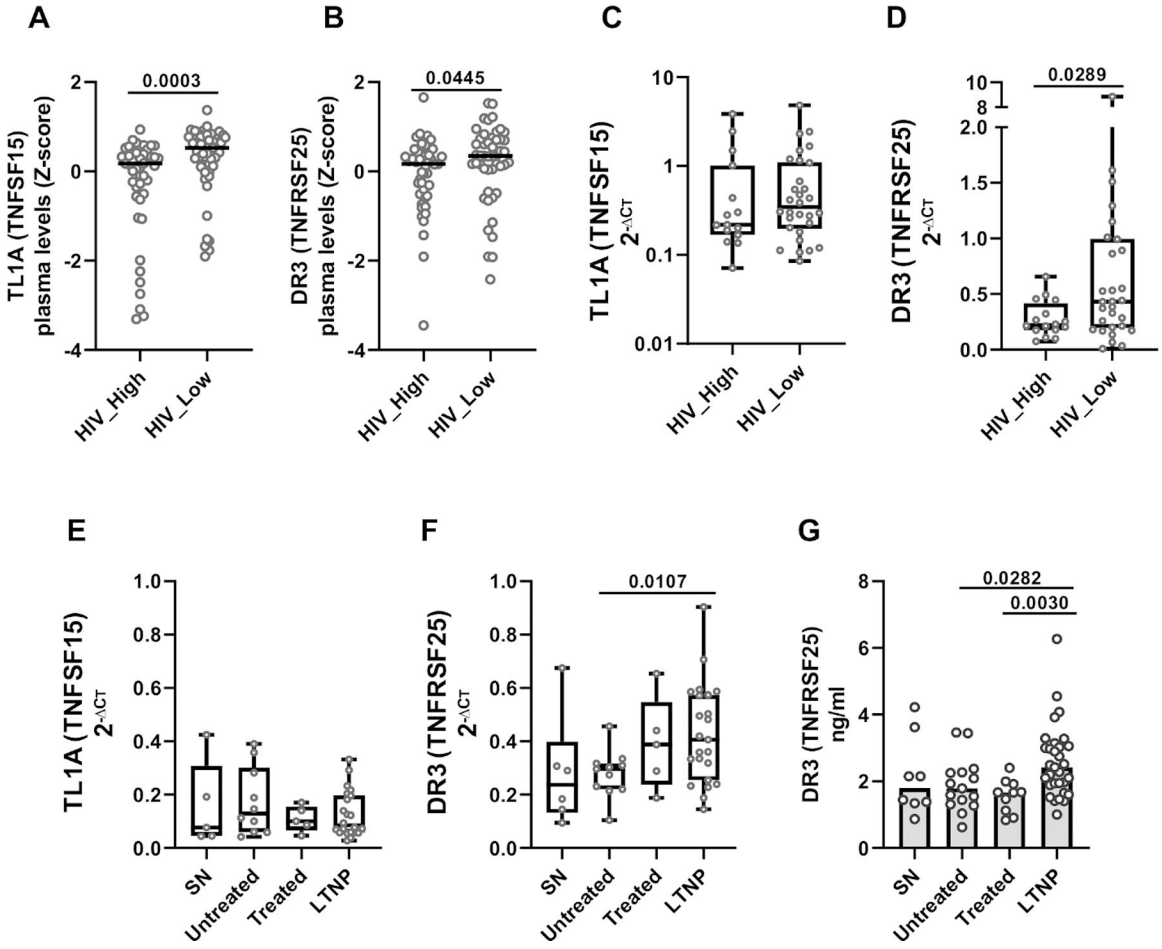

Figure 2: sTL1A and sDR3 plasma levels and PBMC gene expression in chronic HIV-infected individuals.

sTL1A (A) and sDR3 (B) relative plasma levels (Z-score "communicome" values) in HIV-High (n=47) and HIV-Low (n=49) (Table S1). C-D) Gene expression (Table S1) of TL1A (C) and DR3 (D) in HIV-High (n=16) and HIV-Low (n=30). E-F) Unrelated validation cohorts for gene expression of TL1A (E) and DR3 (F) in dry pellet PBMC samples from seronegatives (SN, n=6) and HIV-infected individuals one year before and after initiation of treatment (untreated [n=11] and treated [n=5]) and in LTNP (n=23) (Table S1). G) Absolute DR3 quantifications in plasma (ELISA) in a confirmatory cohort including seronegatives (SN, n=8) and samples from time points one year before and after initiation of treatment (untreated [n=15] and treated [n=10]) and controllers (n=31), Table S1). The Mann-Whitney test was applied for group comparisons, and p-values < 0.05 were considered significant.

