

Soluble CD40-ligand (sCD40L, sCD154) plays an immunosuppressive role via regulatory T cell expansion in HIV infection

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Introduction

Chronic inflammation accompanied with a massive depletion of CD4 T cells in gut mucosa is a hallmark of HIV infection. HIV also alters mucosal immunity which results in microbial translocation from the gut into the periphery and is associated with immune dysfunction and persistent activation [1,2]. Indoleamine 2,3-dioxygenase (IDO), expressed by professional antigen-presenting cells such as

Summary

CD40/CD40-ligand (CD40L) signalling is a key stimulatory pathway which triggers the tryptophan (Trp) catabolizing enzyme IDO in dendritic cells and is immunosuppressive in cancer. We reported IDO-induced Trp catabolism results in a T helper type 17 (Th17)/regulatory T cell (T_{reg}) imbalance, and favours microbial translocation in HIV chronic infection. Here we assessed the link between sCD40L, T_{regs} and IDO activity in HIV-infected patients with different clinical outcomes. Plasmatic sCD40L and inflammatory cytokines were assessed in anti-retroviral therapy (ART)-naive, ART-successfully treated (ST), elite controllers (EC) and healthy subjects (HS). Plasma levels of Trp and its metabolite Kynurenine (Kyn) were measured by isotope dilution tandem mass spectrometry and sCD14 was assessed by enzyme-linked immunosorbent assay (ELISA). IDO-mRNA expression was quantified by reverse transcription–polymerase chain reaction (RT–PCR). The *in-vitro* functional assay of sCD40L on T_{reg} induction and T cell activation were assessed on peripheral blood mononuclear cells (PBMCs) from HS. sCD40L levels in ART-naive subjects were significantly higher compared to ST and HS, whereas EC showed only a minor increase. In ART-naive alone, sCD40L was correlated with T cell activation, IDO-mRNA expression and CD4 T cell depletion but not with viral load. sCD40L was correlated positively with IDO enzymatic activity (Kyn/Trp ratio), T_{reg} frequency, plasma sCD14 and inflammatory soluble factors in all HIV-infected patients. *In-vitro* functional sCD40L stimulation induced T_{reg} expansion and favoured T_{reg} differentiation by reducing central memory and increasing terminal effector T_{reg} proportion. sCD40L also increased T cell activation measured by co-expression of CD38/human leucocyte antigen D-related (HLA-DR). These results indicate that elevated sCD40L induces immunosuppression in HIV infection by mediating IDO-induced Trp catabolism and T_{reg} expansion.

Keywords: CD154, HIV, IDO, regulatory T cells (T_{regs}), soluble CD40-ligand (sCD40L)

macrophages and dendritic cells (DCs), plays a pivotal role in the regulation of the immune system by catabolizing tryptophan (Trp) into kynurenine (Kyn), which favours the differentiation of immunosuppressive regulatory T cells (T_{reg}) [3]. Indeed, IDO-mediated catabolism of Trp limits immune responses in physiological or pathological conditions such as pregnancy, cancer and chronic viral illness [4–6]. We and others have reported that IDO-induced Trp catabolism results in a T helper type 17 (Th17)/T_{reg}

Table 1. Clinical and virological characteristics of study groups.

Characteristics	Study population <i>n</i> = 55			
	Successfully treated (<i>n</i> = 14)	ART-naive (<i>n</i> = 14)	Elite controllers (<i>n</i> = 13)	Healthy controls (<i>n</i> = 14)
Age (years) [mean ± s.d. (range)]	46 ± 9 (29–62)	38 ± 9 (23–52)	47 ± 7 (40–60)	46 ± 8 (30–60)
Male [<i>n</i> (%)]	12 (86%)	13 (93%)	7 (54%)	9 (64%)
Time since HIV-1 diagnosis (years) [mean ± s.d. (range)]	9 ± 4 (3–18)	6 ± 6.5 (0–19)	13 ± 4 (4–20)	n.a.
CD4 T cell count (cells/μl) [mean ± s.d. (range)]	660.0 ± 244 (403–1177)	322 ± 183 (3–523)	624 ± 247 (417–1341)	988 ± 301.5 (519–1559)
CD8 T cell count (cells/μl) [mean ± s.d. (range)]	816 ± 265 (452–1381)	844 ± 354 (272–2180)	596 ± 357 (162–1198)	459 ± 159 (227–843)
CD4 : CD8 ratio [mean ± s.d. (range)]	0.86 ± 0.32 (0.44–1.51)	0.4 ± 0.328 (0.01–1.07)	1.34 ± 0.68 (0.35–2.72)	2.32 ± 0.85 (1.16–3.97)
Viral load (log ₁₀ copies/ml) [mean ± s.d. (range)]	<1.6	4.6 ± 0.8 (2.89–6.35)	<1.6	n.a.
Time since start of ART (years) [mean ± s.d. (range)]	7 ± 3 (3–14)	n.a.	n.a.	n.a.
Monocyte count (10 ⁹ /l) [mean ± s.d. (range)]	0.44 ± 0.15 (0.20–0.71)	0.53 ± 0.22 (0.19–0.91)	0.52 ± 0.17 (0.26–0.90)	0.53 ± 0.17 (0.24–0.76)
Platelet count (10 ⁹ /l) [mean ± s.d. (range)]	234.1 ± 51.4 (178–363)	209.6 ± 55.33 (122–303)	282 ± 121.6 (144–511)	246.5 ± 50.2 (145–323)
sCD40L (pg/ml) [mean ± s.d. (range)]	502 ± 625 (92–2165)	1279 ± 793 (119–3336)	995 ± 977 (16–3049)	601 ± 650 (105–2673)

These include anti-retroviral therapy (ART) successfully treated (ST), ART-naive, elite controllers (EC) and healthy subjects (HS). Results are shown as mean ± standard deviation (s.d.) and (range); n.a. = not applicable.

imbalance and favours microbial translocation in HIV chronic infection [3,7,8]. IDO induction in DCs is regulated by several stimulators such as interferons (IFNs), lipopolysaccharides (LPS), Toll-like receptors (TLRs) and cytotoxic T lymphocyte antigen-4 (CTLA-4) ligation [9–11].

