Interleukin-7 and interleukin-15 drive $CD4^{+}CD28^{null}$ T lymphocyte expansion and function in patients with acute coronary syndrome

Short title: IL-7 & IL-15 drive $CD28^{null}$ T-cell expansion and function

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1. Abstract

Aims: Inflammation has important roles in atherosclerosis. $CD4^+CD28^{null}$ ($CD28^{null}$) T cells are a specialised T lymphocyte subset that produce inflammatory cytokines and cytotoxic molecules. $CD28^{null}$ T cells expand preferentially in patients with acute coronary syndrome (ACS) rather than stable angina and are barely detectable in healthy subjects. Importantly, ACS patients with $CD28^{null}$ T cell expansion have increased risk for recurrent acute coronary events and poor prognosis, compared to ACS patients in whom this cell subset does not expand. The mechanisms regulating $CD28^{null}$ T cell expansion in ACS remain elusive. We therefore investigated the role of cytokines in $CD28^{null}$ T cell expansion in ACS.

Methods and Results: High-purity sorted $CD4^+$ T cells from ACS patients were treated with a panel of cytokines (TNF-α, IL-1β, IL-6, IL-7, IL-15), and effects on the number, phenotype and function of $CD28^{null}$ T cells were analysed and compared to the control counterpart $CD28^+$ T cell subset. IL-7 and IL-15 induced expansion of $CD28^{null}$ T cells from ACS patients, while inflammatory cytokines TNF-α, IL-1β and IL-6 did not. The mechanisms underlying $CD28^{null}$ T cell expansion by IL-7/IL-15 were preferential activation and proliferation of $CD28^{null}$ T cells compared to control $CD28^+$ T cells. Additionally, IL-7/IL-15 markedly augmented $CD28^{null}$ T cell cytotoxic function and interferon-γ production. Further mechanistic analyses revealed differences in baseline expression of component chains of IL-7/IL-15 receptors (CD127 and CD122) and increased baseline STAT5 phosphorylation in $CD28^{null}$ T cells from ACS patients compared to the control $CD28^+$ T cell subset. Notably, we demonstrate that $CD28^{null}$ T cell expansion was significantly inhibited by Tofacitinib, a selective JAK1/JAK3 inhibitor that blocks IL-7/IL-15 signalling.

Conclusions: Our novel data show that IL-7 and IL-15 drive the expansion and function of $CD28^{null}$ T cells from ACS patients suggesting that IL-7/IL-15 blockade may prevent expansion of these cells and improve patient outcomes.

Translational perspective

$CD28^{null}$ T cells expansion in ACS patients is an independent predictor of future acute coronary events and poor prognosis. The precise mechanisms underlying $CD28^{null}$ T cell expansion in ACS remain elusive. We show that IL-7 and IL-15 cytokines cause expansion of $CD28^{null}$ T cells from ACS patients by triggering activation and proliferation, and augment the cytotoxic function of these cells and production of inflammatory cytokines. We demonstrate that $CD28^{null}$ T cell expansion is inhibited by Tofacitinib that specifically blocks IL-7/IL-15 signalling. Further dissection of the roles of IL-7/IL-15 may lead to more effective and specific anti-inflammatory therapies in ACS.

2. Introduction

Coronary artery disease (CAD) and acute coronary syndrome (ACS) remain a major cause of death and morbidity worldwide despite considerable advances in diagnosis, prevention and treatment.[1] T lymphocytes have pivotal roles in atherosclerosis and pathogenesis of CAD.[2, 3] $CD4^{+}CD28^{null}$ ($CD28^{null}$) T cells are an inflammatory subset of T lymphocytes defined by the lack of the co-stimulatory receptor CD28. $CD28^{null}$ T cells are unique as they are not present in mice but have been identified exclusively in humans with chronic inflammatory diseases.[4, 5] These cells preferentially expand in the circulation and atherosclerotic plaques of patients with ACS rather than stable angina[6, 7], and are nearly undetectable in healthy individuals.[4, 8] Importantly, ACS patients with expansion of $CD28^{null}$ T cells have increased risk for recurrent acute coronary events and poor prognosis compared to ACS patients in whom this subset does not expand.[9] Moreover, increased $CD28^{null}$ T cells are an independent predictor of future acute coronary events in ACS patients.[9] We have previously demonstrated that in contrast to the conventional control $CD4^{+}CD28^{+}$ ($CD28^{+}$) T cell subset, $CD28^{null}$ T cells from ACS patients produce high levels of inflammatory cytokines tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) and release cytotoxic molecules (perforin and granzyme B) that could harm the vascular wall by promoting inflammation and plaque rupture.[10] We showed that co-stimulatory receptors OX40 and 4-1BB modulate $CD28^{null}$ T cell function and that co-stimulation blockade reduced the inflammatory and cytotoxic actions of these cells.[10] We have identified that $CD28^{null}$ T cells from ACS patients have defects in proteasomal degradation of pro-apoptotic molecules.[5, 11, 12] However, the precise mechanisms that regulate $CD28^{null}$ T cell expansion in ACS patients are yet to be deciphered.

Inflammatory cytokines such as TNF-α, interleukin-1β (IL-1β) and IL-6 have important roles in driving inflammation in CAD patients.[3, 13-15] Recently, the CANTOS trial showed that targeted cytokine inhibition (IL-1β inhibition with Canakinumab) in CAD patients with a

prior myocardial infarction and residual inflammatory risk reduced further cardiovascular events.[16] Other cytokines that are deregulated in chronic inflammatory diseases (e.g. rheumatoid arthritis, RA) and are currently targeted in patients are IL-7 and IL-15 that belong to the common gamma chain cytokine family.[17-19] Whether these cytokines are involved in the expansion and function of $CD28^{null}$ T cells in ACS patients is unknown.

Here we provide novel evidence that the cytokines IL-7 and IL-15 trigger $CD28^{null}$ T cell expansion in ACS patients, while inflammatory cytokines (TNF-α, IL-1β, IL-6) do not. IL-7 and IL-15 induced preferential activation and proliferation of $CD28^{null}$ T cells and promoted their cytotoxic and inflammatory function. We dissect the mechanistic basis of IL-7 and IL-15 effects on $CD28^{null}$ T cells from ACS patients and demonstrate that Tofacitinib, a selective JAK1/JAK3 inhibitor that blocks IL-7/IL-15 signalling, significantly inhibits $CD28^{null}$ T cell expansion. These new data suggest that targeting IL-7 and IL-15 could potentially provide a therapeutic strategy to prevent expansion of $CD28^{null}$ T cells in ACS patients.

3. Methods

3.1. Study Population

Peripheral blood was collected from patients with ACS (**Supplemental Table 1 and Supplemental Table 2**; samples collected <12 hours from chest pain) admitted at the coronary care unit, St. George's Hospital NHS Trust, London. Patients with malignancies, infectious diseases, autoimmune disorders, on treatment with anti-inflammatory drugs except aspirin or over 80 years old were excluded from the study as previously described.[10, 11] The study conformed to the Declaration of Helsinki principles (study approved by the London & Chelsea Research Ethics Committee, REC 09/H0801/27), and written informed consent was obtained from all study participants prior to inclusion in the study.

3.2. Flow Cytometry

The frequency of $CD28^{null}$ T cells in fresh peripheral blood samples (circulating $CD28^{null}$ T cells) was determined by staining 100 μl blood with CD4-FITC and CD28-APC (BD Biosciences), followed by red blood cells lysis with Lyse/Fix buffer (BD Biosciences), as previously described.[10] The percentage of circulating $CD28^{null}$ T cells was calculated as the percentage of $CD4^+$ T cells as previously described.[10] The gating strategy for quantification of $CD28^{null}$ T cells in fresh peripheral blood samples and cultured peripheral blood mononuclear cells (PBMCs) or $CD4^+$ T cells is depicted in **Supplemental Figure 1**. Where indicated, CD127-PE (Invitrogen), CD122-PE, CD132-PE, CD215-PE (all BioLegend) were added to fresh peripheral blood samples stained with CD4-FITC and CD28-APC. The following monoclonal antibodies were used to identify $CD28^{null}$ T cells in cultured samples and analyse the expression of functional markers on $CD28^{null}$ and $CD28^+$ T cells: CD4-FITC, CD28-APC, CD28-PE, HLA-DR-APC, CXCR3-PE, CCR7-PE, CD45RO-FITC, TNF-α-PE-Cy7 (all BD Biosciences/BD Pharmingen); CD14-PE (Miltenyi Biotec); CD69-APC, CD215-PE, CD45RA-PE-Cy7 (all Biolegend); CD62L-APC, Granzyme B-PE, IFN-γ-APC (all

eBioscience); CCR5-PE, CD127-PE (all Invitrogen). Intracellular levels of IFN-γ and TNF-α were quantified in sorted $CD4^+$ T cells cultured alone or with IL-7 or IL-15 for 3 days following 4 hour stimulation of with phorbol myristate acetate, ionomycin and brefeldin, as previously described.[6, 7, 10] Dead cells were excluded from the analysis by staining with 7-aminoactinomycin D (7-AAD, BD Biosciences) or, for intracellular staining (i.e. granzyme-B, IFN-γ and TNF-α), by labelling with ZombieYellow (Biolegend) or Fixable viability stain 575V (BD Biosciences) prior to fixation and permeabilisation using Cytofix/Cytoperm (BD Biosciences). Phosphorylated STAT5 was assessed via the BD Phosflow method using CD4-H7, CD28-APC, Alexa Fluor 488 anti-STAT5, Alexa Fluor 488 mouse IgG1 κ isotype control, BD Cytofix buffer and BD Perm Buffer III (all BD Biosciences) as per the manufacturer's instructions. Samples were acquired on a FACSCalibur (BD Biosciences) or Navios (Beckman Coulter) flow cytometer and data was analysed using FlowJo software v7.6 (FlowJo, LLC). Mean fluorescence intensity (MFI) was calculated by subtracting the MFI of samples stained with isotype control antibodies from the MFI of samples stained with antibodies against specific markers. The percentage increase in $CD28^{null}$ T cells following cytokine treatment was calculated as: 100x(%$CD28^{null}$ in treated samples - %$CD28^{null}$ in untreated samples)/%$CD28^{null}$ in untreated samples.

3.3. Cell Isolation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples by density gradient centrifugation as described previously.[11] $CD4^+$ T cells were purified by magnetic separation using negative selection kits from Miltenyi Biotec and Invitrogen (MagniSort) as per the manufacturers' instructions. $CD4^+$ T cells were cultured at $2x10^5$ cells per well in U-bottom 96-well plates in RPMI1640 (Life Technologies) containing 100 U/ml penicillin, 100 µg/ml streptomycin and 15 mM L-glutamine (Sigma-Aldrich), supplemented with 5% pooled human AB serum (Corning). Cells were stimulated with the indicated

concentrations of recombinant human cytokines (IL-1β, IL-6, IL-7, IL-15, TNF-α; R&D Systems) and cultured for up to 11 days. Where indicated cells were stimulated with IL-17/IL-15 in the presence or absence of 100 nM Tofacitinib (CP 690550 citrate, Tocris Bioscience). Where indicated culture supernatant from $CD4^+$ T cells stimulated with IL-7, IL-15 and un-stimulated cells were collected and kept frozen until quantification of TNF-α and IFN-γ by DuoSet ELISA (R&D Systems), as per the manufacturer's instructions.

3.4. Proliferation Assay

Sorted $CD4^+$ T cells were labelled with 1 μM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) and cultured in the presence of IL-7 or IL-15 as indicated. Proliferation (assessed as CFSE dilution) was quantified by flow cytometry following staining with antibodies (CD4-APC-H7, CD28-APC) and dead cell exclusion with 7-AAD (all BD Biosciences).

3.5. Degranulation Assay

$2x10^5$ cells $CD4^+$ T cells were cultured alone or stimulated with 50 ng/ml IL-7 or IL-15 for 4 days. Four hours before analysis, CD107a-PE (BD Biosciences) was added to the culture and cells were either left unstimulated or stimulated with 2 μg/ml functional grade anti-human CD3 monoclonal antibody (Invitrogen); BD GolgiStop (BD Biosciences) was added for the last three hours of culture. Cells were then stained with CD4-FITC, CD28-APC and 7-AAD (BD Biosciences) and CD107a expression was quantified by flow cytometry.

3.6. Quantification of plasma cytokine levels

Plasma was separated from fresh EDTA-treated blood samples from ACS patients and stored frozen until quantification of IL-7, IL-15, TNF-α, IL-1β and IL-6 by DuoSet ELISA (R&D Systems) and by IL-7 and IL-15 Quantikine high sensitivity ELISA (R&D Systems).

3.7. Statistics

Statistical analysis was performed using GraphPad Prism v7.02. Cytokine-treated and untreated samples were compared using paired two-tailed Student's t test or two-tailed Wilcoxon matched-pairs signed rank test as indicated. For comparison of more than two groups, statistical significance was determined using one-way or two-way analysis of variance (ANOVA) and Bonferroni post-test for multiple comparisons. Plasma cytokine levels in the two study groups were compared using two-tailed Mann-Whitney test. Categorical data were analysed with the χ^2 test. Probability (p) values of <0.05 were considered statistically significant.

3.8. Study approval

The study conformed to the Declaration of Helsinki principles (study approved by the London & Chelsea Research Ethics Committee, REC 09/H0801/27), and written informed consent was obtained from all study participants prior to inclusion in the study.

4. Results

4.1. Inflammatory cytokines TNF-α, IL-1β and IL-6 do not induce expansion of CD28^{null} T cells from ACS patients

To investigate the role of inflammatory cytokines (TNF-α, IL-1β, IL-6) in CD28^{null} T lymphocyte expansion in ACS we analysed cells from patients without expansion of the CD28^{null} T cell subset defined as less than 2% circulating CD28^{null} T cells out of all CD4^{+} T cells in peripheral blood (quantified as detailed in **Methods** and **Supplemental Figure 1A**).[6, 7, 10] Peripheral blood mononuclear cells (PBMCs) from ACS patients were cultured alone or in the presence of cytokines for up to 7 days. The percentage of CD28^{null} T cells remained unchanged following TNF-α, IL-1β or IL-6 treatment at all concentrations and time points tested (**Supplemental Figure 2**). Similar results were observed with high-purity sorted CD4^{+} T cells treated with TNF-α, IL-1β or IL-6, which did not affect CD28^{null} T cell percentage (**Supplemental Figure 3**). Combinations of inflammatory cytokines (i.e. TNF-α+IL-1β, TNF-α+IL-6, IL-1β+IL-6 and TNF-α+IL-1β+IL-6) had no effect on CD28^{null} T cells (**Supplemental Figure 4**). To further investigate whether inflammatory cytokines have any effect on CD28^{null} T cells, we tested these cytokines on PBMCs (**Supplemental Figure 5A-C**) or high-purity sorted CD4^{+} T cells (**Supplemental Figure 5D-F**) from ACS patients with pre-existing CD28^{null} T cell expansion in peripheral blood, i.e. more than 3% circulating CD28^{null} T cells (detailed in **Methods** and **Supplemental Figure 1A**).[6, 7, 10] In all samples tested TNF-α, IL-1β or IL-6 did not affect CD28^{null} T cell percentage, indicating that these inflammatory cytokines are unlikely to contribute to CD28^{null} T cell expansion in ACS patients.