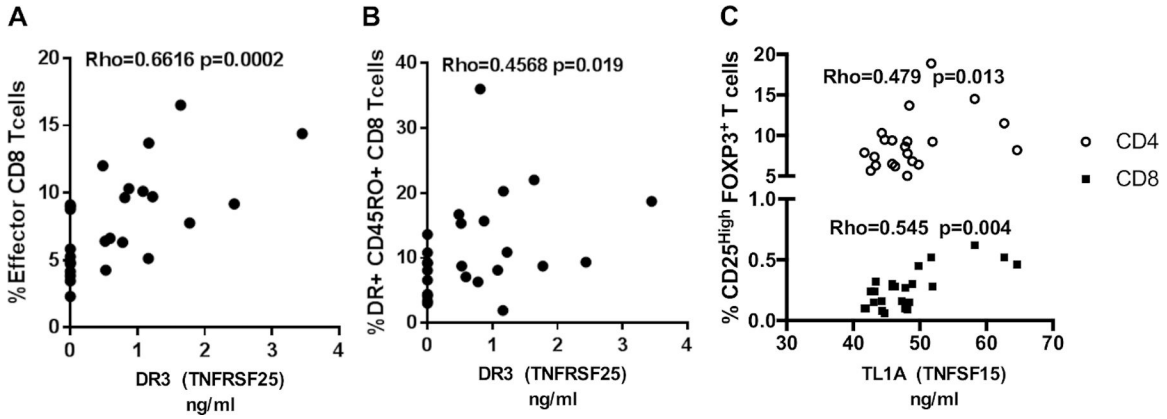

Figure 3: DR3 correlates with effector CD8 T-cells and TL1A, with regulatory CD4 and CD8 phenotypes.

A–B) Correlation between plasma DR3 protein levels and the percentage of effector CD8 T cells (A) and HLA-DR+ CD45RO+ CD8 T cells (B) in a cohort of treated individuals (n=26, Table I). C) Correlation between ELISA-determined plasma TL1A levels and the percentage of $CD25^{high}$ $FOXP3_{+}$ T cells (circles CD4 T cells and squares CD8 T cells) in treated individuals including immune-concordant and -discordant individuals (n=26, Table I). The Spearman rank test was used for the correlations, and p-values <0.05 were considered statistically significant.