More recently, induction of IDO has been observed through CD40L signalling [12,13]. CD40L, also known as CD154, is a membrane glycoprotein and a functional trimer belonging to the tumour necrosis factor (TNF) superfamily, which is expressed on antigen-presenting cells, epithelial cells and haematopoietic progenitor cells [14,15]. The soluble form of CD40L (sCD40L, sCD154) is a functional trimer retaining its ability to bind receptors [16]. sCD40L is shed from activated T cells and platelets [17–19], and plays a crucial role in the co-stimulatory pathways involved in humoral B cell responses [20,21]. The elevated CD40L plays a harmful role in the humoral response due to persistent chronic activation of B cells [22,23]. Levels of sCD40L have been implicated in increasing platelet–monocyte complexes which are normally activated only during inflammatory conditions [24]. Importantly, the CD40–CD40L pathway also contributes to the establishment of gut mucosal inflammation [25,26], as elevated levels of sCD40L are observed in inflammatory bowel disease [27]. Interestingly, increased plasma levels of sCD40L have been reported in HIV-infected patients [14,28,29] with dementia [24,29,30] and impairing peripheral DCs innate immune responses [28]. More recently, high plasma levels of sCD40L in cancer

patients were shown to directly induce immunosuppression via the expansion of T_{reg} and production of inflammatory cytokines [31].

In this study we assessed the immunosuppressive role of sCD40L, making the link between IDO enzyme activity, altered Th17/T_{reg} balance and sCD14, a marker of microbial translocation in HIV-infected patients.

Material and methods

Study population

Peripheral blood mononuclear cells (PBMC) and plasma samples were collected from untreated ART-naive patients, ART-successfully treated patients (ST) and healthy subjects (HS) at the Chronic Viral Illness Service, McGill University Health Centre (MUHC), Montreal, QC, Canada. Samples from elite controllers (EC) were obtained from the FRQ-S slow progressor cohort, Montreal, QC, Canada (Table 1). All study subjects gave their written consent and the study was approved by the Research Ethics Board of MUHC.

Multiplex quantification of plasmatic soluble factors

Prior to analysis, all plasma samples were treated 10:1 with 5% Triton X-100 for 1 h at room temperature to inactivate the HIV-1 particles. Plasma levels of selected inflammatory soluble factors, including IL-1RA, IL-1β, IL-7, IL-8, IL-10, IFN-α2, IFN-γ, TNF-α, TNF-β, IP-10, sCD40L, macrophage

inflammatory protein (MIP)-1 α and MIP-1 β , were measured in duplicate using a MILLIPLEX-MAP kit, according to the manufacturer's instructions (Millipore, Billerica, MA, USA). Mean fluorescence intensities for each analyte in each sample were detected using the MAGPIX instrument (Luminex, Austin, TX, USA) and the results were analysed using the Millipore Analyst software version 3.5.5.

Quantification of IDO-1 mRNA

Total RNA from PBMCs were extracted with RNeasymini kits (Qiagen, Hilden, Germany). cDNA was synthesized from total RNA (500 ng) with oligo-dT and random hexamer primers (Applied Biosystems, Carlsbad, CA, USA) using an Omniscript reverse transcriptase kit (Qiagen). Then, specific mRNAs were amplified using the following primers: IDO-1 (forward: CGCTGTTGGAAATAGCTTCTTGC; reverse: CTTCCAGAACCCCTTCATACACC), and 18 s ribosomal RNA (forward: ATCAACTTTCGATGGTAGTCG; reverse: TCCTTGGATGTGGTAGCCG). Quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed by Rotorgene 3000 thermal cycler (Corbett Life Science, Sydney, Australia) and Quantitect Sybr Green PCR kit (Qiagen). The cycling conditions were 10 min at 95°C, then 35 cycles of 30 s at 94°C, 45 s at 60°C and 30 s at 72°C for 18 s rRNA. For IDO-1 amplification, 40 cycles were performed with 60 s annealing at 55°C followed by 30 s at 72°C. A final melting curve was obtained from 72 to 95°C. The expression of each gene was quantified relative to the housekeeping gene 18 s rRNA.

In-vitro characterization of Th17 cells

PBMCs were cultured in 48-well culture plates at $0.5\text{--}1 \times 10^6$ cells/ml per well and stimulated with 5 ng/ml phorbol myristate acetate (PMA) and 1 $\mu\text{g/ml}$ ionomycin (both from Sigma Aldrich, Oakville, ON, Canada) for 2 h at 37°C. Two $\mu\text{g/ml}$ brefeldin A (Sigma) was then added to block cytokine secretion and cells were cultured for 18 h at 37°C. peripheral blood mononuclear cells (PBMCs), then underwent surface staining followed by fixation and permeabilization using a Cytotfix/Cytoperm Permeabilization kit (BD Bioscience, Mississauga, ON, Canada) for intracellular staining with IL-17A and IFN- γ (positive control).