4.2. IL-7 and IL-15 trigger expansion of CD28^{null} T cells from ACS patients

As inflammatory cytokines did not affect the number of CD28^{null} T cells, we sought whether other cytokines drive expansion of this subset. We focused on IL-7 and IL-15, which belong

to the common gamma chain cytokine family as they are involved in the pathogenesis of chronic inflammatory disorders and are targeted in patients with autoimmune disorders.[17] PBMCs or high-purity sorted $CD4^+$ T cells from ACS patients with more than 3% circulating $CD28^{null}$ T cells were treated with IL-7 and IL-15. $CD28^{null}$ T cell percentage increased significantly on day 4 and 7 of culture (PBMC: **Figure 1A-C**, **Supplemental Figure 6A-D**; $CD4^+$ T cells: **Figure 1D-F, Supplemental Figure 6E-F**). Combinations of IL-7 and IL-15 did not have synergistic effects on $CD28^{null}$ T cell expansion (not shown).

We also examined whether IL-7 and IL-15 expand $CD28^{null}$ T cells from ACS patients with <2% $CD28^{null}$ T cells in peripheral blood. In PBMCs, IL-15 significantly increased $CD28^{null}$ T cell percentage at day 4 and 7 of culture (**Supplemental Figure 7**), while in sorted $CD4^+$ T cells both IL-7 and IL-15 increased $CD28^{null}$ T cell percentage at day 7 and 11 (**Supplemental Figure 8**).

4.3. IL-7 and IL-15 activate $CD28^{null}$ T cells from ACS patients

Next we sought the underlying mechanisms through which IL-7 and IL-15 induced $CD28^{null}$ T cell expansion. T cell activation is a pre-requisite for lymphocyte expansion and therefore we quantified the activation markers CD69 and HLA-DR following cytokine treatment. In both PBMCs (**Figure 2A-D**) and sorted $CD4^+$ T cells (**Figure 2E-H**) IL-7 and IL-15 significantly increased the percentage of CD69-expressing and HLA-DR-expressing $CD28^{null}$ T cells. In contrast, less marked changes were observed in the control $CD28^+$ T cell subset following IL-7 or IL-15 treatment (**Figure 2**). Inflammatory cytokines TNF-α, IL-1β and IL-6 had no effect on CD69 or HLA-DR (**Supplemental Figure 9**), in line with the failure of these cytokines to induce $CD28^{null}$ T cell expansion.

We also investigated the effects of IL-7 and IL-15 on chemokine receptors (CCR5, CXCR3) and memory markers (CD62L, CCR7, CD45RA, CD45RO). IL-7 and IL-15 significantly up-regulated CCR5 expression on $CD28^{null}$ but not $CD28^+$ T cells (**Supplemental Figure**

10A,B), whilst CXCR3 (**Supplemental Figure 10C-D**) and memory markers (**Supplemental Figure 11**) were unchanged.

4.4. IL-7 and IL-15 induce proliferation of CD28^{null} T cells from ACS patients

As IL-7 and IL-15 preferentially activate CD28^{null} T cells (**Figure 2**), we investigated whether this resulted in proliferation of this cell subset, which may explain CD28^{null} T cell expansion in ACS. For this purpose, sorted CD4^{+} T cells were labelled with CFSE and cultured with IL-7 or IL-15; proliferation, assessed as CFSE-dilution, was quantified (detailed in Methods). There was little basal proliferation of CD28^{null} and control CD28^{+} T cells in the absence of IL-7 and IL-15 on day 5 and 7 of culture (**Figure 3**, **Supplemental Figure 12**). IL-7 and IL-15 induced significant proliferation of CD28^{null} T cells, whilst less proliferation was noted in CD28^{+} T cells (**Figure 3**, **Supplemental Figure 12**).

4.5. IL-7 and IL-15 increase granzyme B production and degranulation of CD28^{null} T cells from ACS patients

Next we tested whether IL-7 and IL-15 affect CD28^{null} T cell function. These cells characteristically produce the cytotoxic molecule GzB, which endows them with cell lytic function, in stark contrast to conventional CD28^{+} T cells that do not express this molecule.[10] PBMCs (**Figure 4A-B**) or sorted CD4^{+} T cells (**Figure 4C-D**) from ACS patients were treated with IL-7 or IL-15 for 4 days. In line with previous studies,[10] most CD28^{null} T cells (79% for PBMCs **Figure 4A**; 83% for CD4^{+} T cells **Figure 4C**) expressed GzB at baseline. Both IL-7 and IL-15 significantly increased the percentage of CD28^{null} T cells expressing GzB (**Figure 4A,C**). Moreover, IL-7 and IL-15 also increased the expression levels (mean fluorescence intensity, MFI) of GzB in CD28^{null} T cells (**Figure 4B,D**). Next, we investigated whether IL-7 and IL-15 affect the cytotoxic function of CD28^{null} T cells using a degranulation assay based on CD107a expression as previously described[10]. Degranulation is triggered by T cell receptor (TCR) stimulation either by antigen recognition or, in *in vitro* assays by anti-

CD3 antibodies, and degranulating cells are identified by the expression of CD107a (lysosomal associated membrane protein-1) on the cell surface.[20] Both IL-7 and IL-15 significantly up-regulated the anti-CD3-induced degranulation of $CD28^{null}$ T cells (**Figure 4E**). Notably, IL-15 but not IL-7 induced $CD28^{null}$ T cell degranulation even in the absence of anti-CD3 stimulation (**Figure 4E**).

4.6. IL-7 and IL-15 augment TNF-α and IFN-γ production by $CD28^{null}$ T cells from ACS patients

$CD28^{null}$ T cells from ACS patients produce high levels of TNF-α and IFN-γ.[10] As described previously [10], the percentage of TNF-α (**Figure 5A**) and IFN-γ (**Figure 6A**) producing cells was higher in resting $CD28^{null}$ (TNF-α 94.3%; IFN-γ 83.8%) than in the control $CD28^{+}$ T cell subset (TNF-α 83%; IFN-γ 12.8%). IL-7 or IL-15 did not change the percentage of TNF-α-producing $CD28^{null}$ T cells (**Figure 5A**), however, TNF-α expression levels (MFI) significantly increased in both $CD28^{null}$ and $CD28^{+}$ T cells (**Figure 5B**). This was accompanied by a significant increase in TNF-α in the culture medium of $CD4^{+}$ T cells treated with IL-7 or IL-15 (**Figure 5C**). While IL-7 or IL-15 did not change the percentage of IFN-γ-producing $CD28^{null}$ T cells (**Figure 6A**), they markedly up-regulated IFN-γ expression levels (MFI) in $CD28^{null}$ T cells (**Figure 6B**), whilst this was less prominent in control $CD28^{+}$ T cells (**Figure 6B**). IFN-γ levels in culture medium from cytokine-treated $CD4^{+}$ T cells were significant increased by IL-15 (**Figure 6C**).

4.7. Blocking IL-7 and IL-15 signalling prevents the expansion of $CD28^{null}$ T cells from ACS patients

We next quantified IL-7 and IL-15 in plasma from ACS patients with $CD28^{null}$ T cell expansion (>3% $CD28^{null}$ T cells in peripheral blood) and those without expansion of this cell subset (<2% $CD28^{null}$ T cells). IL-7 and IL-15 levels were very low in plasma (**Supplemental**

Figure 13A), in keeping with other studies that showed that these cytokines are mainly expressed within cells and are barely detectable in circulation.[21, 22] Mean IL-7 and IL-15 levels were higher in ACS patients with $CD28^{null}$ T cell expansion (IL-7: 8.08 pg/ml; IL-15: 11.01 pg/ml) compared to ACS patients without expansion of this cell subset (IL-7: 3.36 pg/ml; IL-15: 3.53 pg/ml), but the differences were not significant (**Supplemental Figure 13A**). TNF-α, IL-1β and IL-6 plasma levels were also low and similar in the two ACS groups (**Supplemental Figure 13B**). We hypothesised that differences in signalling downstream of IL-7/IL-15 receptors may render $CD28^{null}$ T cells more sensitive to these cytokines. The IL-7 receptor (IL-7R) has two chains: CD127 (the α chain, found only in IL-7R); and CD132 (the γ chain) that is shared with the IL-15 receptor.[23] The IL-15 receptor (IL-15R) is composed of CD215 (unique to IL-15R; binds IL-15 but is not required for IL-15 signalling); CD122 (the β chain), which is shared with the IL-2 receptor; and CD132.[24] We assessed the baseline expression of these chains in $CD28^{null}$ and in the control $CD28^{+}$ T cell subset directly in fresh peripheral blood samples from ACS patients. CD127 expression was lower in $CD28^{null}$ T cells compared to $CD28^{+}$ T cells (**Figure 7A,B**; $p<0.01$). CD215 levels were very low in both cell subsets (**Figure 7A,B**), in keeping with previous studies suggesting that CD215 is not expressed in resting T cells but mainly expressed on cells (e.g. dendritic cells) that produce and present IL-15 to responding T cells.[25] Notably, $CD28^{null}$ T cells expressed significantly higher levels of CD122 compared to control $CD28^{+}$ T cells (**Figure 7A,B**; $p<0.0001$), which may explain their increased responsiveness to IL-15. CD132 expression was comparable in the two cell subsets (**Figure 7A,B**). Next, we assessed the effect of IL-7 and IL-15 on the expression of CD127 and CD215 (the chains involved in binding IL-7 and IL-15, respectively) in $CD28^{null}$ and $CD28^{+}$ T cells. IL-7 markedly down-regulated CD127 in both $CD28^{null}$ and $CD28^{+}$ T cells (**Figure 7C,D**). Interestingly, IL-15 also significantly down-regulated CD127 but to a lower extent compared to IL-7 (**Figure 7C,D**). In contrast, CD215

was significantly up-regulated in response to IL-7 and IL-15 mainly in $CD28^{null}$ T cells (**Figure 7E,F**). IL-7 and IL-15 receptors signal preferentially through JAK1/JAK3/STAT5, which associate with CD127, CD122 and CD132.[17, 23, 24, 26] Baseline levels of phosphorylated-STAT5 were significantly higher in un-stimulated $CD28^{null}$ T cells from ACS patients, compared to the control $CD28^{+}$ T cell subset (**Figure 7G**), indicating higher basal activation of IL-7/IL-15 signalling in $CD28^{null}$ T cells. Next, we blocked IL-7/IL-15 signalling with the JAK1/JAK3 selective inhibitor Tofacitinib[27, 28], which significantly inhibited $CD28^{null}$ T cell expansion, whilst not affecting $CD28^{+}$ T cells (**Figure 7H**).

5. Discussion

Here we show that IL-7 and IL-15 cytokines augment the number and function of $CD28^{null}$ T cells from patients with ACS. We demonstrate that IL-7 and IL-15 trigger expansion of $CD28^{null}$ T cells, whilst inflammatory cytokines TNF-α, IL-1β and IL-6 have no effect. The mechanisms underlying $CD28^{null}$ T cell expansion are preferential activation and proliferation of $CD28^{null}$ T cells by IL-7 and IL-15. These cytokines increase the cytotoxic function of $CD28^{null}$ T cells (GzB production and degranulation) and the production of IFN-γ. We provide further mechanistic insights showing marked differences in baseline levels of CD127 and CD122 (component chains of IL-7 and IL-15 receptors) and increased baseline phosphorylation of STAT5 in $CD28^{null}$ T cells compared to their control $CD28^{+}$ T cell subset. Blockade of IL-7/IL-15 signalling with a JAK1/JAK3 selective inhibitor prevents the expansion of $CD28^{null}$ T cells from ACS patients (summarised in the **Graphical abstract**).

Inflammatory $CD28^{null}$ T cells expand in the circulation and atherosclerotic plaques of patients with ACS.[6, 7] Notably, ACS patients with expansion of the $CD28^{null}$ T cell subset have increased risk for recurrent severe acute coronary events and poorer prognosis compared to ACS patients without $CD28^{null}$ T cell expansion.[9] This study also showed that an increase in $CD28^{null}$ T cell frequency is an independent predictor of future acute coronary events in ACS patients.[9] The precise mechanisms driving the expansion of $CD28^{null}$ T cells in ACS are unknown. Here we show that the cytokines IL-7 and IL-15 are involved in the expansion of $CD28^{null}$ T cells from ACS patients, while inflammatory cytokines TNF-α, IL-1β and IL-6 did not have an effect. However, inflammatory cytokines TNF-α, IL-1β and IL-6 are important in atherosclerosis pathogenesis, as recently shown by the CANTOS trial, which found that specifically targeting inflammation (IL-1β inhibition) improves clinical outcomes in patients with a previous myocardial infarction.[16] Our findings underscore that several cytokines and inflammatory networks may be at work in ACS patients, and that a

better characterisation of patients' inflammatory status is needed to improve the efficacy of anti-inflammatory therapies in ACS. Moreover, as $CD28^{null}$ T cells have been exclusively identified in humans and do not exist in mice, this precludes interrogation of their roles in atherosclerosis using currently available animal models. Our work emphasises the need to investigate inflammation and immune responses in patients with ACS as key information on the cellular and molecular pathophysiology of human coronary artery disease may be missed in murine models.

Strikingly, IL-7 and IL-15 drove the activation and proliferation of $CD28^{null}$ T cells from ACS patients in the absence of T cell receptor (TCR)-derived signals (i.e. antigen stimulation). A possible explanation is that $CD28^{null}$ T cells share features with natural killer (NK) cells[29, 30] that also expand in response to IL-15 in the absence of antigen-dependent stimulation. IL-15 has central roles in the development, maintenance, activation and effector function of NK cells (cytotoxicity, IFN-γ production)[31]. Indeed, $CD28^{null}$ T cells express NK cell receptors and exhibit NK-like effector functions such as expression of cytotoxic molecules (GzB, perforin), cytotoxicity and, IFN-γ production[29, 30], which may explain their response to IL-15. In contrast to IL-15, IL-7 does not increase NK cell activation, cytotoxicity and IFN-γ production[32]. Nevertheless, IL-7 triggered activation and markedly augmented the cytotoxic and cytokine production function of $CD28^{null}$ T cells from ACS patients. Moreover, IL-7 had comparable effects to IL-15 on $CD28^{null}$ T cell expansion, activation and function, although only IL-15 directly triggered $CD28^{null}$ T cell degranulation in the absence of anti-CD3 stimulation. Notably, our data that IL-7 and IL-15 drive $CD28^{null}$ T cell activation, proliferation and function independently of TCR signals indicate that these cytokines may sustain the expansion and functions of these lymphocytes in the absence of antigen restimulation in ACS. IL-7 and IL-15 also increased CCR5 expression on $CD28^{null}$ T cells from ACS patients. CCR5 has been suggested to regulate tissue homing to the aorta of

Th1 $CD4^+$ T cells.[33] This suggests that IL-7 and IL-15 may enhance $CD28^{null}$ T cell homing to atherosclerotic plaques, which will be investigated in future studies.

IL-7 and IL-15 have been implicated in several autoimmune disorders, in particular in RA where these cytokines are identified in synovial fluid and tissue.[18, 19] The effect of IL-7 on $CD28^{null}$ T cells has not been previously studied in any disease, while the effect of IL-15 on $CD28^{null}$ T cells has been investigated in multiple sclerosis, elderly individuals and rheumatoid arthritis.[34-36] Whether IL-7 and IL-15 have a role in atherosclerosis is less well established. Expression of IL-15 in lipid-rich human atherosclerotic plaques associated with increased T cell infiltration and plaque-derived T cell lines proliferated to IL-15 *in vitro*.[35] Also IL-15 induced the development of atherosclerotic plaques in $ldlr^{-/-}$ mice, though this is independent of $CD28^{null}$ T cells as this cell subset is not described in mice.[37] While a previous study suggested that plasma IL-7 is raised in unstable or stable angina patients compared to healthy subjects[38], others reported that IL-7 and IL-15 are predominantly expressed within cells (macrophages, dendritic cells, epithelial and stromal cells) with barely detectable circulating stores.[21, 22] We found low plasma levels of IL-7 and IL-15 in ACS patients, suggesting that $CD28^{null}$ T cell expansion in ACS may be driven by IL-7 and IL-15 produced locally in atherosclerotic plaques or artery-associated lymphoid tissue. Our data that baseline expression of CD127 was lower in $CD28^{null}$ T cells from ACS patients compared to control $CD28^+$ T cells, suggest down-regulation of CD127 by *in vivo* exposure to IL-7/IL-15. This is supported by our *in vitro* findings that CD127 is down-regulated in $CD28^{null}$ T cells following IL-7 and IL-15 treatment. Additionally, the higher basal levels of phosphorylated-STAT5 in $CD28^{null}$ T cells indicate higher basal activation of IL-7/IL-15 signalling *in vivo*. The increased responsiveness of $CD28^{null}$ T cells to IL-15 is also supported by our new data that $CD28^{null}$ T cells from ACS patients have higher baseline expression of CD122, the β-chain of IL-15 receptor. Moreover, IL-7 and IL-15 markedly up-regulated the expression of

CD215 (the IL-15 receptor specific chain) in CD28^{null} T cells *in vitro*, which would further augment their responsiveness to IL-15.

