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Inflammation perturbs the IL-7 axis, promoting senescence and exhaustion that broadly characterize immune failure in treated HIV infection

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Abstract

Background—HIV-infected patients who fail to normalize CD4 T cells despite suppressive antiretroviral therapy have impaired immune homeostasis: diminished naïve T cell numbers, elevated T cell turnover, senescence and inflammation.

Methods—Blood samples from immune failures (n=60), immune successes (n=20) and healthy controls (n=20) were examined for plasma IL-7 levels, for cellular expression of the IL-7Rα chain (CD127), for the exhaustion and senescence markers PD-1 and CD57, and for the survival factor Bcl2. As both inflammatory and homeostatic cytokines can induce T cell cycling, we also examined the effects of these mediators on exhaustion and senescence markers.

Results—Plasma levels of IL-7 were elevated and both CD4 and CD8 T cell CD127 expression was decreased in immune failure. Plasma levels of IL-7 correlated directly with naïve CD4 T cell counts in immune success and inversely with T cell cycling (Ki67) in healthy controls and immune success, but not in immune failure. CD4 T cell density of PD-1 was increased and Bcl2+ CD4 T cells were decreased in immune failure but not in immune success, while the proportion of T cells expressing CD57 was increased in immune failure. PD-1 and CD57 were induced on CD4 but not CD8 T cells by stimulation in vitro with inflammatory (IL-1β) or homeostatic (IL-7) cytokines.

Conclusions—Perturbation of the IL-7/IL-7 receptor axis, increased T cell turnover, and increased senescence may reflect dysregulated responses to both homeostatic and inflammatory cytokines in immune failure patients.

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Introduction

Despite an increased survival , as many as 25% of HIV-infected patients with therapy-controlled viremia fail to recover CD4 T cell counts to “normal” levels [1-4]. Patients with low circulating CD4 T cell counts are at increased risk for cardiovascular disease, cancer, and liver disease [5, 6]. In earlier work, we and others found that these immune failure patients had decreased numbers of both CD4 and CD8 naïve T cells, increased activation of CD4 and CD8 T cells as measured by HLADR and CD38 expression, increased cycling of CD4 memory T cells as measured by Ki67 expression, and elevated plasma indices of inflammation and coagulation[7-10]. Immune failure patients may also have perturbations of immune homeostasis as T cell expression of the IL-7 receptor alpha chain (CD127) is diminished [11-13], and there is increased T cell expression of the immune senescence and exhaustion markers, CD57 and PD-1 [14-17]. Recovery of CD4 T cells may be impaired at many stages; naïve T cells may be less responsive to homeostatic signals, mature T cells may display increased turnover or increased sensitivity to death signals and more matured T cells may show signs of exhaustion and senescence. This study was designed to simultaneously explore aspects of CD4 T cell recovery, relating immune senescence, immune homeostasis, and inflammation in a well characterized group of treated patients. We found that markers of immune exhaustion (PD-1) and senescence (CD57) were elevated on T cells from immune failure patients and could be up-regulated when PBMCs from healthy subjects were stimulated with the homeostatic cytokine IL-7 or with the inflammatory cytokine IL-1β that we had shown earlier can drive CD4 T cell turnover [18]. Despite elevated plasma levels of IL-7 in immune failure, there was no association between IL-7 levels and T cell cycling or CD4 T cell numbers in immune failure. In contrast, plasma IL-7 levels correlated directly with naïve CD4 T cell numbers in immune success and inversely with CD4 T cell cycling in immune success and in healthy controls but not in immune failure. These findings support a model where homeostatic failure allows inflammatory mediators to drive cellular turnover in immune failure and both inflammation and homeostatic proliferation contribute to cellular exhaustion/senescence.

Methods

Patients

These studies were approved by the institutional review board (IRB) at University Hospitals/ Case Medical Center and the Cleveland Clinic Foundation and all patients provided written informed consent in accordance with the Declaration of Helsinki. The Cleveland Immune Failure (CLIF) study examined immunologic indices in healthy controls and two groups of patients who had been receiving antiretroviral therapy for at least 2 years with plasma HIV RNA levels below detection using routine clinical assays; typically less than 50 copies/mL. Immune failure patients had CD4 T cells <350/uL and immune success patients had CD4 T cells >500/uL [7].

Cytokines

Plasma levels of IL-7 were measured by high sensitivity IL-7 ELISA (Quantikine HS, R&D Systems, Minneapolis, MN).

Cell preparation

Peripheral blood mononuclear cells (PBMCs) were prepared from whole blood by ficoll-hypaque density sedimentation and were cultured in RPMI medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine at 37°C and 5% CO_2, for functional assays or cryopreserved in 10% DMSO and 90% fetal bovine serum until thawing for phenotypic analysis. Induction of PD-1 and CD57 after stimulation with IL-6, IL-1β, (R&D Systems, Minneapolis, MN), or IL-7 (Cytheris, Issy les Moulineaux, France) was examined on freshly isolated PBMCs from healthy uninfected controls.

CFSE dye dilution

Cell division was assessed by labeling PBMCs with 5(6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes Invitrogen, Grand Island, NY) for 10 minutes at 37° C. Staining was quenched by the addition of FBS for 5 minutes on ice. Cells were then washed and cultured as described.

