Proteasome-mediated reduction in pro-apoptotic molecule Bim renders $CD4^{+}CD28^{null}$ T cells resistant to apoptosis in acute coronary syndrome

Kovalcsik: Apoptosis resistance of $CD28^{null}$ T cells in ACS

Edit Kovalcsik PhD[1], Ricardo F. Antunes PhD[1], Paramita Baruah MD, PhD[1], Juan Carlos Kaski MD, DSc, FRCP, FESC, FACC, FAHA[1], Ingrid E. Dumitriu MD, PhD[1*]

[1]Cardiovascular and Cell Sciences Research Institute, St. George's University of London, Cranmer Terrace, SW17 0RE, London, UK

[*]**Corresponding author:** Ingrid E. Dumitriu, Cardiovascular and Cell Sciences Research Institute, St. George's University of London, Cranmer Terrace, SW17 0RE, London, UK; Phone: 00442087252808; Fax: 00442087253328; e-mail: i.dumitriu@sgul.ac.uk

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Abstract

Background: The number of $CD4^{+}CD28^{null}$ ($CD28^{null}$) T cells, a unique subset of T lymphocytes with proinflammatory and cell-lytic phenotype, increases markedly in patients with acute coronary syndrome (ACS). ACS patients harbouring high numbers of $CD28^{null}$ T cells have increased risk of recurrent severe acute coronary events and unfavourable prognosis. The mechanisms that govern the increase in $CD28^{null}$ T cells in ACS remain elusive. We investigated whether apoptosis pathways regulating T cell homeostasis are perturbed in $CD28^{null}$ T cells in ACS.

Methods and Results: We found that $CD28^{null}$ T cells in ACS were resistant to apoptosis induction via Fas-ligation or ceramide. This was due to a dramatic reduction in pro-apoptotic molecules Bim, Bax and Fas in $CD28^{null}$ T cells, whilst anti-apoptotic molecules Bcl-2 and Bcl-xL were similar in $CD28^{null}$ and $CD28^{+}$ T cells. We also show that Bim is phosphorylated in $CD28^{null}$ T cells and degraded by the proteasome. Moreover, we demonstrate for the first time that proteasomal inhibition restores the apoptosis sensitivity of $CD28^{null}$ T cells in ACS.

Conclusions: We show that $CD28^{null}$ T cells in ACS harbour marked defects in molecules that regulate T cell apoptosis, which tips the balance in favour of anti-apoptotic signals and endows these cells with resistance to apoptosis. We demonstrate that inhibition of proteasomal activity allows $CD28^{null}$ T cells to regain sensitivity to apoptosis. A better understanding of the molecular switches that control apoptosis sensitivity of $CD28^{null}$ T cells may reveal novel strategies for targeted elimination of these T cells in ACS patients.

Key words: apoptosis; atherosclerosis; immunology; T lymphocytes

Introduction

Myocardial infarction and stroke caused by atherosclerosis are the most frequent cardiovascular causes of death and disability in the world despite progress in prevention and therapy[1]. Recent evidence clearly implicates the innate and adaptive immune system in the pathogenesis of atherosclerosis[2]. T lymphocytes, the main orchestrators of adaptive immune responses, have pivotal roles in atherosclerosis. In particular, pro-inflammatory $CD4^+$ T lymphocytes such as T helper 1 (Th1) cells have been shown to promote atherosclerosis in animal models and constitute the predominant T cells in human atherosclerotic plaques[3-5]. We and others have demonstrated that $CD4^+CD28^{null}$ T cells, a unique subset of Th1 lymphocytes characterised by the lack of co-stimulatory receptor CD28, are present in high numbers in the circulation and atherosclerotic plaques of patients with acute coronary syndrome (ACS)[6-8], whilst these cells are not present in healthy individuals. Moreover, $CD4^+CD28^{null}$ T cells (from now on abbreviated as $CD28^{null}$ T cells) produce higher levels of inflammatory cytokines interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) than conventional $CD4^+CD28^+$ ($CD28^+$) T cells[6,9]. In stark contrast to $CD28^+$ T lymphocytes, $CD28^{null}$ T cells release cytotoxic molecules (i.e. perforin and granzymes)[6] that are instrumental in killing endothelial and vascular smooth muscle cells *in vitro*[10]. These properties suggest that $CD28^{null}$ T cells have harmful effects in atherosclerosis, although a direct causal link is yet to be established as this subset of T cells is present only in humans with an equivalent population absent in mice. Recently, we were the first to show that the distinguishing feature between $CD28^{null}$ and conventional $CD28^+$ T cells in ACS patients is a significantly higher expression on $CD28^{null}$ T cells of alternative co-stimulatory receptors 4-1BB and OX40[6]. We further demonstrated that 4-1BB and OX40 have critical roles in regulating the production of inflammatory cytokines IFN-γ and TNF-α and perforin release from $CD28^{null}$ T cells in ACS[6]. In addition to their roles in T cell co-stimulation, 4-1BB and OX40 are important for the survival of T cells[11,12]. As mentioned, ACS patients are known to harbour significantly higher numbers of $CD28^{null}$ T cells compared to stable angina (SA) patients and healthy subjects[8,13]. Importantly, ACS

patients with higher numbers of $CD28^{null}$ T cells have increased risk for recurrent severe acute coronary events and unfavourable prognosis[13]. This study also demonstrated that the number of $CD28^{null}$ T cells does not change during the course of the acute coronary event and up to two years afterwards. Thus understanding the precise mechanisms that lead to accumulation of $CD28^{null}$ T cells in ACS may reveal targets for novel therapies to prevent accumulation of these inflammatory cells.

T cell homeostasis is maintained by elimination of unwanted T cells via apoptotic cell death. Whether a cell survives or succumbs to apoptosis depends on a fine balance between pro- and anti-apoptotic signals[14]. Two main albeit converging apoptosis pathways have been well characterised: extrinsic (death-receptor-dependent) and intrinsic (mitochondrial-dependent) apoptosis[14]. The ultimate event in both pathways is activation of caspases, a family of cysteine proteases that cleave essential cellular targets, ultimately resulting in cell death. Fas (CD95) is the prototypical death receptor in the extrinsic apoptosis pathway[15]. Upon binding to its ligand, FasL (CD95L), Fas transmits a death signal to the cell via its intracellular death domain. The mitochondrial-dependent apoptosis pathway is regulated by Bcl-2 (B cell lymphoma-2) family members[16]. This includes anti-apoptotic (pro-survival) members with Bcl-2 and Bcl-xL as prototypic molecules, and pro-apoptotic members such as Bim and Bax[17,18]. It has been suggested that Bax and its homologues are maintained in an inactive state by association with the anti-apoptotic Bcl-2 members (Bcl-2, Bcl-xL) at the mitochondrial membrane and this 'happy balance' must be overcome for apoptosis to occur[17]. This is primarily accomplished by Bim, which frees Bax from its complex with Bcl-2/Bcl-xL in response to death signals[19], thereby allowing Bax to exert its pro-apoptotic actions.

We hypothesised that induction of apoptosis, which mediates elimination of T cells is dysregulated in $CD28^{null}$ T cells, and this could explain their accumulation in ACS patients. With this in view, we investigated the sensitivity of $CD28^{null}$ T cells from ACS patients to apoptosis. We provide novel evidence that, in ACS, $CD28^{null}$ T cells are resistant to apoptosis. We further demonstrate that the resistance to apoptosis is due to preponderance of anti-apoptotic signals in $CD28^{null}$ T cells as a result of a marked reduction in pro-apoptotic molecules Bim and Bax and the death receptor

Fas. Critically, we propose a novel mechanism that links Bim phosphorylation and its subsequent degradation by the proteasome as a key event that governs the loss of apoptosis sensitivity in $CD28^{null}$ T cells in ACS patients. Our data suggest for the first time that proteasome inhibition restores apoptosis sensitivity in $CD28^{null}$ T cells, which could potentially open new therapeutic avenues to eliminate these cells.

Methods

Patient population

Peripheral blood was obtained from patients with a diagnosis of ACS (STEMI and NSTEMI) and patients with stable angina (SA). The number of patients used in individual experiments is specified in the figure legends. Blood samples were collected 4-12h from the onset of chest pain. The demographic details and clinical characteristics of patients used to compare the levels of apoptotic molecules in $CD28^{null}$ T cells from ACS and SA patients are provided in **Supplemental Table 1**. Patients with evidence of infectious diseases, malignancies, haematologic or immunologic disorders, treatment with anti-inflammatory drugs other than aspirin, and an ejection fraction <40 were excluded. The study was approved by the local research ethics committee; informed consent was obtained from all study subjects.

Flow cytometric analysis

The following antibodies were used: CD4-FITC, CD28-APC, Bcl-2-PE (all from BD Biosciences), Fas/CD95-PE, FasL(CD95L)-PE (eBiosciences), and Bcl-xL-PE (Abcam). Purified Bim (BD Biosciences) and purified Bax (R&D Systems) were used followed by labelling with donkey anti-rabbit-PE (eBiosciences). Surface and intracellular staining were performed in accordance with the manufacturer's instructions. Briefly, 100μl of blood was incubated with antibodies for 30-40min; red blood cells were lysed using Lyse/Fix buffer (BD Biosciences). For intracellular staining, cells were fixed and permeabilised using the Cytofix/Cytoperm buffer (BD Biosciences) prior to addition of antibodies for Bcl-2, Bcl-xL, Bim and Bax. Samples were acquired on a FACSCalibur (BD Biosciences) and data was analysed using the FlowJo software. Mean fluorescence intensity (MFI) was calculated by subtracting the MFI of samples stained with isotype control antibodies from the MFI of samples stained with specific antibodies.

Cell isolation

Histopaque (Sigma) was used to isolate peripheral blood mononuclear cells (PBMCs). $CD4^+$ T cells were sorted by negative selection using the $CD4^+$ T cell isolation kit II (Miltenyi Biotech) as

per the manufacturer's instructions. The purity of sorted $CD4^+$ T cells was 90-95%. $CD28^{null}$ and $CD28^+$ T cells were sorted from the purified $CD4^+$ T cells using the CD28 Microbead kit II (Miltenyi Biotech) as per the manufacturer's instructions. The purity of sorted cells $CD28^{null}$ and $CD28^+$ T cells ranged between 85-95% (**Supplemental Figure 1**).