ACS patients with CD28^{null} T cell expansion have increased risk for recurrent ACS and poor prognosis[9] and may benefit from strategies that prevent expansion of these cells such as IL-7/IL-15 neutralisation or signalling blockade. Indeed we include novel data that Tofacitinib (a selective JAK1/JAK3 inhibitor that blocks IL-7/IL-15 signalling) prevents the expansion of CD28^{null} T cells from ACS patients. This effect of Tofacitinib on CD28^{null} T cells has not been explored in other diseases. Of interest, Tofacitinib is used in patients with RA and active psoriatic arthritis[39, 40] and has been recently approved for use in ulcerative colitis.[41]

A potential limitation of this study is that CD28^{null} T cells have been exclusively identified in humans and do not exist in mice precluding interrogation of their roles in atherosclerosis using currently available animal models. Therefore our study is an in-depth *ex vivo* analysis of fresh peripheral blood and primary cells from ACS patients. However, our work highlights the need to investigate inflammation and immune responses in patients with ACS as key mechanisms of the cellular pathophysiology of human coronary artery disease may be missed in murine models.

6. Conclusions

This study provides new mechanistic insights and identifies novel roles for IL-7 and IL-15 cytokines in CD28^{null} T cell expansion and function in ACS. Our new findings suggest potential clinical applications of IL-7/IL-15 blockade in ACS patients with CD28^{null} T cell expansion for targeted modulation of the inflammation mediated by these cells.

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Author contributions: Research studies design: IED, JB. Conducting experiments, data acquisition and analysis: JB, VM, SK, IC, PD, IED. Original draft writing, reviewing, editing: JB and IED. Funding acquisition and administration: IED. Provision of resources: ZMJA, JCK. Supervision: IED.

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Conflict of interest: none declared.

Data availability: The data underlying this article are available in the article and in its online supplementary material.

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8. Figure legends

Figure 1. Effect of IL-7 and IL-15 on CD28^{null} T cell expansion. PBMCs (**A-C** n=10;) or CD4^{+} T cells (**D-F** n=14) from ACS patients with >3% circulating CD28^{null} T cells were treated with 50 ng/ml IL-7 or IL-15 for 4 days. **(A,D)** Illustrative dot plots show CD28^{null} T cell percentage. **(B,E)** Graphs show CD28^{null} T cell percentage and **(C,F)** % increase in CD28^{null} T cells (calculated as described in Methods) in untreated (w/o) and cytokine-treated samples.* p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001 (paired two-tailed Student's t test)

Figure 2. Effect of IL-7 and IL-15 on CD28^{null} T cell activation. PBMCs **(A-D)** or CD4^{+} T cells **(E-H)** from ACS patients were treated with 50 ng/ml IL-7 or IL-15 for 4 days. The activation markers CD69 and HLA-DR were analysed on CD28^{null} and CD28^{pos} T cells. **(A,E)** Illustrative dot plots display CD69 expression on CD28^{null} and CD28^{pos} T cells; dashed gates, isotype control antibody (Ctrl). **(B,F)** Graphs show the percentage of CD69^{+} cells in untreated samples (w/o) or after cytokine treatment (**B** n=8; **F** n=10). **(C,G)** Illustrative dot plots display HLA-DR expression on CD28^{null} and CD28^{pos} T cells; dashed gates, isotype control antibody (Ctrl). **(D, H)** Graphs show the percentage of HLA-DR^{+} cells in untreated samples (w/o) or after cytokine treatment (**D** n=6; **H** n=10). * p<0.05;** p<0.01; ns, not significant (two-tailed Wilcoxon matched-pairs signed rank test)

Figure 3. Effect of IL-7 and IL-15 on CD28^{null} T cell proliferation. CD4^{+} T cells from ACS patients were labelled with CFSE and cultured in the presence of 10 or 50 ng/ml IL-7 or IL-15 for 5 days. **(A)** Histograms illustrate CFSE fluorescence in CD28^{null} and CD28^{pos} T cells following treatment with 10 ng/ml IL-7 or IL-15. The percentage of cells that have proliferated (diluted CFSE) is indicated above the linear gates. **(B)** Proliferation of CD28^{null} and CD28^{pos} T cells in untreated samples (w/o) and after cytokine treatment (n=8). **(C)**

Histograms show CFSE fluorescence of $CD28^{null}$ (blue) and $CD28^{pos}$ (black) T cells treated with the indicated cytokines. * $p<0.05$; ** $p<0.01$ (paired two-tailed Student's t test)

Figure 4. Effect of IL-7 or IL-15 on the production of granzyme B and degranulation of $CD28^{null}$ T cells. PBMCs (**A,B** n=8) or $CD4^+$ T cells (**C,D** n=10) from ACS patients were treated with 50 ng/ml IL-7 or IL-15 for 4 days. **(A,C)** Illustrative dot plots show Granzyme B (GzB) expression in $CD28^{null}$ T cells; dashed gates, isotype control antibody (Ctrl). The graphs depict percentages of GzB^+ cells in untreated samples (w/o) and after cytokine treatment. **(B,D)** Histograms showing GzB expression in $CD28^{null}$ T cells; dashed histograms, isotype control antibody. The numbers indicate the mean fluorescence intensities (MFI) in cells cultured alone (w/o) or with cytokines. The graphs show GzB levels (MFI) in untreated and cytokine-treated cells. ** $p<0.01$ (two-tailed Wilcoxon matched-pairs signed rank test). a.u., arbitrary units **(E)** $CD4^+$ T cells (n=7) were cultured alone (w/o) or with 50 ng/ml IL-7 or IL-15 for 4 days. On day 4 cells were stimulated with anti-CD3 antibodies (aCD3) for 4 hours as indicated and degranulation was quantified with CD107a (detailed in Methods). Illustrative dot plots show CD107a expression in $CD28^{null}$ T cells; dashed gates, isotype control antibody (Ctrl); the graph displays the percentage of $CD28^{null}$ T cells expressing CD107a (mean±SEM). $*^a$ $p<0.05$ (aCD3 only or IL-15 only vs. w/o); $****^b$ $p<0.0001$ (aCD3+IL-7 or aCD3+IL-15 vs. aCD3) (two-way ANOVA with post-test Tukey for multiple comparisons)

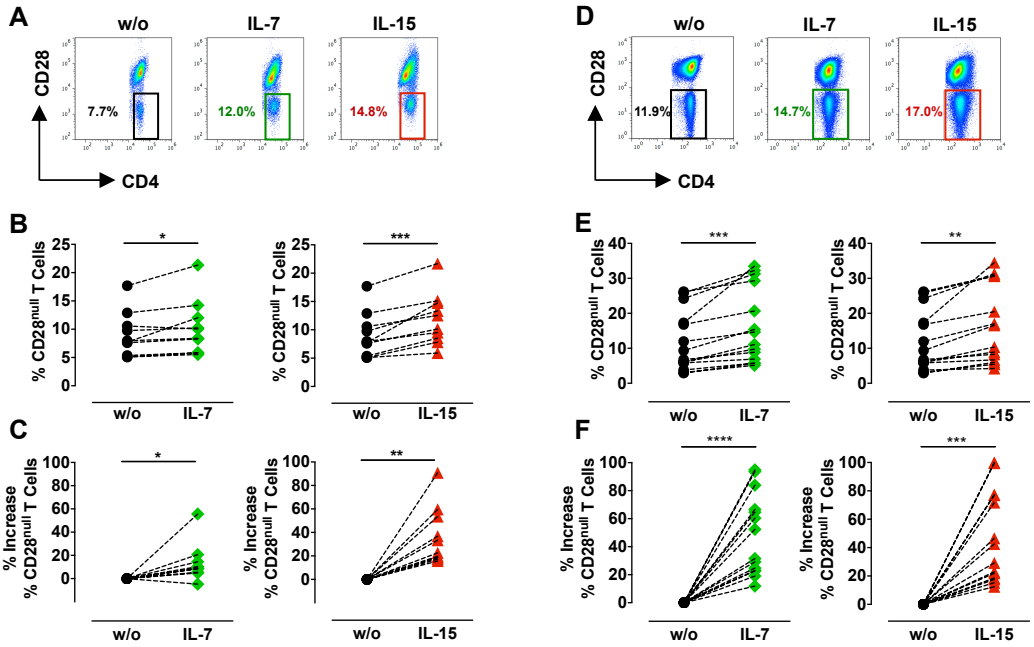
Figure 5. Effect of IL-7 or IL-15 on the production of TNF-α by $CD28^{null}$ T cells. $CD4^+$ T cells (n=10) from ACS patients were treated with 50 ng/ml IL-7 or IL-15 for 3 days. **A**. Illustrative dot plots and graphs display the percentage of $TNF\text{-}\alpha^+CD28^{null}$ (top) and $TNF\text{-}\alpha^+CD28^{pos}$ (bottom) T cells in untreated samples (w/o) and after cytokine treatment; dashed gates, isotype control antibody (Ctrl). **B**. The histograms and graphs show TNF-α expression levels by $CD28^{null}$ (top) and $CD28^{pos}$ (bottom) T cells with or without (w/o)

cytokine treatment; dashed histograms, isotype control antibody; the numbers indicate the mean fluorescence intensities (MFI) for each treatment. **A-B** * $p<0.05$; ** $p<0.01$; ns, not significant (two-tailed Wilcoxon matched-pairs signed rank test). a.u., arbitrary units **C**. The bar graph shows TNF-α levels in media from $CD4^+$ T cells (n=9) cultured with cytokines as above (mean±SEM). ** $p<0.01$ (one-way ANOVA with post-test Bonferroni for multiple comparisons)

Figure 6. Effect of IL-7 or IL-15 on the production of IFN-γ by $CD28^{null}$ T cells. $CD4^+$ T cells from ACS patients (n=10) were treated with 50 ng/ml IL-7 or IL-15 for 3 days. **A**. Illustrative dot plots and graphs display the percentage of IFN-γ^+CD28^{null} (top) and IFN-γ^+CD28^{pos} (bottom) T cells in untreated samples (w/o) and after cytokine treatment; dashed gates, isotype control antibody (Ctrl). **B**. The histograms and graphs show IFN-γ expression levels by $CD28^{null}$ (top) and $CD28^{pos}$ (bottom) T cells with or without (w/o) cytokine treatment; dashed histograms, isotype control antibody; the numbers indicate the mean fluorescence intensities (MFI) for each treatment. **A-B** * $p<0.05$; ** $p<0.01$; ns, not significant (two-tailed Wilcoxon matched-pairs signed rank test). a.u., arbitrary units **C**. The bar graph shows IFN-γ levels in media from $CD4^+$ T cells (n=9) cultured with cytokines as above (mean±SEM). ** $p<0.01$; ns, not significant (one-way ANOVA with post-test Bonferroni for multiple comparisons)

Figure 7. Blockade of IL-7/IL-15 signalling with the JAK1/JAK3 selective inhibitor Tofacitinib prevents $CD28^{null}$ T cell expansion. Fresh peripheral blood samples from ACS patients (n=10) were stained with CD4, CD28, CD122, CD127, CD132 and CD215 monoclonal antibodies. Illustrative dot plots **(A)** and graphs **(B)** show the percentage of $CD28^{null}$ and $CD28^+$ T cells expressing CD122, CD132, CD127 and CD215; dashed gates, isotype control antibody (Ctrl). $CD4^+$ T cells from ACS patients (n=10) were cultured alone (w/o) or treated with 50 ng/ml IL-7 or IL-15 for 3-4 days and expression of CD127 and