Flow cytometry

Flow cytometry was performed using a four-laser LSRII flow cytometer (BD Bioscience). The following antibodies were used: CD3-Pacific blue, CD4-peridinin chlorophyll cyanin 5.5 (PerCpCy5.5), CD4-phycoerythrin (PE)Cy5, CD8-Alexa700, CD25-PE, CD127-PECy7, CD27-Alexa700, human leucocyte antigen D-related (HLA-DR)-allophycocyanin (APC)-Cy7, CD38-APC and IFN- γ -Alexa700

(BD Bioscience); CD45RA-ECD from Beckman Coulter (Mississauga, Ontario, Canada) and IL-17-PE, CD8-APCeFluor780, forkhead box protein 3 (FoxP3)-Alexa488 and FoxP3 staining buffer from eBioscience (San Diego, CA, USA). The viability marker Vivid (Invitrogen, Burlington, ON, Canada) was used in all our cytometry panels to exclude the dead cell population from our analysis. Data were analysed using FlowJo software (version 7.6.5; TreeStar Inc., Ashland, OR, USA). T_{regs} were characterized as CD3⁺CD4⁺CD25^{high}CD127^{low}FoxP3^{high} and Th17 cells as CD3⁺CD4⁺IL-17a⁺ upon PMA/ionomycin stimulation (Supporting information, Fig. S1).

Measurement of plasma levels of IL-6, sCD14 and IL-10

Plasma levels of IL-6 and sCD14 were measured using commercially available human sCD14 and IL-6 enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MN, USA). We quantified IL-10 production in the supernatant of *in-vitro*-stimulated PBMCs using an IL-10 ELISA kit (eBiosciences, San Diego, CA, USA).

In-vitro sCD40L stimulation of PBMCs

PBMCs from 10 healthy subjects were cultured in RPMI containing 10% fetal bovine serum (FBS) and 10 ng/ml recombinant IL-2 (R&D Systems, Minneapolis, MN, USA) with or without 2 $\mu\text{g/ml}$ recombinant soluble CD40 ligand (sCD40L) (Enzo Life Sciences, Farmingdale, NY, USA) for 5 days at 37°C. The cells were cultured at 0.5×10^6 cells/ml per well in a 48-well culture plate. At day 2, the plate was centrifuged at 400 g for 5 min and half the medium was removed and boosted with the same concentrations of IL-2, with or without sCD40L. At day 5, the cells were transferred into fluorescence activated cell sorter (FACS) tubes, then washed and stained for T_{reg} expression and T cell activation.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 5. The Kruskal–Wallis test was performed for comparison between study groups, and Mann–Whitney *U*-tests or Wilcoxon's matched-pairs tests were used for comparison of two variables. Spearman's correlation test was used to identify association among study variables.

Results

Elevated plasmatic sCD40L in ART-naive HIV-infected patients is associated with CD4 T cell depletion but not with HIV viral load

It has been shown previously that HIV-infected patients have a higher plasma level of sCD40L compared to healthy subjects [14,28,29]. Accordingly, our team reported recently

