High Levels of Costimulatory Receptors OX40 and 4-1BB Characterize CD4+CD28null T Cells in Patients With Acute Coronary Syndrome

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Rationale: Patients with acute coronary syndrome (ACS) predisposed to recurrent coronary events have an expansion of a distinctive T-cell subset, the CD4+CD28null T cells. These cells are highly inflammatory and cytotoxic in spite of lacking the costimulatory receptor CD28, which is crucial for optimal T cell function. The mechanisms that govern CD4+CD28null T cell function are unknown.

Objective: Our aim was to investigate the expression and role of alternative costimulatory receptors in CD4+CD28null T cells in ACS.

Methods and Results: Expression of alternative costimulatory receptors (inducible costimulator, OX40, 4–1BB, cytotoxic T lymphocyte associated antigen-4, programmed death-1) was quantified in CD4+CD28null T cells from circulation of ACS and stable angina patients. Strikingly, in ACS, levels of OX40 and 4-1BB were significantly higher in circulating CD4+CD28null T cells compared to classical CD4+CD28+ T lymphocytes. This was not observed in stable angina patients. Furthermore, CD4+CD28null T cells constituted an important proportion of CD4+ T lymphocytes in human atherosclerotic plaques and exhibited high levels of OX40 and 4-1BB. In addition, the ligands for OX40 and 4-1BB were present in plaques and also expressed on monocytes in circulation. Importantly, blockade of OX40 and 4-1BB reduced the ability of CD4+CD28null T cells to produce interferon-γ and tumor necrosis factor-α and release perforin.

Conclusions: Costimulatory pathways are altered in CD4+CD28null T cells in ACS. We show that the inflammatory and cytotoxic function of CD4+CD28null T cells can be inhibited by blocking OX40 and 4-1BB costimulatory receptors. Modulation of costimulatory receptors may allow specific targeting of this cell subset and may improve the survival of ACS patients. (Circ Res. 2012;110:857–869.)

Key Words: atherosclerosis ■ coronary disease ■ immune system ■ lymphocytes ■ receptors

Coronary artery disease continues to be the leading cause of death in the developed world. Recent research has demonstrated that coronary artery disease results from an uncontrolled immune response and T lymphocytes have a central role in the development and progression of the disease. Detailed analysis of CD4+ T cells in coronary artery disease unveiled an increased frequency of a distinctive subset of lymphocytes called CD4+CD28null T cells. These cells are characterized by the lack of CD28, the main costimulatory receptor that regulates the response of T lymphocytes to antigen. The CD4+CD28null T cell subset is present in low frequencies in healthy individuals and has also shown to increase in patients with chronic inflammatory diseases such as autoimmune. In coronary artery disease, the frequency of CD4+CD28null T cells is significantly increased in patients with acute coronary syndrome (ACS) in comparison to stable angina (SA) and healthy subjects. CD4+CD28null T cells accumulate preferentially in unstable ruptured coronary plaques and have been suggested to promote plaque instability. Of note, high frequencies of CD4+CD28null T cells predispose ACS patients to recurrent acute coronary events (ie, myocardial infarction) and indicate a poor prognosis. Interestingly, the CD4+CD28null T cell subset is unique to the human disease and no counterpart has been identified in mice.
Non-standard Abbreviations and Acronyms

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<th>Abbreviation</th>
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<tr>
<td>ACS</td>
<td>acute coronary syndrome</td>
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<tr>
<td>CTLA-4</td>
<td>cytotoxic T lymphocyte associated antigen-4</td>
</tr>
<tr>
<td>ICOS</td>
<td>inducible costimulator</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-γ</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
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<td>PD-1</td>
<td>programmed death-1</td>
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<td>SA</td>
<td>stable angina,</td>
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<td>TNF</td>
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<td>TNFR</td>
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arthritis and ACS suggest that CD4⁺CD28null T cells produce high levels of the proinflammatory cytokine interferon-γ (IFN-γ). Expression of cytolytic molecules (eg, granzymes and perforin), usually found only in cytotoxic CD8⁺ T cells and natural killer cells has also been shown in this cell subset in rheumatoid arthritis patients. These features, together with the preferential accumulation of CD4⁺CD28null T lymphocytes in ruptured plaques, indicate that these cells could tip the balance of the atherosclerotic plaque milieu toward instability and rupture.

The proinflammatory features of CD4⁺CD28null T lymphocytes are surprising, as the activation and survival of T cells depends on costimulatory signals delivered via the CD28 receptor. The activation of T cells requires not only the recognition of antigen, but also a second signal delivered by the interaction of the CD28 costimulatory receptor on T cells with its ligands on antigen presenting cells. CD28 signaling controls the expression of interleukin-2 receptors and the production of interleukin-2 by activated T cells, which enable their proliferation, differentiation into effectors and survival. Indeed, in the absence of CD28 signaling, T cells enter a state of functional inactivation called anergy.

We were therefore intrigued that CD4⁺CD28null T cells retain their ability to proliferate and function in the absence of CD28. We hypothesized that alternative costimulatory receptors may substitute for CD28 functions in CD4⁺CD28null T cells. Various costimulatory receptors other than CD28 are transiently upregulated following activation of T cells: ICOS (inducible costimulator), CTLA-4 (cytotoxic T lymphocyte associated antigen-4), and PD-1 (programmed death-1) belong to the CD28 receptor superfamily; whereas OX40 (CD134) and 4-1BB (CD137) are members of the tumor necrosis factor receptor (TNFR) family. Of these, ICOS, OX40, and 4-1BB stimulate T cell activation, whereas CTLA-4 and PD-1 inhibit it. Optimal equilibrium between costimulatory (positive) and coinhibitory (negative) signals enables effective immune responses, whereas maintaining immunologic tolerance and preventing inflammation-induced tissue damage. Endothelial cells express several ligands for costimulatory receptors (eg, ICOSL, PD-L1, OX40L) that are likely to trigger signaling via the corresponding receptors expressed on CD4⁺CD28null T cells. This could potentially lead to abnormal activation of CD4⁺CD28null T cells in ACS.