T cell activation and proliferation assays

PBMCs were cultured at $5x10^5$ cells per well in 96-well U-bottomed plates (Nunc) in the presence or absence of 2ug/ml plate-bound anti-CD3 antibodies (eBioscience) for 4 days before detection of apoptotic molecules as described for whole blood samples. Cells were cultured in RPMI 1% Penicillin/Streptomycin/L-glutamine (all from Gibco) supplemented with 5% pooled human AB serum (Lonza) and 25IU/ml interleukin-2 (IL-2, Roche). To assess the proliferation of $CD28^{null}$ and $CD28^+$ T cells from ACS patients, T cells were labelled with 1μM CFSE (carboxyfluorescein succinimidyl ester) and stimulated with 2ug/ml plate-bound anti-CD3 antibodies (eBioscience), as previously described[20]. Cells were cultured in RPMI 1% Penicillin/Streptomycin/L-glutamine (all from Gibco) supplemented with 5% pooled human AB serum (Lonza) and 25IU/ml interleukin-2 (IL-2, Roche) for 4 days. Proliferation as visualised by CFSE-dilution was quantified by flow cytometry.

PhosFlow analysis of ERK1/2 signalling and Bim phosphorylation

The following phospho site specific antibodies were used for detection of phosphorylated ERK1/2 and Bim: anti-phospho-ERK1/2 (pT202/pY204) (BD Biosciences) and anti-phospho-Bim (pS65, Abcam). $CD28^{null}$ and $CD28^+$ T cells purified by magnetic sorting were either left unstimulated or stimulated by CD3 cross-linking with monoclonal antibodies for 15min at 37ºC. Where indicated, cells were pre-incubated for 1 hour with the ERK1/2 specific inhibitor PD184352 (10μM, Sigma) or the proteasomal inhibitor MG-132 (10μM, Merck Millipore) followed by activation with anti-CD3 antibodies in the continuous presence of the inhibitor. Cells were fixed with BD Cytofix Fixation buffer for 10min at 37ºC and then permeabilised with BD PhosFlow Perm buffer III for 30min on ice prior to staining with phospho site specific antibodies, as per the manufacturer's recommendations.

Apoptosis assays

Sorted $CD28^{null}$ and $CD28^{pos}$ T cells were cultured at $5x10^4$ cells per well in 96-well U-bottomed plates (Nunc) in serum-free X-Vivo medium (Lonza) supplemented with 1% Penicillin/Streptomycin/L-glutamine and 25IU/ml IL-2. For apoptosis induction cells were treated with 100ng/ml of anti-Fas clone CH11 antibody (Millipore) or 50μM C2-ceramide (Enzo Life Sciences). Apoptotic cells were measured at 20 hours using annexin V-FITC and 7-AAD (BD Biosciences) as per the manufacturer's instructions. In some experiments, cells were treated with 5μg/ml, 10μg/ml, 30μg/ml and 90ug/ml Atorvastatin®Pfizer (Selleckchem) or Rosuvastatin (Selleckchem) and cultured for 48 hours prior to apoptosis detection. Statins were purchased as reconstituted pure active compound. Where indicated, cells were pre-treated with the ERK1/2 specific inhibitor PD184352 (5μM, Sigma) or the proteasomal inhibitor MG-132 (10nM, Merck Millipore) prior to CH11 treatment. Induction of apoptosis as a percentage was calculated as follows: 100x(percent apoptotic cells with CH11 - percent apoptotic cells without CH11)/(percent apoptotic cells without CH11). Where indicated, normalisation of apoptosis was performed to pool data from different patients. The percentage of $CD28^{pos}$ apoptotic cells in samples treated with CH11 alone was normalised to 100. All other samples were normalised accordingly.

Statistical analysis

Categorical data were analysed by the χ^2-test. Statistically significant differences were determined using the Student's *t*-test, ANOVA with post-test multiple comparisons or Wilcoxon matched-pairs signed rank test (as indicated in the Figure legends). Probability values (p) of less than 0.05 were considered statistically significant. Statistical analysis was performed using the GraphPad Prism software version 5.02 and 6.04.

Results

CD28^{null} T cells from ACS patients are resistant to apoptosis.

Two features led us to hypothesise that CD28^{null} T cells in ACS patients have alterations in apoptosis sensitivity: i) their number is increased[8]; and ii) these cells express higher levels of alternative co-stimulatory receptors 4-1BB and OX40[6], that have been shown to promote survival of other subsets of T lymphocytes[11,12]. To verify this hypothesis we examined the response of CD28^{null} T cells to apoptosis induction. For this purpose, we treated purified CD28^{null} and CD28^{+} T cells with CH11, a widely used agonistic anti-Fas antibody that triggers apoptosis in T cells by mimicking the effect of Fas ligation by FasL[21,22]. Apoptosis induction was quantified using Annexin-V (AxV) and 7-AAD. As expected, CH11 treatment induced apoptosis in conventional CD28^{+} T cells, as quantified by an increase in both early (AxV^{+}7-AAD^{-}) and late (AxV^{+}7-AAD^{+}) apoptotic cells (**Figure 1A,B**). In contrast, CD28^{null} T cells exhibited significantly lower induction of apoptosis (both early and late) upon CH11 treatment in comparison to conventional CD28^{+} T cells (n=18, $p<0.001$, **Figure 1A-C**). We next examined whether the resistance of CD28^{null} T cells to apoptosis induction via Fas ligation extended to other triggers of apoptosis. To test this, purified CD28^{null} and CD28^{+} T cells were treated with C2-ceramide, a membrane-permeable ceramide analogue that has been suggested to induce apoptosis via the mitochondrial pathway through release of cytochrome c from the mitochondria[23]. As observed with CH11, C2-ceramide treatment resulted in a good induction of early and late apoptosis in CD28^{+} T cells (**Figure 2A,B**). In keeping with our results with CH11, CD28^{null} T cells from ACS patients underwent significantly less apoptosis in response to C2-ceramide compared to CD28^{+} T cells (n=10, $p<0.005$ for early apoptosis and $p<0.05$ for late apoptosis, **Figure 2A-C**).

It has been suggested that statins could induce apoptosis of CD28^{null} T cells[24]. To test this, we cultured purified CD28^{null} and CD28^{+} T cells either alone or in the presence of increasing concentrations of Atorvastatin or Rosuvastatin for 48 hours and quantified apoptosis induction. At all concentrations tested (5, 10, 30 and 90μg/ml), Atorvastatin and Rosuvastatin did not induce significant apoptosis in either CD28^{null} or CD28^{+} T cells (**Supplemental Figure 2A,B**).

Noteworthy, the concentration of statins used in these experiments corresponded to plasma concentrations reached in patients on treatment with statins[25]. Furthermore, the highest concentration tested was well above the maximum recommended dose of 80mg Atorvastatin or 40mg Rosuvastatin. These results demonstrate that statins do not induce apoptosis in $CD28^{null}$ T cells.

Another reason for an increase in the number of $CD28^{null}$ T cells in ACS patients may be differences in the proliferation of $CD28^{null}$ and $CD28^{+}$ T cells. Therefore, we quantified the proliferation of sorted $CD28^{null}$ and $CD28^{+}$ T cells following stimulation with plate-bound anti-CD3 cross-linking antibodies using the CFSE-dilution method. As seen in **Supplemental Figure 3**, $CD28^{+}$ T cells proliferated efficiently in response to anti-CD3 antibodies. Indeed, more than 80% of $CD28^{+}$ T cells exhibited CFSE dilution and had undergone 1 to 5 division rounds. In contrast, $CD28^{null}$ T cells had a slower division rate and proliferated significantly less than $CD28^{+}$ T cells.

$CD28^{null}$ T cells from ACS patients express low levels of death receptor Fas and pro-apoptotic molecules Bim and Bax.

Following on our findings that $CD28^{null}$ T cells in ACS are resistant to apoptosis, we further investigated the mechanisms underlying the apoptosis resistance of this cell subset. Whether cells undergo apoptosis or survive depends on the balance between pro- and anti-apoptotic signals. We therefore examined the expression of death receptor Fas and its ligand FasL, as well as pro-apoptotic (Bim and Bax) and anti-apoptotic (Bcl-2, Bcl-xL) molecules in circulating $CD28^{null}$ T cells from ACS patients (n=25). We analysed $CD28^{null}$ T cells directly *ex vivo* by staining fresh blood samples with monoclonal antibodies. We found that $CD28^{null}$ T cells have significantly reduced levels of Fas compared to conventional $CD28^{+}$ T cells, while there was no difference in FasL expression ($p<0.001$ for Fas, **Figure 3A,B**). In addition, levels of pro-apoptotic proteins Bim and Bax were significantly decreased in $CD28^{null}$ T cells in comparison to $CD28^{+}$ T cells ($p<0.001$, **Figure 3A,B**). In contrast, the anti-apoptotic molecules Bcl-2 and Bcl-xL were expressed at similar levels in $CD28^{null}$ T cells and their conventional $CD28^{+}$ counterparts (**Figure 3A,B**). These results

demonstrate that the balance between pro- and anti-apoptotic molecules is altered in favour of anti-apoptotic signals in $CD28^{null}$ T cells in ACS.

$CD28^{null}$ T cells fail to upregulate pro-apoptotic molecules Bim and Bax upon activation.

Activation of T cells is known to sensitise them to apoptosis[14,26]. We therefore investigated the impact of T cell activation on the expression of Fas, Bim and Bax pro-apoptotic molecules in $CD28^{null}$ T cells from ACS patients. For this purpose, cells were cultured *in vitro* for four days in the presence or absence of signals that activate T cells (i.e. anti-CD3 cross-linking antibodies). We showed that unstimulated $CD28^{null}$ T cells from ACS patients maintained low levels of Fas, Bim and Bax following 4 days of culture (**Supplemental Figure 4A-C**), in line with our observations on fresh cells (**Figure 3**). Following activation, Fas expression reached similar levels on the two T lymphocyte subsets (**Supplemental Figure 4A**). Noteworthy, even upon activation, $CD28^{null}$ T cells from ACS patients failed to up-regulate pro-apoptotic molecules Bim and Bax to levels comparable to conventional $CD28^{+}$ T cells (**Supplemental Figure 4B,C**). No differences were noted in the expression of FasL and anti-apoptotic molecules Bcl-2 and Bcl-xL in cultured $CD28^{null}$ and $CD28^{+}$ T cells whether the cells were activated or not, similar to our data on fresh T cells.

Bim and Bax are markedly lower on $CD28^{null}$ T cells in ACS compared to SA.

In comparison to healthy individuals, the number of circulating $CD28^{null}$ T cells increases not only in ACS but also in SA patients, albeit at lower levels than in ACS[13]. We therefore investigated whether the alterations in apoptotic molecules that we identified in ACS were also present in $CD28^{null}$ T cells from patients with SA. There was no significant difference in the demographic and clinical characteristics of the two patient groups (**Supplemental Table 1**). Similar to our findings in ACS patients, the levels of Fas, Bim and Bax were significantly lower in $CD28^{null}$ T cells from SA patients in comparison to conventional $CD28^{+}$ T cells (**Supplemental Figure 5**). We next compared the levels of these pro-apoptotic molecules on $CD28^{null}$ T cells from ACS versus SA patients. Importantly, we found that Bim and Bax levels were significantly lower in $CD28^{null}$ T cells from ACS patients compared to SA patients ($p<0.01$ for Bim and $p<0.001$ for Bax, ACS (n=25) vs. SA (n=18), **Figure 4A**) and that the anti-apoptotic molecule Bcl-2 was higher in

$CD28^{null}$ T cells from ACS patients ($p<0.05$, **Figure 4B**). No differences were detected in the expression of Fas, FasL and anti-apoptotic molecule Bcl-xL in $CD28^{null}$ T cells between ACS and SA patients (**Figure 4A,B**). Our findings suggest that the reduction in Bim and Bax is more pronounced in $CD28^{null}$ T cells in ACS patients compared to SA and that this is accompanied by higher levels of the anti-apoptotic protein Bcl-2.