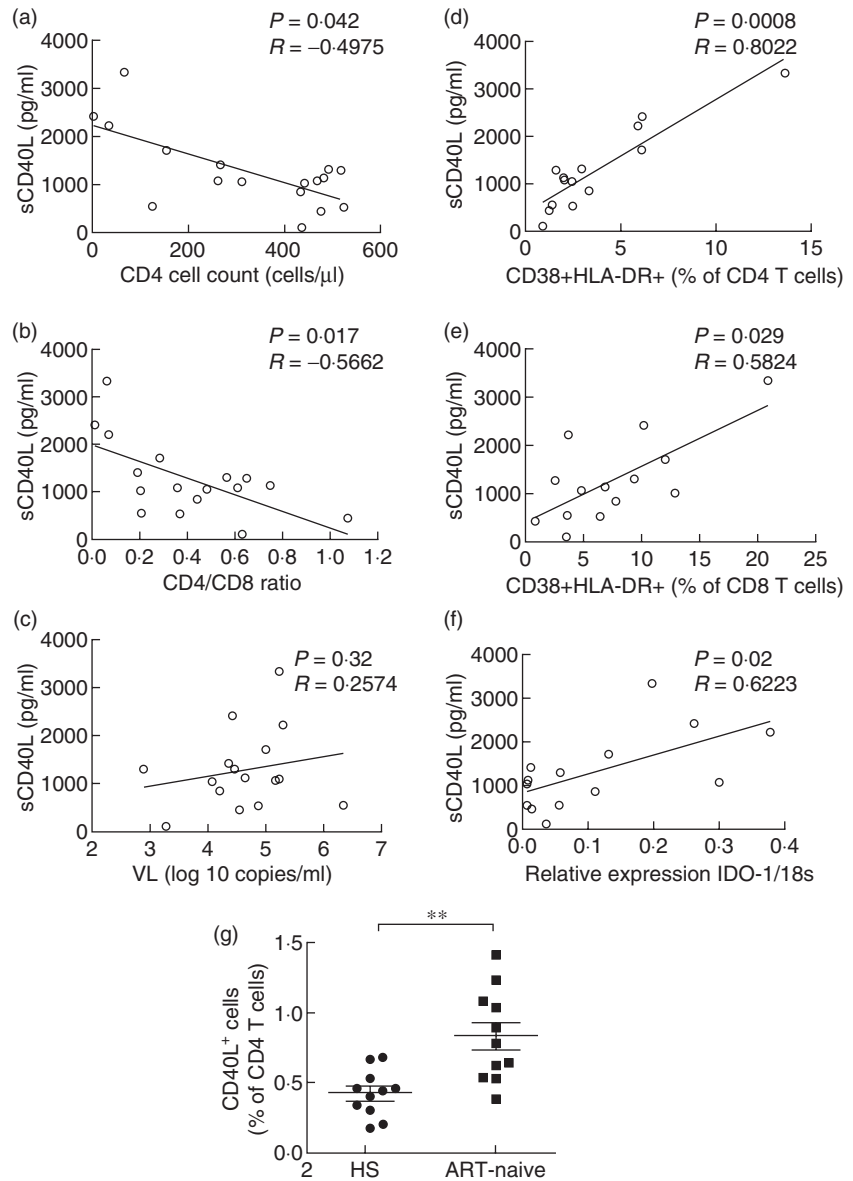


Fig. 1. Plasma sCD40L is associated with CD4 T cell counts and indoleamine 2,3-dioxygenase (IDO)-1 mRNA expression but not HIV viral load in anti-retroviral therapy (ART)-naive patients. Association of plasma sCD40L levels are shown with (a) CD4 T cell count, (b) CD4:CD8 T cell ratio (c) HIV viral load (VL), (d) CD4 and (e) CD8 T cell activation as well as (f) IDO-1 mRNA expression. (g) Increased CD40L expression by CD4 T cells in ART-naive patients. Significant values determined by either Spearman's correlation or Mann–Whitney *U*-tests; ** $P < 0.01$.

that ART-naive HIV-infected patients displayed significantly elevated plasmatic sCD40L when compared to ST and HS, whereas, following ART in ST patients, plasmatic sCD40L levels became identical to EC and HS [7] and Table 1. For the ART-naive group, sCD40L was found to be correlated negatively with CD4 T cell counts (Fig. 1a) and CD4 : CD8 T cell ratio (Fig. 1a), but not correlated with viral load (Fig. 1c). Positive correlation was observed between sCD40L plasma levels and T cell immune activation defined by co-expression of CD38/HLA-DR on CD4 and CD8 T cells (Fig. 1d,e), as well as IDO-mRNA expression in ART-naive patients ([7] and Fig. 1f). However, no correlation was observed between sCD40L and the expression of two other Trp catabolizing enzymes, IDO-2 and TDO mRNA (Spearman's $P > 0.05$, data not shown). In line with a previous study [28], we also observed a significant increase in the

CD40L expression by CD4 T cells in ART-naive patients (Fig. 1g). As CD40L is highly expressed by platelets, which could be a major contributor of circulating sCD40L [32–34], we compared platelet counts in the study groups and no significant difference was observed (Kruskal–Wallis test $P > 0.05$, Table 1).

Plasma levels of sCD40L are associated with altered Th17/T_{reg} balance, microbial translocation and systemic inflammation in HIV-infected patients

It has been proved previously that sCD40L is involved in induction of IDO expression and activity [20]. In line with this, we showed recently that plasma sCD40L levels are associated with a higher IDO enzyme activity (Trp/Kyn) ratio in HIV-infected patients [7]. Indeed, in the patients

studied here, a strong positive correlation was observed between levels of plasma sCD40L and IDO enzyme activity when evaluated in all HIV-infected patients (Spearman's $P = 0.005$, $r = 0.4317$, Fig. 2a). We and others have shown that higher IDO-induced Trp catabolism is involved in the imbalance of Th17/T_{reg} in favour of T_{reg} by stimulation of T_{reg} differentiation in HIV infection [3,7]. Recently, it has been shown in cancer that sCD40L can induce T_{reg} expansion [31]. We therefore evaluated the possible association between sCD40L, T_{reg} frequency and the Th17/T_{reg} ratio. ART-naïve patients had the highest T_{reg} absolute number with lowest Th17 frequencies when compared to ST, and the proportion of T_{reg} as well as the Th17/T_{reg} ratio was normalized following ART (data not shown). Interestingly, plasma sCD40L levels were correlated with increased T_{reg} frequency ($P = 0.01$, $r = 0.4001$) (Fig. 2b) and a low Th17/T_{reg} ratio in all HIV-infected patients ($P = 0.008$, $r = -0.4210$) (Fig. 2c). Such a correlation was not observed with Th17 cell frequency ($P = 0.37$, data not shown). Altogether, these results suggest the involvement of sCD40L in T_{reg} induction via IDO enzyme activity. Furthermore, a low Th17/T_{reg} ratio is harmful to gut mucosal immunity, resulting in microbial translocation in HIV infection [35–37]. Importantly, elevated levels of the monocyte activation marker sCD14, a reliable marker of microbial translocation in HIV infection [37], was also correlated positively with plasma levels of sCD40L when evaluated in all HIV-infected patients (Fig. 2d).

We evaluated which inflammatory soluble factors were most associated with sCD40L levels among HIV-infected patients. Results presented in Table 2 showed that sCD40L was correlated positively with multiple inflammatory soluble factors, including markers of HIV disease progression such as IL-6 and IP-10 [2,38,39].