With this in view, we investigated the presence of alternative costimulatory receptors and their contribution to the function of CD4⁺CD28null T cells in ACS patients. We provide evidence that in ACS, CD4⁺CD28null T cells in the circulation and in atherosclerotic plaques express increased levels of costimulatory receptors OX40 and 4-1BB belonging to the TNFR family, which mediate the inflammatory and cytotoxic function of this T cell subset. These alterations in costimulatory receptors may provide novel therapeutic targets to inhibit tissue damage mediated by CD4⁺CD28null T cells in ACS.

Methods

An expanded Methods section is provided in the online-only Data Supplement.

Study Population

Peripheral blood was obtained from 48 patients with a diagnosis of ACS (STEMI and NSTEMI), 24 patients with SA and 30 healthy subjects. The demographic details and clinical characteristics of patients and controls are provided in Online Tables I and II. Patients with evidence of infectious diseases, malignancies, hematologic or immunologic disorders, treatment with anti-inflammatory drugs other than aspirin, and an ejection fraction <40 were excluded. The demographic and clinical characteristics of the patient groups did not differ. The study was approved by the local research ethics committee and informed consent was obtained from all study subjects.

Intracellular Staining

Intracellular cytokine content was determined after stimulation of peripheral blood mononuclear cells (PBMCs) with 50 ng/mL PMA (Sigma-Aldrich) and 1 μg/mL Ionomycin (Calbiochem) as previously described. The protein transport inhibitor Brefeldin A was added during the last 2 hours of culture to prevent cytokine secretion. Following surface staining with monoclonal antibodies anti-CD4 and anti-CD28 (for identification of CD4⁺CD28null T cells) and classical CD4⁺CD28⁺ T cells, cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) and then stained with monoclonal antibodies for IFN-γ, tumor necrosis factor-α (TNF-α, BD Biosciences). PBMCs cultured alone or in the presence of anti-CD3 antibodies were also assayed for intracellular levels of perforin and granzyme using monoclonal antibodies specific for perforin and Granzyme B (eBioscience).

Degranulation Assay

Degranulating cells can be identified by the expression of CD107a (lysosomal associated membrane protein-1) on the cell surface. CD107a resides in the membrane of cytoplasmic cytolytic granules and following activation-induced granule exocytosis, is mobilized to the cell surface. This is accompanied by a loss of perforin from degranulating cells. We analyzed the cell surface expression of CD107a 4 hours following activation, a time point where CD107a peaks, together with intracellular expression of perforin and granzyme B. PBMCs cultured alone or activated with anti-CD3 antibodies were stained on the cell surface with monoclonal antibodies anti-CD4 and anti-CD28 and with antibodies specific for CD107a (BD Biosciences) as per the manufacturer’s instructions. Intracellular expression of perforin and granzyme B was assessed as described above.

Statistical Analysis

Categorical data were analyzed by the χ²-test. Mann-Whitney U test was used for comparison of T cell frequency between ACS, SA,
ACS Patients Have an Increased Frequency of CD4\(^+\)CD28\(^{null}\) T Cells

It has been previously reported by others and by us that the frequency of CD4\(^+\)CD28\(^{null}\) T cells increases in patients with ACS.\(^3\) We verified the frequency of CD4\(^+\)CD28\(^{null}\) T cells in our study groups of ACS patients, SA patients, and healthy individuals. We confirmed that ACS patients had significantly higher percentages of CD4\(^+\)CD28\(^{null}\) T cells in the circulation compared to healthy subjects and patients with SA (Figure 1). This was also true when we quantified absolute numbers of CD4\(^+\)CD28\(^{null}\) T cells (Online Figure IA). Furthermore the percentage and absolute numbers of all CD4\(^+\) T lymphocytes were not significantly different between the studied groups (Online Figure IB and IC), indicating that the observed CD4\(^+\)CD28\(^{null}\) T cells differences were not on account of differences in the whole CD4\(^+\) T cell population.

**CD4\(^+\)CD28\(^{null}\) T Cells From ACS Patients Express Higher Levels of the Alternative Costimulatory Receptors OX40 and 4-1BB Compared to Classical CD4\(^+\)CD28\(^{+}\) T Cells**

CD28 has pivotal roles in the activation of T cells and is constitutively expressed on these cells.\(^4\) In contrast, other costimulatory receptors are induced following T cell activation (eg, OX40, CTLA-4).\(^12,14\) We therefore investigated whether CD4\(^+\)CD28\(^{null}\) T cells, which are characteristically devoid of CD28, express alternative costimulatory receptors to compensate the loss of CD28. We analyzed the expression of costimulatory (OX40, 4-1BB, ICOS) as well as coinhibitory (CTLA-4, PD-1) receptors on circulating CD4\(^+\) T cells in the resting state and after activation. As expected, low levels of costimulatory receptors were found on resting classical CD4\(^+\)CD28\(^{+}\) and CD4\(^+\)CD28\(^{null}\) T cells. Following stimulation with anti-CD3 antibodies, the expression of OX40, 4-1BB, and ICOS was upregulated in both T cell subsets (Figure 2A). Interestingly, in ACS patients the up-regulation of OX40 and 4-1BB was much more marked in the CD4\(^+\)CD28\(^{null}\) T cell subset compared to classical CD4\(^+\)CD28\(^{+}\) T lymphocytes (Figure 2B). Of note, this finding was specific to the ACS patient group as CD4\(^+\)CD28\(^{null}\) T cells from SA patients showed similar levels of OX40 and 4-1BB to their classical counterparts (Figure 2C). The levels of the costimulatory receptor ICOS did not differ between CD4\(^+\)CD28\(^{null}\) and classical CD4\(^+\)CD28\(^{+}\) T cells in both ACS and SA (Figure 2B and C). OX40 and 4-1BB belong to the TNFR family, whereas ICOS is a member of the CD28 receptor family along with CTLA-4 and PD-1 which are coinhibitory receptors. As seen with ICOS, we detected similar levels of CTLA-4 and PD-1 on CD4\(^+\)CD28\(^{null}\) and classical CD4\(^+\)CD28\(^{+}\) T cells in ACS and SA patients (Online Figure II). These data demonstrate that CD4\(^+\)CD28\(^{null}\) T cells from ACS patients characteristically express higher levels of OX40 and 4-1BB than classical CD4\(^+\)CD28\(^{+}\) T cells and that this phenomenon is not observed in SA. We further tested the expression of OX40 and 4-1BB on CD4\(^+\)CD28\(^{null}\) T cells from healthy individuals. This subset is present at very low frequency in healthy subjects and is thus difficult to characterize in a large number of individuals. In healthy subjects with detectable frequencies of CD4\(^+\)CD28\(^{null}\) T cells, no difference was found in the levels of costimulatory receptors compared to classical CD4\(^+\)CD28\(^{+}\) T cells (Online Figure III). Furthermore, the levels of OX40, 4-1BB, and ICOS on CD4\(^+\)CD28\(^{null}\) T cells from ACS patients were higher compared to CD4\(^+\)CD28\(^{null}\) T cells from healthy subjects (Online Figure IIIIB).