Flow cytometry

Viable cells were gated using live/dead- yellow viability dye (Invitrogen, Grand Island, NY). Lymphocytes were identified by forward and side scatter and T cell phenotype was assessed using the following fluorochrome-conjugated monoclonal antibodies: anti-CD3 peridinin chlorophyll protein (Percp), anti-CD8 allophycocyanin-cy7 (APC-Cy7), anti-CD127 phycoerythrin (PE), anti-CD45RA phycoerythrin cy7 (PE-Cy7), anti-CD27 AlexaFluor 700, (anti-CD279) anti-PD-1 allophycocyanin (APC), anti-CD57 fluorescein isothiocyanate (FITC), (all from BD Biosciences, San Jose, CA); and anti-CD4 Pacific Blue (Biolegend, San Diego, CA). Cells were incubated with monoclonal antibodies for 20 minutes in the dark at room temperature, washed, fixed in PBS containing 0.5% formaldehyde before analysis. For detection of the intracellular protein Bcl2, cells were surface stained, fixed, and permeabilized with a saponin-based buffer (BD Biosciences, San Jose, CA) followed by incubation with anti-Bcl2-FITC (BD Biosciences) for 40 minutes on ice. Samples were acquired on an LSRII flow cytometer (Becton Dickinson, San Jose, CA) and 30,000-50,000 live-gated events were collected. Data were analyzed using FACSDIVA, (Version 6.2 BD Biosciences) or Flow-Jo software (TreeStar Ashland, OR).

Statistics

Continuous variables were compared between groups using the Mann-Whitney U test. Correlations between groups were assessed using a correlation matrix of the Spearman's rank correlation method (GraphPad Prism software, Version 5.04). *P* values of less than 0.05 were considered significant.

Results

This study was designed to ask if dysregulated immune homeostasis, and increased exhaustion and senescence might be related to an elevated inflammatory environment in well-characterized groups of patients with therapy-controlled viremia.

Immune failure is associated with perturbations in the IL-7/IL-7 receptor axis

As we had shown earlier that immune failure is associated with profound decreases in numbers of circulating naïve CD4 and CD8 T cells [7], it was important to monitor IL-7 and its receptor, key mediators of naïve T cell homeostasis [19-22].

We found that plasma levels of IL-7 were higher in immune failure than in immune success (Fig. 1a; p=0.05). Earlier studies found decreased T cell expression of CD127 associated with low CD4 T cell numbers [11, 13, 23-25]. We found a significant reduction in CD127 density on CD4 T cells from both immune success and immune failure patients when compared to CD127 density among healthy controls (p=0.03; p=0.0005 respectively), but there was no difference between CD127 density in immune failure and immune success (p=0.25) (Fig. 1b, c). CD127 density on CD8 T cells was decreased significantly in immune failure when compared to CD127 density in healthy controls (p=0.002) and in immune success (p= 0.02) (Fig. 1d, e). We also found that in immune failure, CD8 CD127 density was inversely correlated with plasma levels of IL-6 (r= -0.270, p= 0.042), LPS (r= -0.277, p=0.037), and IP-10 (r= -0.276, p= 0.037) (not shown). We had previously reported that IL-6 or IL-1β exposure decreases T cell CD127 expression in vitro[18] but type1 interferon does not [26].

Discordant relationships among plasma IL-7, T cell numbers and cycling in immune failure

Iinverse relationships between plasma IL-7 and CD4 T cell numbers [24, 27, 28] have been reported largely among patients with incomplete virologic control. Here, we find that in persons with ART-mediated control of HIV replication, plasma levels of IL-7 are higher in immune failure than in immune success; nonetheless, CD4 T cell counts and plasma IL-7 levels were not correlated in immune failure (r= 0.19, p=0.15); or when immune failures and immune successes were combined (r= 0.03, p=0.77). Plasma IL-7 rises dramatically only as CD4 T cell numbers fall below 100 cells/uL and, here CD4 T cell numbers in immune failure averaged 252 cells/uL and were not as low as in the earlier studies where this relationship was identified [24, 27, 28]. Interestingly, although plasma IL-7 levels correlated inversely with cycling (Ki67+) CD4 and CD8 T cells in immune success (r= -0.71, p=0.001; r= -0.56, p=0.01 respectively) and in healthy controls (r= -0.43, p=0.05; r= -0.62, p=0.003 respectively), they did not correlate in the immune failure patients (Fig. 2) uncovering a novel and interesting relationship between IL-7 and T cell cycling in health and successfully treated HIV infection that is perturbed in the setting of immune failure.

Immune exhaustion and senescence markers, PD-1 and CD57, are elevated on most T cell subpopulations in immune failure

As immune failure is associated with naïve T cell restoration failure and sustained turnover and relative expansion of more mature T cells, we examined T cell expression of PD-1 and CD57. Programmed death 1 (PD-1) is an inhibitory receptor expressed after T cell activation [29] and in HIV infection increased T cell expression of PD-1 is associated with functional exhaustion and disease progression [15, 16, 29-31]. Immune failure patients had greater PD-1 density on all CD4 T cell subsets than did healthy controls and immune successes indicating that irrespective of maturation phenotype, activation and exhaustion was characteristic of all helper subpopulations in immune failure (Fig. 3a, b). PD-1 density was

greater on naïve CD8 T cells in immune failure than among immune successes (p= 0.013) and among healthy controls and was greater on central memory CD8 T cells in immune failure than among healthy controls (p= 0.024) and tended to also be greater than among immune successes but not significantly (p= 0.129, Fig. 3c,d), while among more matured effector memory CD8 T cells, PD-1 densities were comparable in all patient groups (Fig 3d).