In-vitro sCD40L stimulation induces T_{reg} expansion and differentiation

It has been shown that sCD40L plays an immunosuppressive role in cancer via induction of T_{regs} [31]. To investigate the direct role of sCD40L on T_{reg} expression, a 5-day *in-vitro* sCD40L stimulation of PBMCs from healthy donors ($n = 10$) was performed in cell culture. T_{reg} subsets were characterized according to the expression of CD45RA and CD27 (Fig. 3a). Results presented in Fig. 3b show a significant increase of T_{reg} frequency following sCD40L stimulation compared to non-stimulated PBMCs (1.67 ± 0.57 versus $1.37 \pm 0.6\%$, Wilcoxon's matched-pairs $P = 0.037$). To evaluate the potential impact of sCD40L on T_{reg} differentiation, we further assessed the changes in T_{reg} subsets and phenotypes following *in-vitro* sCD40L stimulation. Interestingly, sCD40L favoured T_{reg} differentiation by reducing central memory T_{regs} (44.41 ± 18.45 versus $51.53 \pm 17.24\%$, Wilcoxon's matched-pairs $P = 0.019$; Fig. 3c) and increasing terminal effector T_{regs} (6 ± 3.6 versus $1.2 \pm 1.33\%$,

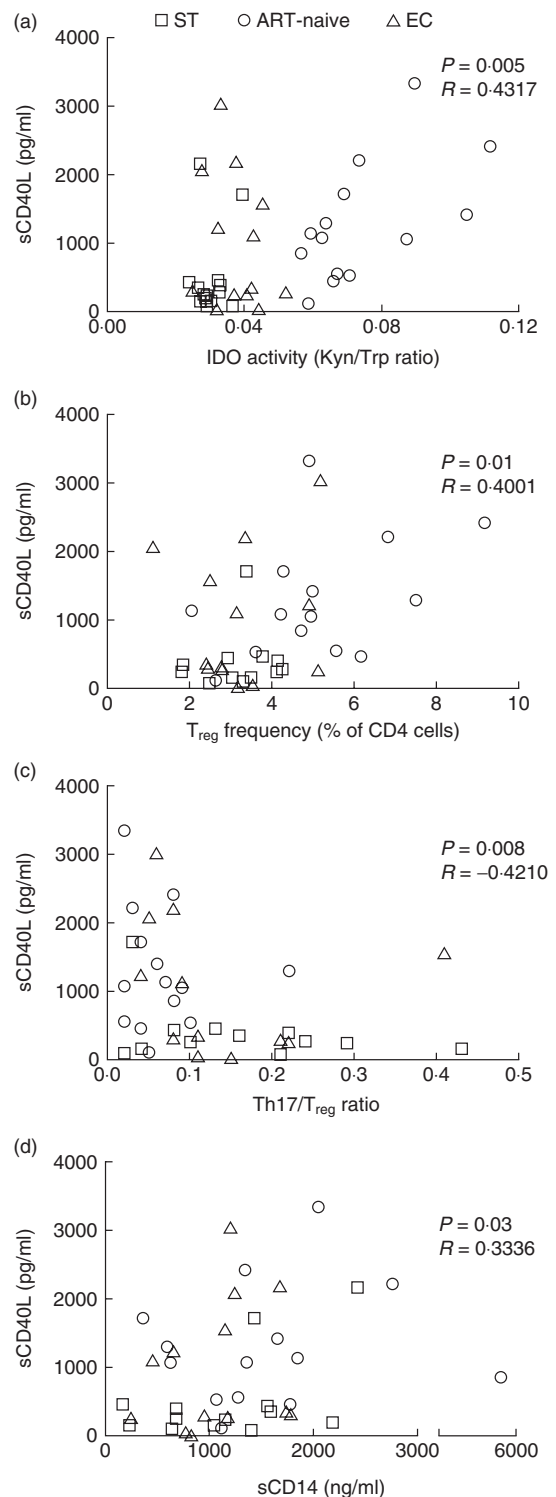


Fig. 2. Plasma sCD40L levels correlates with indoleamine 2,3-dioxygenase (IDO) enzyme activity, T helper type 17 (Th17)/regulatory T cell (T_{reg}) imbalance and microbial translocation in HIV-infected patients. Correlations are shown between plasma sCD40L levels and (a) IDO enzyme activity, (b) T_{reg} frequency, (c) Th17/T_{reg} ratio and (d) marker of microbial translocation sCD14 in HIV-infected patients. *P*-values determined by Spearman's test. EC = elite controllers; ST = anti-retroviral therapy (ART) treated successfully.

Table 2. Correlations between plasma sCD40L levels and other inflammatory soluble markers in all HIV-infected patients. Spearman’s test was used for statistical analysis.

	IL-1RA	IL-1β	IL-6	IL-7	IL-8	IL-10	IP-10	IFN-γ	TNF-α	TNF-β	MIP-1α	MIP-1β
P-value	<0.0001	<0.0001	0.04	<0.0001	0.0003	0.004	0.05	0.017	0.038	0.0003	0.03	<0.0001
Spearman’s r	0.5737	0.5820	0.3121	0.7269	0.4734	0.4389	0.3038	0.3701	0.3254	0.5363	0.3352	0.4872

IL = interleukin; IFN = interferon; TNF = tumour necrosis factor; IP = IFN gamma-induced protein 10; MIP = macrophage inflammatory protein.