CD215 was analysed on $CD28^{null}$ and $CD28^{pos}$ T cells. Illustrative dot plots and graphs display the percentage of $CD127^{+}$ cells **(C,D)**, and $CD215^{+}$ cells **(E,F)** in $CD28^{null}$ and $CD28^{pos}$ T cells; dashed gates, isotype control antibody (Ctrl). (**G**) Baseline levels of phosphorylated STAT5 (pSTAT5) were quantified using the PhosFlow Method (described in Methods); black and dashed histograms, isotype control antibody; the numbers indicate the mean fluorescence intensity (MFI). Illustrative histograms and bar graph display the expression levels (MFI) of pSTAT5 in $CD28^{null}$ and $CD28^{+}$ T cells (n=6; mean±SEM). (**H**) $CD4^{+}$ T cells from ACS patients (n=10) were cultured alone (w/o) or treated with 50 ng/ml IL-7 or IL-15 in the presence or absence of 100 nM Tofacitinib (Tofa) for 7 days. Graphs show the fold change in the percentage of $CD28^{null}$ or $CD28^{+}$ T cells (mean±SEM). * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$ **(B)** two-tailed Mann-Whitney test; **(D,F)** two-tailed Wilcoxon matched-pairs signed rank test; **(G)** paired two-tailed Student's t test; **(H)** two-way ANOVA with post-test Bonferroni for multiple comparisons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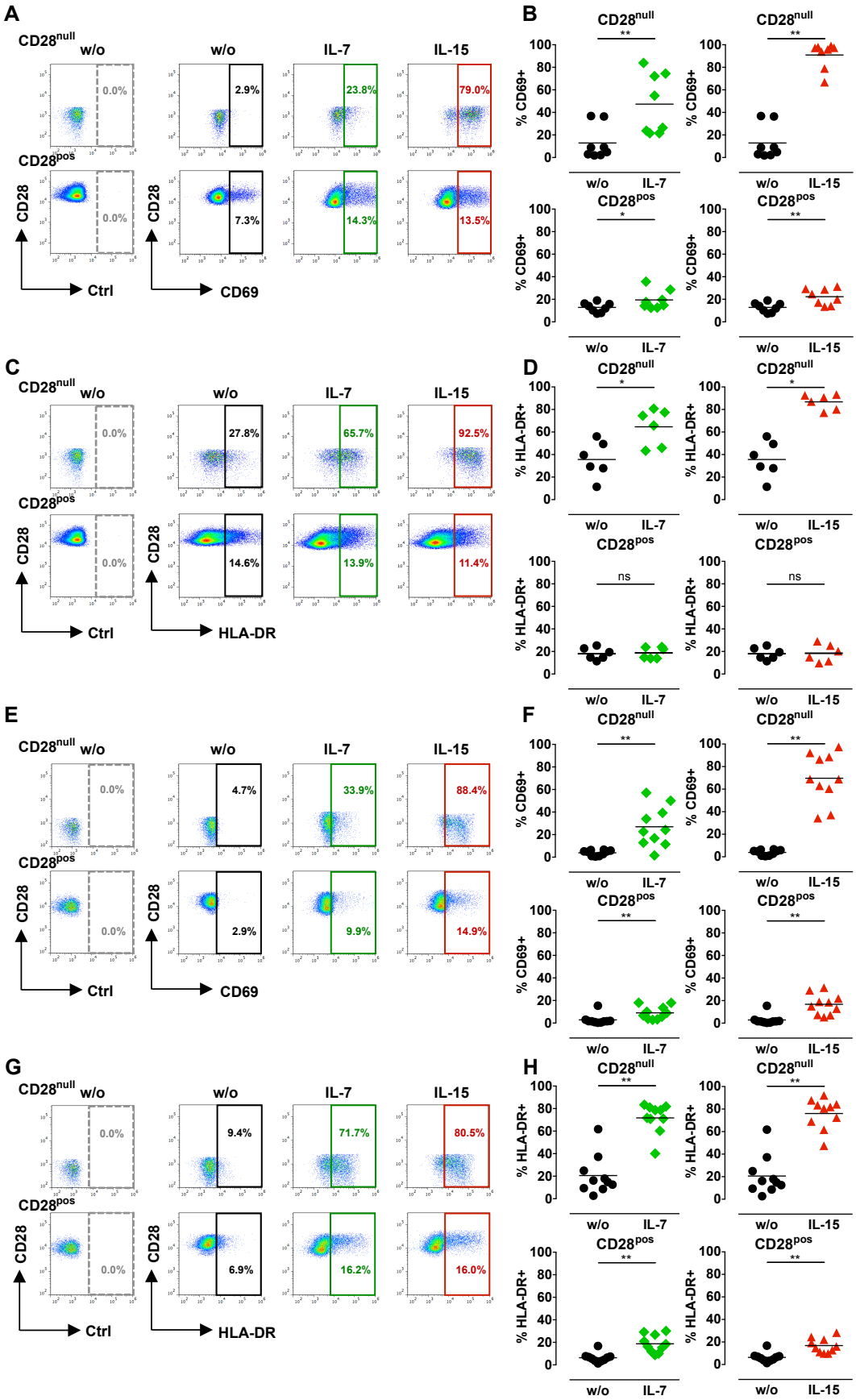
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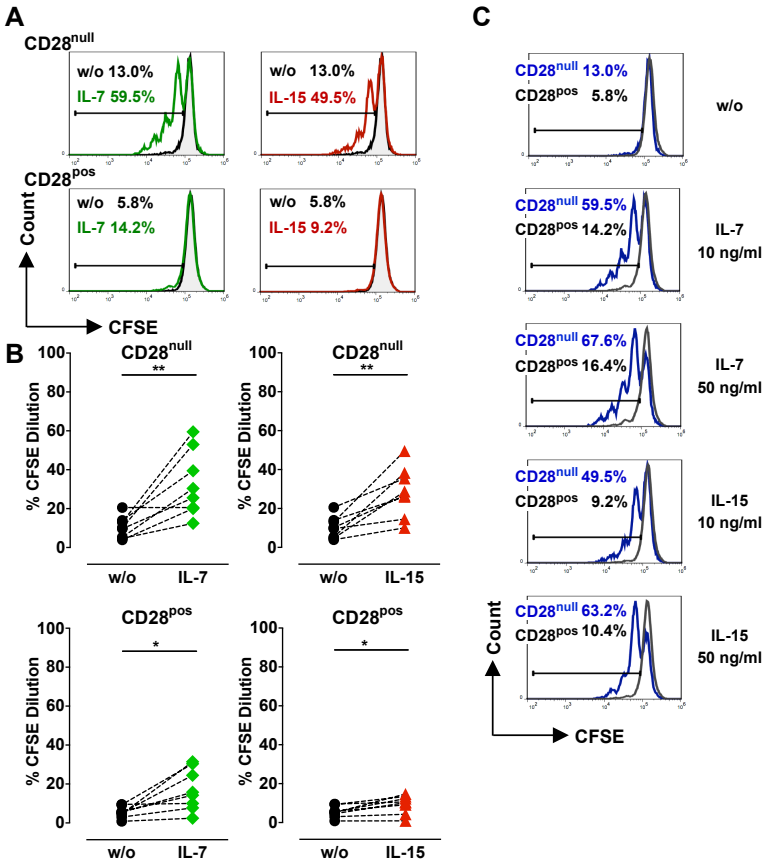
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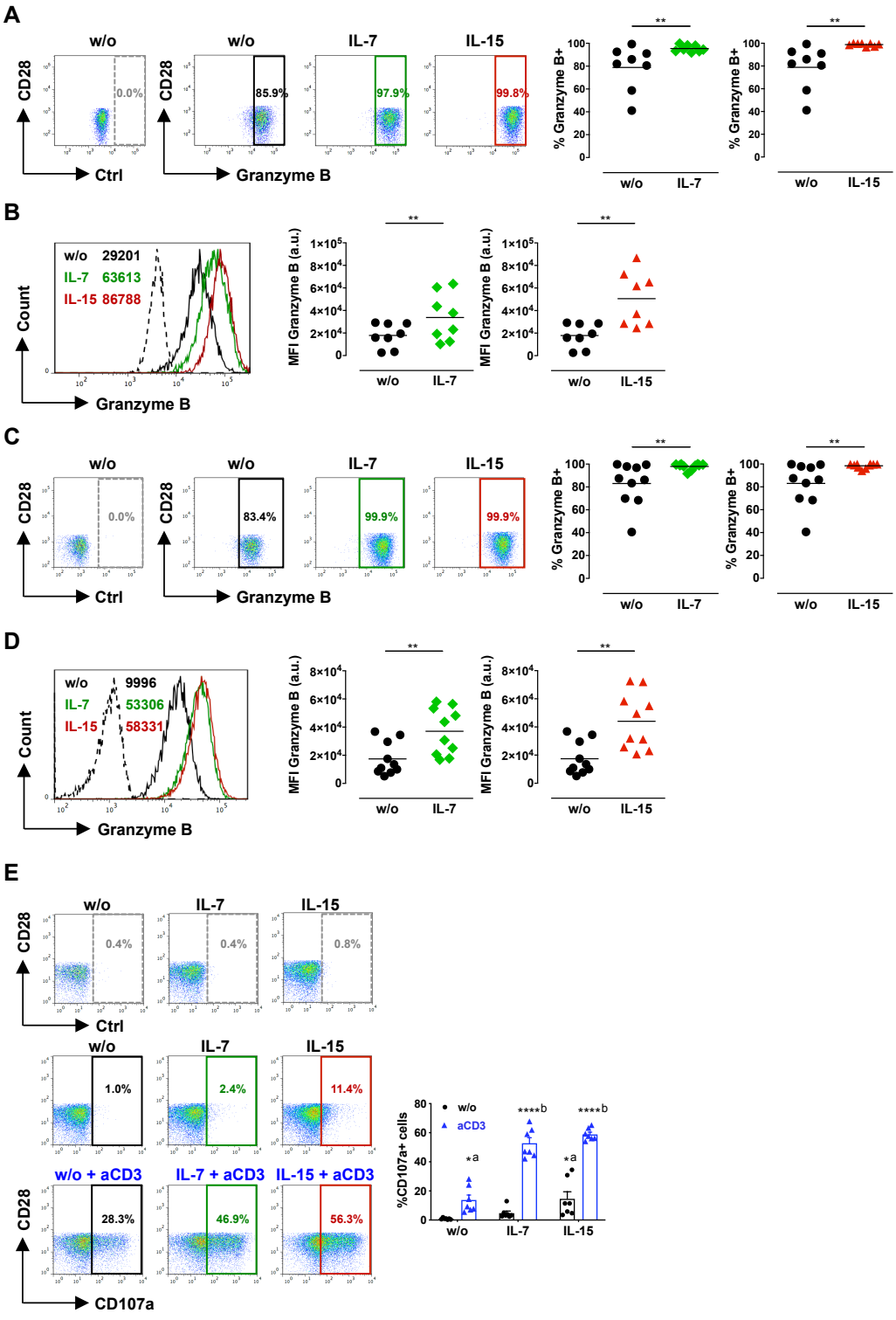
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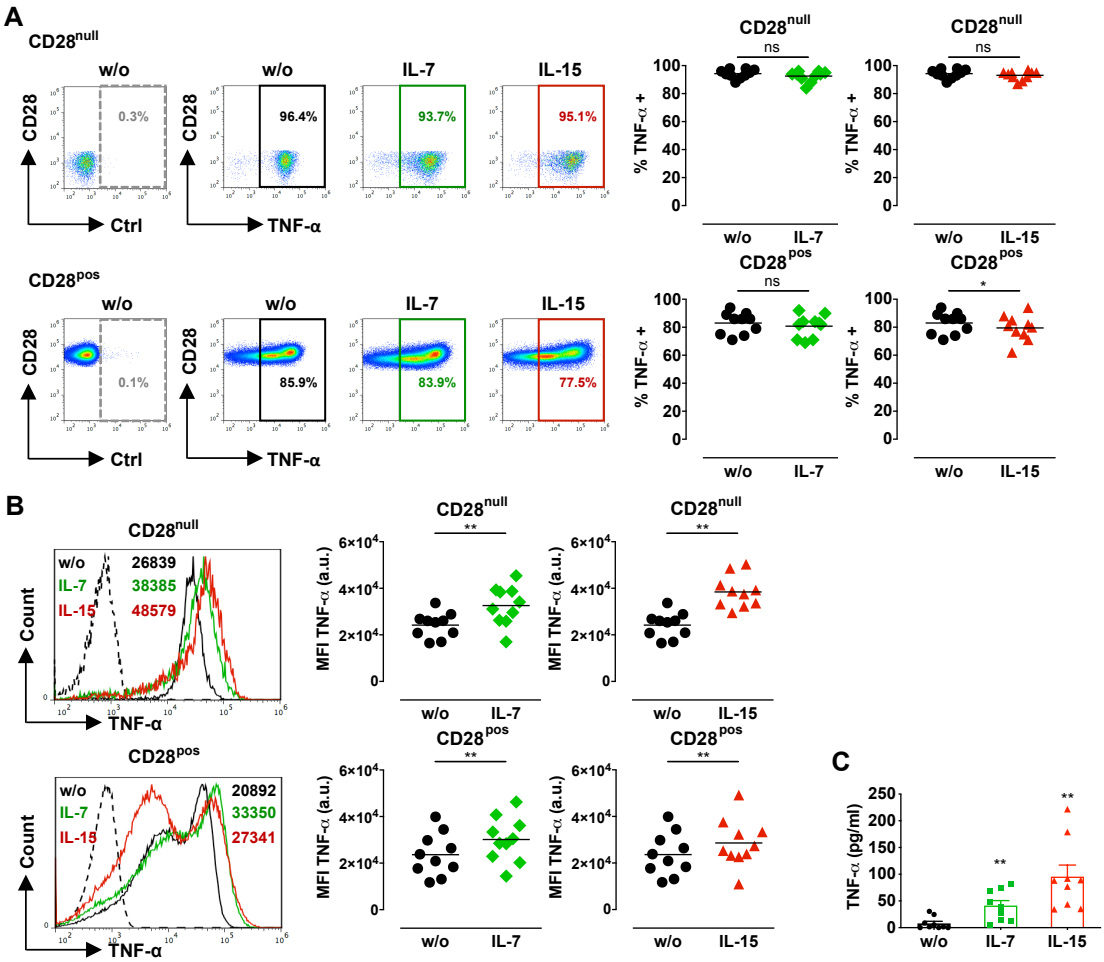
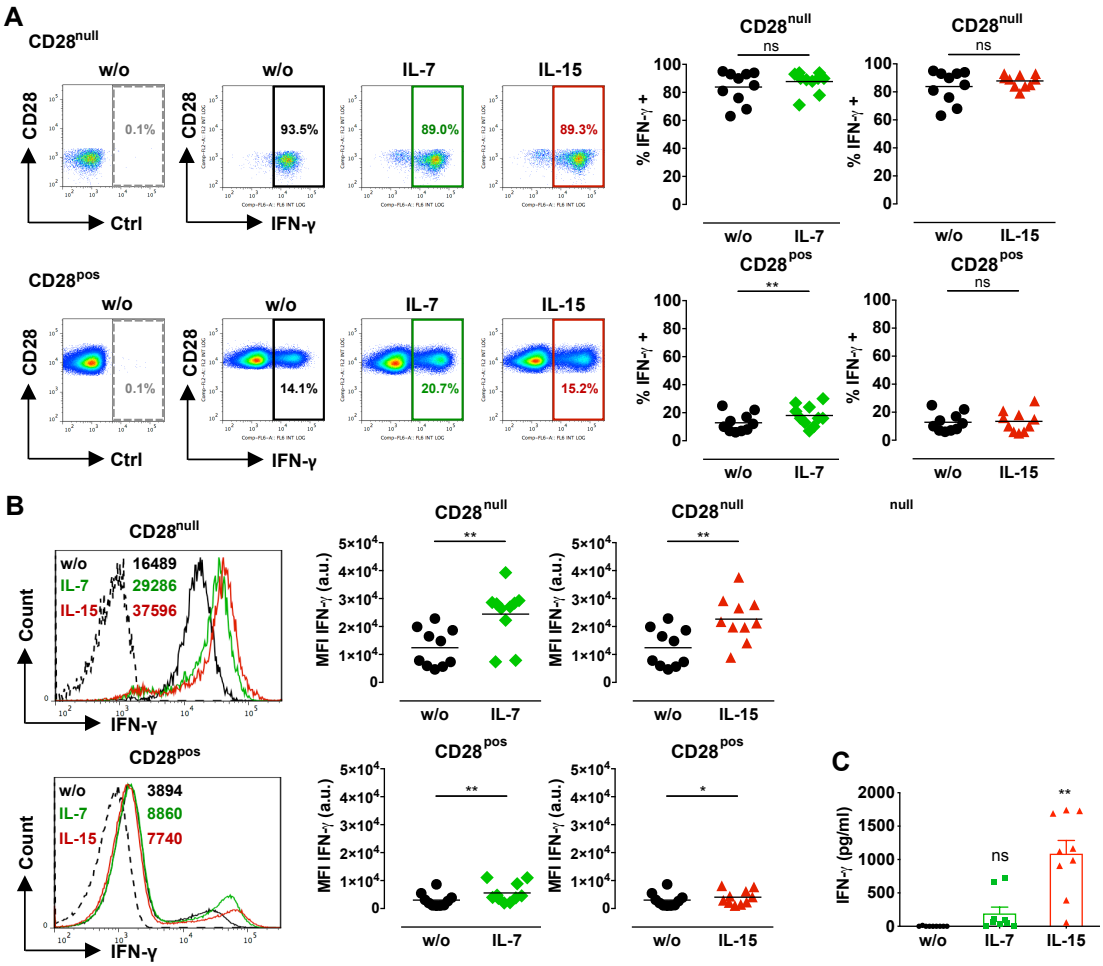
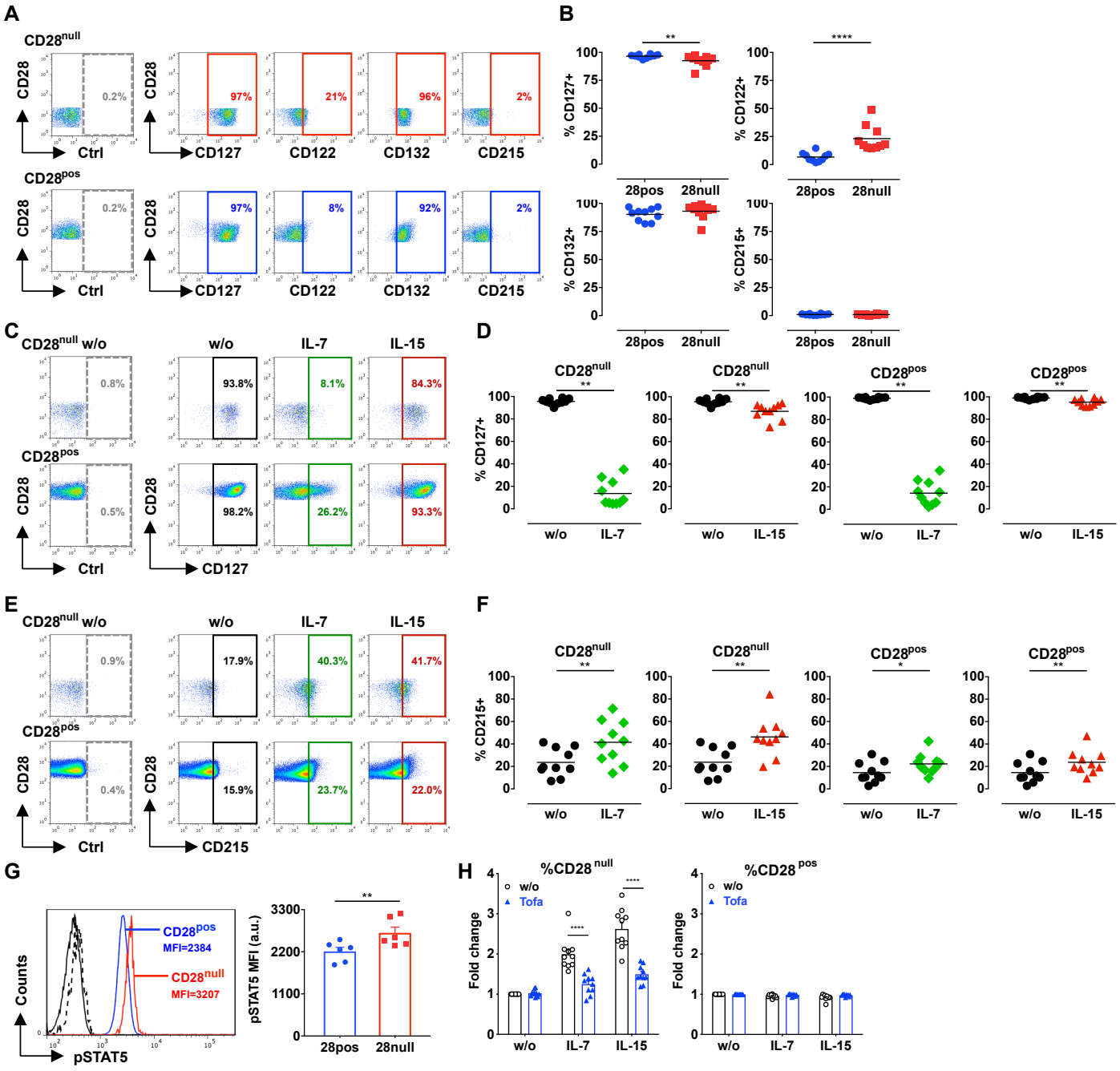
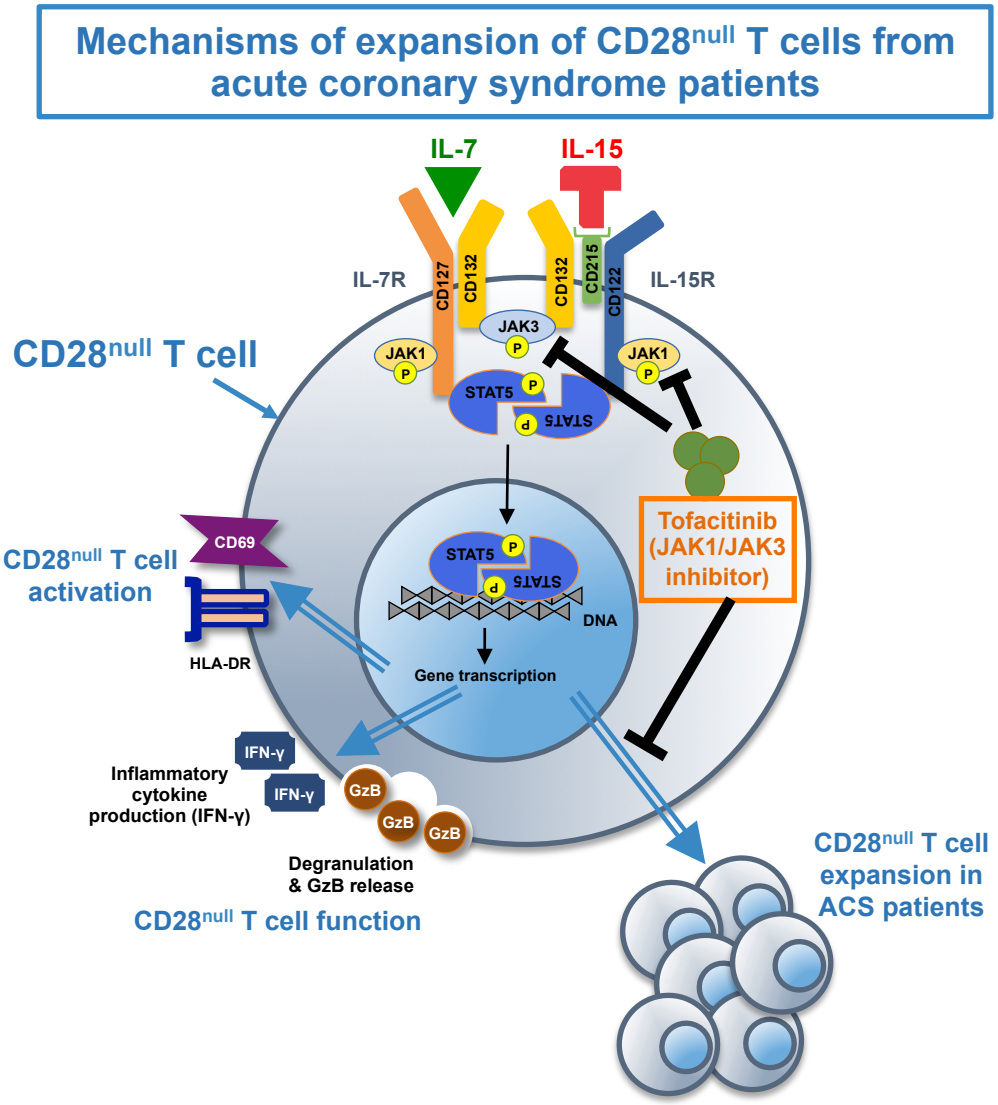
w/o
IL-7
IL-15
B
w/o 29201
IL-7 63613
IL-15 86788
Count
Granzyme B
MFI Granzyme B (a.u.)
C
0.0%
83.4%
99.9%
99.9%
D
w/o 9996
IL-7 53306
IL-15 58331
E
0.4%
0.4%
0.8%
1.0%
2.4%
11.4%
w/o + aCD3
IL-7 + aCD3
IL-15 + aCD3
28.3%
46.9%
56.3%
CD107a
%CD107a+ cells
aCD3
*a
****b