CD57 is a cell surface marker of cells that have undergone multiple rounds of cell division, have shortened telomeres, and are unable to proliferate [32]. In HIV infection, T cell expression of CD57 is elevated [13, 14, 16, 32, 33]. Here, we found that populations of CD57 negative and positive cells were readily distinguished (Fig 3 e, g) and so report these results as proportions of expressing and non-expressing cells. The proportion of CD57 positive CD4 and CD8 T cells was elevated in all maturation subsets in immune failure patients compared to proportions in healthy controls (Fig. 3e-h) and when compared to immune successes, was increased in naïve and central memory CD4 and CD8 T cells, but not among effector memory cells. (Fig. 3 e-h). In immune failures the proportion of CD4 T cells expressing CD57 was correlated with plasma levels of IL-6 (r= 0.341, p= 0.009) and CD4 and CD8 T cell activation (r= 0.397, p=0.002; r= 0.267, p=0.045, respectively) and inversely with absolute naïve CD4 and CD8 counts (r= -0.454, p= <0.001; r= -0.432, p= 0.001, respectively) (not shown).

Both homeostatic and inflammatory cytokines drive expression of exhaustion/senescence markers on CD4 T cells in vitro

While elevated T cell expression of PD-1 and CD57 has been demonstrated in HIV infection [14-16, 30, 31, 34] and here we report elevated expression of these markers in distinct T cell maturation subsets in immune failure patients. The drivers of exhaustion and senescence in this setting are not well-defined. As elevated levels of IL-7 [27, 28] and IL-6 levels are seen in immune failure [7] predictmorbid outcomes [35-37] and as increased and as increased expression of IL-1β in lymphoid tissues may drive memory CD4 T cell cycling[18], we examined the effects of these cytokines on exhaustion and senescence marker expression.

PBMCs from healthy uninfected controls were labeled with CFSE dye, stimulated with IL-6 (10ng/mL), IL-1β (10ng/mL), or IL-7 (5ng/mL) for 7 days, and then dilution of dye and proportions of PD-1 and CD57 positive cells were measured. Both IL-1β and IL-7 increased proportions of PD-1 (Fig. 4a, b) and CD57 (Fig. 4c, d) positive CD4 T cells and these increases were greater in cells that had proliferated than among cells that had not divided (Fig 4e, f). A more modest increase in the proportion of PD-1 expressing cells was seen after IL-6 exposure, and there was no effect of IL-6 on CD57. In contrast, CD8 T cells were unaffected after stimulation with IL-1β, IL-6 or IL-7 (not shown). Both CD4 and CD8 T cells divide after exposure to IL-7 while IL-6 and IL-1β induce division of CD4 but not CD8 T cells [18].

T cell Bcl2 levels are diminished in immune failure

Reduced levels of the pro-survival factor Bcl2 have been found in CD4 T cells from viremic HIV-infected patients [11] and immune failure patients [38], and CD4 T cells from these

patients have a reduced ability to increase Bcl2 after IL-7 stimulation [11, 24]. Most CD4 T cells expressed detectable levels of Bcl2, ranging from 88%-98% in healthy controls, 89%-98% in immune success patients, and 81%-98% in immune failure patients; however the median proportion of Bcl2+ CD4 T cells was lower in immune failure patients (92%) than among healthy controls (97%, p = <0.0001) or among immune success patients (96%, p= 0.0006) (Fig. 5).

Discussion

The responsiveness of T cells to IL-7 is essential for the maintenance of T cell homeostasis and for recovery from CD4 T cell deficiency [19, 21, 28, 39]. In HIV infection, plasma IL-7 levels are often elevated and correlate inversely with CD4 T cell counts [19, 24, 27]. Although we found elevated levels of plasma IL-7 in the immune failure patients, there was no correlation between plasma levels of IL-7 and CD4 T cell counts, perhaps because the CD4 T cell counts in our study were not as low as those seen in earlier studies[19, 24, 27]. Yet, in immune success but not in immune failure, IL-7 levels correlated with naïve CD4 T cell counts (r= 0.47, p= 0.04).

Reduced thymic output [40], lymph node fibrosis [41-43], and increased T cell turnover [8, 18] are all plausible contributors to immune restoration failure. Earlier studies also found decreased CD4 T cell expression of CD127 in immune failure [11, 23, 24] and linked decreased receptor expression and function to immune activation [11, 25]. We found decreased expression of the IL-7Rα chain (CD127) in T cells from both immune failure and immune success patients and that expression of CD127 on CD8 T cells was inversely correlated with plasma levels of inflammatory mediators, IL-6, LPS, and IP-10 in immune failure. In immune success, expression of CD127 on both CD8 and CD4 T cells was inversely correlated with plasma levels of IP-10. This suggests that inflammation may be driving decreased CD127 expression and inhibiting responsiveness to IL-7 and CD4 recovery in immune failure as suggested earlier by our in vitro studies where both IL-1β and IL-6 decreased CD127 expression and these cytokines as well as type 1 interferon could impair IL-7 responsiveness [18, 26]. Other studies have demonstrated that T cells from immune failure patients show reduced proliferation [11, 12] and fail to up-regulate Bcl2 [11, 12, 38] in response to IL-7. Here, we saw significantly lower proportions of Bcl2 positive CD4 T cells in immune failure, but *in vitro* responses to IL-7 were not examined. Colle et. al. found that CD4 T cell expression of CD127 predicted the IL-7-induced increase of Bcl2 and CD25 among healthy controls [44]. This association was lost in viremic and ART treated patients [44].