**Enhanced Expression of Costimulatory Receptors on CD4\(^+\)CD28\(^{null}\) T Cells Is Persistent Over Time**

We next investigated whether the increased expression of alternative costimulatory receptors on CD4\(^+\)CD28\(^{null}\) T cells is a transient phenomenon. In short-term kinetic
experiments, we found that the expression of costimulatory receptors became detectable 24 hours following stimulation (not shown). The expression of costimulatory receptors peaked around 72 hours for both CD4+CD28null and classical CD4+CD28+ T cells, and remained elevated at late time points (7 days) (Online Figure IVA). Of note, at all time points studied, the expression of costimulatory receptors was higher on CD4+CD28null T cells compared to classical CD4+CD28+ T lymphocytes (Online Figure IVA).

We also evaluated the effect of stimulating the cells with anti-CD3 antibodies alone or in combination with anti-CD28 antibodies. As expected, addition of anti-CD28 antibodies did not influence the effects observed with anti-CD3 alone on the expression of costimulatory receptors by CD4+CD28null T cells (Online Figure IVB). Interestingly, though anti-CD28 antibodies did increase the expression of costimulatory receptors on classical CD4+CD28+ T cells compared to anti-CD3 alone, the levels of costimulatory receptors on classi-

Figure 2. Expression of costimulatory receptors in CD4+CD28null T cells. Peripheral blood mononuclear cells (PBMCs) from acute coronary syndrome (ACS, n=25) and stable angina (SA, n=21) patients were cultured alone (w/o) or activated with anti-CD3 antibodies (aCD3) and the expression of costimulatory receptors (OX40, 4–1BB, inducible costimulator [ICOS]) was analyzed on day 4. A, Illustrative histograms show the expression of OX40, 4-1BB, and ICOS (green histograms) and control staining with isotype-matched antibodies (red histograms) by activated CD4+CD28null (28null) and classical CD4+CD28+ (28pos) T cells from an ACS patient. B, Scatter plots show the distribution and mean fluorescence intensity (MFI) of cells expressing costimulatory receptors in the ACS group. C, Scatter plots show the distribution and mean MFI of cells expressing costimulatory receptors in the SA group. Probability values (P) were calculated by 2-tailed t test. a.u. indicates arbitrary units.
cal T cells remained lower than those observed on CD4+CD28null T cells even in the presence of anti-CD28 antibodies (Online Figure IVB).

**CD4^+CD28null T Cells Express Higher Levels of Inflammatory Cytokines Compared to Classical CD4^+CD28^+ T Cells**

We then assessed the functional attributes of CD4^+CD28null T cells in ACS patients. Following stimulation with anti-CD3 antibodies, intracellular levels of IFN-γ and TNF-α increased in both classical and CD4^+CD28null T cells (Figure 3A and 3B). We found that CD4^+CD28null T cells had significantly higher levels of IFN-γ than their classical CD4^+CD28^+ counterparts (Figure 3B). Of note, this was true both in the resting state as well as following activation (Figure 3B). Furthermore, similar results were observed for the inflammatory cytokine TNF-α (Figure 3A and 3B, right panels). These results show that CD4^+CD28null T cells have a potent proinflammatory cytokine signature. We next verified whether this decrease in perforin and granzyme was a consequence of activation-induced degranulation, a prerequisite for cytolysis. A recent study showed that degranulation can be evaluated by quantifying the expression of CD107a (lysosomal associated membrane protein-1) on the cell surface of cytotoxic cells. We found that activation of CD4^+CD28null T cells increased the expression of CD107a on the cell surface (Figure 4C), and this coincided with decreased expression of perforin (Figure 4D) suggesting that activation of CD4^+CD28null T cells induced degranulation. In contrast, CD107a was not present on the surface of classical CD4^+CD28^+ T cells (Figure 4C), which do not contain perforin and granzyme (Figure 4A and B) and are not expected to degranulate.

**CD4^+CD28null T Cells Are Present in Atherosclerotic Plaques and Express OX40 and 4-1BB**

We next investigated the presence of CD4^+CD28null T cells in human atherosclerotic plaques and the expression of costimulatory receptors. Lymphocytes were isolated ex vivo from atherosclerotic plaques from carotid and femoral endarterectomies and analyzed by flow cytometry.
CD4^+CD28null T cells were present in all atherosclerotic plaques studied and constituted between 5% to 23% of all CD4^+ T cells resident in the plaque (Figure 5A and Online Tables III and IV). Importantly, freshly isolated plaque-associated CD4^+CD28null T cells expressed OX40 and 4-1BB costimulatory receptors (Figure 5A). In addition, using immunohistochemistry we found strong expression of OX40 in the atherosclerotic plaque (Figure 5B).
Figure 5. Analysis of CD4⁺CD28null T cells, costimulatory receptors and ligands in atherosclerotic plaques. A, The frequency of CD4⁺CD28null (CD28neg), CD4⁺CD28⁺ (CD28pos) T cells and the expression of OX40 and 4-1BB costimulatory receptors was quantified in cells isolated from human endarterectomy atherosclerotic plaques using flow cytometry. Representative plots show expression of OX40 and 4-1BB on plaque-derived T cells from atherosclerotic plaques B, Paraffin embedded tissue sections were immunohistochemically labeled.

