The life (and death) of $CD4^{+}CD28^{null}$ T cells in inflammatory diseases

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Abstract

Inflammation contributes to the development and perpetuation of several disorders and T lymphocytes orchestrate the inflammatory immune response. Although the role of T cells in inflammation is widely recognised, specific therapies that tackle inflammatory networks in disease are yet to be developed. $CD4^{+}CD28^{null}$ T cells are a unique subset of helper T lymphocytes that recently shot back in the limelight as potential catalysts of inflammation in several inflammatory disorders such as autoimmunity, atherosclerosis and chronic viral infections. In contrast to conventional helper T cells, $CD4^{+}CD28^{null}$ T cells have an inbuilt ability to release inflammatory cytokines and cytotoxic molecules that can damage tissues and amplify inflammatory pathways. It comes as no surprise that patients who have high numbers of these cells have more severe disease and poor prognosis. In this review, I provide an overview on the latest advances in the biology of $CD4^{+}CD28^{null}$ T cells. Understanding the complex functions and dynamics of $CD4^{+}CD28^{null}$ T cells may open new avenues for therapeutic intervention to prevent progression of inflammatory diseases.

Introduction

Several subsets of T and B-lymphocytes with highly specialized functions have been characterized so far. Some lymphocytes promote inflammation, whilst others have anti-inflammatory roles, and an optimal balance between these two opposing sets of lymphocytes is critical for immune homeostasis. We have recently characterized a pro-inflammatory subset of $CD4^+$ T helper 1 (Th1) lymphocytes known as $CD4^+CD28^{null}$ ($CD28^{null}$) T cells as they characteristically lack CD28, a co-stimulatory receptor critical for the activation and function of T cells.[1, 2] $CD4^+CD28^{null}$ T cells expand in several diseases that associate with chronic inflammation (e.g. autoimmunity, atherosclerosis, etc.), whilst in healthy individuals they are almost undetectable.[3] High frequencies of $CD4^+CD28^{null}$ T cells correlate with disease severity and poor prognosis, which led to suggestions that these lymphocytes may be involved in the pathogenesis of chronic inflammatory disorders.[4] This review provides an overview on the 'signature' features that distinguish $CD4^+CD28^{null}$ T cells from conventional $CD4^+CD28^+$ T lymphocytes, the diseases in which these cells have been identified, the mechanisms that underlie expansion of this cell subset and potential therapeutic strategies that could be employed to target $CD4^+CD28^{null}$ T cells.

The ABC of $CD4^+CD28^{null}$ T cell biology

The main feature of $CD4^+CD28^{null}$ T cells is the loss of CD28, a co-stimulatory receptor that is critical for the activation, proliferation and survival of $CD4^+$ T cells.[5] For most $CD4^+$ T cells and especially for naive $CD4^+$ T lymphocytes, lack of CD28-transduced signals during activation induces an anergic state and renders lymphocytes unable to respond to antigen at subsequent encounters.[6] However, this is not the case with $CD4^+CD28^{null}$ T cells that instead of being anergic have enhanced effector

functions and can be (re)-stimulated by antigens.[7] Indeed, in several diseases in which this subset expands it has been shown that $CD4^+CD28^{null}$ T cells have oligoclonal antigen receptors with restricted antigen diversity, suggesting that repeated antigen stimulation may facilitate expansion of these lymphocytes.[8, 9] Moreover, $CD4^+CD28^{null}$ T lymphocytes display potent effector functions such as secretion of inflammatory cytokines interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α).[1, 10, 11] Another feature that markedly differentiates $CD4^+CD28^{null}$ from conventional $CD4^+CD28^+$ T lymphocytes is the expression of cytotoxic molecules perforin and granzyme B[1, 12] that are physiologically employed by natural killer cells and cytotoxic $CD8^+$ T lymphocytes to kill target cells (**Table 1**). Not only that $CD4^+CD28^{null}$ T cells express cytotoxic molecules but they release them and kill endothelial cells, at least *in vitro*.[13] Another contrasting feature to conventional helper $CD4^+CD28^+$ T lymphocytes is the expression of activating natural killer cell receptors such as NKG2D.[14] Moreover, $CD4^+CD28^{null}$ T cells lose their sensitivity to apoptosis induction[2] and are resistant to the suppressive actions of regulatory T cells (Tregs),[15] compared to $CD28^+$ counterparts. Tregs have pivotal roles in keeping pro-inflammatory lymphocytes in check and, by this, maintaining immunological tolerance and preventing excessive immune responses.[16] By becoming less susceptible to suppression, $CD4^+CD28^{null}$ T cells may escape from the control of Tregs and thus drive inflammation unimpeded.