We found that in both healthy controls and in immune success, plasma IL-7 levels correlated inversely with T cell cycling and in immune success, plasma IL-7 directly correlated with naïve CD4 T cell counts as might be expected in the setting of a successful homeostatic response. These associations were lost in immune failure. It may seem counterintuitive that plasma levels of IL-7 are inversely correlated with T cell cycling in health and immune success. Yet, as IL-7-induced cycling is initiated in lymphoid tissues, Ki-67+ cells in blood have mostly completed division and in these two settings, have consumed IL-7 in order to do so. As IL-7 levels are regulated largely by receptor mediated uptake [21], this impact on

plasma levels of IL-7 may be more demonstrable at the lower levels of IL-7 seen in healthy controls and in immune success patients than in patients with immune failure where low levels of CD127 keep IL-7 levels high. Also, the lack of correlation between plasma IL-7 levels and T cell cycling in immune failure patients suggests that other factors may be driving T cell cycling in immune failure and are masking any relationship among IL-7 levels, T cell cycling and CD127 expression. In the setting of immune failure, cycling of memory CD4 T cells has the characteristics of broad bystander activation [45] and may be driven at least in part by exposure to IL-1β [18] and other inflammatory mediators such as IL-2 and IL-15, common gamma receptor cytokines whose expression is also elevated in lymphoid tissues [46]. Moreover, our new data demonstrating an inverse correlation between plasma levels of IL-6, LPS, and IP-10 with CD127 expression on CD8 T cells in immune failure suggests that the IL-7 axis is impaired in this inflammatory environment. Indeed, our previous studies suggest that the inflammatory cytokines IL-6 and IL-1β can down-regulate the expression of CD127 on T cells and both these cytokines and type 1 interferons can block T cell responses to IL-7 [18, 26]. Expression of each of these cytokines is increased in treated HIV infection [4, 9, 18, 35, 37, 47]. Thus, failure to reconstitute CD4 T cells on therapy may be related at least in part to inflammation-driven impaired responses to IL-7.

Memory T cells in HIV infection often express markers of senescence/exhaustion [13-16, 30-33] that is seen in settings of sustained inflammation and T cell activation [14, 48]. We refer to the PD-1+ T cells as exhausted and the CD57+ T cells as senescent , yet there is overlap between these phenotypes that is associated with growth arrest [49-51]. One difference, however, is that while “exhausted” cells demonstrate decreased cytokine production [49], “senescent cells may divide poorly, yet continue to produce inflammatory cytokines [48, 51], and by filling “T cell space” may interfere with the homeostatic regulation of T cell numbers. Markers of immune senescence and exhaustion are particularly elevated on T cells from immune failure patients [13-15] and our measures of PD-1 and CD57 on circulating T cells are confirmatory. We showed a greater proportion of CD57+ “naïve” and “central memory” CD4 and CD8 T cells in immune failure. An earlier study also showed an increased proportion of CD57+ “central memory” and “naïve” CD4 T cells in HIV infection and that patients with CD4 T cells < 200/uL had the highest proportions of senescent cells [33]. We also found elevated expression of PD-1 on all maturation subsets of CD4 T cells and on “naïve” and “central memory” CD8 T cells in immune failure. Increased PD-1 expression was reported earlier in treated and untreated HIV infection but those patients were not segregated according to the success of immune restoration [29]. Here we show that immune failure is associated with dramatic increases in PD-1 expression on T lymphocyte subpopulations despite virologic control, while among immune successes PD-1 expression is normal. As both CD57 and PD-1 expression are associated with decreased proliferative capability, their elevated expression on “naïve” and “central memory” CD4 T cells in the immune failure patients may implicate an additional mechanism impairing CD4 T cell recovery in these patients that does not resolve with ART. It is likely that other factors are more important in driving senescence and exhaustion in CD8 T cells and it should be noted that CD8 T cell numbers do not durably increase during immune recovery on ART [52].

Earlier studies have shown that common gamma chain cytokines such as IL-7 could increase expression of senescence markers PD-1 and Tim-3 on T cells, but IL-1β could not [53, 54] . Here, we demonstrate the novel finding that the inflammatory cytokine IL-1β, as well as the homeostatic cytokine IL-7 could induce expression of the exhaustion and senescence markers PD-1 and CD57 on CD4 T cells from healthy uninfected subjects while IL-6 increased CD4 T cell expression of PD-1 only modestly. These effects were most dramatic on cells that had undergone cell division. Further study will be needed to compare the functional phenotype of CD4 T cells that have proliferated in response to these inflammatory and homeostatic stimuli. By simultaneously examining these indices, in the same study, we could demonstrate associations among inflammation, exhaustion/senescence, and the IL-7/IL-7 receptor axis in patients who had recovered CD4 T cells and those who had not.

Thus, immune failure in treated HIV infection is characterized by increased levels of circulating IL-7, decreased cell surface expression of the IL-7 receptor alpha chain (CD127) in a setting characterized by naïve T cell restoration failure and increased turnover and senescence of memory CD4 T cells. A heightened inflammatory environment in treated HIV infection [35, 37] may contribute to this scenario, with inflammatory mediators IL-1β and IL-6 blunting responses to IL-7 by decreasing expression of CD127 [18] and increasing both CD4 T cell turnover [18] and senescence, and with type 1 interferon providing additional blockade of IL-7 signaling [55].