A
CD28null
w/o
w/o
IL-7
IL-15
CD28
0.3%
96.4%
93.7%
95.1%
Ctrl
TNF-α
CD28null
ns
ns
% TNF-α +
w/o
IL-7
IL-15
CD28pos
0.1%
85.9%
83.9%
77.5%
CD28pos
ns
*
B
CD28null
w/o 26839
IL-7 38385
IL-15 48579
Count
TNF-α
MFI TNF-α (a.u.)
**
CD28pos
w/o 20892
IL-7 33350
IL-15 27341
C
TNF-α (pg/ml)


A
CD28null
w/o
w/o
IL-7
IL-15
CD28
Ctrl
IFN-γ
0.1%
93.5%
89.0%
89.3%
CD28null
ns
% IFN-γ +
w/o
IL-7
IL-15
CD28pos
0.1%
14.1%
20.7%
15.2%
**
B
CD28null
Count
w/o 16489
IL-7 29286
IL-15 37596
IFN-γ
MFI IFN-γ (a.u.)
CD28pos
w/o 3894
IL-7 8860
IL-15 7740
*
C
IFN-γ (pg/ml)
2000
1500
1000
500
0


A
CD28null
CD28
Ctrl
0.2%
CD127
97%
CD122
21%
CD132
96%
CD215
2%
CD28pos
0.2%
97%
8%
92%
2%
B
% CD127+
% CD122+
% CD132+
% CD215+
28pos
28null
**
****
C
CD28null w/o
w/o
IL-7
IL-15
0.8%
93.8%
8.1%
84.3%
CD28pos
0.5%
98.2%
26.2%
93.3%
CD127
D
CD28null
CD28pos
% CD127+
w/o
IL-7
IL-15
E
CD28null w/o
0.9%
17.9%
40.3%
41.7%
CD28pos
0.4%
15.9%
23.7%
22.0%
CD215
F
% CD215+
*
G
Counts
pSTAT5
CD28pos
MFI=2384
CD28null
MFI=3207
pSTAT5 MFI (a.u.)
3300
2200
1100
0
H
%CD28 null
%CD28 pos
w/o
Tofa
Fold change
IL-7
IL-15

Graphical abstract



Supplementary Data

Interleukin-7 and interleukin-15 drive $CD4^{+}CD28^{null}$ T lymphocyte expansion and function in patients with acute coronary syndrome

Short title: IL-7 & IL-15 drive $CD28^{null}$ T-cell expansion and function

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Supplementary Data Inventory:

1. Supplemental Tables 1 and 2
2. Supplemental Figure legends
3. Supplemental Figures 1-13

Supplemental Table 1. ACS patients' characteristics (data included in Figures 1-7 and Supplemental Figures 1-12)

	ACS patients
Number (n)	83
Age, years (mean±SD)	64±13
Gender % (male/female)	77/23
Ethnicity % (C/A/B/O)*	64/24/4/8
Body mass index (mean±SD)	26.74±5.63
Family history of CAD (%)	36
Diabetes (type 2) (%)	24
Hypertension (%)	48
Current smoking (%)	33
Hypercholesterolemia (%)	36
Prior MI† (%)	14
Prior CABG‡ (%)	4
Prior PCI§ (%)	8
Type of MI % (STEMI/NSTEMI)‖	90/10
Cholesterol (mmol/L) (mean±SD)	4.85±1.14
LDL# (mmol/L) (mean±SD)	2.81±1.00
HDL** (mmol/L) (mean±SD)	1.35±0.61
Triglycerides	1.54±1.70
Aspirin (prior to admission, %)	20
Statin (prior to admission, %)	23

*C/A/B/O, Caucasian/Asian/Black/Other; †MI, myocardial infarction; ‡CABG, coronary artery bypass grafting; §PCI, percutaneous coronary intervention; ‖STEMI, ST-segment elevation MI; #LDL, low-density lipoprotein; **HDL, high-density lipoprotein

Supplemental Table 2. ACS patients' characteristics (data included in Supplemental Figure 13)

	ACS>3% CD28^{null}	**ACS<2% CD28^{null}**
Number (n)	38	38
Age, years (mean±SD)	65±13	60±13
Gender % (male/female)	71/29	79/21
Ethnicity % (C/A/B)*	58/40/2	82/16/2
Body mass index (BMI, mean±SD)	25.24±4.01	27.42±4.42
Family history of CAD (%)	50	45
Diabetes (type 2) (%)	26	29
Hypertension (%)	42	55
Current smoking (%)	34	42
Hypercholesterolemia (%)	24	26
Prior MI† (%)	11	16
Prior CABG‡ (%)	5	8
Prior PCI§ (%)	5	10
Type of MI % (STEMI/NSTEMI)‖	95/5	97/3
Cholesterol (mmol/L) (mean±SD)	4.71±1.02	4.67±0.93
LDL# (mmol/L) (mean±SD)	2.79±0.88	2.95±0.91
HDL** (mmol/L) (mean±SD)	1.32±0.37	1.10±0.23
Triglycerides	1.28±0.88	1.53±1.23
Aspirin (prior to admission, %)	16	13
Statin (prior to admission, %)	18	23

*C/A/B, Caucasian/Asian/Black; †MI, myocardial infarction; ‡CABG, coronary artery bypass grafting; §PCI, percutaneous coronary intervention; ‖STEMI, ST-segment elevation MI; #LDL, low-density lipoprotein; **HDL, high-density lipoprotein; BMI ($p<0.05$); no other significant differences were identified between the two groups

Supplemental Figures Legends

Supplemental Figure 1. Illustration of the gating strategy for quantification of $CD28^{null}$ T cells in fresh peripheral blood samples and cultured cells from acute coronary syndrome (ACS) patients. (A) Circulating $CD4^{+}CD28^{null}$ ($CD28^{null}$) T cells were quantified in fresh peripheral blood samples from ACS patients by staining with CD4-FITC and CD28-APC monoclonal antibodies. Illustrative dot plots showing gating of lymphocytes, $CD4^{+}$ T cells and $CD28^{null}$ T cells in samples with >3% (top) and <2% (bottom) circulating $CD28^{null}$ T cells. The frequency of $CD28^{null}$ T cells was calculated as percentage of $CD4^{+}$ T cells (details in Supplemental Methods). **(B)** Peripheral blood mononuclear cells from ACS patients were cultured alone (w/o, top) or in the presence IL-7 (middle) or IL-15 (bottom) for 4 days and stained with CD4-FITC, CD28-APC, CD14-PE and 7-AAD. Illustrative dot plots showing the gating strategy: following gating of lymphocytes, $CD4^{+}$ T cells and exclusion of dead cells (live $CD4^{+}$ T cells), CD28 expression was used to gate $CD28^{null}$ T cells. **(C)** $CD4^{+}$ T cells from ACS patients were cultured alone (w/o, top) or in the presence IL-7 (middle) or IL-15 (bottom) for 4 days and stained with CD4-FITC, CD28-APC, CD14-PE and 7AAD. Illustrative dot plots showing the gating strategy: following gating of lymphocytes, $CD4^{+}$ T cells and exclusion of dead cells, CD28 expression was used to gate $CD28^{null}$ T cells. Data included in panels **A**, **B**, **C** is representative of more than n=10 different ACS patients

Supplemental Figure 2. Effect of inflammatory cytokines on $CD28^{null}$ T cell expansion. Peripheral blood mononuclear cells from ACS patients with <2% circulating $CD28^{null}$ T cells were cultured alone or in the presence of **(A)** TNF-α (n=8), **(B)** IL-1β (n=6) or **(C)** IL-6 (n=7) as indicated for up to 7 days. Illustrative dot plots show the percentage of $CD28^{null}$ T cells in the analysed samples. Graphs show the percentage of

$CD28^{null}$ T cells for each cytokine and time point. No significant differences were identified (one-way ANOVA with post-test Bonferroni for multiple comparisons)

Supplemental Figure 3. Effect of inflammatory cytokines on $CD28^{null}$ T cell expansion. $CD4^+$ T cells from ACS patients with <2% circulating $CD28^{null}$ T cells were cultured alone or in the presence of the indicated concentrations of **(A)** TNF-α (n=7), **(B)** IL-1β (n=6) or **(C)** IL-6 (n=6) for up to 7 days. Illustrative dot plots show the percentage of $CD28^{null}$ T cells in each sample. Graphs show the percentage of $CD28^{null}$ T cells for each time point. No significant differences were identified (one-way ANOVA with post-test Bonferroni for multiple comparisons)

Supplemental Figure 4. Effect of inflammatory cytokine combinations on $CD28^{null}$ T cell expansion. Peripheral blood mononuclear cells from ACS patients (n=5) with <2% circulating $CD28^{null}$ T cells were cultured alone (w/o) or in the presence of different combinations of inflammatory cytokines (10 ng/ml TNF-α, 10 ng/ml IL-1β, 100 ng/ml IL-6) as indicated for up to 7 days. **(A)** Illustrative dot plots show the percentage of $CD28^{null}$ T cells in each sample. **(B)** Graphs show the percentage of $CD28^{null}$ T cells for each time point (mean±SEM)