ERK1/2 is activated in $CD28^{null}$ T cells from ACS patients.

As the pro-apoptotic molecule Bim has a pivotal role in regulating apoptosis, we next examined the mechanisms that underlie the reduction in Bim levels in $CD28^{null}$ T cells in ACS. It has been suggested that post-translational modifications of Bim, such as phosphorylation, target it for proteasomal degradation[27], thereby promoting cell survival. Activation of the ERK1/2 signalling pathway has been shown to promote Bim phosphorylation and proteasome-dependent degradation of phosphorylated Bim[28]. We therefore analysed ERK1/2 signalling in $CD28^{null}$ and $CD28^{+}$ T cells from ACS patients. We used the PhosFlow technique, which, in comparison to standard western blotting, provides a direct cell-based assessment of signalling events when the number of cells obtainable for analysis is low, as is the case with $CD28^{null}$ T cells. Purified $CD28^{null}$ and $CD28^{+}$ T cells were analysed either directly (resting T cells) or following activation by cross-linking CD3 with monoclonal antibodies. We observed consistently high baseline ERK1/2 phosphorylation in resting $CD28^{null}$ T cells (**Figure 5A,C**). In contrast, resting $CD28^{+}$ T cells exhibited low levels of ERK1/2 phosphorylation, which were significantly lower than those observed in resting $CD28^{null}$ T cells ($p<0.001$, **Figure 5A,C**). These results clearly demonstrate constitutive activation of ERK1/2 in resting $CD28^{null}$ T cells in ACS. *In vitro* stimulation with anti-CD3 antibodies led to upregulation of phosphorylated ERK1/2 in both $CD28^{null}$ and $CD28^{+}$ T cells (**Figure 5B,C**). Of note, even following activation the levels of phosphorylated ERK1/2 remained significantly higher in $CD28^{null}$ T cells compared to their conventional $CD28^{+}$ counterparts ($p<0.001$, **Figure 5A,C**).

Bim is phosphorylated in $CD28^{null}$ T cells from ACS patients.

As mentioned, ERK1/2 activation has been shown to induce phosphorylation of Bim, which targets Bim for degradation via the proteasome[28]. We therefore assessed the expression of phosphorylated

Bim (phospho-Bim) in $CD28^{null}$ T cells in ACS patients using an antibody that recognises solely the phosphorylated form of human Bim. Phospho-Bim was detected in both resting $CD28^{null}$ and $CD28^{+}$ T cells (**Figure 6A,C**). However, resting $CD28^{null}$ T cells expressed significantly higher levels of phospho-Bim than conventional $CD28^{+}$ T cells ($p<0.001$, **Figure 6A,C**). Following activation with anti-CD3 cross-linking antibodies, phospho-Bim increased further in $CD28^{null}$ T cells, whilst in $CD28^{+}$ T cells it remained unchanged (**Figure 6B,C**). These results support our hypothesis that Bim is phosphorylated in $CD28^{null}$ T cells, which may tag it for proteasomal degradation. To determine whether ERK activation plays a role in Bim phosphorylation in $CD28^{null}$ T cells, we pre-incubated purified $CD28^{null}$ and $CD28^{+}$ T cells from ACS patients with PD184352, a specific ERK1/2 inhibitor[29] followed by activation with anti-CD3 cross-linking antibodies in the continuous presence of the inhibitor and quantified phospho-Bim expression. As shown previously (**Figure 6B**), activation of $CD28^{null}$ T cells increased phospho-Bim levels (**Figure 6E**). This increase was abrogated by ERK1/2 inhibition with PD184352 ($p<0.05$, **Figure 6D,E**). ERK1/2 inhibition did not affect phospho-Bim levels in $CD28^{+}$ T cells (**Figure 6D,E**). Bim phosphorylation has been shown to tag the protein for degradation by the proteasome[28]. We therefore examined whether proteasome inhibition affects the levels of phospho-Bim in $CD28^{null}$ T cells. Treatment with the cell-permeable proteasomal inhibitor MG-132[27] increased phospho-Bim levels in both resting and activated $CD28^{null}$ T cells ($p<0.05$, **Figure 6E**), whilst it did not have an effect on $CD28^{+}$ T cells.

Proteasomal inhibition restores apoptosis sensitivity of $CD28^{null}$ T cells in ACS.

We have demonstrated that $CD28^{null}$ T cells have higher levels of activated ERK1/2 and this associates with Bim phosphorylation and proteasomal degradation in $CD28^{null}$ T cells. It has been shown that proteasomal degradation of phosphorylated Bim, confers apoptosis resistance by leaving Bcl-2 and Bcl-xL unhampered to exert their anti-apoptotic effects[27]. We therefore hypothesised that inhibiting Bim phosphorylation by blocking ERK1/2 or preventing phospho-Bim degradation by blocking the proteasome should restore the apoptosis sensitivity of $CD28^{null}$ T cells. To test this hypothesis we investigated the effect of the ERK1/2 inhibitor PD184352 on apoptosis

sensitivity of $CD28^{null}$ T cells in ACS patients. Purified $CD28^{null}$ and $CD28^{+}$ T cells were induced to enter apoptosis by treatment with CH11 in the presence or absence of PD184352. We found that ERK1/2 inhibition with PD184352 did not significantly affect the apoptosis of $CD28^{null}$ T cells in response to CH11 (**Figure 7**). Even though we observed a trend towards an increase in the percentage of early apoptotic $CD28^{null}$ T cells this did not reach statistical significance (**Figure 7**).

Next we investigated the effect of proteasome inhibition on $CD28^{null}$ T cell apoptosis. For this, cells were treated with CH11 in the presence or absence of the cell-permeable proteasomal inhibitor MG-132. MG-132 on its own did not affect the viability of $CD28^{null}$ and $CD28^{+}$ T cells which were not treated with CH11 (**Figure 8A,B**). Importantly, MG-132 significantly increased apoptosis of $CD28^{null}$ T cells treated with CH11 ($p<0.01$, **Figure 8A,B**). This was true for both early and late apoptotic $CD28^{null}$ T cells. Of note, MG-132 did not influence the apoptosis of $CD28^{+}$ T cells treated with CH11 (**Figure 8A,B**), suggesting that it did not affect the viability of conventional $CD28^{+}$ T cells.

Discussion

Here we report that $CD28^{null}$ T cells from ACS patients harbour important alterations in apoptosis that could explain their accumulation. We demonstrate for the first time that in ACS patients $CD28^{null}$ T cells are endowed with resistance to apoptosis induction both via the death receptor (Fas-mediated) and mitochondrial (ceramide-mediated) pathways. Moreover, we show that the molecular basis for the loss of apoptosis sensitivity in $CD28^{null}$ T cells is a marked reduction in pro-apoptotic molecules Bim and Bax and death receptor Fas. Critically, we propose a novel mechanism that links Bim phosphorylation and its degradation by the proteasome as the key event that governs the loss of apoptosis sensitivity in $CD28^{null}$ T cells. In addition, we present promising new results that demonstrate for the first time that apoptosis sensitivity of $CD28^{null}$ T cells can be reinstated by proteasomal inhibition.

T cell sensitivity to apoptosis is tightly regulated and the intricate mechanisms controlling this process have not been completely deciphered. Here we show for the first time that $CD28^{null}$ T cells from ACS patients are resistant to apoptosis induction and that they exhibit reduced levels of pro-apoptotic molecules Bim and Bax and death receptor Fas, compared to conventional $CD28^{+}$ T cells. Changes in levels of members of the Bcl-2 family and their interactions have long been recognised to regulate survival of T cells[16]. In particular, Bim has pivotal roles in controlling the fine balance between pro-apoptosis and pro-survival molecules in various cells, including T cells[30]. However, the role of Bim in $CD28^{null}$ T cells has not been previously investigated. Bim acts as an initiator of apoptosis and although it does not trigger apoptosis itself, it enables Bax to induce cell death[30]. In viable cells, the pro-apoptotic function of Bax is inhibited by binding to anti-apoptotic molecules (Bcl-2 and Bcl-xL)[31]. In response to death signals, Bim translocates from the cytosol/cytoskeleton to the mitochondria and binds to Bcl-2, displacing it from its complex with Bax, which in turn releases Bax to trigger apoptosis. Thus Bim is crucial for the pro-apoptotic action of Bax[30]. Our finding that $CD28^{null}$ T cells in ACS have significantly reduced levels of both Bim and Bax suggests that in these cells the anti-apoptotic activity of Bcl-2/Bcl-xL is left unopposed by pro-apoptotic signals from Bim and Bax, which may endow $CD28^{null}$ T cells with

apoptosis resistance and give these cells a survival advantage. This is supported by other studies which report that induction of either Bim or Bax counteracts Bcl-2 anti-apoptotic signals and can enable apoptosis even when Bcl-2 levels are maintained[30]. Furthermore, deletion of Bim and Bax inhibited the death of murine T cells in knock-out models[32,33]. Bim has also been suggested to collaborate with Fas in the control of T cell homeostasis and elimination of unwanted T cells[34,35]. Mice deficient in both Bim and Fas accumulate more T cells and display severe autoimmunity compared to those deficient in either Bim or Fas that develop mild autoimmune phenomena[36]. Of note, we found that freshly analysed $CD28^{null}$ T cells from ACS patients had low levels of both Bim and Fas, which may suggest a pronounced defect in apoptosis sensitivity in these cells. Moreover, the levels of Bim, Bax and Fas stayed low even when $CD28^{null}$ T cells were cultured *in vitro*. Interestingly, following *in vitro* activation, which is known to increase the sensitivity of T cells to apoptosis, Fas increased on $CD28^{null}$ T cells to levels comparable to $CD28^{+}$ T cells, while Bim and Bax remained significantly low. These findings suggest that Bim and Bax are the major drivers of apoptosis resistance in $CD28^{null}$ T cells.