It remains to be seen whether cellular division induced by IL-7 is more likely to result in cell death in the presence of these inflammatory mediators. Markers of immune exhaustion/senescence, PD-1 and CD57, are broadly elevated on both CD8 and CD4 maturation subsets in immune failure patients and the inflammatory cytokine IL-1β and the homeostatic cytokine IL-7, that can both drive memory cell turnover, also can increase expression of PD-1 and CD57 on CD4 T cells in vitro. Thus, inflammatory cytokines may further disrupt T cell homeostasis at multiple stages of CD4 T cell recovery by promoting T cell activation and immune senescence.

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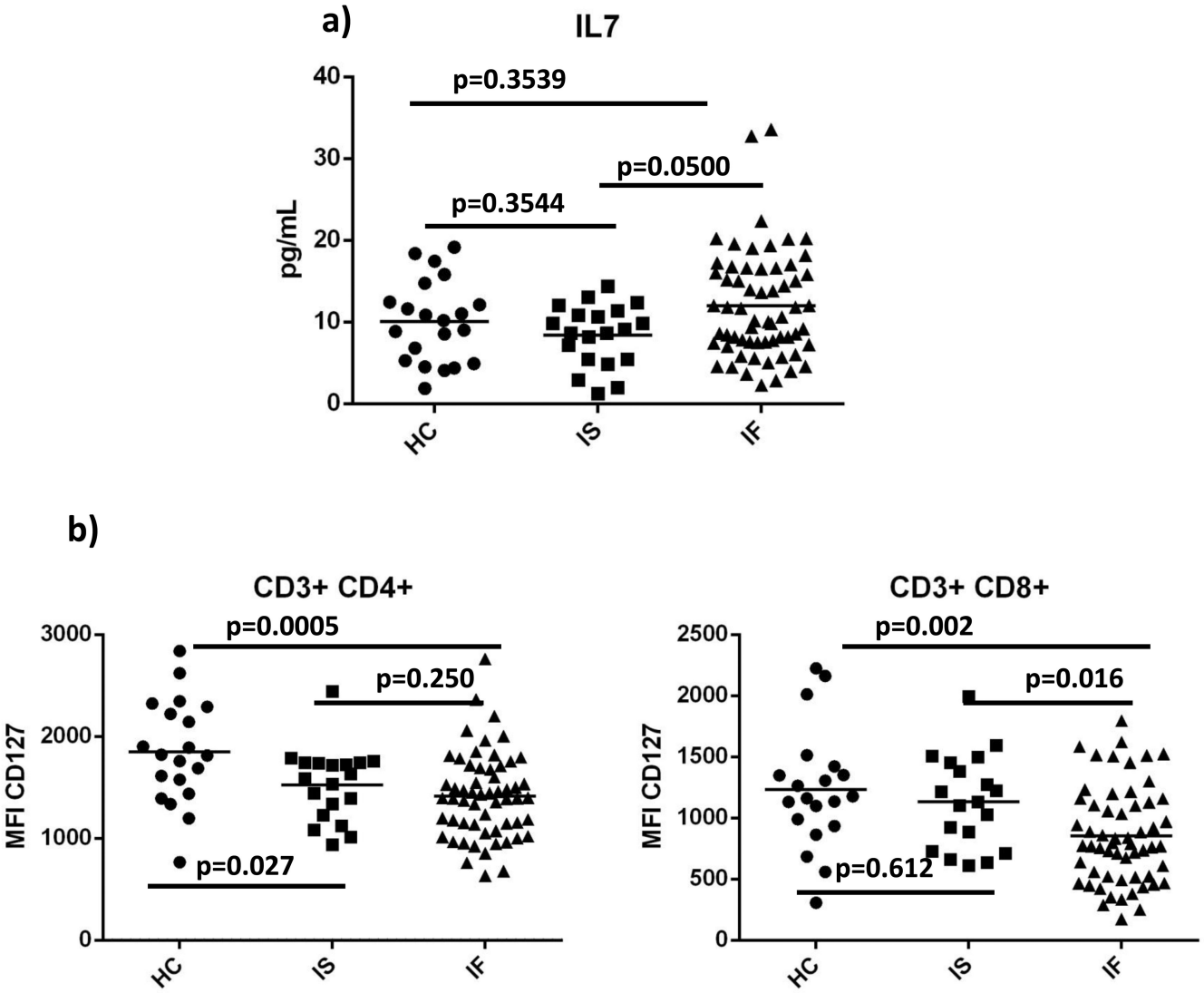

Figure 1. Plasma levels of IL-7 are elevated in immune failure and expression of the IL-7Rα chain (CD127) is decreased

Plasma levels of IL-7 were measured by ELISA (a). Surface expression of CD127 on gated CD4 and CD8 T cells was measured by flow cytometry on cryopreserved PBMC (b,d; representative samples; c,e; summary data). HC= healthy controls (n=20); IS= immune success (n=20); IF= immune failure (n=60) Bars represent medians; groups compared by Mann-Whitney U test.