Supplemental Figure 5. Effect of inflammatory cytokines on $CD28^{null}$ T cell expansion. PBMCs **(A-C**, n=6**)** or $CD4^+$ T cells **(D-F**, n=6**)** from ACS patients with >3% circulating $CD28^{null}$ T cells were cultured alone (w/o) or in the presence of **(A,D)** TNF-α, **(B,E)** IL-1β or **(C,F)** IL-6 as indicated for up to 7 days. Illustrative dot plots show the percentage of $CD28^{null}$ T cells in the analysed samples. Graphs show the percentage of $CD28^{null}$ T cells for each cytokine and time point. No significant differences were identified (one-way ANOVA with post-test Bonferroni for multiple comparisons)

Supplemental Figure 6. Effect of different concentrations of IL-7 and IL-15 on CD28^{null} T cell expansion at different time points. Peripheral blood mononuclear cells (**A-D** n=7) or CD4^{+} T cells (**E,F** n=7) from ACS patients with >3% circulating CD28^{null} T cells were treated with 10 ng/ml or 50 ng/ml IL-7 or IL-15 for up to 7 days. **(A,C,E)** Illustrative dot plots show the percentage of CD28^{null} T cells. **(B,D,F)** Graphs show the percentage of CD28^{null} T cells in untreated samples (w/o) or after treatment with IL-7 or IL-15.* p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001 (paired two-tailed Student's t test)

Supplemental Figure 7. Effect of IL-7 and IL-15 on CD28^{null} T cell expansion. Peripheral blood mononuclear cells (n=10) from ACS patients with <2% circulating CD28^{null} T cells were treated with 10 ng/ml or 50 ng/ml IL-7 or IL-15 for 4 and 7 days. **(A,C)** Illustrative dot plots display CD28^{null} T cell percentage following **(A)** IL-7 and **(C)** IL-15 treatment at the indicated time points. **(B,D)** Graphs show CD28^{null} T cell percentage in untreated samples (w/o) or after treatment with the indicated concentrations of **(B)** IL-7 or **(D)** IL-15. * p<0.05; ** p<0.01; ns, not significant (paired two-tailed Student's t test)

Supplemental Figure 8. Effect of IL-7 and IL-15 on CD28^{null} T cell expansion. CD4^{+} T cells from ACS patients with <2% CD28^{null} T cells were treated with 10 ng/ml or 50 ng/ml IL-7 or IL-15 for 4 (n=8), 7 (n=8) or 11 (n=6) days. **(A,C)** Illustrative dot plots show the percentage of CD28^{null} T cells following **(A)** IL-7 and **(C)** IL-15 treatment at the indicated time points. Graphs display the percentage of CD28^{null} T cells in untreated samples (w/o) or after treatment with the indicated concentration of **(B)** IL-7 or **(D)** IL-15. * p<0.05; ** p<0.01; ns, not significant (paired two-tailed Student's t test)

Supplemental Figure 9. Effect of inflammatory cytokines on CD28^{null} T cell activation. CD4^{+} T cells from ACS patients (n=6) were cultured alone (w/o) or treated with 10 ng/m TNF-α, 10 ng/m IL-1β, or 100 ng/m IL-6 for 4 days. The activation markers

CD69 and HLA-DR were analysed on $CD28^{null}$ and $CD28^{pos}$ T cells. Graphs show the percentage of $CD69^+$ cells **(A)** or HLA-DR^+ cells **(B)** in cytokine-treated and untreated samples. No significant differences were identified (two-tailed Wilcoxon matched-pairs signed rank test)

Supplemental Figure 10. Effect of IL-7 and IL-15 on expression of chemokine receptors by $CD28^{null}$ T cells. $CD4^+$ T cells from ACS patients (n=10) were cultured alone (w/o) or treated with 50 ng/ml IL-7 or IL-15 for 3-4 days. Expression of CCR5 and CXCR3 was analysed on $CD28^{null}$ and $CD28^{pos}$ T cells. Illustrative dot plots and graphs display the percentage of $CCR5^+$ cells **(A,B)** and $CXCR3^+$ cells **(C,D)** in $CD28^{null}$ and $CD28^{pos}$ T cells; dashed gates, isotype control antibody (Ctrl). ** $p<0.01$ (two-tailed Wilcoxon matched-pairs signed rank test)

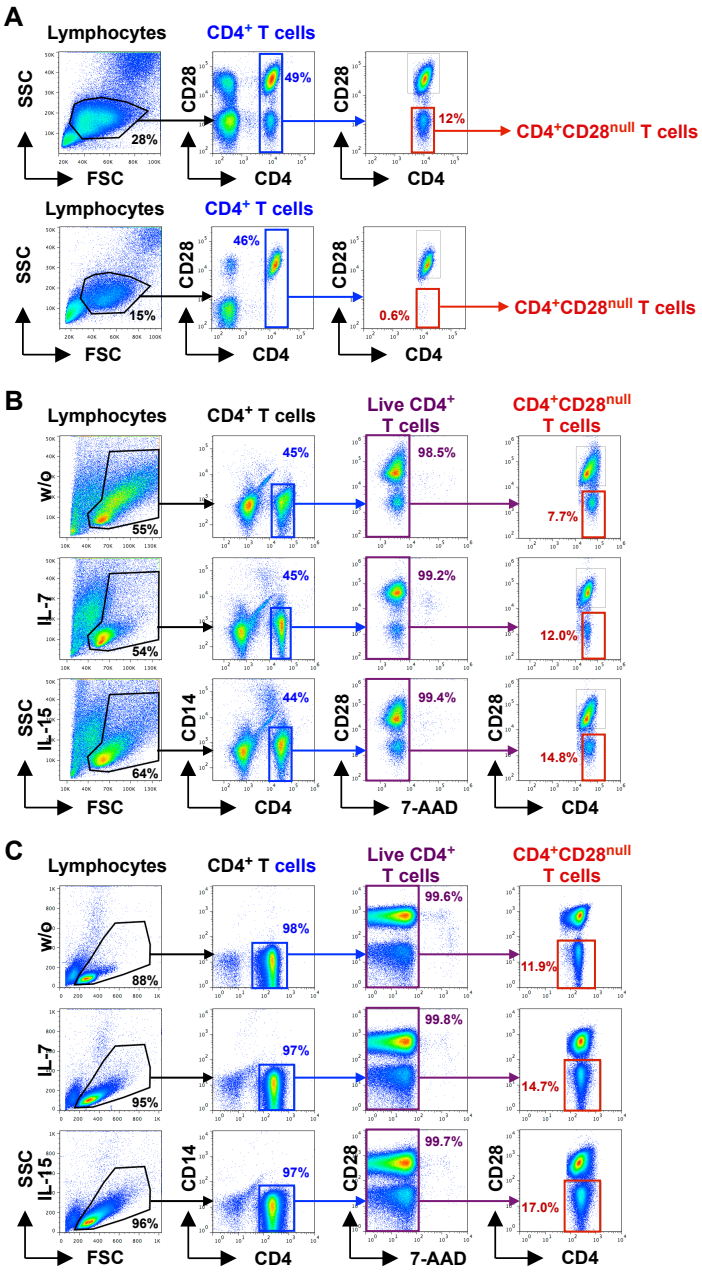
Supplemental Figure 11. Effect of IL-7 and IL-15 on expression of memory markers by $CD28^{null}$ T cells. $CD4^+$ T cells from ACS patients (n=10) were cultured alone (w/o) or treated with 50 ng/ml IL-7 or IL-15 for 3-4 days. Expression of CD62L, CCR7, CD45RA and CD45RO was analysed on $CD28^{null}$ and $CD28^{pos}$ T cells. Illustrative dot plots and graphs display the percentage of $CD45RA^+CD62L^+$, $CD45RA^-CD62L^+$, $CD45RA^-CD62L^-$ and $CD45RA^+CD62L^-$ cells **(A,B)**; $CD45RA^+CCR7^+$, $CD45RA^-CCR7^+$, $CD45RA^-CCR7^-$ and $CD45RA^+CCR7^-$ cells **(C,D)**; and $CD45RA^+CD45RO^-$, $CD45RA^+CD45RO^+$ and $CD45RA^-CD45RO^+$ cells **(E,F)** in $CD28^{null}$ and $CD28^{pos}$ T cells (mean±SEM); dashed gates, isotype control antibody (Ctrl). No significant differences were identified (two-way ANOVA with post-test Bonferroni for multiple comparisons)

Supplemental Figure 12. Comparison of different concentrations of IL-7 or IL-15 on $CD28^{null}$ T cell proliferation at different time points. $CD4^+$ T cells from ACS patients (n=5) were labelled with CFSE and cultured in the presence of 10 or 50 ng/ml IL-7 **(A)** or IL-15 **(B)** up to 7 days. Graphs show proliferation of $CD28^{null}$ and $CD28^{pos}$ T cells in

untreated samples (w/o) and after cytokine treatment at the indicated time points. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$; ns, not significant (paired two-tailed Student's t test)

Supplemental Figure 13. Cytokine plasma levels in ACS patients. Cytokine levels were quantified in EDTA-plasma samples from ACS patients with >3% circulating $CD28^{null}$ T cells (with $CD28^{null}$ T cell expansion; n=38) and ACS patients with <2% circulating $CD28^{null}$ T cells (without $CD28^{null}$ T cell expansion; n=38). Graphs show the concentration of the indicated cytokines in the two study groups (**A.** IL-7 and IL-15; **B.** TNF-α, IL-1β and IL-6). The horizontal bar indicates the mean concentration. No significant differences were identified (two-tailed Mann-Whitney test)

Supplemental Figure 1



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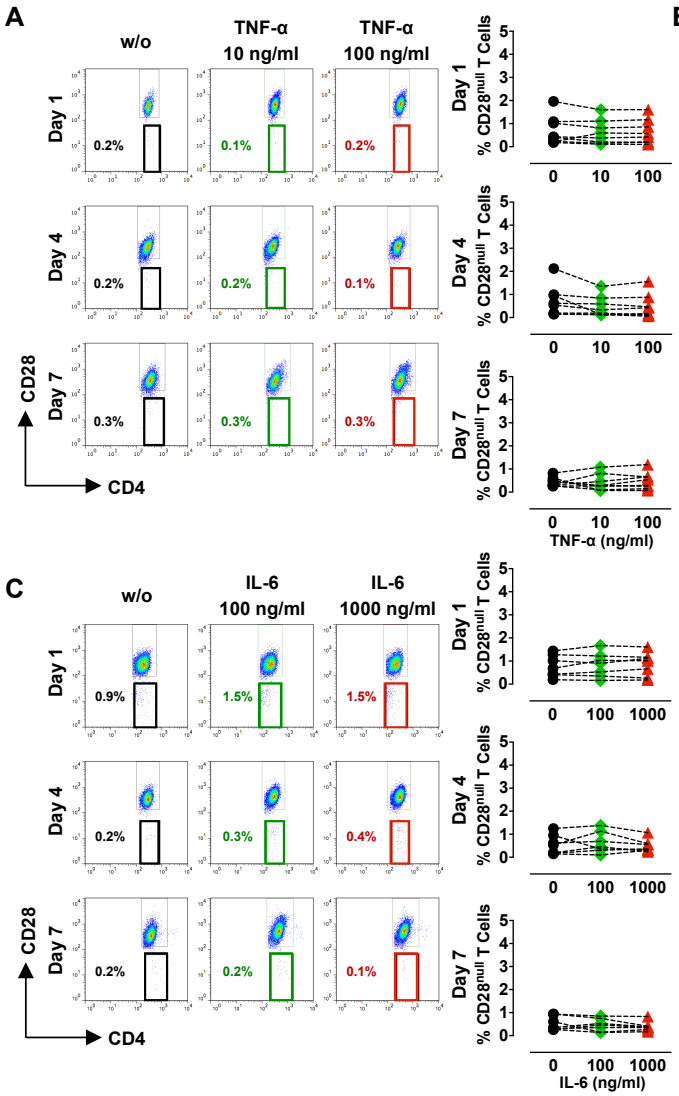

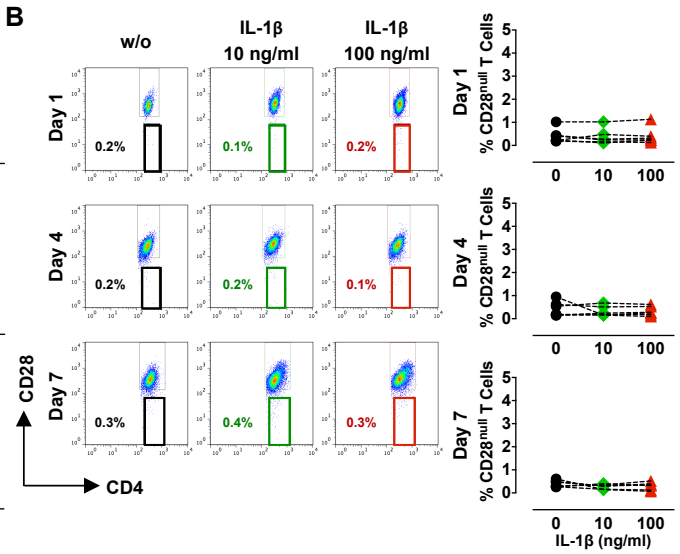

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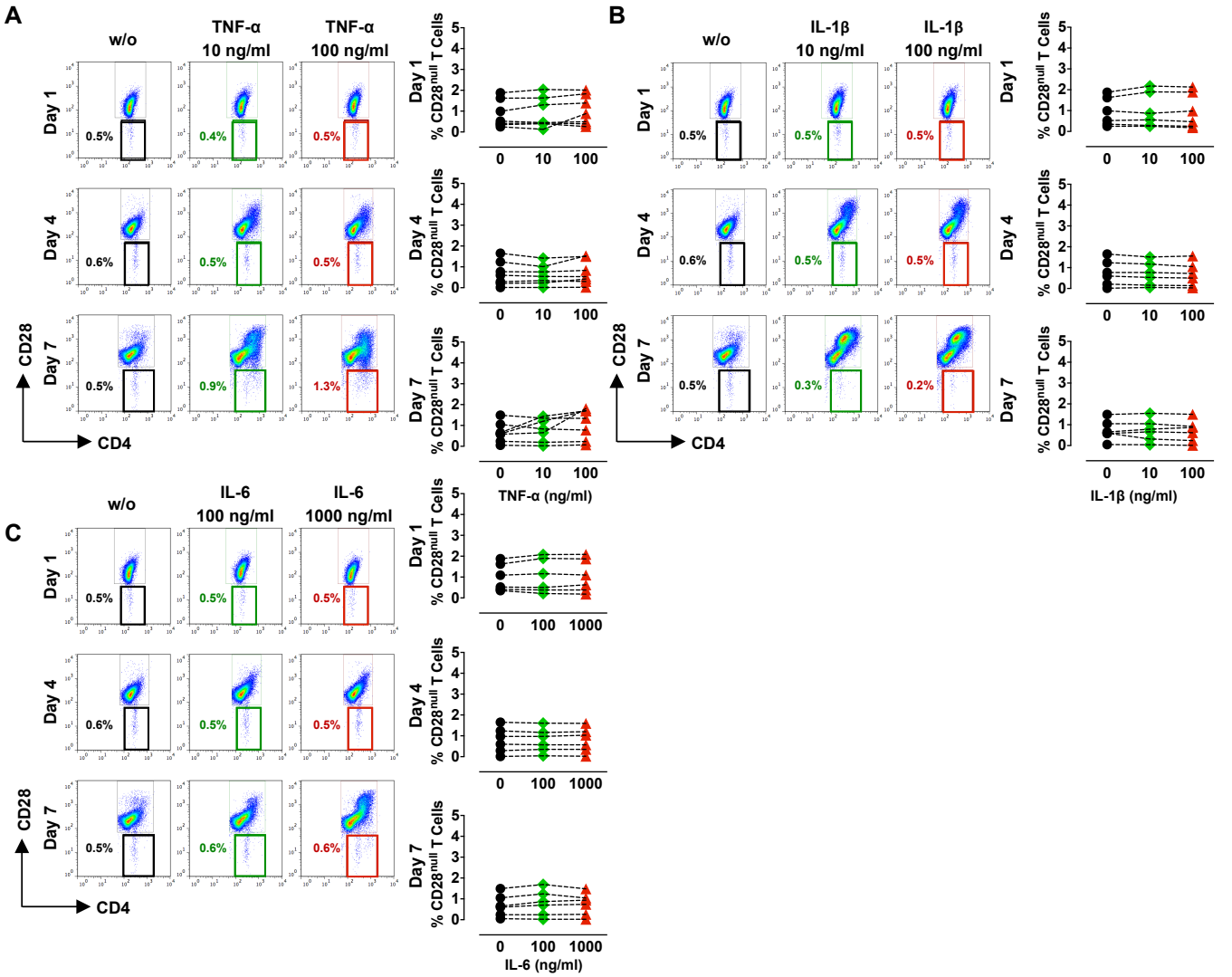