Our results show that anti-apoptotic molecules Bcl-2 and Bcl-xL are expressed at similar levels in $CD28^{null}$ and $CD28^{+}$ T cells in ACS patients, implying that these molecules do not have a central role in apoptosis resistance of $CD28^{null}$ T cells. Our findings are in contrast to data on $CD28^{null}$ T cell clones from patients with rheumatoid arthritis (RA) [37,38], another disease in which this subset expands. These studies reported an increase in Bcl-2 in $CD28^{null}$ T cell lines and clones compared to lines and clones derived from $CD28^{+}$ T cells. In addition, they did not identify any differences in Fas, Bcl-xL and Bax between $CD28^{null}$ and $CD28^{+}$ T cell lines, whilst Bim was not investigated. Bcl-2 upregulation reported in these studies on RA patients could be explained by use of $CD28^{null}$ T cell lines/clones, instead of analysing fresh primary T cells (as was done in our study on ACS patients). Clonal expansion alters the levels of pro- and anti-apoptotic molecules, as cells are subjected to several rounds of activation *in vitro*, aimed at extending their life in culture and therefore may not be representative of the apoptosis sensitivity of these cells *in vivo*.

It has been suggested that Rosuvastatin may induce apoptosis of $CD28^{null}$ T cells in ACS patients[37]. In our hands, direct treatment of pure populations of $CD28^{null}$ and $CD28^{+}$ T cells from ACS patients with increasing doses of Atorvastatin or Rosuvastatin did not induce apoptosis. Indeed many ACS patients who are already on statin therapy still exhibit high numbers of $CD28^{null}$ T cells and previous reports showed that $CD28^{null}$ T cell frequency remained unchanged for up to two years after an acute coronary event although statins are routinely prescribed following ACS[13], which indicate that statins do not induce apoptosis in $CD28^{null}$ T cells, in line with our *in vitro* findings.

Given Bim's central role in controlling the balance between pro- and anti-apoptotic signals, we investigated the mechanisms responsible for Bim reduction in $CD28^{null}$ T cells. Previous studies on cancer cell lines, fibroblasts and $CD8^{+}$ T cells from OT-I transgenic mice have suggested that Bim can be degraded by the proteasome, following post-transcriptional modifications such as phosphorylation[27,39]. We demonstrate that ERK1/2, a protein kinase that has been implicated in regulating Bim levels by phosphorylating it, is constitutively activated (phosphorylated) in resting $CD28^{null}$ T cells from ACS patients, whilst conventional $CD28^{+}$ T cells exhibited significantly lower ERK1/2 activation. In addition, we also detected higher levels of phosphorylated ERK1/2 following activation of $CD28^{null}$ T cells, well above those present in $CD28^{+}$ T cells. Another novel finding was that $CD28^{null}$ T cells express significantly higher levels of phosphorylated Bim than conventional $CD28^{+}$ counterparts. In support of a role for ERK1/2 in Bim phosphorylation, we present data showing that ERK1/2 inhibition reduced phosphorylated Bim levels in $CD28^{null}$ T cells following activation via CD3 cross-linking. Bim phosphorylation has been shown to tag this protein for degradation by the proteasome, which may explain the marked reduction in Bim that characterises $CD28^{null}$ T cells in ACS patients. In support of this, we demonstrated that proteasome inhibition increased phosphorylated Bim levels in both resting and activated $CD28^{null}$ T cells, confirming a central role for the proteasome in Bim regulation.

Next we sought a method to restore apoptosis sensitivity of $CD28^{null}$ T cells while sparing the conventional T cells. We tested two strategies: (i) ERK1/2 inhibition to prevent Bim

phosphorylation; and (ii) proteasome inhibition to prevent phospho-Bim degradation. We demonstrate that treatment with the proteasomal inhibitor MG-132 restored apoptosis sensitivity of $CD28^{null}$ T cells to CH11 in ACS patients. Encouragingly, proteasomal inhibition did not alter apoptosis of conventional T cells. Proteasomal inhibitors have been used to sensitise cancer cells to apoptosis. Of note, the dose of proteasomal inhibitor that restored apoptosis sensitivity in $CD28^{null}$ T cells was in the nano molar range, approximately a thousand times lower than the doses used to induce apoptosis in cancer cells, suggesting that re-sensitisation of $CD28^{null}$ T cells to apoptosis could potentially be achieved *in vivo* in ACS at much lower doses of proteasomal inhibitors than those used for elimination of malignant cells, with lower side effect profile. This makes the proteasome an attractive target for selective elimination of $CD28^{null}$ T cells, while sparing their conventional counterparts and preventing bystander immunosupression.

In our hands, ERK inhibition failed to significantly alter apoptosis sensitivity of $CD28^{null}$ T cells from ACS patients. This suggests that proteasomal degradation of Bim in $CD28^{null}$ T cells is not solely controlled by ERK1/2, as is the case for other cells such as $CD8^{+}$ T cells from OT-I transgenic mice. Indeed, we found that ERK1/2 inhibition failed to affect the levels of phospho-Bim in resting $CD28^{null}$ T cells, in which we showed that ERK is constitutively activated. Interestingly, ERK inhibition abrogated de novo induced Bim phosphorylation upon activation of $CD28^{null}$ T cells. In contrast, proteasomal inhibition increased phospho-Bim levels in both resting and activated $CD28^{null}$ T cells. This may explain why proteasomal inhibition was more effective at restoring apoptosis sensitivity in $CD28^{null}$ T cells than ERK1/2 inhibition. Additionally, one cannot exclude that other protein kinases may contribute to Bim phosphorylation in resting $CD28^{null}$ T cells, which will be explored in future work.

In summary, we show that $CD28^{null}$ T cells in ACS patients exhibit resistance to apoptosis as a result of a reduction in pro-apoptotic molecules Bim, Bax and Fas and we propose a novel strategy to restore apoptosis sensitivity in $CD28^{null}$ T cells via proteasome inhibition. As ACS patients who harbour high numbers of $CD28^{null}$ T cells have increased risk for recurrent severe acute coronary events and poor prognosis, deciphering the regulation of life and death decisions in $CD28^{null}$ T cells

will be useful not only in understanding the behaviour of this unique T cell subset but may also have potential therapeutic implications.

Contributions

E.K. and R.A. designed and performed experiments and analysed data. P.B. designed experiments, analysed data and edited the manuscript. J.C.K. provided critical discussion, access to patients and clinical data. I.E.D designed the study, designed and performed experiments, analysed data and wrote the manuscript.

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Disclosures

None.

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Figure legends

Figure 1. $CD4^+CD28^{null}$ T cells from ACS patients display reduced apoptosis in response to CH11. $CD4^+CD28^{null}$ (28null) and conventional $CD4^+CD28^+$ (28pos) T cells isolated from ACS patients (n=18) were cultured alone (w/o) or in the presence of the Fas ligating antibody CH11 antibody for 20 hours. Apoptosis was quantified using Annexin-V (AxV) and 7-AAD; cells in early stages of apoptosis were identified as $AxV^+7\text{-}AAD^-$, while cells in late stages of apoptosis were $AxV^+7\text{-}AAD^+$; total apoptosis was calculated as the sum of early and late apoptotic cells. **A.** Representative dot plots depicting AxV/7-AAD staining. Numbers indicate percentage of cells in each quadrant. **B.** Percentage of apoptotic cells in the same patient as in A. **C.** Apoptosis induction (calculated as described in Methods) in $CD28^{null}$ and $CD28^{pos}$ T cell subsets (n=18). **p<0.005; ***p<0.001 (Wilcoxon matched-pairs signed rank test)

Figure 2. $CD4^+CD28^{null}$ T cells from ACS patients display reduced apoptosis in response to ceramide. $CD4^+CD28^{null}$ (28null) and conventional $CD4^+CD28^+$ (28pos) T cells isolated from ACS patients (n=10) were cultured alone (w/o) or in the presence of the apoptosis inducing reagent C2-ceramide for 20 hours. Apoptosis was detected as in Figure 1. **A.** Representative dot plots depicting AxV/7-AAD staining. Numbers indicate percentage of cells in each quadrant. **B.** Percentage of apoptotic $CD28^{null}$ and $CD28^{pos}$ T cells in the same patient as in A. **C.** Apoptosis induction (calculated as described in Methods) in $CD28^{null}$ and $CD28^{pos}$ T cell subsets (n=10). *p<0.05; **p<0.005 (Wilcoxon matched-pairs signed rank test)

Figure 3. $CD4^+CD28^{null}$ T cells from patients with acute coronary syndrome (ACS) have reduced levels of Fas, Bim and Bax. Blood samples from ACS patients (n=25) were stained directly *ex vivo* for the expression of CD4, CD28, pro-apoptotic (Fas, FasL, Bim, Bax) and anti-aapoptotic (Bcl-2, Bcl-xL) molecules and analysed by flow cytometry (as detailed in Methods). **A.** Representative dot plots display the gating strategy for $CD4^+CD28^{null}$ (28null) and conventional $CD4^+CD28^+$ (28pos) T cells, with numbers indicating percentage of cells in each gate. Histogram plots depict expression of apoptotic molecules on gated 28^{null} (grey) and 28^+ (white) T cells or control samples labelled with isotype-matched antibodies (28null, dotted; 28pos, dashed). Numbers

represent the mean fluorescence intensity (MFI) for the indicated apoptotic molecules (grey, 28null; black, 28pos). **B.** MFI of pro- and anti-apoptotic molecules in 28null and 28pos T cells (n=25; horizontal bar, mean value). ***p<0.001 (*t*-test). a.u.=arbitrary units; ns=not significant

Figure 4. $CD4^+CD28^{null}$ T cells from ACS patients have lower levels of Bim and Bax compared to SA patients. Levels of pro- (Fas, FasL, Bim, Bax) and anti- apoptotic (Bcl-2, Bcl-xL) molecules on $CD4^+CD28^{null}$ (28null) T cells from ACS (n=25) and SA (n=18) patients were compared. **A.** MFI values for Fas, Bim and Bax in 28^{null} T cells. **B.** MFI values for FasL, Bcl-2 and Bcl-xL in 28^{null} T cells. *p<0.05; **p<0.01; ***p<0.001 (*t*-test); horizontal bar, mean value; MFI=mean fluorescence intensity; a.u.=arbitrary units; ns=not significant

Figure 5. $CD4^+CD28^{null}$ T cells from ACS patients exhibit ERK1/2 activation. $CD4^+CD28^{null}$ (28null) and conventional $CD4^+CD28^+$ (28pos) T cells were isolated from ACS patients (n=6). Phosphorylation of ERK1/2 was quantified by PhosFlow method before (w/o) and after activation of cells by CD3 cross-linking (aCD3). **A.** ERK1/2 phosphorylation in resting (w/o) and activated (aCD3) 28^{null} (grey histograms) and 28^{pos} (white histograms) T cells. Dotted or dashed line histograms indicate isotype controls for 28^{null} and 28^{pos} T cells, respectively. **B.** Histograms depict the effect on ERK1/2 phosphorylation of T cell activation via CD3 cross-linking in 28^{null} and 28^{pos} T cells. **C.** Percentage (left panel) and MFI (right panel) of phosphorylated ERK1/2 (mean±SD) in resting (w/o) and activated (aCD3) 28^{null} and 28^{pos} T cells in the patients studied (n=6). ***p<0.001 (ANOVA and post-test Bonferroni multiple comparisons); MFI=mean fluorescence intensity; a.u.=arbitrary units