Wilcoxon’s matched-pairs $P = 0.004$; Fig. 3d). To test the hypothesis that sCD40L can directly impact T_{reg} , we evaluated the expression of CD40 on T_{reg} . The expression of CD40 on T_{reg} was significantly higher compared to non- T_{reg} conventional CD4 T cells (4.4 ± 1 versus 2.6 ± 0.8 , Wilcoxon’s matched-pairs $P = 0.001$, Fig. 4a). Interestingly, we observed the highest expression of CD40 on the terminal effector (TE) subset of T_{reg} , suggesting a potential direct impact of sCD40L on T_{reg} (Kruskal–Wallis $P = 0.0015$, Fig. 4b). To assess whether the generation of T_{reg} in this model was induced by T cell activation, we evaluated the potential impact of sCD40L stimulation on the co-expression of activation markers CD38 and HLA-DR on CD4 and CD8 T cells. Results showed that sCD40L increased both CD4 and CD8 T cell activation in our *in-vitro* model (2.55 ± 2 versus 3.36 ± 2.6 , $P = 0.01$ and

4.75 ± 5.1 versus 6.32 ± 5.5 , $P = 0.05$, respectively, Fig. 5a,b). To evaluate if sCD40L was able to change the milieu of common secreted cytokines between T_{reg} and monocytes, we quantified the production of IL-10 in our model. Therefore, either total PBMCs or monocyte-depleted PBMCs (depletion performed by cell sorting) were stimulated with sCD40L over 5 days, as described in the Methods section. At day 5, supernatants were removed and the IL-10 production was quantified by ELISA. Our results showed no significant difference in IL-10 production by total PBMCs between non-stimulated and sCD40L-stimulated conditions (28.3 ± 33.4 versus 32 ± 34.9 pg/ml, respectively, Wilcoxon’s matched-pairs $P = 0.31$, data not shown). As expected, the IL-10 production by monocyte-depleted PBMCs was lower, but no significant difference was observed between non-stimulated and sCD40L-stimulated conditions (2.23 ± 2.78

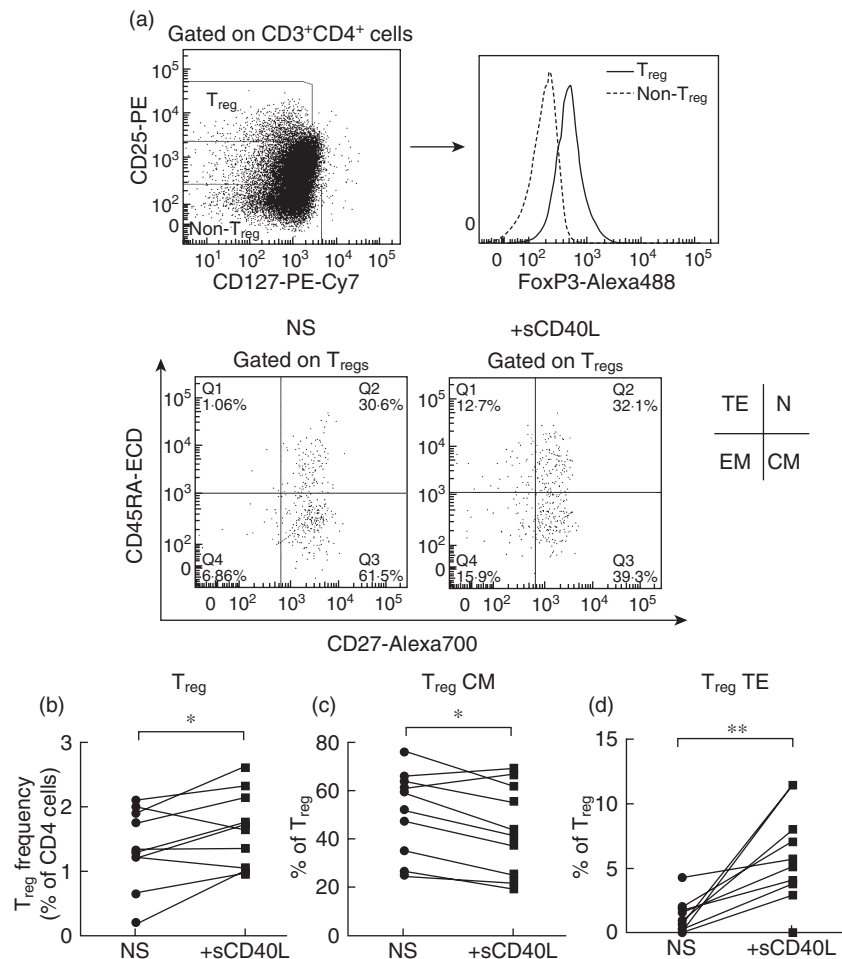


Fig. 3. *In-vitro* sCD40L stimulation causes regulatory T cell (T_{reg}) expansion and differentiation. (a) Gating strategy used for characterization of T_{reg} s and T_{reg} subsets including naive (N), central memory (CM), effector memory (EM) and terminal effector (TE). (b) Impact of *in-vitro* stimulation by sCD40L on (b) total T_{reg} s, (c) central memory T_{reg} and (d) terminal effector T_{reg} . $n = 10$; P -values determined by Wilcoxon’s matched-pairs test; * $P < 0.05$; ** $P < 0.01$.