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Ligands for OX40 and 4-1BB Are Present Both in Atherosclerotic Plaques and on Cells From Peripheral Circulation
Given our findings with the expression of OX40 and 4-1BB costimulatory receptors on circulating and plaque-associated CD4⁺CD28null T cells from ACS patients, we investigated the availability of ligands for these receptors in vivo. Immunohistochemistry on human atherosclerotic plaques from endarterectomy specimens revealed strong expression of OX40L and 4-1BL (Figure 5B). We also found that the ligands for OX40 and 4-1BB are expressed on cells in the peripheral circulation, with highest levels observed on circulating monocytes (Figure 5C).

Blockade of OX40 and 4-1BB Costimulatory Receptors Reduces the Production of Inflammatory Cytokines and the Degranulation of CD4⁺CD28null T Cells
One of the striking differences we found between CD4⁺CD28null and classical CD4⁺CD28⁺ T cells characteristic to ACS patients is the increased expression of the costimulatory receptors OX40 and 4-1BB. We next investigated whether these costimulatory receptors endow CD4⁺CD28null T cells with inflammatory and cytotoxic function. To test this hypothesis, cells from ACS patients were activated with anti-CD3 alone or in the presence of blocking antibodies against 4-1BB or OX40. We found that blockade of 4-1BB or OX40 significantly downregulated the production of IFN-γ from CD4⁺CD28null T cells (Figure 6A), whereas the effects on classical CD4⁺CD28⁺ T cells were negligible. Similarly, blockade of 4-1BB and OX40 significantly reduced the production of TNF-α from CD28null T cells, whereas the classical T cells remained unaffected (Figure 6B). Isotype-matched control antibodies did not influence the production of IFN-γ or TNF-α from CD4⁺CD28null T cells (Figure 6A and 6B). These findings were further substantiated by using stimulating antibodies against OX40 and 4-1BB and recombinant ligands. We found increased production of IFN-γ and TNF-α on activating CD4⁺CD28null T cells concomitantly with triggering OX40 and 4-1BB receptors using stimulating antibodies or the corresponding ligands (Online Figure VA).

We next tested the effects of blocking 4-1BB and OX40 on the expression of perforin and granzyme B and on the activation-induced degranulation of CD4⁺CD28null T cells. We found that activation of CD4⁺CD28null T cells induced expression of CD107a on the cell surface (a marker for degranulation) and a decrease in perforin (Figure 7A and 7B). Interestingly, blocking antibodies against 4-1BB or OX40 inhibited surface expression of CD107a and reversed the downregulation of perforin (Figure 7A and 7B). Minimal effects were observed with costimulation blockade on the expression of granzyme B by CD4⁺CD28null T cells (Figure 7C). As expected, stimulating antibodies or recombinant ligands had opposite effects and significantly increased the release of perforin and granzyme B (Online Figure VB). Isotype-matched control antibodies did not have any effect on all parameters investigated (not shown). These results suggest that 4-1BB and OX40 are important regulators of degranulation and perforin release from CD4⁺CD28null T cells.

Discussion
In this study we demonstrate that CD4⁺CD28null T cells from ACS patients harbor important alterations in costimulatory receptors. Our novel finding is that circulating CD4⁺CD28null T cells from ACS patients express significantly higher levels of OX40 and 4-1BB costimulatory receptors compared to classical CD4⁺CD28⁺ T lymphocytes and that this is a characteristic of ACS, as SA patients do not have these alterations. Notably, we demonstrate that CD4⁺CD28null T cells are present in human atherosclerotic plaques and also exhibit the same costimulatory receptors as observed in the circulation. We also show that CD4⁺CD28null T cells produce potent inflammatory cytokines (IFN-γ and TNF-α) and contain significant intracellular stores of perforin and granzyme B, all of which are regulated by OX40 and 4-1BB.

OX40 and 4-1BB, the costimulatory receptors that are higher on CD4⁺CD28null T cells from ACS patients are both members of the TNFR family. In contrast, members of CD28 family of costimulatory receptors (ICOS, CTLA-4, and PD-1) did not show any alterations in CD4⁺CD28null T cells (Figure 2 and Online Figure II). Costimulatory receptors belonging to the TNFR family have emerged as key mediators of survival and effector function in T cells.OX40 and 4-1BB control inflammatory cytokine production, survival, and proliferation of T cells via NF-κB and MAP kinase pathways. Indeed, we show that CD4⁺CD28null T cells express high levels of OX40 and 4-1BB and produce higher amounts of IFN-γ and TNF-α than their classical counterparts (Figure 3). Additionally, blocking OX40 or 4-1BB significantly reduces the production of IFN-γ and TNF-α from CD4⁺CD28null T cells but not classical T cells (Figure 6). These results suggest that OX40 and 4-1BB deregulation in CD4⁺CD28null T cells could have important implications in ACS (Figure 8). Our findings are interesting, as polymorphisms in the genes for OX40 and its ligand (OX40L) identified in genome-wide association studies, have recently been shown to correlate with increased risk of myocardial infarction in humans. So far, no human polymorphisms have been described for 4-1BB. However, 4-1BB is expressed in human atherosclerotic plaques.