It remains controversial whether $CD4^+CD28^{null}$ T cells are antigen specific and which are the precise antigens that trigger and/or drive their expansion. It has been suggested that $CD4^+CD28^{null}$ T lymphocytes are auto-reactive and that repeated stimulation by auto-antigens drives the expansion of this cell subset. However, $CD4^+CD28^{null}$ T cells often respond to ubiquitous antigens such as heat shock proteins

(HSP) and viral antigens, whilst failing to respond to well-known auto-antigens such as collagen in rheumatoid arthritis (RA) or oxidised LDL in atherosclerosis.[15, 17] Indeed, some studies suggested that infection with cytomegalovirus might drive expansion of $CD4^+CD28^{null}$ T cells, as this virus is well known to induce loss of CD28 in $CD8^+$ T cells.[18] However, other studies failed to find any relationship between $CD4^+CD28^{null}$ T cells proliferation and CMV-seropositivity.[17, 19] Another proposed antigen is human HSP60, as $CD4^+CD28^{null}$ T cells from patients with myocardial infarction were found to respond to this antigen *in vitro*.[17] Myelin basic protein (MBP), that is the target of the autoimmune response in multiple sclerosis (MS) has also been suggested to induce proliferation of $CD4^+CD28^{null}$ T cells isolated from MS patients and to enhance IFN-γ production from these cells.[20] However, other studies failed to identify MBP reactivity in $CD4^+CD28^{null}$ T cells.[15] An alternative hypothesis for what drives $CD4^+CD28^{null}$ T cell expansion is that other cues (e.g. ligands for co-stimulatory and/or NK cell receptors, chemokines, adhesion molecules) rather than antigens may be sufficient to activate and induce effector functions in $CD4^+CD28^{null}$ T lymphocytes in the disease setting.

It is tempting to speculate that $CD4^+CD28^{null}$ T cells cross the classical boundaries of innate and adaptive immune cells and, by doing so, share features with innate-like T lymphocytes. Several populations of innate-like T cells have been described, including invariant natural killer T (iNKT) cells, γδ T cells, and mucosa-associated invariant T (MAIT) cells.[21-23] Responses mediated by innate-like T cells occur in the early stages of infectious and inflammatory disorders and shape the subsequent adaptive responses.[24] The main characteristics of innate-like T cells that set them apart from traditional adaptive T lymphocytes are: relatively restricted antigen receptor repertoire; potent and rapid cytokine production (due to constitutive

transcription of cytokine genes); and cytolytic activity. Indeed, in patients with inflammatory disorders it has been shown that $CD4^{+}CD28^{null}$ T cells have oligoclonal antigen receptors,[8, 9] produce high levels of inflammatory cytokines and express cytotoxic molecules, features similar to those of innate-like T cells.

$CD4^{+}CD28^{null}$ T cells - senescent vs. divergent?

Highly proliferative cells such as fibroblasts and T lymphocytes are susceptible to entering a state of arrested cell division termed cellular senescence. Characteristically, senescent cells irreversibly lose their capacity to proliferate, whilst remaining viable and metabolically active. Senescent T lymphocytes have been suggested to accumulate with age. In addition to growth arrest, senescent cells are often resistant to apoptosis, have altered expression of genes that regulate cell cycle entry and progression and express senescence markers (e.g. β-galactosidase, p16).[25] Additionally, loss of CD28 has been proposed to identify senescent T lymphocytes. At birth nearly all T lymphocytes express CD28 in humans, whilst with ageing, $CD8^{+}CD28^{null}$ T cells and, to a lesser extent $CD4^{+}CD28^{null}$ T cells, are detected in increasing numbers in the circulation.[7] Interestingly, for reasons that are not known, the increase in circulating $CD28^{null}$ T lymphocytes occurs only in humans and primates but has not been detected in aged mice. Previous studies have shown that $CD4^{+}CD28^{null}$ T cells lack CD28 mRNA suggesting that CD28 loss is due to a block in transcription, which is regulated by binding of nuclear protein complexes to α and β motifs in the minimal promoter of the CD28 gene.[26] However, loss of CD28 is not a specific senescence marker as $CD4^{+}CD28^{null}$ T cells are a heterogeneous population including not only senescent but also different types of non-senescent effector T lymphocytes.[27] Importantly, in contrast to the marked expansion of $CD8^{+}CD28^{null}$ T cells in aged individuals, $CD4^{+}CD28^{null}$ T cell expansion is rarely detected in most