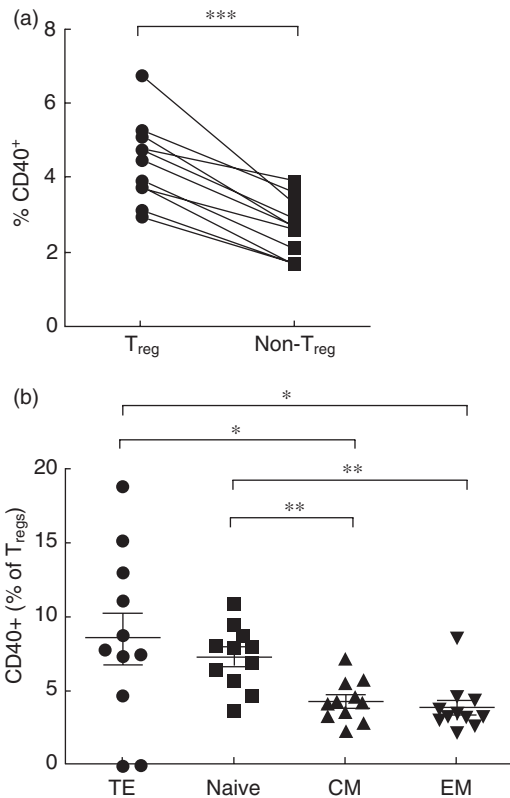


Fig. 4. Regulatory T cells (T_{reg}) express higher levels of extracellular CD40. (a) T_{reg} express higher levels of extracellular CD40 compared to non- T_{reg} CD4 T cells. Wilcoxon’s matched-pairs test was used for statistical analysis. (b) *Ex-vivo* expression of CD40 on T_{reg} subsets, including naive (N), central memory (CM), effector memory (EM) and terminal effector (TE), Kruskal–Wallis and Mann–Whitney *U*-tests were used for statistical analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

versus 6.8 ± 11.72 pg/ml, respectively, Wilcoxon’s matched-pairs $P = 0.5$, data not shown). Our results are in line with previously published articles, which indicated that neither IL-2 [40] nor sCD40L [28,31] are strong stimuli capable of inducing large amounts of IL-10.

Discussion

The co-stimulatory molecule CD40L is expressed mainly on the surface of activated T cells [41], and to a lesser extent by B cells and platelets. CD40L plays a key role in T cell-dependent B cell responses and stimulating antigen-presenting cells [21,42]. CD40L signalling has been implicated in diseases associated with immune activation such as HIV [43,44]. Over-expression of CD40L on the surface of activated CD4 T cells promotes HIV replication, contributing to CD4 T cell depletion [43,44]. The soluble form of CD40L (sCD40L) is cleaved and released from the cell surface, where it retains its ability to bind to the CD40 receptor [33]. Elevated sCD40L in HIV-infected patients compared to healthy controls have been observed previously [14,28,29]. In line with these findings, our results showed a higher level of plasma sCD40L in ART-naive chronically infected patients that became comparable to healthy subjects in ART-treated patients in association with CD4 T cell lymphopenia and the CD4 : CD8 T cell ratio, but not HIV viral load. It was not surprising that sCD40L was elevated in ART-naive subjects with low CD4 T cell counts, as CD40L is expressed on activated T cells, which are the main targets for HIV infection [15,41]. Furthermore, it has been shown recently that the cell cycling CD4 memory T cells in chronically HIV-infected patients contains lower intracellular CD40L compared to healthy subjects [45]. Surprisingly, however, in the study by Sipsas *et al.*, despite the higher plasma sCD40L levels evaluated in 77 patients with a mean CD4 T cell count of 335 ± 248 cells/ μ l compared to healthy subjects, the correlation between CD4 T cell count and plasma sCD40L was seen only for patients having CD4 T cell count < 130 cells/ μ l, and they observed an unexpected positive correlation between CD4 count and sCD40L [14]. Moreover, platelets represent one of the main sources of plasmatic sCD40L released upon stimulation in a variety of inflammatory disorders [17]. In the Sipsas *et al.* study, platelet count was not assessed and it may, in part, explain the different findings. We observed no differences in platelet counts between study groups, despite

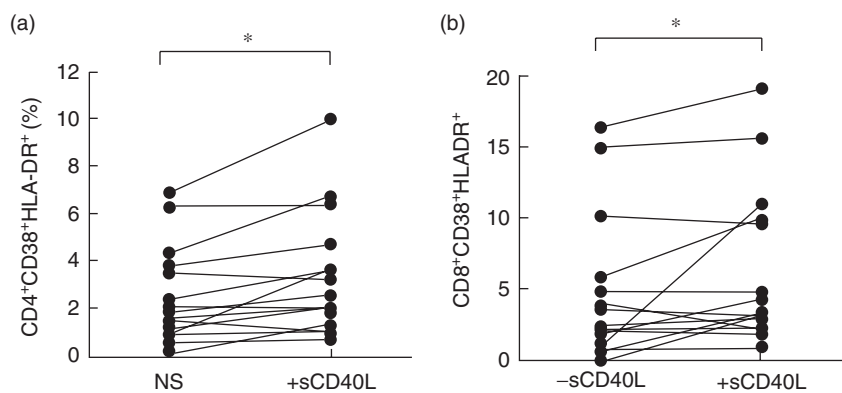


Fig. 5. Increase of T cell activation following sCD40L stimulation. Increase of T cell immune activation defined by the co-expression of CD38 and human leucocyte antigen D-related (HLA-DR) on (a) CD4 and (b) CD8 T cells following *in-vitro* sCD40L stimulation. Wilcoxon’s matched-pairs test was used for statistical analysis; * $P < 0.05$.

important differences in plasmatic sCD40L indicating that the activated T cells were most probably the source of elevated sCD40L in HIV infection [10,22]. Indeed, we observed strong associations between sCD40L levels and T cell immune activation defined by co-expression of CD38/HLA-DR in ART-naive patients. Accordingly, we observed a higher membrane expression of CD40L in ART-naive patients compared to HS, which confirms a previous study [28].