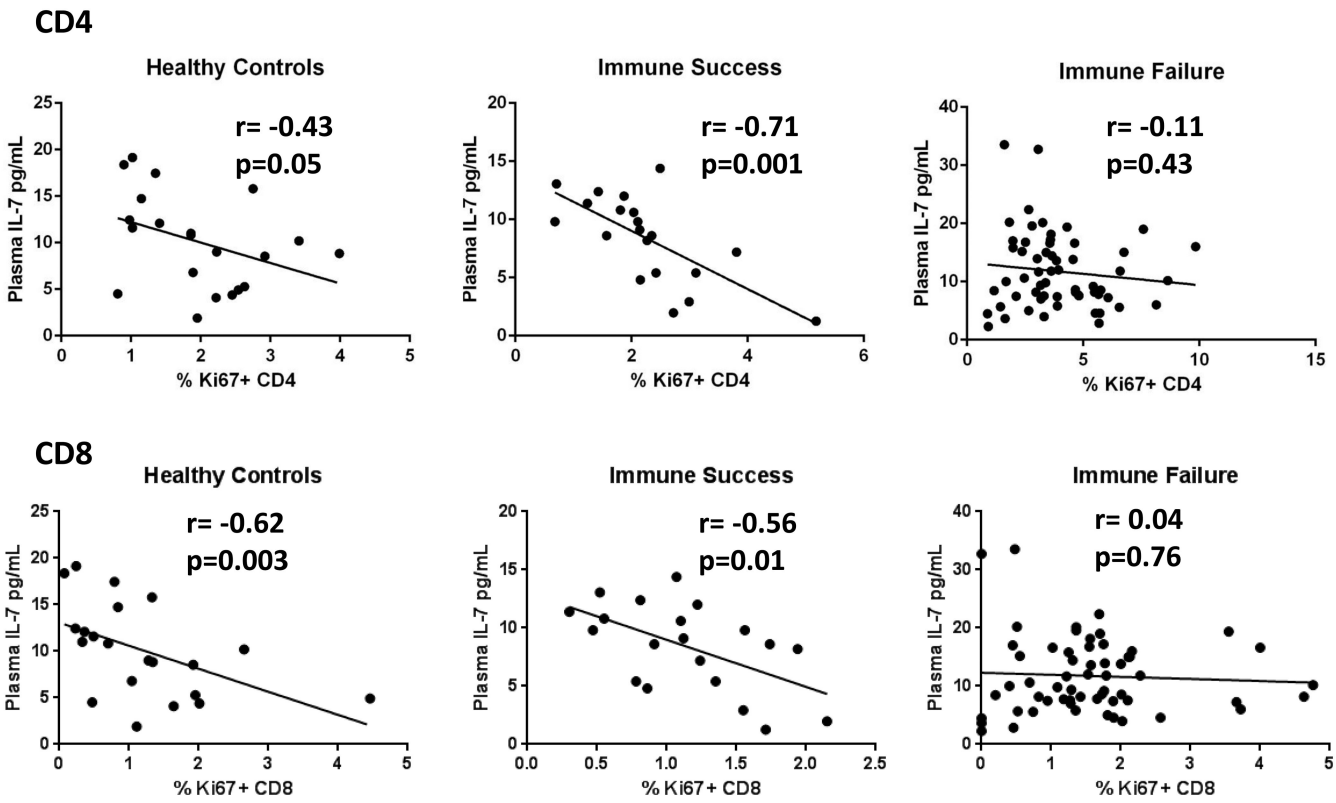

Figure 2. Plasma IL-7 correlates inversely with cycling T cells in immune success and healthy controls but not in immune failure

Correlations between plasma IL-7 levels and % Ki67+ CD4 and CD8 T cells were assessed by Spearman's rank test. Linear regression curves are displayed in graphs. *P* values of 0.05 or less were considered significant.