elderly subjects in absence of inflammatory comorbidities[7], suggesting that $CD8^+$ T cells are more susceptible to replicative senescence. Reduced binding of nuclear proteins to the β but not α motif of the CD28 promoter is characteristic of replicative senescence.[26] In comparison to $CD4^+$ T cells, $CD8^+$ T cells contain a single β-bound protein complex, which explains why they are more susceptible to complete loss of nuclear proteins bound to the β motif of the CD28 promoter and subsequent CD28 down-regulation.[26] CD27 is also progressively lost during T cell differentiation and it has been proposed to identify senescent lymphocytes that have lost the ability to proliferate.[7] $CD4^+CD28^{null}$ T cells that lose expression of CD27 have been suggested to represent end-stage senescent lymphocytes that have marked telomere shortening and impaired proliferation. $CD4^+CD28^{null}CD27^-$ T cells have been described in CMV (Cytomegalo virus)-seropositive individuals but were absent in CMV-seronegative subjects.[28] The inability of $CD4^+CD27^-$ T cells to proliferate is mediated, at least in part, by activation of the p38 kinase.[27] However, not all $CD4^+CD28^{null}$ T cells lose CD27,[29] and what is the CD27 expression profile on $CD4^+CD28^{null}$ T cells in patients with autoimmunity or atherosclerosis has not been investigated. Previous studies suggested that although proliferation may be affected in senescent lymphocytes, certain effector functions (e.g. production of inflammatory cytokines, cytotoxicity) are preserved, which may enable these cells to damage tissues and amplify inflammation. Of note, we recently found that $CD4^+CD28^{null}$ T cells maintain their ability to proliferate *in vitro* in response to anti-CD3 antibodies, albeit with a slower division rate compared to $CD4^+CD28^+$ T cells, which indicates that $CD4^+CD28^{null}$ T cells do not have replicative senescence.[2] Whether truly senescent or not, it is clear that $CD4^+CD28^{null}$ T cells have different properties than those ascribed to immune-exhausted senescent lymphocytes induced by chronic re-stimulation by viruses.

Another important aspect is that T cell senescence may not always be irreversible. Indeed, senescence of a subset of effector memory $CD4^+CD27^-$ T cells characterised by re-expression of CD45RA (also known as EMRA $CD4^+$ T cells) was reversed *in vitro* by inhibition of p38 signalling.[30] In view of these recent findings, it remains to be clarified whether $CD4^+CD28^{null}$ T cells that expand in various inflammatory diseases are indeed senescent or simply diverge phenotypically and functionally from helper $CD4^+$ T lymphocytes. Additionally, if $CD4^+CD28^{null}$ T cells are senescent, it would be important to know whether their senescence is irreversible or whether it can be reversed by inflammatory cues.

$CD4^+CD28^{null}$ T cells in inflammatory diseases

$CD4^+CD28^{null}$ T cells were originally identified in patients with rheumatoid arthritis (RA).[9, 31] Since then this lymphocyte subset has been found in the circulation and/or tissues in several chronic inflammatory disorders (e.g. autoimmunity, atherosclerosis, viral infections). A high proportion of RA patients have increased frequencies of circulating $CD4^+CD28^{null}$ T cells and in some patients this subset has also been identified in the synovial fluid.[12] However, subsequent studies failed to confirm the presence of $CD4^+CD28^{null}$ T cells in the synovial fluid or membrane.[32] Moreover, the frequency of $CD4^+CD28^{null}$ T cells correlated with RA severity and extra-articular involvement (e.g. vascular inflammation).[33, 34] *In vitro* experiments showed that $CD4^+CD28^{null}$ T cells from RA patients support synoviocyte proliferation better than conventional $CD4^+CD28^+$ T lymphocytes.[35, 36] Excessive proliferation of synovial cells causes cartilage erosion and bone destruction in RA, indicating that $CD4^+CD28^{null}$ T cells could potentially contribute to this process. Another autoimmune disorder in which $CD4^+CD28^{null}$ T cells have been described is multiple sclerosis.[20] In this disease, $CD4^+CX3CR1^+$ T lymphocytes had similar functional

features to those ascribed to $CD4^+CD28^{null}$ T cells in RA (i.e. IFN-γ production, expression of perforin and granzyme).[37] $CD4^+CD28^{null}$ T cells have also been identified in systemic lupus erythematosus, ankylosing spondylitis, Crohn's disease, Wegener's granulomatosis (currently known as granulomatosis with polyangiitis), Grave's disease, and autoimmune myopathy.[38-44] In addition to autoimmunity, $CD4^+CD28^{null}$ T cells have been characterised in chronic viral infections (e.g. CMV,[18] HIV,[45] and hepatitis B[29]), end stage and chronic kidney disease, including chronic rejection of kidney transplants.[46]