The catabolism of Trp into Kyn via IDO enzyme expressed by DCs plays a detrimental immunosuppressive role in HIV infection by favouring T_{reg} expansion [3,7]. Of particular interest, IDO-1 activity has been observed through CD40L signalling after T cell interactions. CD40L may further heighten IDO responses, behaving as a second stimulus [10,13,20,46]. Indeed, stimulation and maturation of immature DC by sCD40L induces IDO expression [10,13], resulting in inhibition of T cell proliferation [10]. Furthermore, sCD40L as well as Cholera toxin increased the expression of IDO mRNA in mDC in association with a higher Kyn/Trp ratio *in vitro* [12]. Accordingly, we observed a positive correlation between sCD40L levels and IDO-1 mRNA and not with the two other Trp catabolizing enzymes IDO-2 and TDO in ART-naive viraemic patients. In addition, our results showed that plasma sCD40L was associated positively with IDO enzyme activity as defined by the Kyn/Trp ratio in all HIV-infected patients. As emphasized earlier, IDO-induced Trp catabolism favours T_{reg} expansion and has a harmful impact on the gut mucosal immunity by decreasing the Th17/ T_{reg} ratio [3,7,8,35]. Our results showed a positive association between sCD40L levels and T_{regs} but not with Th17 frequency, resulting in a lower Th17/ T_{reg} ratio. An impaired Th17/ T_{reg} balance favours microbial translocation from gut to peripheral blood in HIV infection [1,8,37]. In line with this, our results show a positive correlation between higher sCD40L and a reliable marker of microbial translocation, sCD14 [37]. In addition, a higher expression of CD40L has been observed in inflammatory bowel disease [25,26], where the CD40–CD40L pathway contributes to the proinflammatory function of intestinal epithelial cells [47].

More recently, CD40L was shown to be immunosuppressive in cancer via the direct expansion of T_{regs} [31]. To further explore its possible immunosuppressive role in HIV infection, we performed an *in-vitro* functional assay that demonstrated an expansion of T_{reg} frequency upon sCD40L stimulation in HIV-negative subjects. Moreover, we showed that sCD40L not only induced an expansion of T_{regs} , but also favoured T_{reg} differentiation by reducing central memory cells that are important for antigen-specific immune responses and increasing terminal effector cells. One of the potential impacts of sCD40L on T_{reg} expansion and differentiation could be due to a direct effect of sCD40L on these cells, as we observed a higher expression of CD40 on T_{regs} versus non- T_{reg} conventional CD4 T cells

and the highest expression of CD40 by the TD subset of T_{regs} . In addition, we observed a higher T cell activation defined by the co-expression of CD38 and HLA-DR on CD4 and CD8 T cells following sCD40L stimulation. However, no significant change in IL-10 production was observed following sCD40L treatment, as sCD40L is not a strong stimulus for the induction of large amount of IL-10 [28,31]. During inflammatory conditions possibly influenced by elevated sCD40L, further signalling mediated by this co-stimulatory molecule may play an immunosuppressive role in HIV infection by favouring DC-induced IDO stimulation associated with T_{reg} expansion and differentiation. Interestingly, it has been reported recently that T_{reg} cells have the lowest intracellular CD40L among all CD4 T cell subsets to avoid compromising their suppressive activity [48].

Our results showed that the levels of sCD40L correlated with multiple inflammatory soluble factors, including HIV disease progression IL-6 [2,38] and IP-10 [38,39]. We also observed a correlation between sCD40L and IL-1RA, which is strongly associated with T cell activation whose level is linked with the altered Th17/ T_{reg} balance [49]. It has been shown recently by the Maggirwar S.B. team that CD40–CD40L signalling has been implicated in HIV-associated neuroinflammation, as the HIV Tat protein can increase blood–brain barrier (BBB) permeability in a CD40L-dependent manner [30,50]. sCD40L has been implicated in increasing platelet–monocyte complexes, which are normally activated only during inflammatory conditions [50–52]. In addition, Tat was shown to induce platelet activation *in vivo*, leading to an increase in plasma sCD40L concentration [30]. Moreover, an increase in CD40L in both blood and cerebrospinal fluid of cognitively impaired HIV-infected patients was implicated in central nervous inflammation while impacting neuronal survival in alliance with the HIV-1 Tat protein [29]. Indeed, CD40L, together with Tat, increased TNF- α release from primary monocytes and microglia in a nuclear factor kappa B (NF- κ B)-dependent manner [29]. CD40L may also stimulate IL-8 production in HIV-infected patients via NF- κ B activation [53]. Notably, we observed an association between sCD40L and IL-8 levels. sCD40L-induced IL-8 production was shown to also be involved in neuroinflammation in HIV-infected patients [54]. Furthermore, previous studies have also reported that CD40L expressed by HIV-1 virions may augment virus transfer from CD40-expressing B cells, which are not HIV-reservoirs, to CD4 T cells upon entry into lymphoid tissues [23]. Studies have also shown that sCD40L favours the HIV-induced dysfunction of humoral immunity by activation of B cells via CD40 signalling [22].

Together, our results indicate that elevated sCD40L induces immunosuppression in HIV infection by mediating IDO-induced Trp catabolism and T_{reg} expansion. Therapeutic strategies which decrease sCD40L levels, such as valproic acid [50,55,56], or other compounds reversing

inflammation could be helpful to overcome sCD40L release in the periphery in the context of HIV infection.

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Disclosures

No conflicts of interest to declare.

Author contributions

M. A. J. and J. P. R. designed the study; M. A. J., I. K., M. P., K. V., P. T. and C. T. performed the experiments; M. A. J., D. R., P. A., N. G. and J. P. R. analysed and discussed the data; D. R., R. L., C. T., P. A. and J. P. R. contributed reagents/materials/analysis tools; M. A. J. and J. P. R. wrote the paper.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Gating strategy used for characterization of T helper type 17 (Th17) cells.