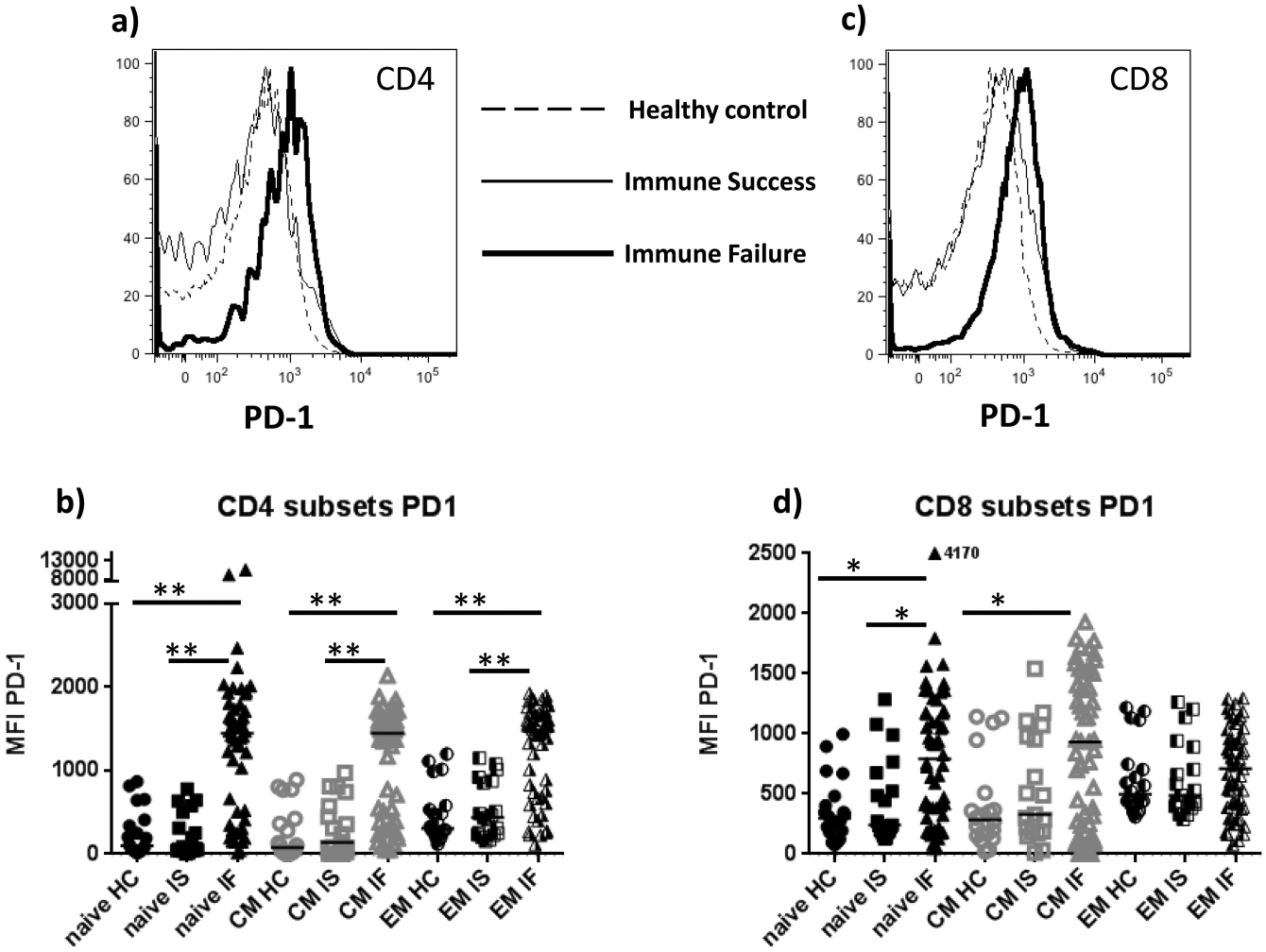

Figure 3. Immune senescence markers PD-1 and CD57 are elevated on T cells in immune failure

Surface expression of PD-1 is expressed as mean fluorescent intensity (MFI) (a,c, representative samples; b,d, summary data). Surface expression of CD57 is shown as proportion of stained cells (e,g, representative samples; f,h, summary data). Maturation subsets defined using CD45RA and CCR7 staining (naïve= CD45RA+CCR7+; central memory (CM) CD45RA-CCR7+; effector memory (EM) CD45RA-CCR7-) HC= healthy controls (n=20); IS= immune success (n=20); IF= immune failure (n=60) Bars represent medians; groups are compared by Mann-Whitney U test. **p= <0.001; *p= ≤0.05