Advances in the biology and role of $CD4^+CD28^{null}$ T cells in atherosclerosis

Following their identification in RA, $CD4^+CD28^{null}$ T cells have been found to expand in patients with myocardial infarction or unstable angina (often grouped under the term of acute coronary syndrome, ACS).[10] Patients with stable angina also have higher frequencies of $CD4^+CD28^{null}$ T cells compared to healthy individuals, albeit lower than the frequencies identified in patients with myocardial infarction.[10] Interestingly, $CD4^+CD28^{null}$ T cells were identified preferentially in unstable compared to stable atherosclerotic lesions, suggesting that they may trigger plaque rupture and life-threatening acute coronary events.[8] The role of $CD4^+CD28^{null}$ T cells in plaque rupture was later reinforced by observations that these cells can kill endothelial cells *in vitro*.[13] Moreover, activated $CD4^+$ T lymphocytes isolated from the circulation or atherosclerotic plaques of patients with myocardial infarction are known to kill vascular smooth muscle cells,[47, 48] which may also be the case for $CD4^+CD28^{null}$ T cells, although direct experimental proof is lacking. Another study implicating these lymphocytes in the pathogenesis of myocardial infarction found that patients with high percentages of $CD4^+CD28^{null}$ T cells have increased risk of recurrent acute coronary events and poor outcome compared to patients with low

$CD4^+CD28^{null}$ T cell frequencies.[49] Patients with diabetes, a well-known risk factor for myocardial infarction, also have increased circulating $CD4^+CD28^{null}$ T cells. Moreover, high frequencies of these lymphocytes associate with the occurrence of the first acute cardiovascular event and poor outcome following myocardial infarction in diabetic patients.[50] We demonstrated that $CD4^+CD28^{null}$ T cells from ACS patients have features that markedly distinguish them from conventional $CD4^+CD28^+$ T cells (**Figure 1**). Firstly, both resting and *in vitro* activated $CD4^+CD28^{null}$ T cells produce more IFN-γ and TNF-α than their $CD4^+CD28^+$ counterparts.[1] Secondly, in stark contrast to conventional $CD4^+CD28^+$ T cells, we showed that resting $CD4^+CD28^{null}$ T cells express perforin and granzymes. Moreover, activation of $CD4^+CD28^{null}$ T cells induced a decrease in perforin and granzyme, which was accompanied by expression of the degranulation marker CD107a on the cell surface. These findings indicate that in patients with myocardial infarction $CD4^+CD28^{null}$ T cells are equipped with a potent pro-inflammatory and cytotoxic arsenal that may enable them to amplify the inflammatory response and kill cells in the vascular wall with potentially devastating consequences for the stability of atherosclerotic lesions.

We demonstrated for the first time that alternative co-stimulatory receptors are crucial for the pro-inflammatory and tissue-damaging functions of $CD4^+CD28^{null}$ T cells in atherosclerosis. We found that the expression of alternative co-stimulatory receptors OX40 and 4-1BB is significantly higher in $CD4^+CD28^{null}$ T cells compared to conventional $CD4^+CD28^+$ T cells.[1] The up-regulation of co-stimulatory receptors was present only in $CD4^+CD28^{null}$ T cells from patients with myocardial infarction but was absent in stable angina, suggesting that this altered phenotype was specific for myocardial infarction. Furthermore, using blocking antibodies we clearly demonstrated that OX40 and 4-1BB control the production of inflammatory cytokines

and perforin from $CD4^+CD28^{null}$ T cells. Alternative co-stimulatory receptors may facilitate direct stimulation of $CD4^+CD28^{null}$ T lymphocytes cells in peripheral tissues and render them independent of activation by professional dendritic cells in secondary lymphoid organs. Indeed, we have shown that atherosclerotic plaques express OX40L and 4-1BBL and that the phenotype of $CD4^+CD28^{null}$ T lymphocytes isolated from plaques resembled that of $CD4^+CD28^{null}$ T cells activated *in vitro*, with high levels of OX40 and 4-1BB.[2] Local activation of $CD4^+CD28^{null}$ T cells in peripheral tissues may amplify inflammation in targeted organs and lead to breakage of self-tolerance and autoimmune responses. Overall, our identification of alternative co-stimulatory receptors in $CD4^+CD28^{null}$ T cells provided a mechanism that regulates the harmful actions of $CD4^+CD28^{null}$ T cells in atherosclerosis and a proof of concept that therapeutic agents targeting OX40 and 4-1BB co-stimulation may be beneficial in this disease.[51]

How do $CD4^+CD28^{null}$ T cells expand?