Additional evidence on OX40 and 4-1BB in atherosclerosis...
sis comes from murine models of the disease. Interruption of OX40-OX40L pathway has been shown to significantly decrease the extent of atherosclerosis in ApoE⁻/⁻ or Ldlr⁻/⁻ mice.²⁶ Also 4-1BB deficiency reduced atherosclerotic plaques in ApoE⁻/⁻ or Ldlr⁻/⁻ mice, via decreased production of IFN-γ and TNF-α and MCP-1.²⁸

Classically the expression of OX40 and 4-1BB is induced by TCR signals, further enhanced by CD28 and is usually transient.¹⁴ Interestingly, our results show a sustained expression of OX40 and 4-1BB on CD4⁺CD28null T cells in ACS, up to 7 days following activation (Online Figure IV). In vivo, a sustained expression of these costimulatory receptors has been observed in diseases associated with persistent inflammation: OX40 and its ligand are expressed for prolonged periods during chronic infection and are found in inflamed tissues in a number of human autoimmune diseases such as lupus.²⁹ We show here that CD4⁺CD28null T cells from human atherosclerotic plaques have high levels of OX40 and 4-1BB receptors. Furthermore we demonstrate that ligands to OX40 and 4-1BB are readily available in atherosclerotic lesions (Figure 5) suggesting that CD4⁺CD28null T cells could get activated in the plaque microenvironment. This observation is in line with previous evidence that T cells in ACS acquire an activated status,³,³⁰ which may be one of the contributing factors to plaque instability.

A striking feature of CD4⁺CD28null T cells is that, in contrast to classical CD4⁺CD28⁺ T cells, they are equipped with potent cytotoxic machinery. We show that even in the resting state virtually all CD4⁺CD28null T cells express granzyme B and a high proportion of cells harbor perforin (Figure 4). Furthermore, activation of CD4⁺CD28null T cells resulted in decreased intracellular perforin and granzyme B coupled with the expression of CD107a on the cell surface (Figure 4). CD107a usually resides in the membrane of cytolytic granules located in the cytoplasm and is mobilized to the cell surface following granule exocytosis.¹⁹ These results suggest that activation of CD4⁺CD28null T cells induces degranulation and release of perforin and granzyme B, which endows them with a strong cytotoxic potential. Remarkably, blockade of OX40 or 4-1BB had potent inhibitory effects on degranulation and release of perforin (Figure 7). Our results are in keeping with previous reports suggesting thatOX40 and 4-1BB drive cytotoxicity in antiviral CD8⁺ T cells.³¹,³² In one report, triggering of OX40 and 4-1BB enhanced the expansion, perforin levels and cytolytic activity of CD8⁺ T
cells, with 4-1BB having more potent effects. It is of note that in our hands 4-1BB blockade had stronger effects than OX40 blockade on inhibiting the production of proinflammatory cytokines and the cytotoxic potential of CD4<sup>+</sup>/CD28<sup>-</sup> null T cells (Figures 6 and 7). It has been reported that 4-1BB deficient mice have a reduced number of natural killer (NK) cells and natural killer T (NKT) cells. Notably, CD4<sup>+</sup>/CD28<sup>-</sup> null T cells express a variety of receptors belonging to the KIR family, a feature they share with NK and NKT cells, which may also link to the potent effect observed with 4-1BB blockade on the function of CD4<sup>+</sup>/CD28<sup>-</sup> null T cells.

Interestingly, although blockade of OX40 and 4-1BB strongly affected perforin release from CD4<sup>+</sup>/CD28<sup>-</sup> null T cells, only marginal effects were observed on the intracellular levels of granzyme B (Figure 7). Perforin has been suggested to act as a gatekeeper for granzymes, enabling access of granzymes to the target cell cytosol where they induce apoptosis. Thus perforin has been proposed to have an essential role in the induction of granzyme-
mediated cytotoxicity. Indeed, perforin-deficient mice have significantly altered cytotoxic T cell function, whereas granzyme-deficient mice show minor effects.37,38 Also mice and humans have a single copy of the perforin gene but redundant genes for granzymes.36 Therefore, it is likely that inhibition of perforin release by costimulation blockade will impact greatly on the cytotoxic activity of CD4⁺CD28null T cells even if granzymes remain unchanged.

An interesting finding of our study is that alterations in OX40 and 4-1BB are present in CD4⁺CD28null T cells from ACS patients but not from SA patients. The life-threatening acute coronary events that characterize ACS are often the result of sudden rupture of unstable atherosclerotic plaques. Previous reports have shown that CD4⁺CD28null T cells accumulate preferentially in unstable ruptured coronary plaques and may promote plaque instability.6 This may be mediated by IFN-γ, which is produced in large amounts by CD4⁺CD28null T cells, and is known to trigger macrophage activation and secretion of matrix metalloproteinases.39 Metalloproteinases have been involved in plaque rupture via degradation of the fibrous cap.40 In addition, CD4⁺CD28null T cells could directly lyse vascular smooth muscle and endothelial cells and induce rupture of atherosclerotic lesions.41 In view of the above, our results showing that blockade of OX40 and 4-1BB can decrease the proinflammatory and cytotoxic function of CD4⁺CD28null T cells carry therapeutic implications for treatment of ACS patients. A feature that makes OX40 and 4-1BB attractive targets for immunomodulation is their absence from naive and resting T cells and preferential expression on activated/effector T cells. This implies that targeting these mediators could potentially enable CD4⁺CD28null T cells to trigger plaque rupture via macrophage activation and endothelial and vascular smooth muscle cell lysis. VSMC indicates vascular smooth muscle cell.

In summary, our data show that CD4⁺CD28null T cells from ACS patients have striking alterations in costimulatory receptors of the TNFR family, OX40, and 4-1BB. Blocking these receptors significantly impairs the production of IFN-γ and TNF-α and the release of perforin from CD4⁺CD28null T cells. Our novel results suggest that therapeutic targeting of
OX40 and 4-1BB to modulate CD4<sup>+</sup>CD28<sup>+</sup> T cell function in ACS could potentially inhibit the progression of atherosclerosis.

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**Disclosures**

None.

**References**


**Novelty and Significance**

**What Is Known?**
- Immune cells like T lymphocytes are present in atherosclerotic plaques and can contribute to plaque rupture that leads to acute coronary syndrome (ACS).
- A peculiar subset of T lymphocytes, the CD4+/CD28null T cells, which are characterized by the absence of the costimulatory receptor CD28, expand in ACS patients.
- The frequency of CD4+/CD28null T cells correlates with the clinical severity and recurrence of ACS.