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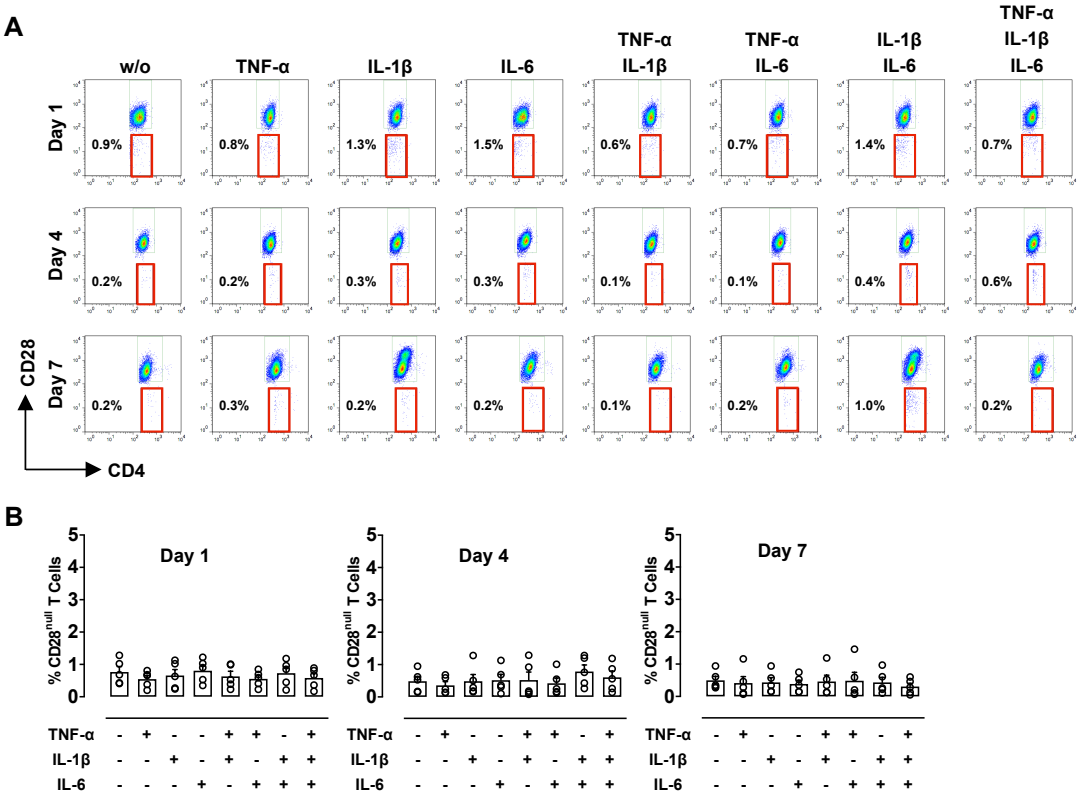

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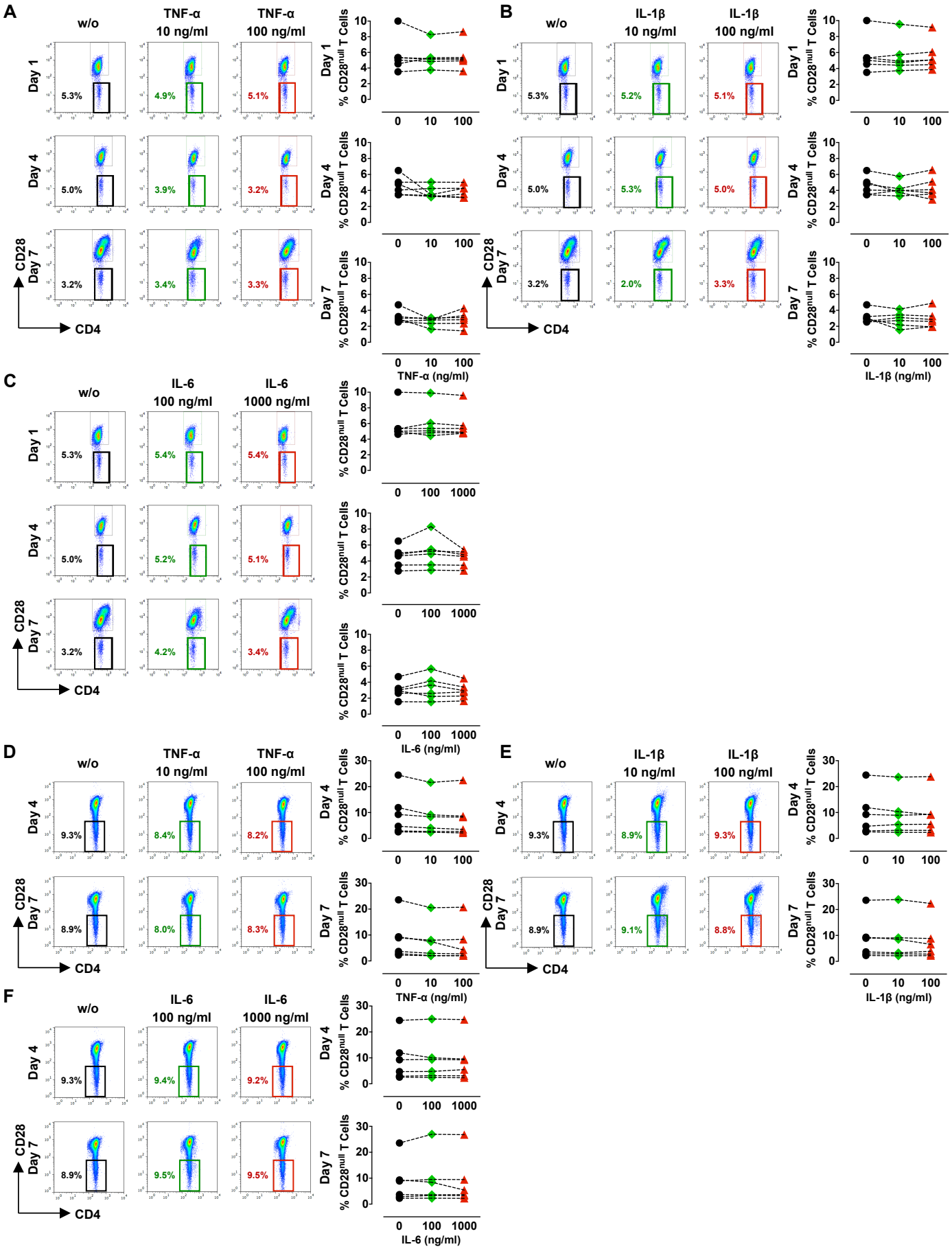

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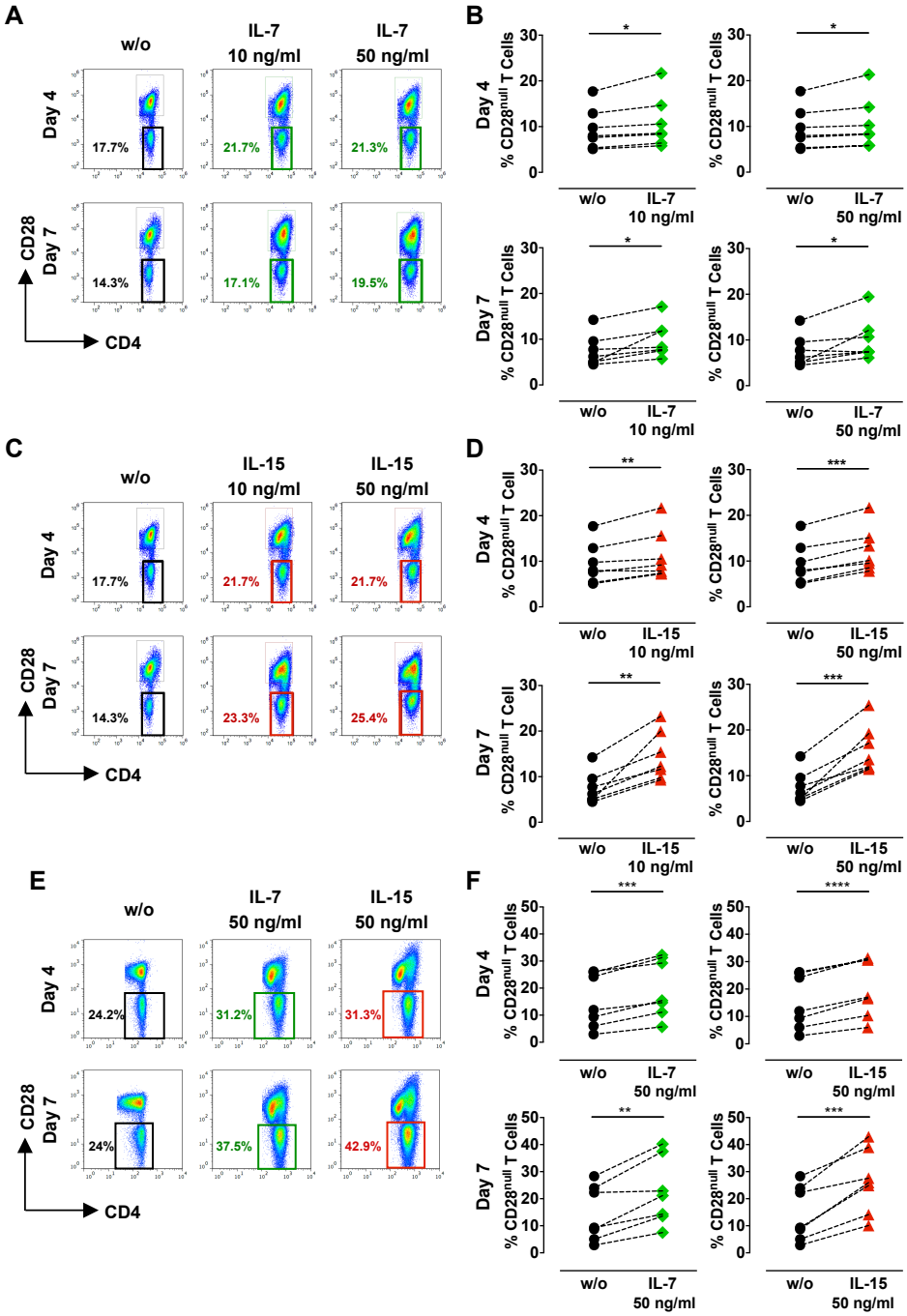

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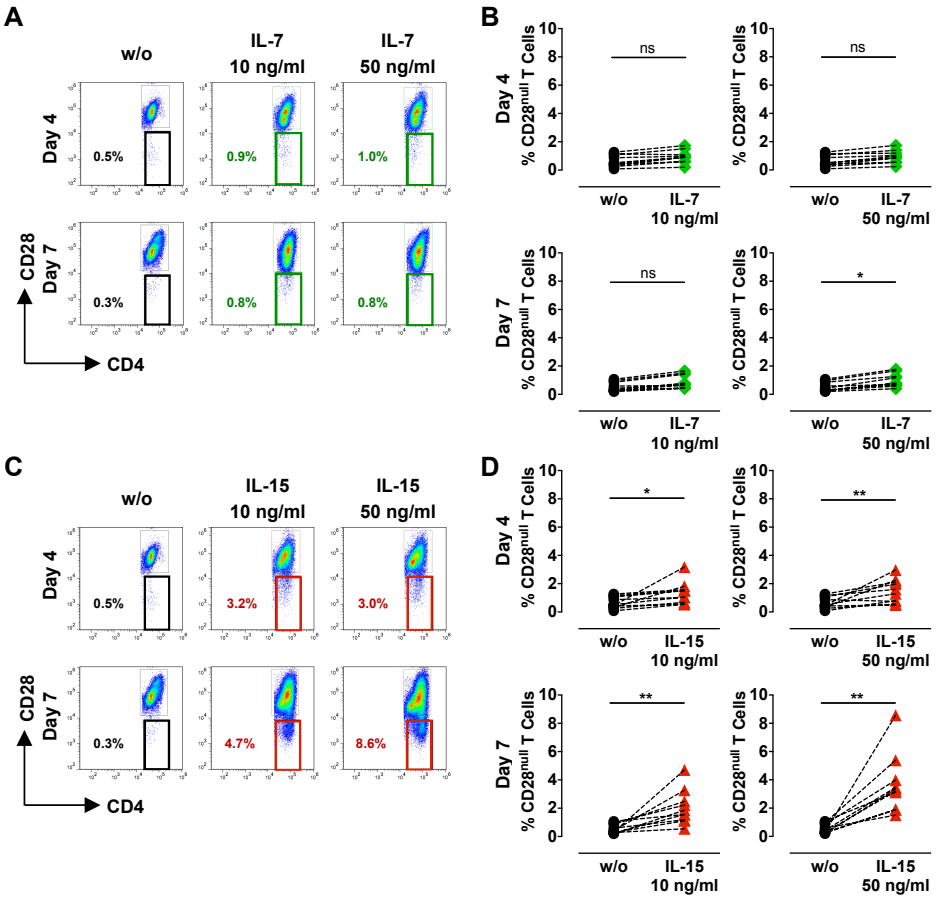