As previously mentioned, expansion of the $CD4^+CD28^{null}$ T cell subset in patients affected by autoimmune disorders or ACS has been linked to the severity of disease and an unfavourable prognosis.[52, 53] Thus, deciphering the mechanisms responsible for the accumulation of these cells may lead to the identification of novel strategies to target $CD4^+CD28^{null}$ T cells. Following T cell activation and expansion, unwanted and potentially harmful T lymphocytes are purged through apoptosis, which ensures homeostasis and prevents a build-up of inflammatory T cells. Apoptosis induction is tightly regulated by the balance of pro- and anti-apoptotic signals induced in response to environmental queues.[54] Apoptotic cell death regularly proceeds either via the extrinsic (death-receptor-dependent) or intrinsic (mitochondrial-dependent) pathway, both of which culminate in activation of caspases that cleave DNA and other essential

cellular components, followed by the demise of the cell.[54] The extrinsic apoptosis pathway is initiated by ligation of death receptors such as Fas (CD95) that relay death signals through proteins that associate with their intracellular death domain.[55] The central molecules in the mitochondrial pathway belong to the Bcl-2 (B cell lymphoma-2) family,[56] which includes anti-apoptotic (e.g. Bcl-2, Bcl-xL) and pro-apoptotic proteins (e.g. Bim, Bax).[57, 58] One model proposes that anti-apoptotic Bcl-2 molecules form complexes with the pro-apoptotic Bax in the mitochondrial membrane, which prevents apoptosis induction unless these complexes are disintegrated.[57] This function is carried out by the pro-apoptotic molecule Bim, which in response to death triggers, displaces Bcl-2/Bcl-xL and releases Bax to exercise its death-inducing effects.[59]

We have recently identified that $CD4^+CD28^{null}$ T cells from patients with myocardial infarction have defects in apoptosis regulation. We demonstrated that, in contrast to conventional $CD4^+CD28^+$ T lymphocytes, $CD4^+CD28^{null}$ T cells resist apoptosis induction through Fas ligation or ceramide treatment *in vitro*.[2] The loss of apoptosis sensitivity in $CD4^+CD28^{null}$ T cells was mediated by a marked reduction in Fas and the pro-apoptotic mitochondrial molecules Bim and Bax. Previous studies in RA suggested that $CD4^+CD28^{null}$ T cell expansion is primarily due to up-regulation of the anti-apoptotic molecule Bcl-2.[60, 61] Moreover, in this disease $CD4^+CD28^{null}$ T cells displayed similar levels of Fas, Bcl-xL and Bax compared to $CD4^+CD28^+$ T cells. Noteworthy, these results were generated using $CD4^+CD28^{null}$ T cell lines/clones instead of direct analysis of primary $CD4^+CD28^{null}$ T cells. The levels of molecules regulating apoptosis pathways are perturbed in T cells expanded in culture, as cells are subjected to several rounds of re-activation to extend their survival and therefore may not model with sufficient fidelity the *in vivo* status of apoptotic molecules.

Interestingly, in patients with myocardial infarction we did not identify any differences in anti-apoptotic molecules Bcl-2 and Bcl-xL in freshly analysed primary $CD4^+CD28^{null}$ T cells, implying that these molecules do not have a central role in apoptosis resistance of $CD4^+CD28^{null}$ T lymphocytes in this disease. Whether the mechanisms that underlie the apoptosis resistance of $CD4^+CD28^{null}$ T cells diverge in RA and myocardial infarction or are the result of using different types of cells remains to be established in future studies.

Tissue tropism of $CD4^+CD28^{null}$ T cells

$CD4^+CD28^{null}$ T cells have been identified not just in the circulation of patients with inflammatory diseases but also in target tissues. A better understanding of the mechanisms that guide tissue recruitment and re-circulation of this cell subset bears potential translational implications. Chemokine receptors and adhesion molecules tightly regulate the migration of T lymphocytes during physiological immune responses and have crucial roles in inflammatory diseases.[62] Some studies suggest that expression of chemokine receptors and adhesion molecules by $CD4^+CD28^{null}$ T cells drives their ability to infiltrate tissues, which may enable these cells to cause local inflammation and tissue damage. $CD8^+CD28^{null}$ T cells have been found to express high levels of adhesion molecule LFA-1 (lymphocyte function-associated antigen-1), which is possibly the result of changes in DNA methylation induced by ageing.[63] LFA-1 is an integrin that regulates T cell recruitment at inflammatory sites and their interaction with antigen presenting cells *via* binding to ICAM-1 (intercellular adhesion molecule-1) and it lowers the activation threshold of T cells. Moreover, as ICAM-1 is also expressed by cells in various tissues (e.g. synoviocytes), it has been proposed that LFA-1 may enable $CD8^+CD28^{null}$ T cells to stimulate synoviocytes. Whether LFA-1 carries out similar functions in $CD4^+CD28^{null}$ T cells