**What New Information Does This Article Contribute?**
- In ACS but not stable angina patients, circulating CD4+/CD28null T cells express high levels of alternative costimulatory receptors.
- Atherosclerotic plaques have CD4+/CD28null T cells expressing high levels of alternative costimulatory receptors; ligands for these receptors are also present in the plaques.
- Inhibition of the alternative costimulatory receptors decreases the production of inflammatory cytokines and cytotoxic enzymes from CD4+/CD28null T cells.

ACS are triggered by rupture of atherosclerotic plaques. T lymphocytes contribute to plaque rupture via production of inflammatory cytokines or cytotoxic enzymes that lyse endothelial or smooth muscle cells. A peculiar subset of T lymphocytes, the CD4+/CD28null T cells, are highly inflammatory and cytotoxic, despite lacking the costimulatory receptor CD28, which is crucial for optimal function of T lymphocytes. CD4+/CD28null T cells increase predominantly in ACS patients and less markedly in patients with stable angina, and they correlate with disease severity. This study examines whether CD4+/CD28null T cells express alternative costimulatory receptors that possibly regulate their damaging effects on atherosclerotic plaques. We show that circulating CD4+/CD28null T cells from ACS patients, but not stable angina, express high levels of alternative costimulatory receptors. Increased levels of alternative costimulatory receptors are also present on CD4+/CD28null T cells from human atherosclerotic plaques. These plaques additionally express ligands that on binding to alternative costimulatory receptors could trigger production of inflammatory cytokines and cytotoxic enzymes from CD4+/CD28null T cells. Inhibition of alternative costimulatory receptors reduced the release of inflammatory cytokines and cytotoxic enzymes from CD4+/CD28null T cells. Modulation of alternative costimulatory receptors could possibly dampen the tissue damaging effects of CD4+/CD28null T cells in ACS.
High Levels of Costimulatory Receptors OX40 and 4-1BB Characterize CD4^+CD28^nul T Cells in Patients With Acute Coronary Syndrome

Ingrid E. Dumitriu, Paramita Baruah, Caroline J. Finlayson, Ian M. Loftus, Ricardo F. Antunes, Pitt Lim, Nicholas Bunce and Juan Carlos Kaski

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Supplemental material

Supplemental Materials and Methods

Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from blood by density gradient centrifugation using Histopaque (Sigma-Aldrich). Cells were cultured in RPMI 1640 (Invitrogen) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 15mM L-glutamine, 25 U/ml IL-2 (Roche) and 5% heat inactivated pooled human serum (BioWhittaker). For T cell activation, cells were stimulated with 1-2 µg/ml immobilised anti-CD3 antibodies (eBioscience). Where indicated, 1 µg/ml soluble anti-CD28 antibodies (eBioscience) were added to the cultures. Unless otherwise specified, T cells analysis was performed on day 4 following activation. For co-stimulation blockade, PBMCs were activated with anti-CD3 antibodies alone or in the presence of 10 µg/ml purified blocking antibodies for 4-1BB (Biolegend, clone 4B4-1), OX40 (R&D Systems, clone 443318) and isotype-matched control antibodies (eBioscience). Stimulation of 4-1BB and OX40 was done with 10 µg/ml stimulating antibodies for 4-1BB (R&D Systems, AF838) or OX40 (AF3388) or with 5 µg/ml recombinant ligands 4-1BBL (R&D Systems, 2295-4L) and OX40L (R&D Systems, 1054-OX).

Flow cytometry

Percentages of CD4⁺CD28null and total CD4⁺ T cells in blood samples were determined by flow cytometry. To accurately assess the frequency of T cell subsets, whole blood was stained with FITC-labelled anti-CD4 and APC-labelled anti-CD28 monoclonal antibodies (BD Biosciences), followed by lysis of red blood cells with Lyse/Fix buffer (BD Biosciences). Isotype-matched control antibodies were used to assess non-specific staining. Absolute cell numbers were quantified using BD Biosciences Trucount tubes as
per the manufacturer’s instructions. Additional phenotypic characterisation of co-stimulatory receptors and ligands expression was performed by staining with monoclonal antibodies specific for: CTLA-4, ICOS, PD-1, OX40 and 4-1BB, OX40L, 4-1BBL (BD Biosciences). The samples were acquired on a FACSCalibur (BD Biosciences) flow cytometer and data analysis was performed using FlowJo software (Tree Star).

**Analysis of CD4⁺CD28null T cells, co-stimulatory receptors and ligands in human atherosclerotic plaques.** The tissue was washed extensively three times in RPMI to remove the blood. Infiltrating leucocytes were isolated after enzymatic digestion of minced endarterectomy specimens in 200U/ml DNAase I (Sigma Aldrich) and 500U/ml collagenase I (Gibco) in serum-free RPMI at 37°C for 2 hours. The digested material was passed through a 70μm sieve and washed by low-speed centrifugation. Dead cells and debris were removed by density gradient centrifugation using Histopaque (Sigma-Aldrich). Cells were labelled with monoclonal antibodies for CD4, CD28, OX40 and 4-1BB (BD Biosciences) and analysed by flow cytometry.

**Immunohistochemistry**

Serial sections of formalin-fixed paraffin embedded carotid endarterectomy were used for immunohistochemistry using the Leica Bond III IHC staining system. Antigen retrieval was carried out at 100°C for 30min, followed by incubation with primary antibodies against OX40 (Abcam, clone MM0505-8S23; 1:40), OX40L (R&D Systems, clone 159403; 1:40), 4-1BBL (Abcam, ab64912; 1:200) for 15min, followed by peroxide block for 5min, post-primary reagent for 8min, HRP-conjugated polymer reagent for 8min and 3,3’-diaminobenzidine (DAB) for 10min. Slides were rinsed with Bond Wash in between steps and at the end were counterstained with Haematoxylin, dehydrated, cleared and mounted in Pertex mounting media. Images were captured using a Olympus BX50 microscope equipped with a Nikon camera and the NIS elements 2.30 software.
Supplemental Table I. Demographic characteristics of study groups.