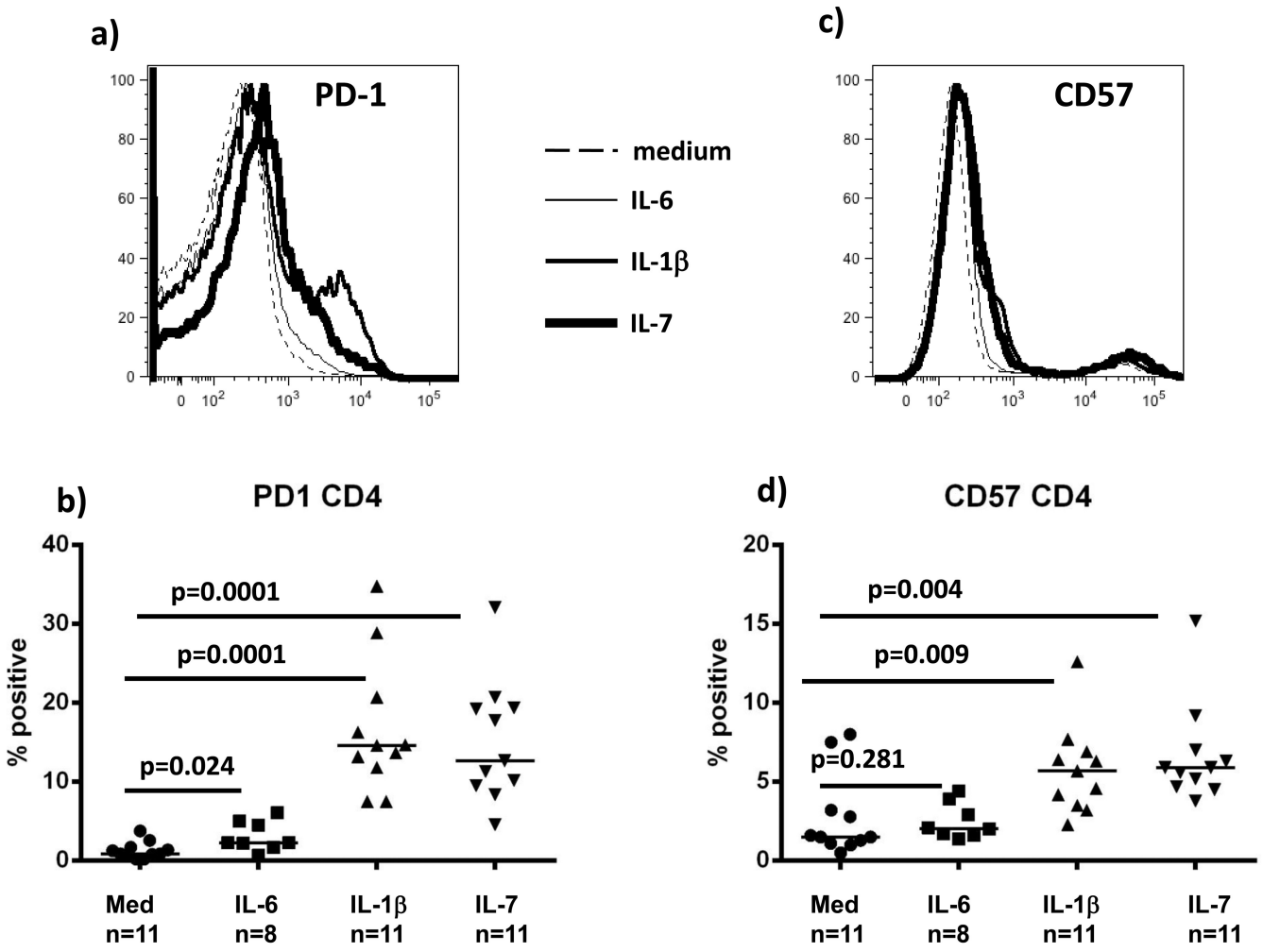

Figure 4. Immune exhaustion/senescence markers PD-1 and CD57 are inducible by IL-1β and IL-7 in vitro

PBMCs from healthy uninfected subjects were labeled with CFSE, stimulated in vitro for 7 days with IL-6 (10ng/mL), IL-1β (10ng/mL), or IL-7 (5ng/mL), then examined for proliferation by dye dilution and for surface protein expression by flow cytometry on gated CD4 T cells (a,c representative samples; b,d summary data; e,f surface antigen expression among non-proliferating (CSFE high) and proliferating (CFSE low cells) cells) Bars represent medians; groups are compared by Mann-Whitney U test.

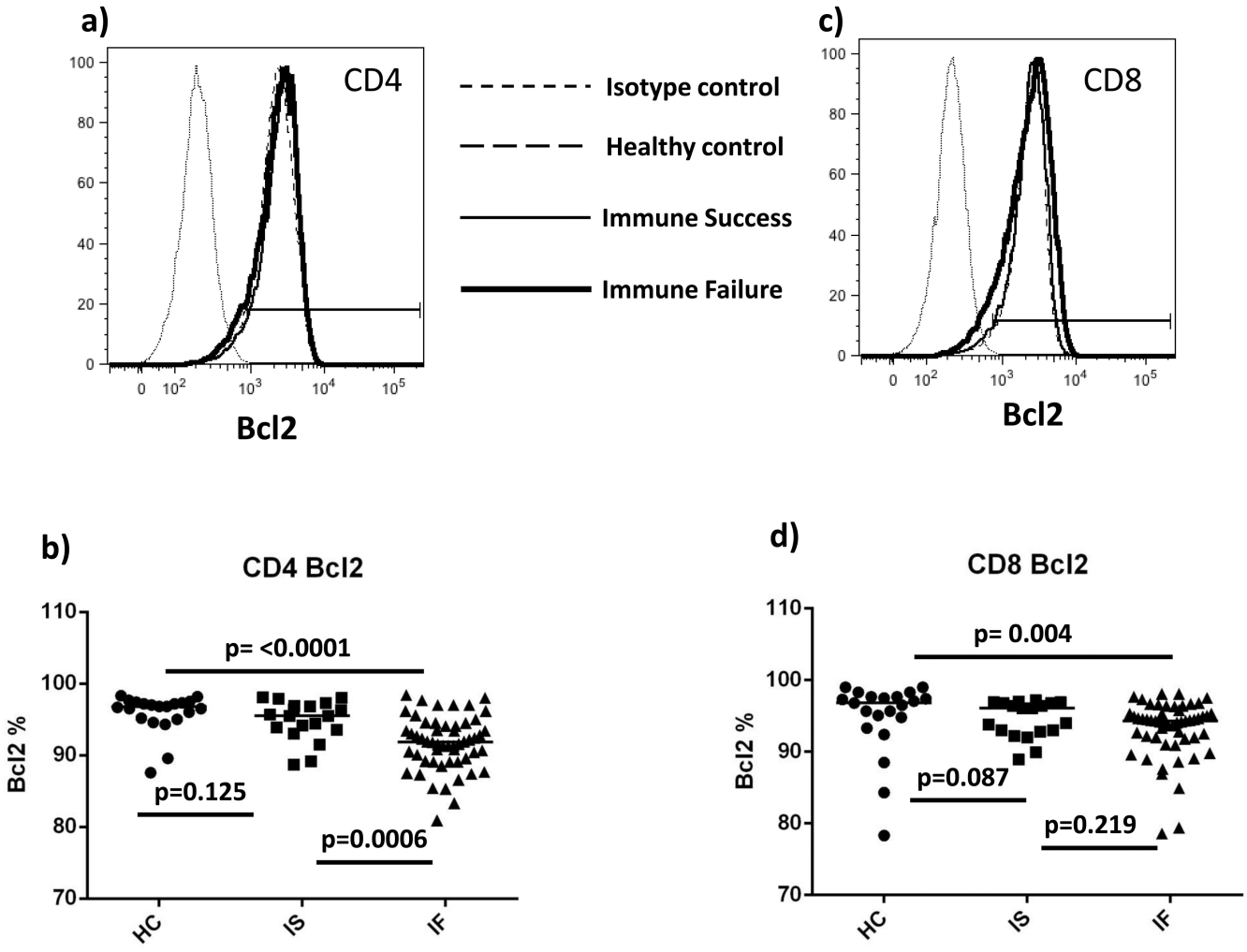

Figure 5. Fewer CD4 T cells express detectable Bcl2 in immune failure

(a,c, representative samples; b,d, summary data) HC= healthy controls (n=20); IS= immune success (n=20); IF= immune failure (n=60) Bars represent medians; groups are compared by Mann-Whitney U test.