Figure 6. Bim is phosphorylated in $CD4^+CD28^{null}$ T cells from ACS patients. $CD4^+CD28^{null}$ (28null) and conventional $CD4^+CD28^+$ (28pos) T cells were isolated from ACS patients (n=6) and Bim phoshorylation was detected with a phosphorylation site-specific antibody before (w/o) and after activation of cells by CD3 cross-linking (aCD3) (see Methods). **A.** Histograms depict Bim phoshorylation (pBim) in resting (w/o) and activated (aCD3) 28^{null} (grey histograms) and 28^{pos} (white histograms) T cells. Dotted or dashed line histograms indicate isotype controls for 28^{null} and 28^{pos} T cells, respectively. **B.** Histograms depict the effect on Bim phosphorylation of T cell

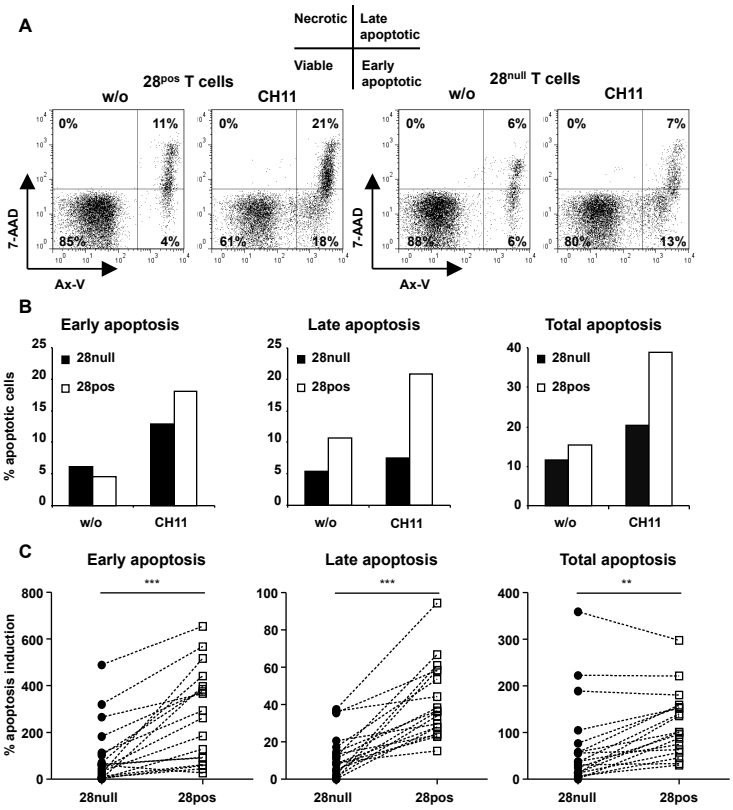
activation via CD3 cross-linking in 28^{null} and 28^{pos} T cells. **C.** MFI of phosphorylated Bim (pBim) (mean±SD) in resting (w/o) and activated (aCD3) 28^{null} and 28^{pos} T cells in the patients studied (n=6). Cells were pre-treated with the ERK1/2 specific inhibitor PD184352 (PD) or with the proteasomal inhibitor MG-132 (MG) followed by activation with anti-CD3 antibodies (aCD3) in the continued presence of the inhibitors. **D.** Histograms depict the effect of PD treatment (grey histogram) on Bim phophorylation in resting (w/o) and activated (aCD3) 28^{null} and 28^{pos} T cells. **E.** MFI of phosphorylated Bim (pBim) in 28^{null} and 28^{pos} T cells treated as indicated in the patients studied. ***p<0.001 (ANOVA and post-test Bonferroni multiple comparisons); MFI=mean fluorescence intensity; a.u.=arbitrary units

Figure 7. ERK1/2 inhibition did not alter apoptosis sensitivity of $CD4^+CD28^{null}$ T cells from ACS patients. $CD4^+CD28^{null}$ (28null) and conventional $CD4^+CD28^+$ (28pos) T cells were isolated from ACS patients (n=6). Cells cultured alone (w/o) or treated with CH11 to induce apoptosis were incubated in the presence or absence of the ERK1/2 specific inhibitor PD184352 (PD). Apoptosis was detected as in Figure 1. **A.** Representative dot plots depicting AxV/7-AAD staining. Numbers indicate percentage of cells in each quadrant. Bar graphs display percentage of apoptotic 28^{null} and 28^{pos} T cells with various treatments in the same patient as in A. **B.** Graphs depict the normalised percentage of apoptotic cells (mean±SD; n=6). The percentage of apoptotic 28^{pos} T cells in samples treated with CH11 alone was normalised to 100 (as detailed in Methods). ns=not significant (ANOVA and post-test Bonferroni multiple comparisons)

Figure 8. Proteasome inhibition restores apoptosis sensitivity of $CD4^+CD28^{null}$ T cells from ACS patients. $CD4^+CD28^{null}$ (28null) and conventional $CD4^+CD28^+$ (28pos) T cells were isolated from ACS patients (n=6). Cells cultured alone (w/o) or treated with CH11 to induce apoptosis were incubated in the presence or absence of the proteasomal inhibitor MG-132 (MG). Apoptosis was detected as in Figure 1. **A.** Representative dot plots depicting AxV/7-AAD staining. Numbers indicate percentage of cells in each quadrant. Graph bars display the percentage of apoptotic 28^{null} and 28^{pos} T cells with various treatments in the same patient as in A. **B.** Graphs depict the normalised percentage of apoptotic cells (mean±SD; n=6). The percentage of apoptotic 28^{pos} T

cells in samples treated with CH11 alone was normalised to 100 (as detailed in Methods). **p<0.01 (ANOVA and post-test Bonferroni multiple comparisons)

Figure 1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80%
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B
Early apoptosis
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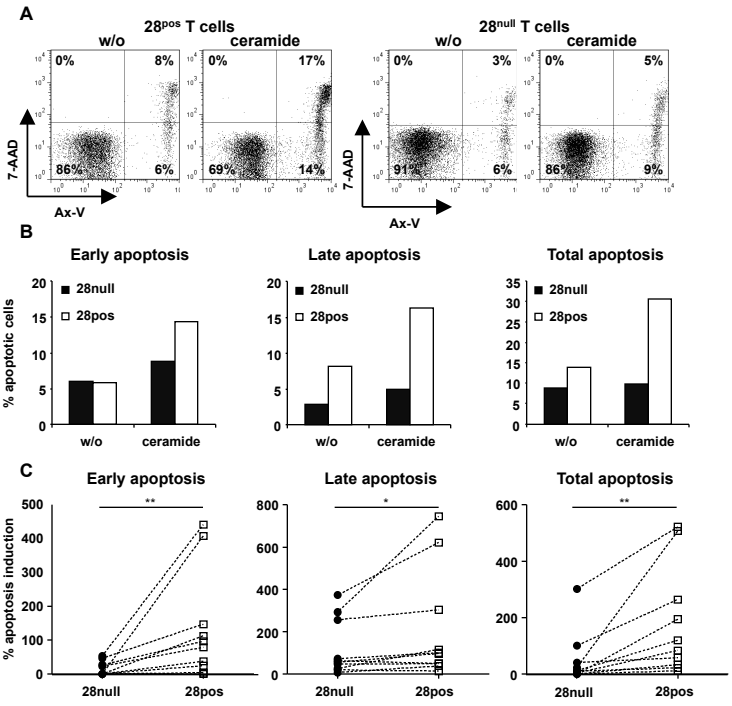
Figure 2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6%
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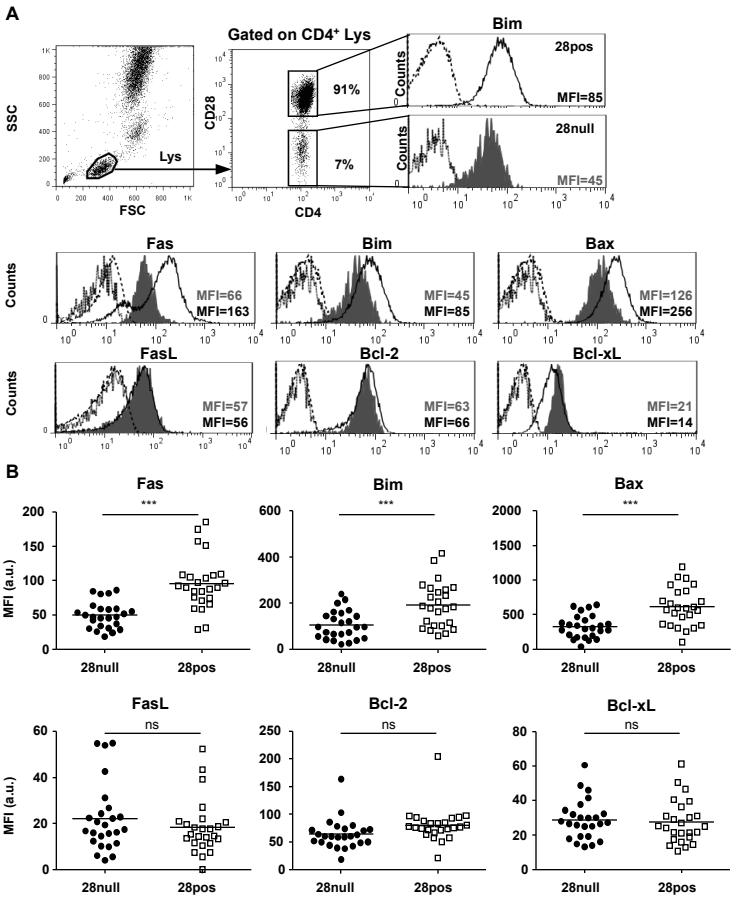
Figure 3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Bim
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Bax
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Figure 4