remains to be investigated. A chemokine receptor that has been identified preferentially on $CD4^+CD28^{null}$ T cells is CX3CR1 (the fractalkine receptor).[36] This receptor enables $CD4^+CD28^{null}$ T cells to interact with synoviocytes which express fractalkine, which in turn increases proliferation, IFN-γ production and release of cytotoxic molecules from $CD4^+CD28^{null}$ T lymphocytes *in vitro*. Moreover, this interaction enhanced the proliferation of synoviocyte cell lines generated from the synovium of RA patients.[35] CX3CR1 has also been suggested to facilitate $CD4^+CD28^{null}$ T cell infiltration in peripheral target tissues. However, direct identification of these cells *in situ* is less accurate compared to the peripheral circulation as their main feature is the lack of the CD28 receptor and other unique markers that could distinguish $CD4^+CD28^{null}$ T cells from other $CD4^+$ T lymphocytes are not available. It has been suggested that $CD4^+CD28^{null}$ T cells are present in the brain of some patients with multiple sclerosis, based on detection of $CD4^+CX3CR1^+$ T cells, which should include predominantly of $CD4^+CD28^{null}$ T cells as other subsets of $CD4^+$ T cells do not usually express this chemokine receptor.[64] Noteworthy, fractalkine the ligand for CX3CR1, is upregulated in the serum and cerebrospinal fluid of MS patients as well as in MS brain lesions.[64] Fractalkine levels are also elevated in urine in kidney transplant recipients during graft rejection,[65] suggesting that this chemokine receptor/ligand pair has important role in guiding the migration of $CD4^+CD28^{null}$ T cells into target tissues. $CD4^+CD28^{null}$ T cells have also been identified in the lung in RA patients with pneumonitis[66], in the muscle in patients with autoimmune myopathies[39, 42] as well as lamina propria of patients with Crohn's disease,[67] primarily based on identification of $CD4^+NKG2D^+$ T cells in these tissues, as NKG2D is a marker preferentially expressed by $CD4^+CD28^{null}$ and not

$CD4^+CD28^+$ T cells), although the mechanisms that guide recruitment of this cell subset in these tissues remain unknown.

Potential strategies to target $CD4^+CD28^{null}$ T cells

Blockade of inflammatory cytokines

Considering that $CD4^+CD28^{null}$ T cell expansion is a consistent feature of chronic inflammatory conditions, several attempts have been made to identify strategies for targeting this cell subset. One of the initial studies targeted the pro-inflammatory cytokine TNF-α. Bryl et al. found that *in vitro* treatment with TNF-α down-regulated the expression of CD28 on $CD4^+CD28^+$ T cell clones by affecting the transcription of the CD28 gene.[68] In a later study, the same group described that TNF-α blockade with Infliximab induced a recovery of CD28 expression on peripheral blood mononuclear cells from RA patients.[69] Similar effects of Infliximab on CD28 expression were generated by treatment of whole blood from patients with unstable angina and elevated frequencies of $CD4^+CD28^{null}$ T cells, which resulted in an increase in CD28 levels on T cells.[70] Based on these findings TNF-α blockade has been proposed to reduce the frequency of $CD4^+CD28^{null}$ T cells in RA patients. However, a recent study found that the percentage of $CD4^+CD28^{null}$ T cells remained unchanged in RA patients treated with Infliximab or Etanercept over a period of one year.[71] Interestingly, we have recently found that TNF-α treatment failed to down-regulate CD28 in primary $CD4^+CD28^+$ T cells from both healthy individuals and patients with myocardial infarction (Bullenkamp J. and Dumitriu I.E., manuscript in preparation). Moreover, due to severe adverse effects, use of TNF-α inhibitors is currently not justified in patients with myocardial infarction and the potential atheroprotective effects of TNF-α blockade remain to be precisely established in this disease. Another cytokine that has been proposed to enable CD28 re-expression is IL-12.

$CD4^+CD28^{null}$ T cell clones re-stimulated in the presence of IL-12 were also shown to up-regulate CD28.[72] In contrast to TNF-α, IL-12 on its own failed to up-regulate CD28. Whether IL-12 could induce CD28 re-expression in primary $CD4^+CD28^{null}$ T cells is not known. Importantly, whether inflammatory cytokine blockade alters the function of $CD4^+CD28^{null}$ T cells in addition to re-expression of CD28 has not been investigated.