<table>
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<tr>
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<th>SA</th>
<th>Healthy</th>
<th>p</th>
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<tr>
<td>Patients (n)</td>
<td>48</td>
<td>24</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Age, years (mean±SD)</td>
<td>66.9±13.5</td>
<td>68.1±9.7</td>
<td>54.1±8.5</td>
<td>0.68</td>
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<tr>
<td>Gender % (male/female)</td>
<td>69/31</td>
<td>58/42</td>
<td>57/43</td>
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<tr>
<td>Ethnicity % (C/A)*</td>
<td>74/26</td>
<td>79/21</td>
<td>87/13</td>
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*C/A, Caucasian/Asian

Supplemental Table II. Clinical characteristics of coronary artery disease patients.

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<th>SA</th>
<th>p</th>
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<tr>
<td>Family history of CAD (%)</td>
<td>12</td>
<td>8</td>
<td>0.85</td>
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<tr>
<td>Diabetes (type 2) (%)</td>
<td>21</td>
<td>29</td>
<td>0.73</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>41</td>
<td>75</td>
<td>0.43</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>28</td>
<td>50</td>
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<tr>
<td>Hypercholesterolemia (%)</td>
<td>41</td>
<td>54</td>
<td>0.59</td>
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<tr>
<td>Prior MI* (%)</td>
<td>8</td>
<td>25</td>
<td>0.16</td>
</tr>
<tr>
<td>Prior CABG† (%)</td>
<td>3</td>
<td>17</td>
<td>0.13</td>
</tr>
<tr>
<td>Prior PCI‡ (%)</td>
<td>5</td>
<td>25</td>
<td>0.07</td>
</tr>
<tr>
<td>Cholesterol (mmol/L) (mean±SD)</td>
<td>4.7±1.22</td>
<td>4.3±1.22</td>
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<tr>
<td>LDL (mmol/L) (mean±SD)</td>
<td>3±1.16</td>
<td>2.5±1.03</td>
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</tr>
<tr>
<td>HDL (mmol/L) (mean±SD)</td>
<td>1.4±0.52</td>
<td>1.17±0.44</td>
<td>0.21</td>
</tr>
<tr>
<td>Aspirin (%)</td>
<td>74</td>
<td>54</td>
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<tr>
<td>Statin (%)</td>
<td>56</td>
<td>46</td>
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</tr>
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</table>

*MI, myocardial infarction; †CABG, coronary artery bypass grafting; ‡PCI, percutaneous coronary intervention
### Supplemental Table III. Percentage of CD4+CD28null and CD4+CD28+ T cells in human atherosclerotic plaques (endarterectomy samples).

<table>
<thead>
<tr>
<th>Endarterectomy sample</th>
<th>%CD4+CD28+ T cells</th>
<th>%CD4+CD28null T cells</th>
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<tr>
<td>Patient 1</td>
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<td>23</td>
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<tr>
<td>Patient 2</td>
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<tr>
<td>Patient 3</td>
<td>88</td>
<td>10</td>
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<tr>
<td>Patient 4</td>
<td>76</td>
<td>20</td>
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<tr>
<td>Patient 5</td>
<td>90</td>
<td>5</td>
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<td>Patient 6</td>
<td>89</td>
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</table>

### Supplemental Table IV. Clinical details of endarterectomy patients.

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<tr>
<td>Age (years)/sex(M/F)</td>
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<td>56/M</td>
<td>61/M</td>
<td>73/M</td>
<td>85/M</td>
<td>71/M</td>
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<tr>
<td>Diabetes</td>
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<td>Yes</td>
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<td>Hypertension</td>
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<td>Smoking</td>
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<tr>
<td>Hypercholesterolemia</td>
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<td>Prior CABG‡</td>
<td>Yes</td>
<td>Yes</td>
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</table>

*F, femoral artery; †C, carotid artery; ‡CABG, coronary artery bypass grafting
Supplemental Figure I. Absolute number and frequency of CD4^+CD28^{null} and CD4^+ T cells in patients with ACS or SA and healthy subjects. Whole blood from patients with acute coronary syndrome (ACS, n=40), stable angina (SA, n=24) and healthy controls (ctrl, n=16) was stained ex vivo for the expression of CD4 and CD28 and analysed by flow cytometry. Counting beads were used for quantification of absolute numbers of cells. 

A. The scatter plot shows the distribution and mean absolute number of CD4^+CD28^{null} T cells.

B. The scatter plot shows the distribution and mean percentage of CD4^+ T cells. 

C. The scatter plot shows the distribution and mean absolute number of CD4^+ T cells. Probability value (p) was calculated using the Mann-Whitney U test.

Supplemental Figure II. Expression of co-inhibitory receptors in CD4^+CD28^{null} T cells. PBMCs from ACS (n=20) and SA (n=21) patients were cultured alone (w/o) or activated with anti-CD3 antibodies (aCD3) and the expression of co-inhibitory (CTLA-4, PD-1) receptors was analysed on day 4. 

A. Scatter plots show the distribution and mean MFI of CD4^+CD28^{null} (28^{null}) and classical CD4^+CD28^{+} (28^{pos}) T cells expressing co-inhibitory receptors in the ACS group.

B. Scatter plots show the distribution and mean MFI of T cells expressing co-inhibitory receptors in the SA group. Probability values (p) were calculated by two-tailed t test. a.u., arbitrary units; MFI, mean fluorescence intensity; n.s., not significant

Supplemental Figure III. Expression of co-stimulatory receptors in CD4^+CD28^{null} T cells from healthy subjects. PBMCs from healthy subjects (ctrl, n=5) or ACS patients (n=24) were cultured alone (w/o) or activated with anti-CD3 antibodies (aCD3) and the expression of co-stimulatory receptors (OX40, 4-1BB, ICOS) was analysed on day 4. 