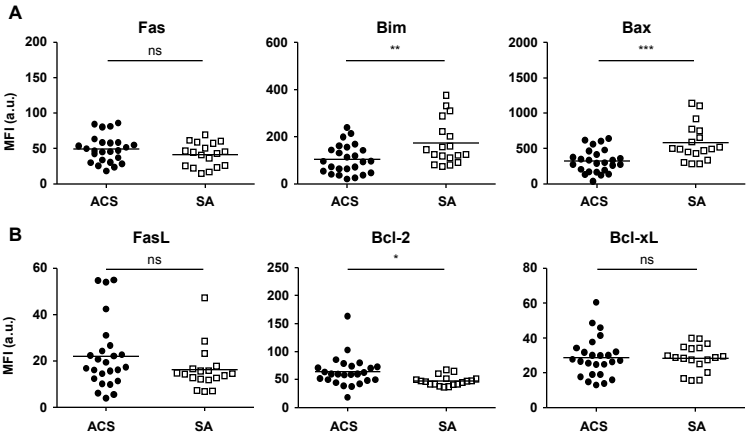
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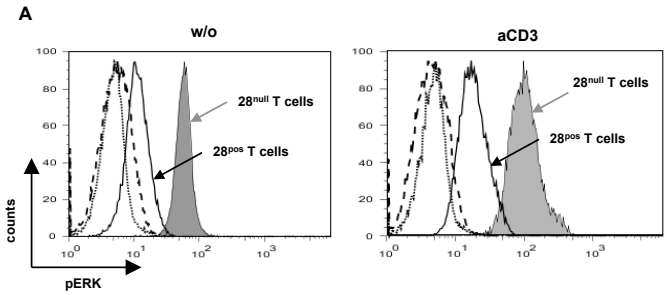
Figure 5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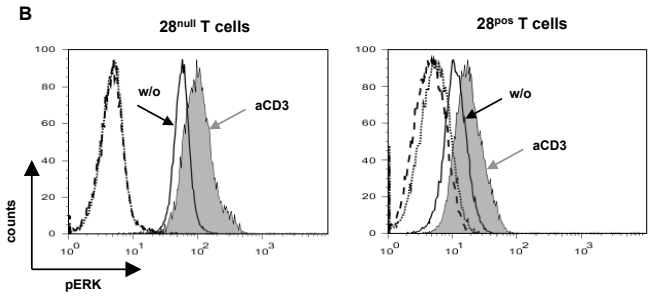
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counts
pERK

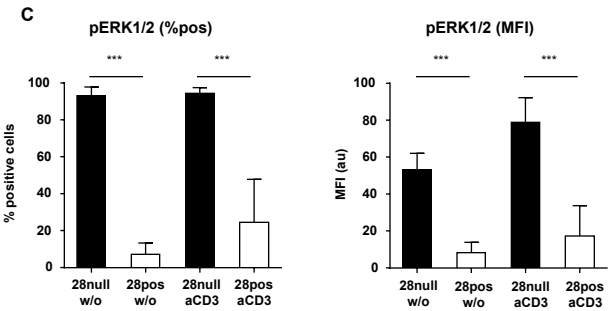

C
pERK1/2 (%pos)
pERK1/2 (MFI)
% positive cells
MFI (au)
***
28null w/o
28pos w/o
28null aCD3
28pos aCD3

Figure 6

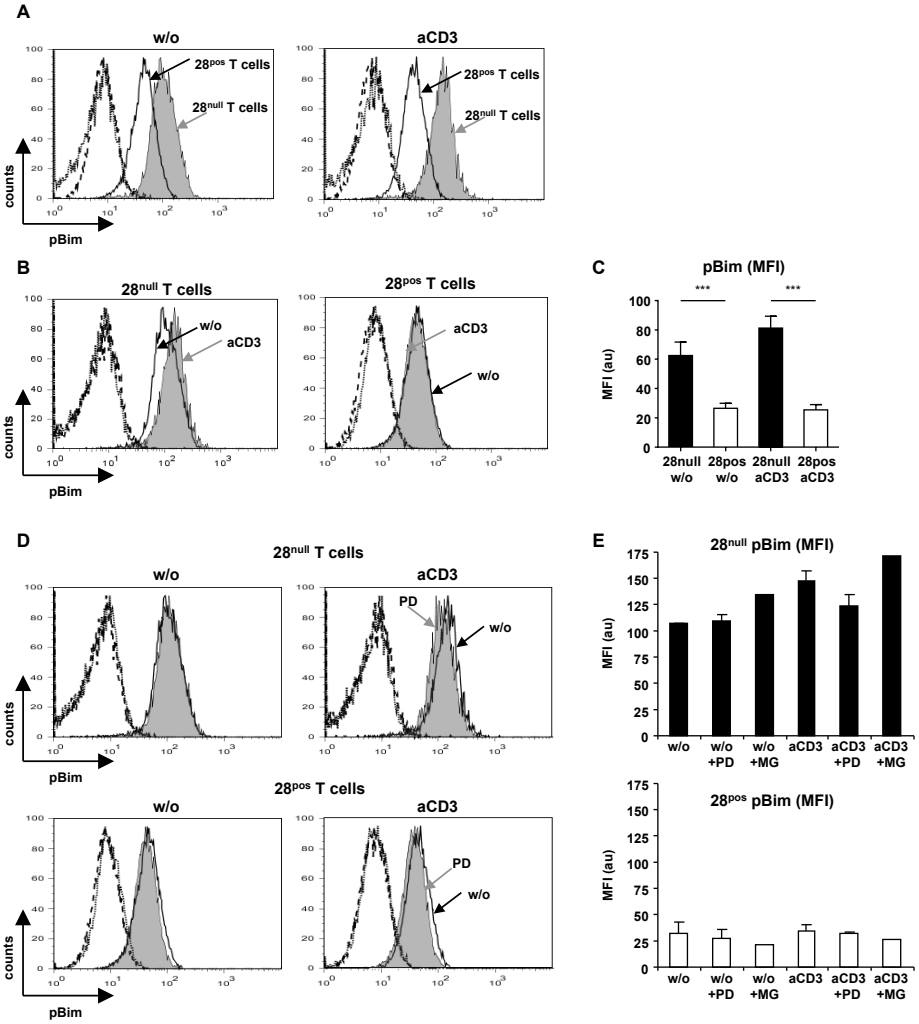
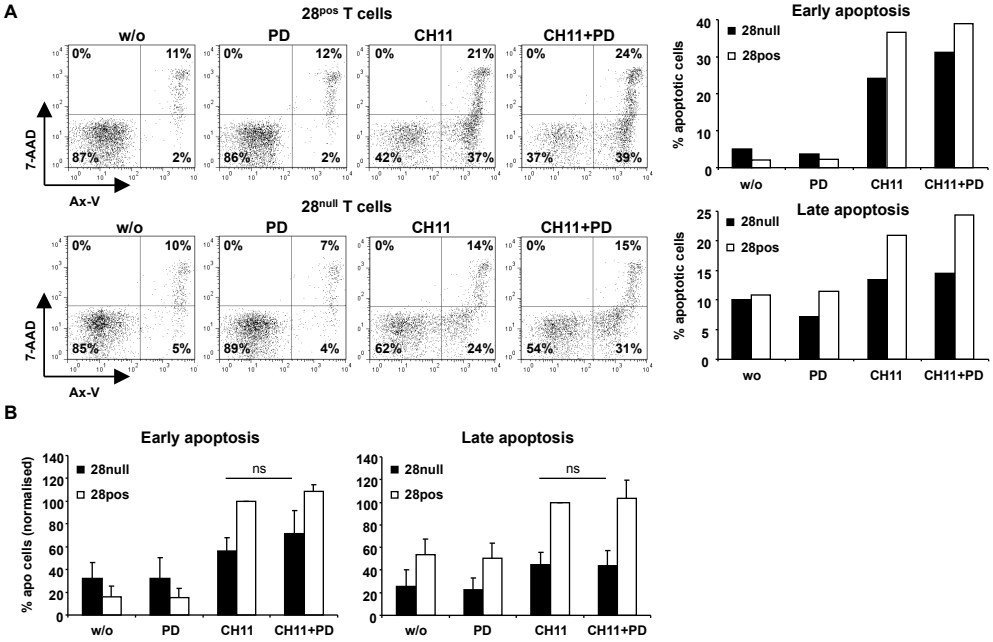
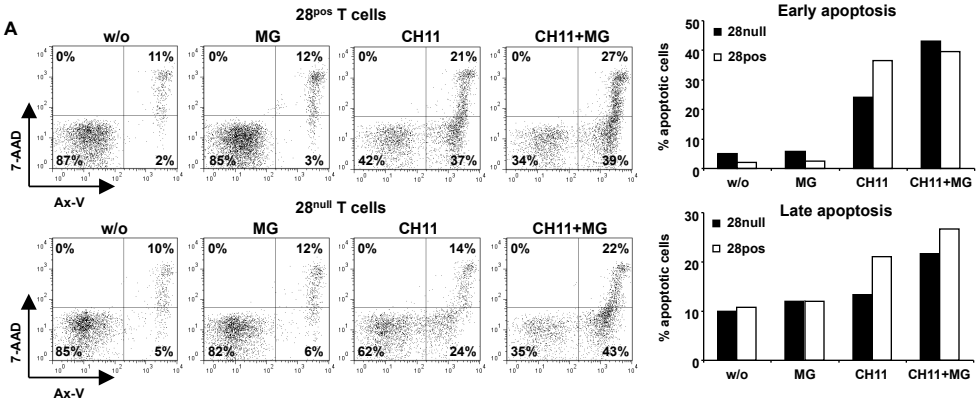

A
w/o
aCD3
28pos T cells
28null T cells
counts
pBim
B
28null T cells
28pos T cells
w/o
aCD3
C
pBim (MFI)
***
MFI (au)
28null w/o
28pos w/o
28null aCD3
28pos aCD3
D
28null T cells
PD
E
28null pBim (MFI)
28pos pBim (MFI)
w/o
w/o +PD
w/o +MG
aCD3
aCD3 +PD
aCD3 +MG

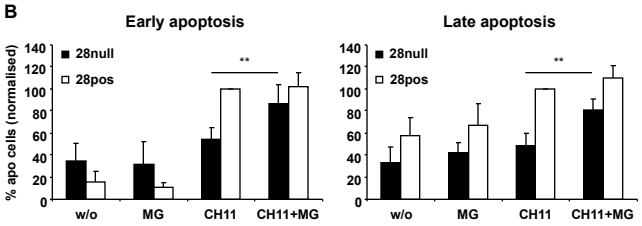
Figure 7


A
28pos T cells
w/o
PD
CH11
CH11+PD
0%
11%
87%
2%
0%
12%
86%
2%
0%
21%
42%
37%
0%
24%
37%
39%
7-AAD
Ax-V
28null T cells
0%
10%
85%
5%
0%
7%
89%
4%
0%
14%
62%
24%
0%
15%
54%
31%
Early apoptosis
28null
28pos
% apoptotic cells
w/o
PD
CH11
CH11+PD
Late apoptosis
wo

B
Early apoptosis
Late apoptosis
28null
28pos
ns
% apo cells (normalised)
w/o
PD
CH11
CH11+PD

Figure 8


A
28pos T cells
w/o
MG
CH11
CH11+MG
0%
11%
87%
2%
0%
12%
85%
3%
0%
21%
42%
37%
0%
27%
34%
39%
7-AAD
Ax-V
28null T cells
0%
10%
85%
5%
0%
12%
82%
6%
0%
14%
62%
24%
0%
22%
35%
43%
Early apoptosis
28null
28pos
% apoptotic cells
50
40
30
20
10
0
w/o
MG
CH11
CH11+MG
Late apoptosis
28null
28pos
% apoptotic cells
30
20
10
0
w/o
MG
CH11
CH11+MG


B
Early apoptosis
28null
28pos
% apo cells (normalised)
140
120
100
80
60
40
20
0
**
w/o
MG
CH11
CH11+MG
Late apoptosis
28null
28pos
140
120
100
80
60
40
20
0
**
w/o
MG
CH11
CH11+MG

SUPPLEMENTAL MATERIAL

Proteasome-mediated reduction in pro-apoptotic molecule Bim renders $CD4^{+}CD28^{null}$ T cells resistant to apoptosis in acute coronary syndrome

Edit Kovalcsik PhD[1], Ricardo F. Antunes PhD[1], Paramita Baruah MD, PhD[1], Juan Carlos Kaski MD, DSc, FRCP, FESC, FACC, FAHA[1], Ingrid E. Dumitriu MD, PhD[1*]

[1]Cardiovascular and Cell Sciences Research Institute, St. George's University of London, Cranmer Terrace, SW17 0RE, London, UK

[*]**Corresponding author:** Ingrid E. Dumitriu, Cardiovascular and Cell Sciences Research Institute, St. George's University of London, Cranmer Terrace, SW17 0RE, London, UK; Phone: 00442087252808; Fax: 00442087253328; e-mail: i.dumitriu@sgul.ac.uk

Supplemental Material Inventory:

Supplemental Table 1. Baseline patients' characteristics of the study population.