Statins

In addition to their well-documented ability to down-regulate LDL-cholesterol, statins have anti-inflammatory and immune-modulatory effects.[73] Statin treatment in patients with unstable angina was suggested to reduce the percentage of $CD4^+CD28^{null}$ T cells, although the effect was rather limited (from an average of 3% to 2.3%, p=0.022).[74] Another group suggested that Rosuvastatin triggers apoptosis of $CD4^+CD28^{null}$ T cells in patients with acute myocardial infarction and induces a contraction of this cell subset[37]. We recently investigated the effect of statins on $CD4^+CD28^{null}$ T cells from patients with myocardial infarction. Treatment of sorted $CD4^+CD28^{null}$ T cells with increasing doses of Atorvastatin or Rosuvastatin failed to induce apoptosis.[2] This is not surprising as many patients with acute myocardial infarction or stable angina who are already on statin therapy still exhibit high numbers of $CD4^+CD28^{null}$ T cells. Additionally, $CD4^+CD28^{null}$ T cell frequency remained unchanged for up to two years after an acute coronary event although statins are routinely prescribed following myocardial infarction[13], indicating that statins do not affect the frequency of $CD4^+CD28^{null}$ T cells, in line with our *in vitro* findings.

Modulation of co-stimulatory pathways

Another class of drugs that have the potential to modulate the deleterious functions of $CD4^+CD28^{null}$ T cells target co-stimulation. Blockade of T cell co-stimulation is being

used in patients with RA. Abatacept, a CTLA-4Ig fusion protein that works by binding to B7 ligands CD80/CD86 and blocking their interaction with CD28 on T cells has shown some results in RA. Treatment with Abatacept for one year reduced the frequency of circulating $CD8^{+}CD28^{null}$ T cells but had only a marginal and not statistically significant effect on $CD4^{+}CD28^{null}$ T cells in RA patients, although a small correlation between $CD4^{+}CD28^{null}$ T cells and the disease activity score DAS28 was found.[75] Moreover, a study on a small group of RA patients showed that Abatacept did no alter the frequency of $CD4^{+}CD28^{null}$ T cells in RA patients on long-term therapy with this drug (> 5 years),[76] suggesting that any effects on this cell subset induced by Abatacept may be transient or the mere result of fluctuations in disease activity. In view of our findings that OX40 and 4-1BB co-stimulatory receptors are up-regulated on $CD4^{+}CD28^{null}$ T cells in myocardial infarction, targeting these molecules may prove a more successful approach, especially as OX40 and 4-1BB belong to a different family of co-stimulatory receptors than CD28 and CTLA-4.[77] Clinical targeting of OX40 and 4-1BB is being investigated in rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, asthma, transplantation and graft versus host disease.[78] What makes OX40 and 4-1BB such attractive targets for modulation of co-stimulation pathways is their preferential expression on activated/effector T cells, whilst they are absent in naive/resting lymphocytes. This should facilitate specific targeting of effector T cells that mediate tissue damage without compromising the ability of naive T lymphocytes to mount appropriate immune responses to exogenous antigens.

Proteasomal inhibitors

Our findings that $CD4^{+}CD28^{null}$ T cells have markedly reduced levels of the pro-apoptotic mitochondrial protein Bim, which has central roles in controlling apoptosis

induction, made us investigate mechanisms responsible for Bim reduction in $CD4^+CD28^{null}$ T cells to identify tools to revert their resistance to apoptosis. We showed that ERK1/2, a protein kinase that has been implicated in regulating Bim levels by phosphorylating it, was constitutively activated in resting $CD4^+CD28^{null}$ T cells from ACS patients.[2] Moreover, ERK1/2 activation was further enhanced by $CD4^+CD28^{null}$ T cell activation. In line with this, $CD4^+CD28^{null}$ T lymphocytes expressed significantly higher levels of phosphorylated Bim than conventional $CD4^+CD28^+$ counterparts. Moreover, ERK1/2 inhibition reduced phosphorylated Bim levels in activated $CD4^+CD28^{null}$ T cells. Bim phosphorylation has been shown to tag this protein for degradation by the proteasome, which may explain the marked reduction in Bim that characterises $CD4^+CD28^{null}$ T cells in ACS. Proteasome inhibition increased phosphorylated Bim levels in both resting and activated $CD4^+CD28^{null}$ T cells, confirming its central role in regulation of Bim levels.[2] We were the first to show that treatment with the proteasome inhibitor MG-132 restored apoptosis sensitivity of $CD4^+CD28^{null}$ T cells in ACS patients. Noteworthy, the dose of proteasome inhibitor that restored apoptosis sensitivity in $CD4^+CD28^{null}$ T cells was approximately a thousand times lower than doses needed for induction of apoptosis in cancer cells, suggesting that re-sensitisation of $CD4^+CD28^{null}$ T cells to apoptosis could potentially be achieved *in vivo* in ACS at much lower doses than in cancer, with lower side-effect profile. Encouragingly, proteasome inhibition did not cause apoptosis in conventional T cells, indicating that the proteasome is an attractive target for selective elimination of $CD4^+CD28^{null}$ T cells, while sparing their conventional counterparts and reducing bystander immunosupression.