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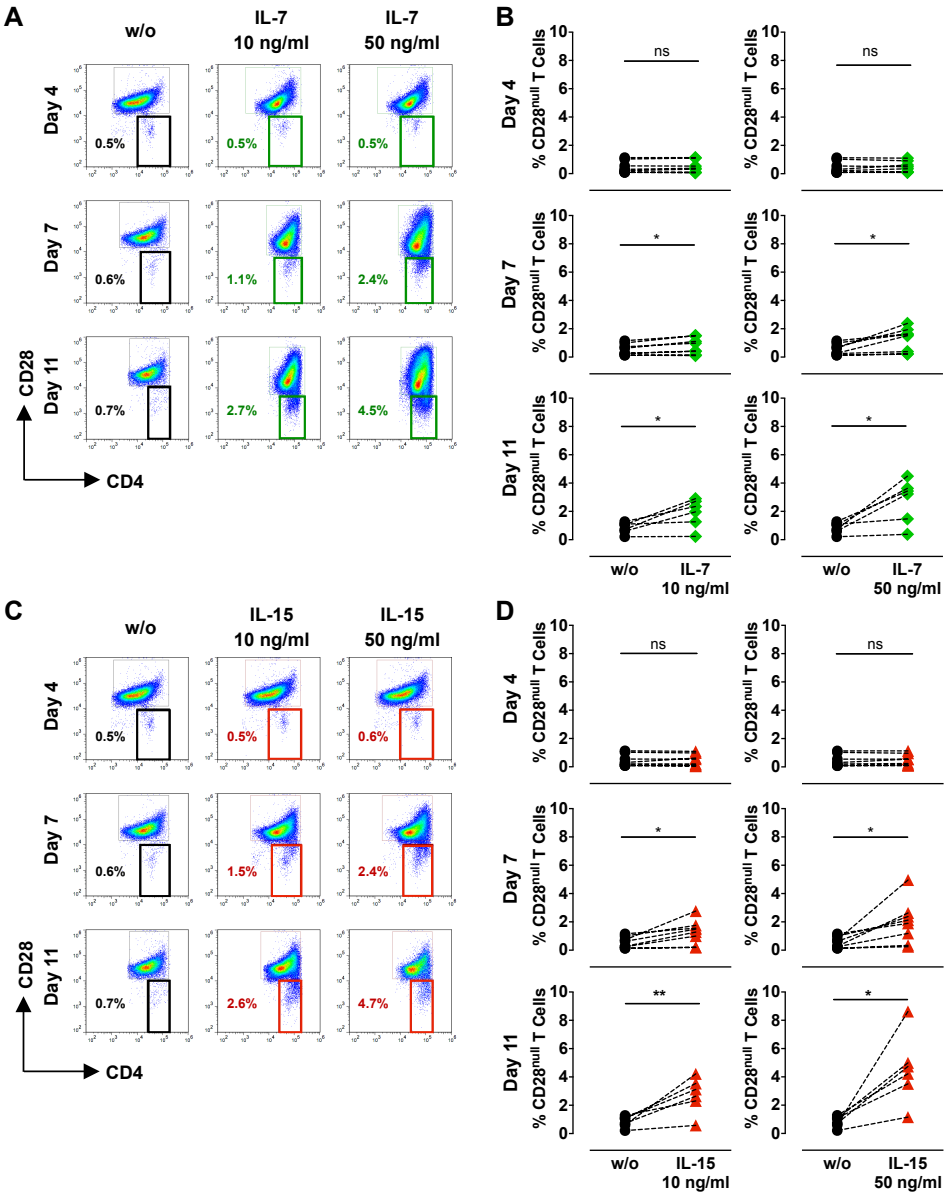

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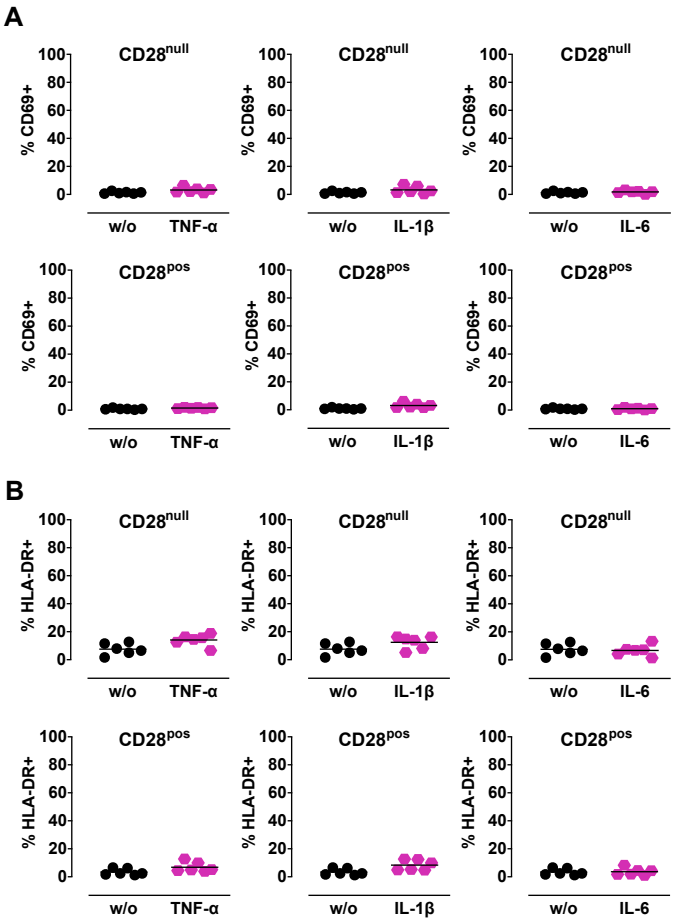

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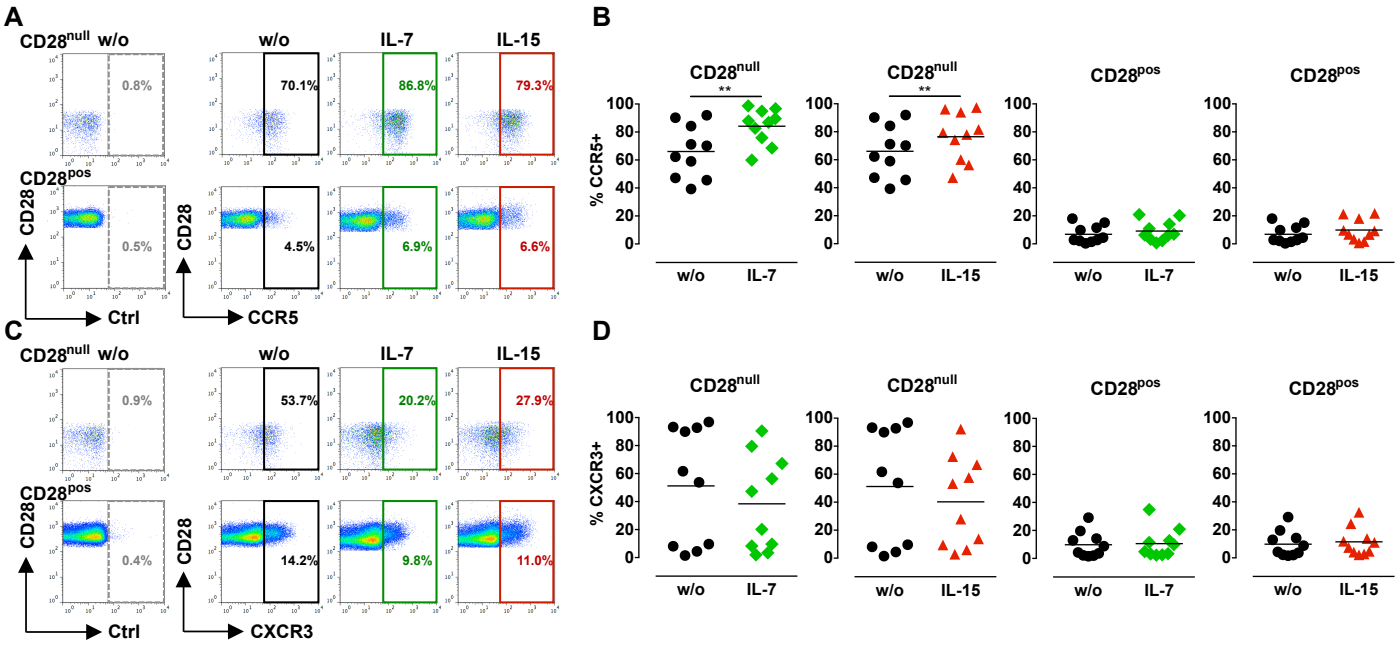

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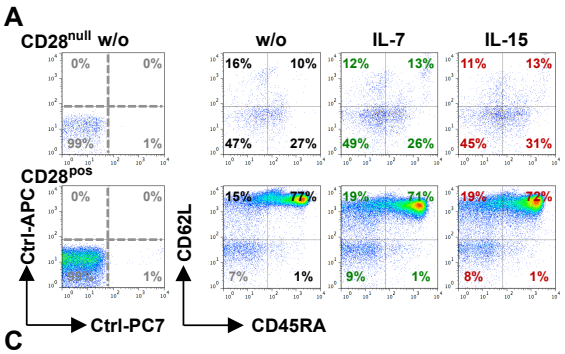

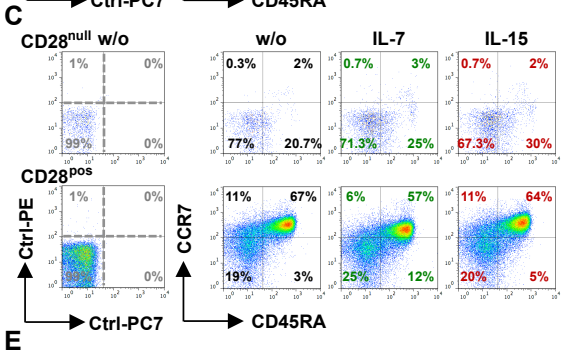

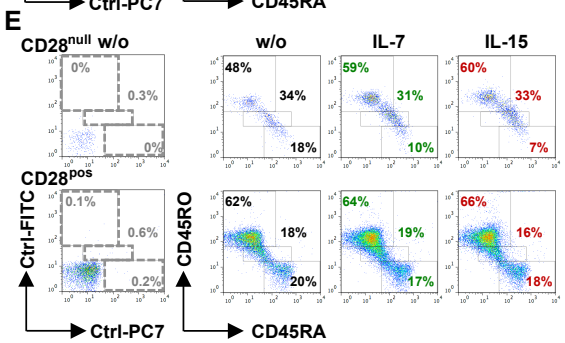

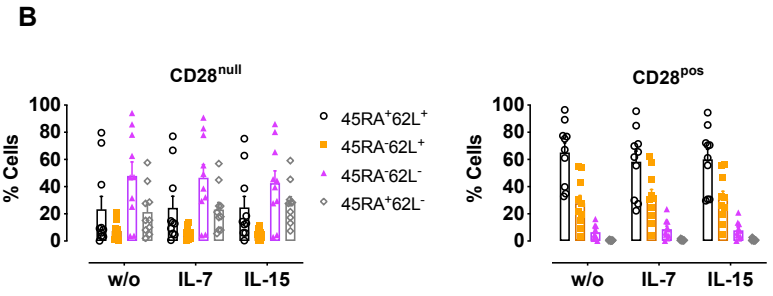

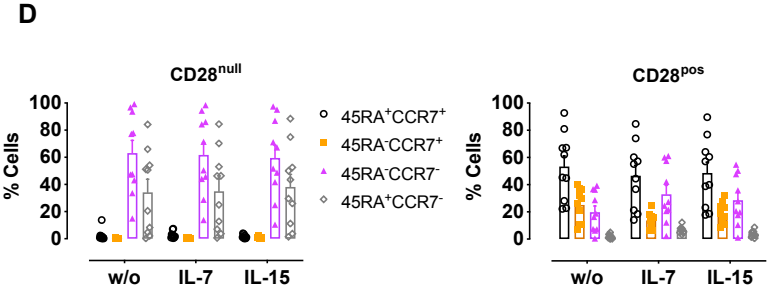

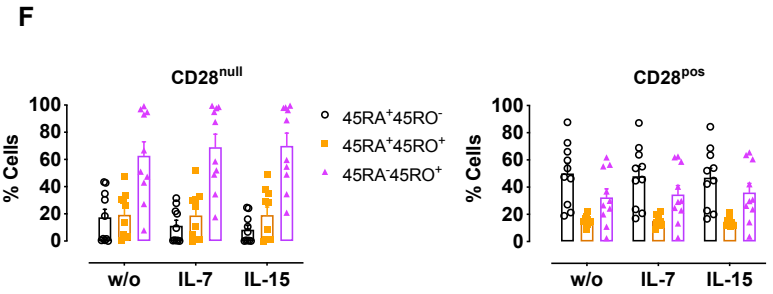

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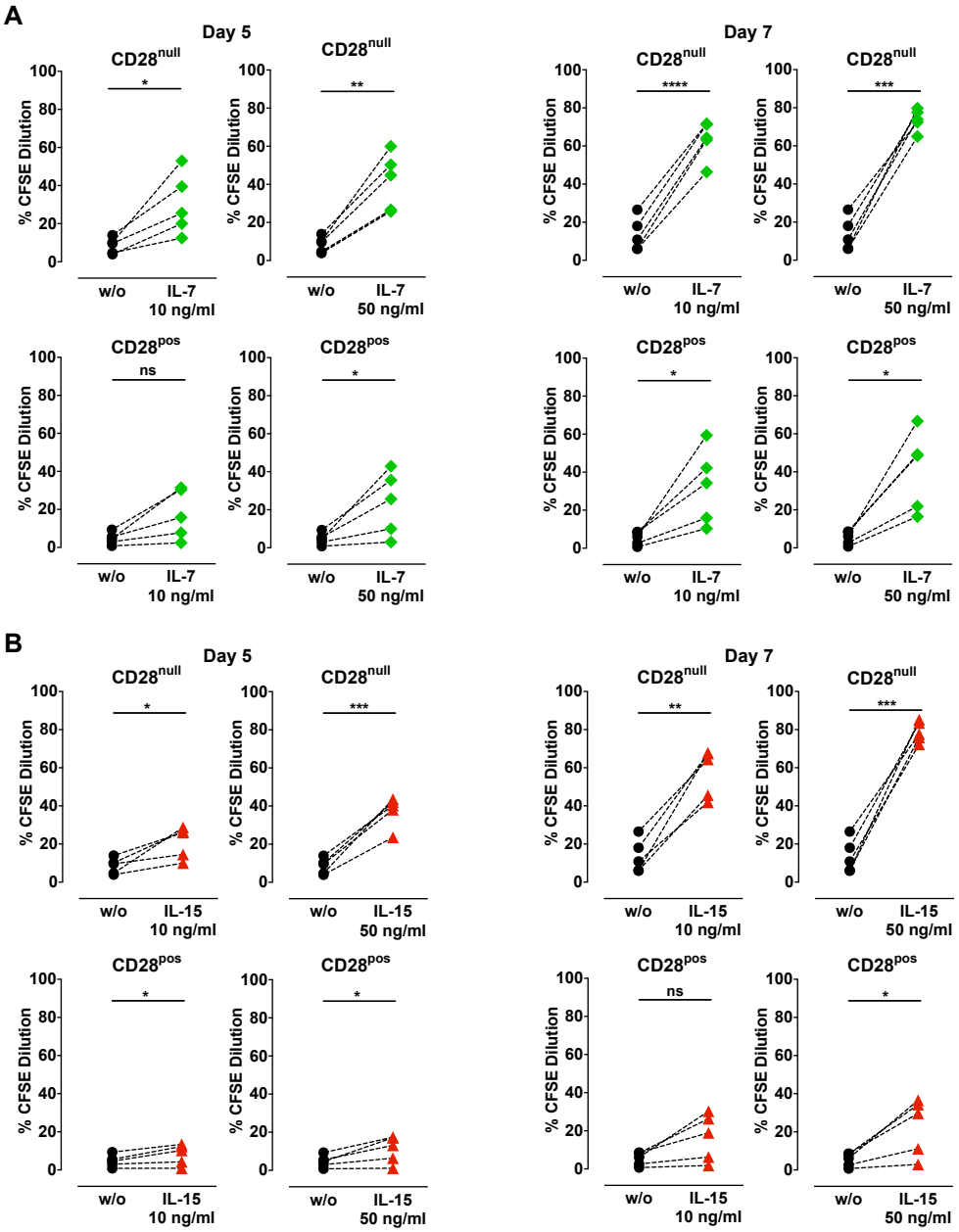

Supplemental Figure 13