	ACS	SA	p-value
Patients (n)	25	18	
Age, years (mean±SD)	67.7±15.7	72±7.8	0.25
Gender % (male/female)	58/42	72/28	0.35
Ethnicity % (C/A)[*]	87/13	66/33	0.10
Family history of CAD (%)	30	16	0.05
Diabetes (type 2) (%)	34	61	0.07
Hypertension (%)	63	88	0.05
Smoking (%)	30	5	0.05
Hypercholesterolemia (%)	55	78	0.12
Prior MI[†] (%)	25	28	0.84
Prior CABG[‡] (%)	4	17	0.17
Prior PCI[§] (%)	21	44	0.10
Cholesterol (mmol/L) (mean±SD)	4.1±1.14	3.7±0.81	0.29
LDL (mmol/L) (mean±SD)	2.5±0.95	1.92±0.74	0.05
HDL (mmol/L) (mean±SD)	1.1±0.36	1.14±0.24	0.65
Triglycerides	1.24±0.75	1.5±0.83	0.33
Aspirin (%)	83	72	0.39
Statin (%)	70	55	0.30

[*]C/A, Caucasian/Asian; [†]MI, myocardial infarction; [‡]CABG, coronary artery bypass grafting; [§]PCI, percutaneous coronary intervention

Supplemental figure legends.

Supplemental Figure 1. Purity of sorted $CD4^+CD28^{null}$ and conventional $CD4^+CD28^+$ T cells from ACS patients. $CD4^+CD28^{null}$ (28null) and conventional $CD4^+CD28^+$ (28pos) T cells were isolated from ACS patients using negative selection of $CD4^+$ T cells followed by positive selection of 28^+ T cells (as detailed in Supplemental Methods). The percentages of 28^{null} and 28^{pos} T cells in fresh blood samples and the enriched fractions were determined by staining with monoclonal antibodies for CD4 and CD28. The plots illustrate data from two ACS patients (**A**, ACS001 and **B**, ACS002).

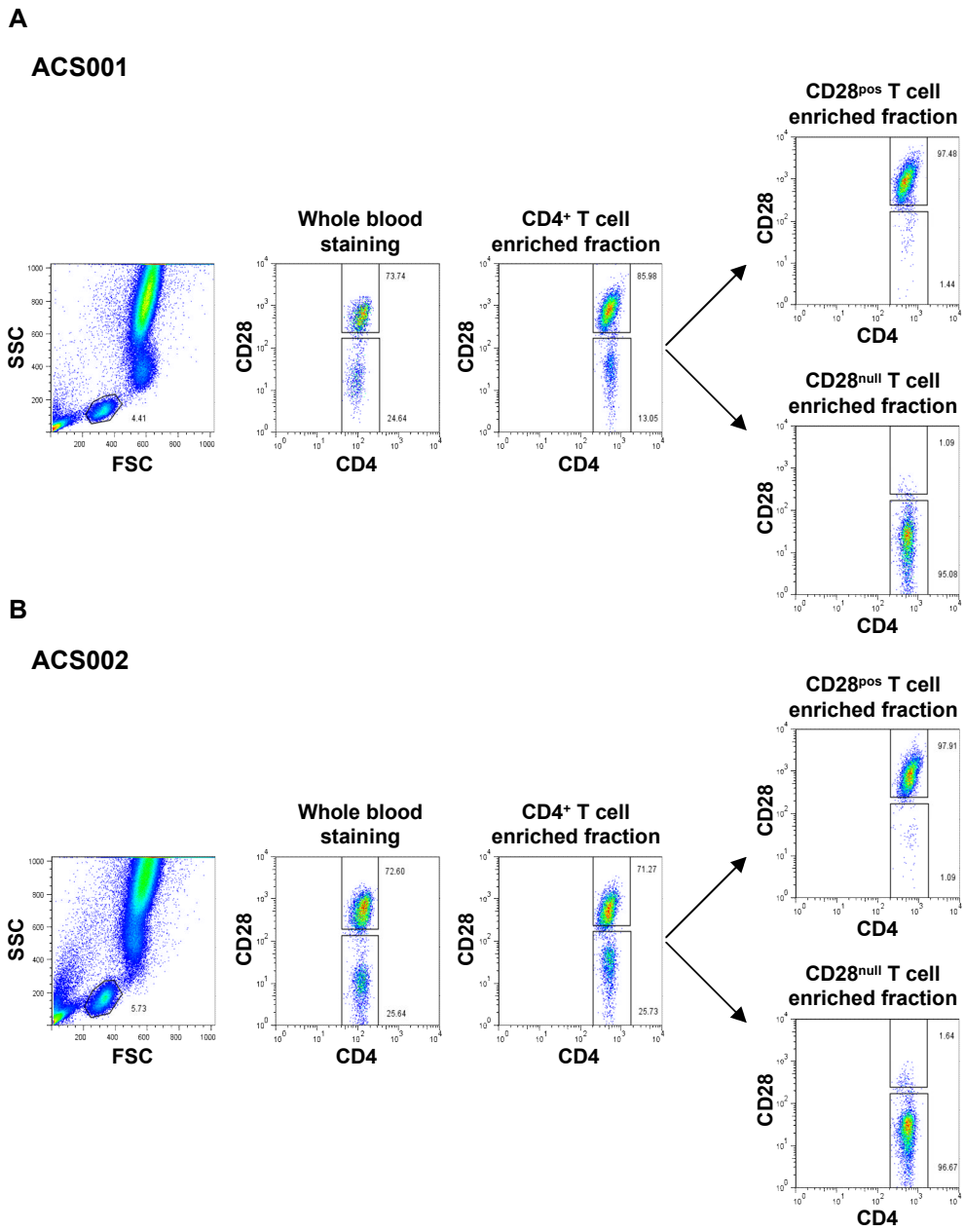
Supplemental Figure 2. Statins do not induce apoptosis in $CD4^+CD28^{null}$ T cells from ACS patients. $CD4^+CD28^{null}$ (28null) and conventional $CD4^+CD28^+$ (28pos) T cells were isolated from ACS patients (n=4). Cells were cultured alone (w/o) or treated with increasing concentrations (5μg/ml, 10μg/ml, 30μg/ml and 90ug/ml) of Atorvastatin (AT) or Rosuvastatin (RO). Apoptosis was quantified using Annexin-V (AxV) and 7-AAD following 48h of culture. **A.** Representative plots from 28^{null} and 28^{pos} T cells treated with Atorvastatin or incubated alone (w/o). Percentage of apoptotic cells (mean±SD) in 28^{null} and 28^{pos} T cells for the specified treatments in the patients studied (n=4). **B.** Representative plots and cumulative data from 28^{null} and 28^{pos} T cells incubated alone (w/o) or in the presence of Rosuvastatin.

Supplemental Figure 3. $CD4^+CD28^{null}$ T cells from ACS patients proliferate less than conventional CD4+CD28+ T cells. $CD4^+CD28^{null}$ (28null) and conventional $CD4^+CD28^+$ (28pos) T cells were isolated from ACS patients (n=5). T cells were labelled with CFSE and stimulated with anti-CD3 antibodies to induce T cell activation. Cells were cultured for four days and CFSE dilution was assessed by flow cytometry. **A.** Histogram plots illustrate CFSE dilution; the linear gates indicate the percentage of cells that have undergone CFSE dilution. **B.** Percentage of cells that have undergone CFSE dilution (mean±SD) from pooled experiments (n=5). *p,0.05; ***p<0.001 (ANOVA and post-test Tukey multiple comparisons)

Supplemental Figure 4. Bim and Bax failed to be induced on $CD4^+CD28^{null}$ T cells following activation. Peripheral blood mononuclear cells from ACS patients (n=18) were cultured alone (w/o) or stimulated with anti-CD3 antibodies (aCD3) to induce T cell activation. Levels of pro- (Fas, FasL, Bim, Bax) and anti-apoptotic (Bcl-2, Bcl-xL) molecules were detected on day four of culture. **A, B, C.** MFI of Fas, Bim and Bax in $CD4^+CD28^{null}$ (28null) and conventional $CD4^+CD28^+$ (28pos) T cells in the patients studied (n=18; horizontal bar, mean values). **D, E, F.** MFI of FasL, Bcl-2 and Bcl-xL in the two T cell subsets. *p<0.05; **p<0.01; ***p<0.001 (ANOVA and post-test Tukey multiple comparisons); MFI=mean fluorescence intensity; a.u.=arbitrary units; ns=not significant