A. Scatter plots show the distribution and mean MFI of CD4^+CD28^{null} (28^{null}) and
CD4⁺CD28⁺ (28pos) T cells expressing co-stimulatory receptors. **B.** Scatter plots show the distribution and mean MFI of CD4⁺CD28null from healthy subjects (Ctrl-28null) or ACS patients (ACS-28null) expressing co-stimulatory receptors. Probability values (p) were calculated by two-tailed t test. a.u., arbitrary units; MFI, mean fluorescence intensity.

**Supplemental Figure IV. Time kinetics of co-stimulatory receptor expression. A.** PBMCs from ACS patients were activated with anti-CD3 antibodies (aCD3) and the expression of CTLA-4 and OX40 by CD4⁺CD28⁺ (28pos) and CD4⁺CD28null (28null) T cells was measured at the indicated time points. **B.** PBMCs were activated with anti-CD3 antibodies alone (aCD3) or in combination with antibodies against CD28 (aCD3aCD28). OX40 levels were quantified on CD4⁺CD28⁺ (28pos) and CD4⁺CD28null (28null) T cells at the indicated time points. Data (mean ± SD) are representative of three independent experiments with cells from different ACS patients. Probability values (p) were calculated by two-tailed t test. **p<0.01, *p<0.05; a.u., arbitrary units; MFI, mean fluorescence intensity.

**Supplemental Figure V. Effects of OX40 and 4-1BB stimulation on cytokine, perforin and granzyme B production by CD4⁺CD28null T cells.** PBMCs from ACS patients (n=5) were activated with anti-CD3 antibodies alone (aCD3) or in the presence of stimulating antibodies for 4-1BB (s4-1BB) and OX40 (sOX40), recombinant ligands 4-1BBL and OX40L, or control antibodies (ctrl Ig) for 4 days. **A.** Bar graphs display percentage (mean ± SD) of IFN-γ⁺ or TNF-α⁺ CD4⁺CD28null T cells. **B.** The graphs show percentage (mean ± SD) of perforin⁺ and granzyme⁺ CD4⁺CD28null T cells. Probability values (p) were calculated by two-tailed t test. ***p<0.001, **p<0.01, *p<0.05
Supplemental Figure I

Supplemental Figure I. Absolute number and frequency of CD4\(^+\)CD28\(^{\text{null}}\) and CD4\(^+\) T cells in patients with ACS or SA and healthy subjects. Whole blood from patients with acute coronary syndrome (ACS, n=40), stable angina (SA, n=24) and healthy controls (ctrl, n=16) was stained directly ex vivo for the expression of CD4 and CD28 and analysed by flow cytometry. Counting beads were used for quantification of absolute numbers of cells. A. The scatter plot shows the distribution and mean absolute number of CD4\(^+\)CD28\(^{\text{null}}\) T cells. B. The scatter plot shows the distribution and mean percentage of CD4\(^+\) T cells. C. The scatter plot shows the distribution and mean absolute number of CD4\(^+\) T cells. Probability value (p) was calculated using the Mann-Whitney U test.
**Supplemental Figure II**

**A.** Scatter plots show the distribution and mean MFI of CD4⁺CD28⁻null (28null) and classical CD4⁺CD28⁺ (28pos) T cells expressing co-inhibitory receptors in the ACS group.

**B.** Scatter plots show the distribution and mean MFI of T cells expressing co-inhibitory receptors in the SA group. Probability values (p) were calculated by two-tailed t test. a.u., arbitrary units; MFI, mean fluorescence intensity; n.s.=not significant.

**Supplemental Figure II.** Expression of co-inhibitory receptors in CD4⁺CD28null T cells. PBMCs from ACS (n=20) and SA (n=21) patients were cultured alone (w/o) or activated with anti-CD3 antibodies (aCD3) and the expression of co-inhibitory (CTLA-4, PD-1) receptors was analysed on day 4. **A.** Scatter plots show the distribution and mean MFI of CD4⁺CD28null (28null) and classical CD4⁺CD28⁺ (28pos) T cells expressing co-inhibitory receptors in the ACS group. **B.** Scatter plots show the distribution and mean MFI of T cells expressing co-inhibitory receptors in the SA group. Probability values (p) were calculated by two-tailed t test. a.u., arbitrary units; MFI, mean fluorescence intensity; n.s.=not significant.
Supplemental Figure III

Expression of co-stimulatory receptors in CD4⁺CD28null T cells from healthy subjects. PBMCs from healthy subjects (ctrl) or ACS patients were cultured alone (w/o) or activated with anti-CD3 antibodies (aCD3) and the expression of co-stimulatory receptors (OX40, 4-1BB, ICOS) was analysed on day 4. A. Scatter plots show the distribution and mean MFI of CD4⁺CD28null (28null) and CD4⁺CD28⁺ (28pos) T cells expressing co-stimulatory receptors. B. Scatter plots show the distribution and mean MFI of CD4⁺CD28null from healthy subjects (Ctrl-28null) or ACS patients (ACS-28null) expressing co-stimulatory receptors. Probability values (p) were calculated by two-tailed t test. a.u., arbitrary units; MFI, mean fluorescence intensity.
Supplemental Figure IV

**A**

CTLA-4 - aCD3

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OX40 - aCD3

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**B**

OX40 - aCD3

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OX40 - aCD3aCD28

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Supplemental Figure IV. Time kinetics of co-stimulatory receptor expression. A. PBMCs from ACS patients were activated with anti-CD3 antibodies (aCD3) and the expression of CTLA-4 and OX40 by CD4+CD28null (28null) and CD4+CD28+ (28pos) T cells was measured at the indicated time points. B. PBMCs were activated with anti-CD3 antibodies alone (aCD3) or in combination with antibodies against CD28 (aCD3aCD28). OX40 levels were quantified on CD4+CD28null (28null) and CD4+CD28+ (28pos) T cells at the indicated time points. Data (mean ± SD) are representative of three independent experiments with cells from different ACS patients. Probability values (p) were calculated by two-tailed t test. **p<0.01, *p<0.05; a.u., arbitrary units; MFI, mean fluorescence intensity.
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