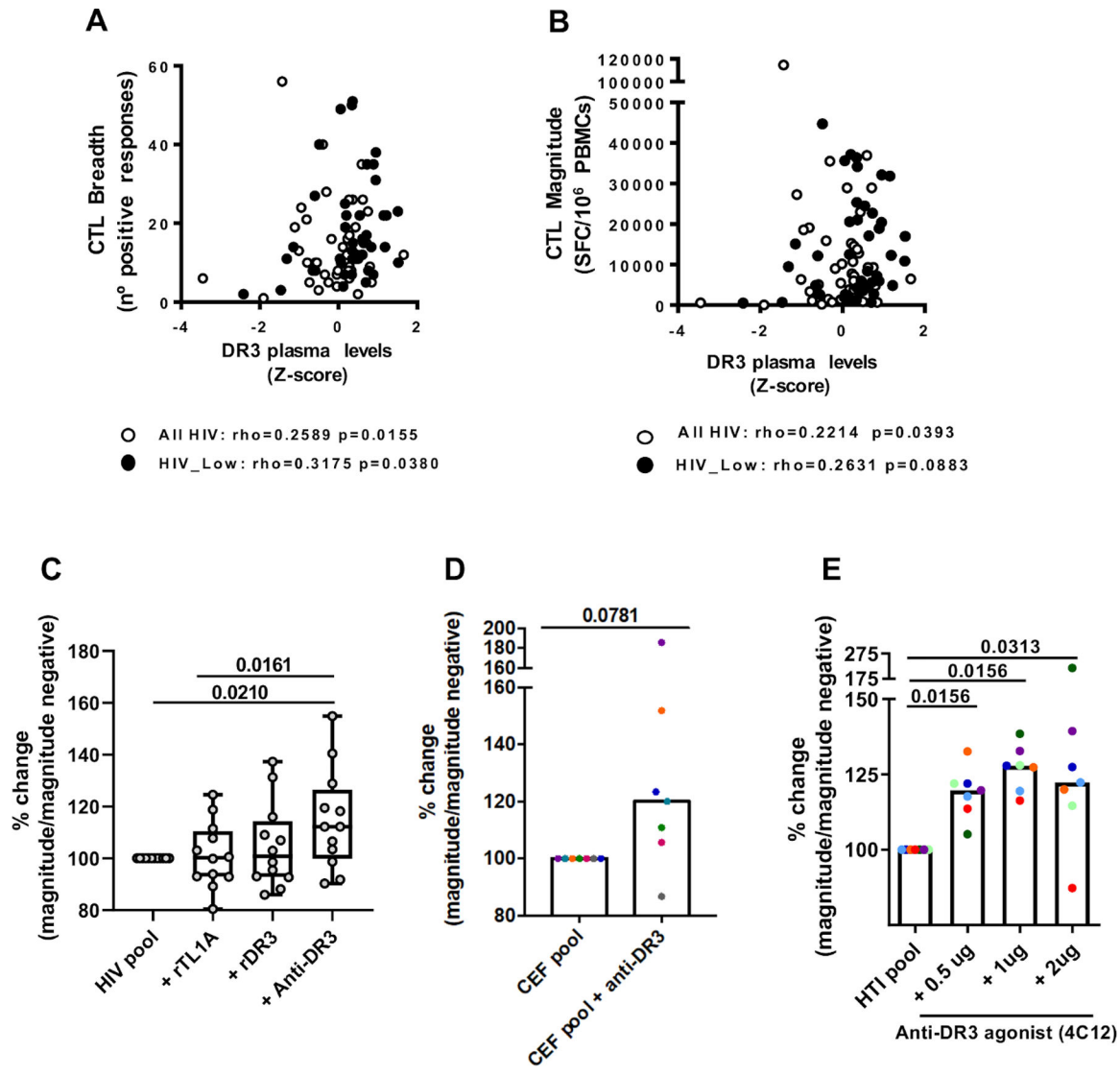

Figure 4: TL1A/DR3 axis role in HIV-specific T-cell responses.

A-B) sDR3 relative plasma levels (Z-score communicome) correlate with T-cell breadth (A) and magnitude (B) of HIV-High (n=46) and HIV-Low (n=41) individuals (Table S1). C) IFN-g-ELISPOT (percentage increase in the magnitude of T-cell) after HIV-specific peptide pool stimulation in the presence of recombinant TL1A and DR3 and specific anti-DR3 mAb in PBMCs from untreated PLWH (Table S1). D) Percentage increase in the magnitude of CEF-specific responses upon co-stimulation with anti-DR3 mAb. E) Increase in magnitude of DNA.HTI-specific T cell responses in isolated splenocytes from HTI-vaccinated mice after stimulation with the mouse DR3 specific antibody 4C12 (agonist DR3). The Spearman rank test was used for correlation analysis and the Wilcoxon signed rank test for group comparisons. p-values <0.05 were considered statistically significant.

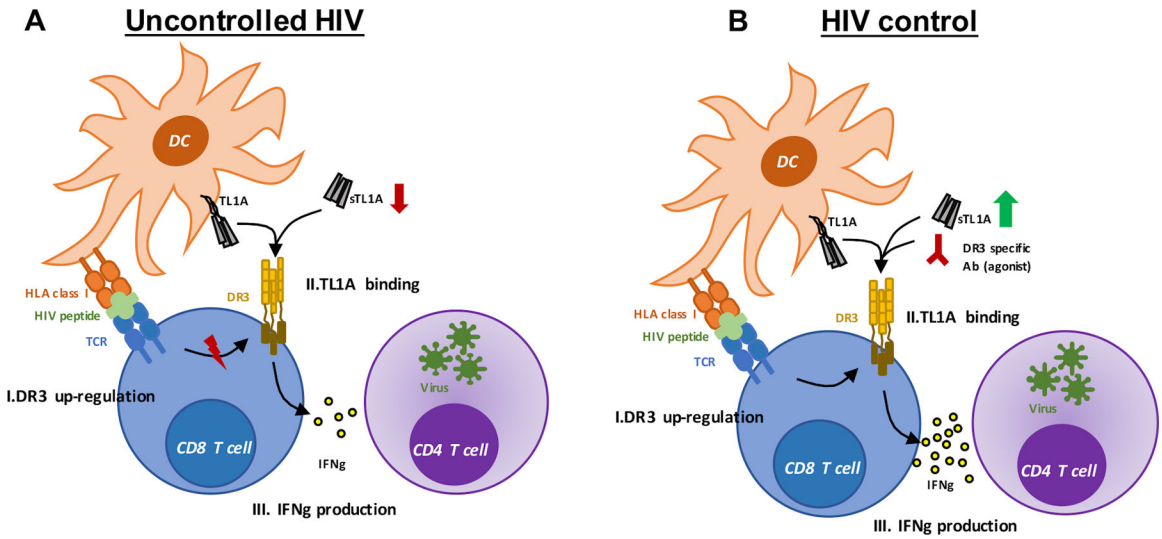

Figure 5. Model of TL1A-DR3 axis in HIV infection.