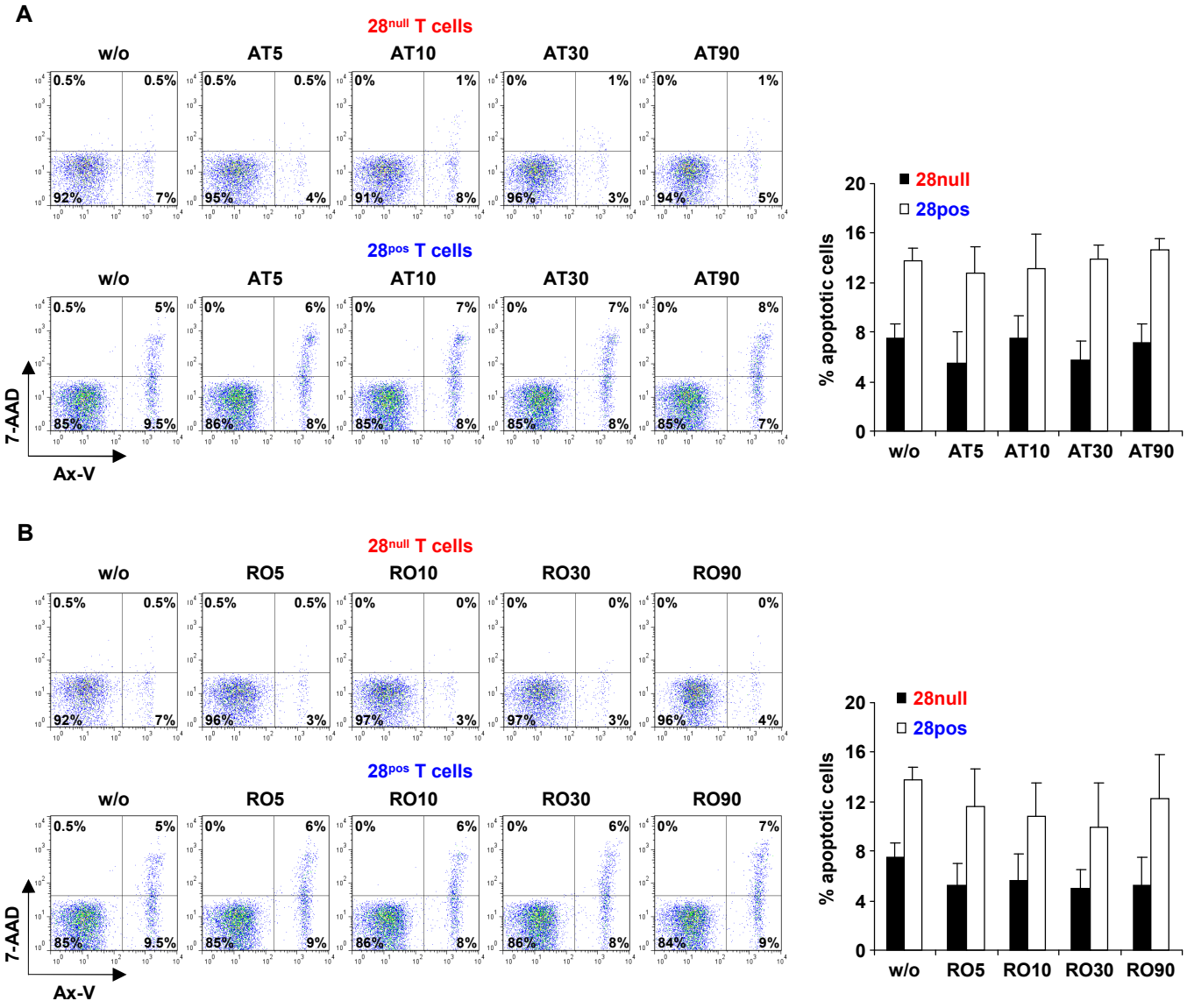
Supplemental Figure 5. Pro-apoptotic molecules Fas, Bim and Bax levels are reduced on $CD4^+CD28^{null}$ T cells in patients with stable angina (SA). Levels of pro- (Fas, FasL, Bim, Bax) and anti-apoptotic (Bcl-2, Bcl-xL) molecules on $CD4^+CD28^{null}$ (28null) and conventional $CD4^+CD28^+$ (28pos) T cells were quantified in fresh blood samples from SA patients (n=18). **A.** MFI of Fas, Bim and Bax in 28null and 28pos T cells in the patients studied (n=18; horizontal bars, mean values). **B.** MFI of FasL, Bcl-2 and Bcl-xL in the two T cell subsets. *p<0.05; **p<0.01; ***p<0.001 (*t*-test); MFI=mean fluorescence intensity; a.u.=arbitrary units; ns=not significant

Supplemental Figure 1



Supplemental Figure 1. Purity of sorted CD4+CD28null and conventional CD4+CD28+ T cells from ACS patients. CD4+CD28null (28null) and conventional CD4+CD28+ (28pos) T cells were isolated from ACS patients using negative selection of CD4+ T cells followed by positive selection of CD28+ T cells (as detailed in Supplemental Methods). The percentages of CD28null and CD28pos T cells in fresh blood samples and the enriched fractions were determined by staining with monoclonal antibodies for CD4 and CD28. The plots illustrate data from two ACS patients (**A**, ACS001 and **B**, ACS002).

Supplemental Figure 2



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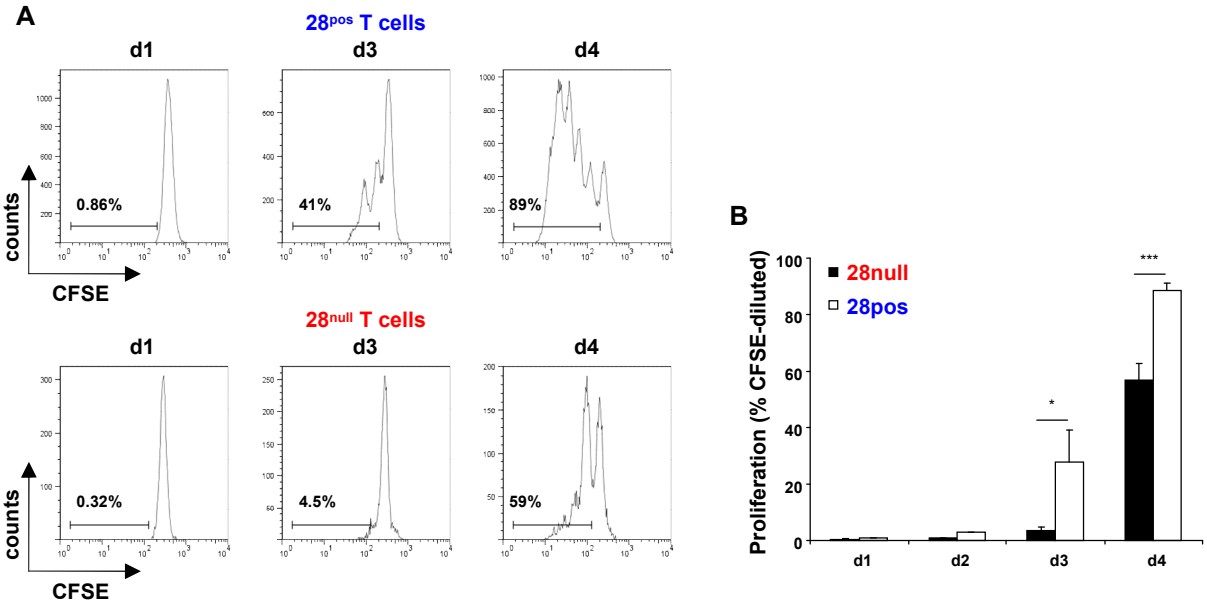
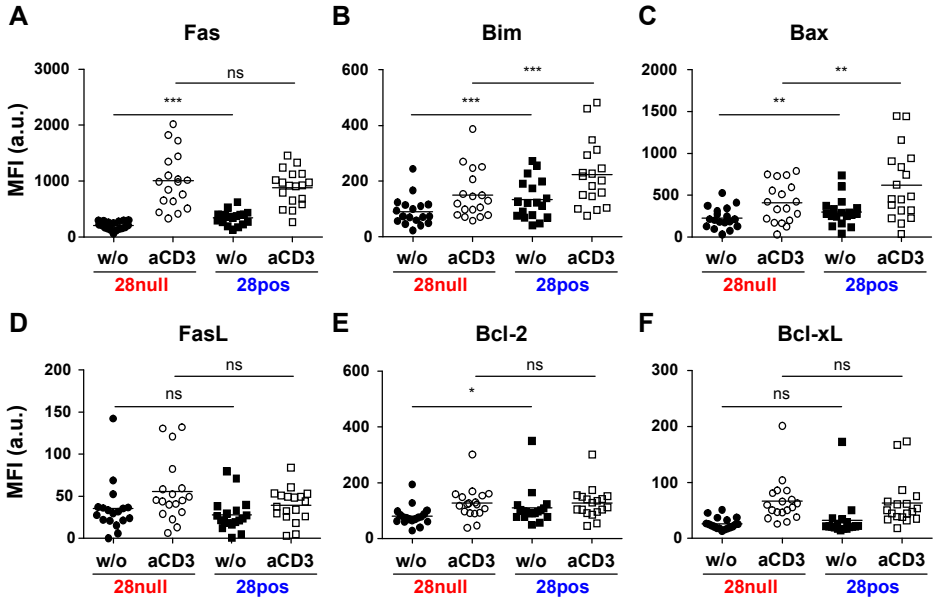
Supplemental Figure 3



Supplemental Figure 3. CD4+CD28null T cells from ACS patients proliferate less than conventional CD4+CD28+ T cells. CD4+CD28null (28null) and conventional CD4+CD28+ (28pos) T cells were isolated from ACS patients (n=5). T cells were labelled with CFSE and stimulated with anti-CD3 antibodies to induce T cell activation. Cells were cultured for four days and CFSE dilution was assessed by flow cytometry. **A.** Histogram plots illustrate CFSE dilution; the linear gates indicate the percentage of cells that have undergone CFSE dilution. **B.** Percentage of cells that have undergone CFSE dilution (mean±SD) from pooled experiments (n=5). *p,0.05; ***p<0.001 (ANOVA and post-test Tukey multiple comparisons)

Supplemental Figure 4



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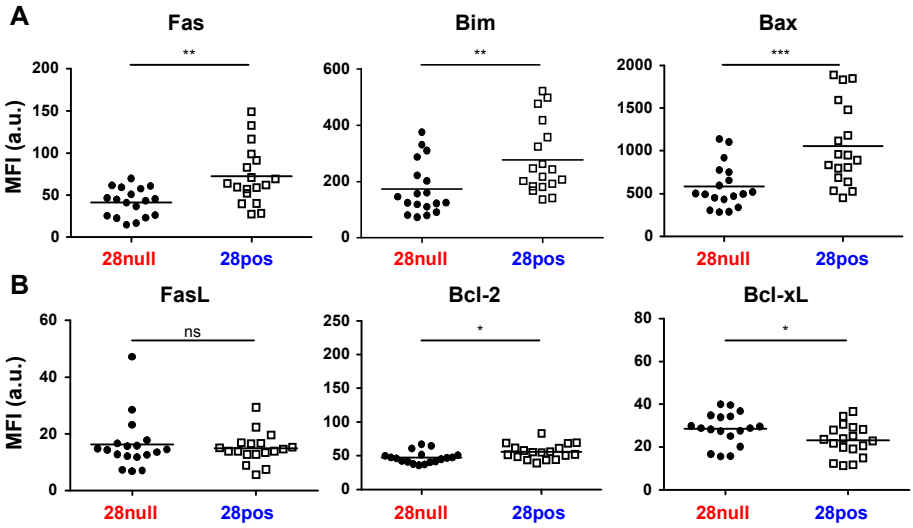
Supplemental Figure 5



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