A) Failure of TL1A-DR3 axis during uncontrolled HIV infection. Upon HIV-specific activation, there is no upregulation of DR3 in surface membrane of CD8 T cells (A.1). The reduced levels of sTL1A do not allow the signaling through DR3 receptor (A.2) that would weaken the IFNg production (A.3). B) HIV control. Upon HIV-specific activation, DR3 protein is upregulated in cell membrane (B.1) that together with higher plasma levels of sTL1A and/or the presence of a DR3-agonist would ensure the signaling of the TL1A-DR3 axis (B.2). That co-stimulatory effect would enhance the HIV-specific IFNg responses (B.3)

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Table I.

CD4 and CD8 cell markers of maturation, Tregs, and activation evaluated by flow cytometry[1]

		PARAMETER	Median Dis	Median Conc	p-value (Mann-Whitney)	Plasma TL1A (pg/ml) correlation Spearman Rho	P (two-tailed)	Plasma DR3 (ng/ml) correlation Spearman Rho	P (two-tailed)
Clinical Info		Age	49	45	n.s	0.221	ns	0.264	ns
		Gender (Male : Female)	11 M : 1 F	13 M: 1 F	n.s	0.164	ns	0.040	ns
		Absolute CD4 (cells/μl)	213.5	828.5	<0.0001	−0.226	ns	−0.116	ns
MATURATION	**CD4 T cells**	Naïve (CD27+CD45RA+CCR7+)	13.2	41.9	<0.0001	−0.357	ns	−0.369	ns
		Central Memory (CD27+CD45RA −CCR7+)	25.2	26.0	ns	0.390	ns	0.121	ns
		Transitionals (CD27+CD45RA −CCR7−)	32.4	14.2	0.001	0.488	0.011	0.293	ns
		Effector (CD45RA−CCR7−CD27−CD28+)	13.8	6.4	<0.001	0.036	ns	0.167	ns
		TEMRA (CD45RA +CCR7−CD28−CD27−)	8.8	1.2	ns	0.057	ns	0.100	ns
	CD8 T cells	**Naïve (CD27+CD45RA+CCR7+)**	10.7	28.4	0	−0.290	ns	−0.512	0.008
		Central_Memory (CD27+CD45RA −CCR7+)	6.0	6.8	ns	0.237	ns	0.114	ns
		Transitionals (CD27+CD45RA −CCR7−)	15.2	11.0	ns	0.010	ns	0.279	ns
		Effector (CD27+CD28−)	8.3	5.5	ns	0.036	ns	0.662	<0.001
		TEMRA (CD28−CD27−)	46.2	40.7	ns	0.129	ns	0.113	ns
TREGS	**CD4 T cells**	**CD4 Treg CLASSIC (CD25hiFOXP3+)**	8.4	6.4	ns	0.479	0.013	0.094	ns
		Treg classic ki67+	9.7	5.4	0.001	0.134	ns	0.056	ns
	CD8 T cells	**CD8 Treg CLASSIC (CD25hiFOXP3+)**	0.2	0.3	ns	0.545	0.004	0.192	ns
		Treg CD8 Ki67+	11.9	9.1	ns	−0.120	ns	−0.056	ns
ACTIVATION	**CD4 T cells**	CD38+ CD45RA-CD4 T cells	39.7	25.5	<0.001	0.302	ns	0.294	ns
		HLADR+ CD95+ CD4 T cells	18.7	6.0	0.002	0.390	ns	0.413	ns
		HLADR+CD38+	11.2	5.5	0.005	0.261	ns	0.121	ns

		PARAMETER	Median Dis	Median Conc	p-value (Mann-Whitney)	Plasma TL1A (pg/ml) correlation Spearman Rho	P (two-tailed)	Plasma DR3 (ng/ml) correlation Spearman Rho	P (two-tailed)
		CD45RO+CD38+	12.4	8.2	0.020	0.183	ns	0.093	ns
		HLA-DR+CD45RO+	15.1	5.8	<0.001	0.227	ns	0.262	ns
	CD8 T cells	CD38+ CD45RA- CD8 T cells	29.0	24.8	ns	0.088	ns	0.166	ns
		HLADR+ CD95+ CD8 T cells	**15.2**	**10.4**	ns	0.280	ns	0.434	0.034
		HLADR+CD38+	15.3	15.6	ns	0.066	ns	0.065	ns
		CD45RO+CD38+	8.9	6.7	ns	0.246	ns	0.152	ns
		HLADR+CD45RO+	**9.2**	**7.4**	ns	0.147	ns	0.457	0.019

*1*Table containing the percentage of CD4 and CD8 T cells expressing cell markers of maturation, Tregs, or activation evaluated by flow cytometry. ns = not significant. Cells shaded in green indicate a significant Spearman correlation.