Other approaches to target $CD4^+CD28^{null}$ T cells

Polyclonal anti-lymphocyte globulins have been recently suggested to reduce $CD4^+CD28^{null}$ T cell frequency in transplant recipients, possibly via triggering apoptosis as demonstrated *in vitro*.[79] Additionally, a recent study described that expression of Kv1.3 channels may confer the potential to target $CD4^+CD28^{null}$ T cells, as specific blockade of these channels reduced the ability of $CD4^+CD28^{null}$ T lymphocytes to produce IFN-γ and perforin *in vitro*.[80] Interestingly, another recent *in vitro* study suggested that $CD4^+CD28^{null}$ T cells might be resistant to the effects of immunosuppressive drugs Tacrolimus and Everolimus, which may explain the expansion of this lymphocyte subset in chronic kidney allograft rejection.[19] Better characterisation of $CD4^+CD28^{null}$ T cells should unveil other strategies to modulate the expansion and/or the function of this subset.

Gaps in knowledge and future perspectives

Although $CD4^+CD28^{null}$ T cells have been identified in several inflammatory disorders wherein they have enhanced effector function characterised by production of inflammatory factors and cytotoxicity, their precise contribution to the pathogenesis of these diseases is yet to be completely understood. Important questions that remain unanswered are which mechanisms drive the expansion of $CD4^+CD28^{null}$ T cells, how are these cells recruited in tissues, and how can their deleterious effector function be down-modulated. Recent identification of molecules that regulate the production of inflammatory cytokines, cytotoxic proteins and the loss of apoptosis sensitivity in $CD4^+CD28^{null}$ T cells may provide novel strategies for targeting $CD4^+CD28^{null}$ T cells to tame this subset of lymphocytes and tackle inflammation.

Table 1. The ABC of $CD4^+CD28^{null}$ T cell biology.

	$CD4^+CD28^{null}$ T cells	$CD4^+CD28^+$ helper T cells	Ref.
CD28 co-stimulatory receptor	absent	present	[3]
OX40/4-1BB co-stimulatory receptors	up-regulated	absent*	[1]
T cell receptors (TCR)	oligoclonal	polyclonal	[8, 9]
Activating NK cell receptors (e.g. NKG2D)	present	absent	[14]
CX3CR1 (fractalkine receptor)	present	absent	[36]
'Signature' cytokines	IFN-γ, TNF-α	variable#	[1, 11]
Cytolytic enzymes (perforin, granzymes)	present	absent	[1, 12]
Cytotoxic function	present	absent	[1, 13]
Resistance to apoptosis induction	yes	no	[2, 60]
Ability to provide help signals to B cells	no	yes	[11]
Sensitivity to suppression by Treg cells	decreased	yes	[15]

*, OX40 and 4-1BB expression are induced only after T cell activation; #, depending on the Th cell subset (e.g. IFN-γ for Th1; IL-4 for Th2; IL-17 for Th17, etc.)

Legends to figures.

Figure 1. Characteristics of $CD4^+CD28^{null}$ T cells in atherosclerosis. In patients that develop myocardial infarction due to coronary atherosclerosis $CD4^+CD28^{null}$ T cells have features that distinguish them from conventional helper $CD4^+CD28^+$ T lymphocytes. Specifically, $CD4^+CD28^{null}$ T cells express higher levels of alternative co-stimulatory receptors OX40 and 4-1BB, whilst co-inhibitory receptors (CTLA-4 and PD-1) are present in similar levels to those on $CD28^+$ counterparts. OX40 and 4-1BB regulate the production of inflammatory cytokines (TNF-α and IFN-γ) and the release of cytotoxic molecules (perforin, granzymes) by $CD4^+CD28^{null}$ T cells. Moreover, in these patients the expression of the death receptor Fas and of pro-apoptotic mitochondrial proteins Bim and Bax are significantly reduced in $CD4^+CD28^{null}$ T cells. This endows $CD4^+CD28^{null}$ T lymphocytes with resistance to apoptosis, which may allow these cells to accumulate and amplify inflammation and cause vessel wall damage, which may contribute to atherosclerotic plaque rupture.

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Disclosures

None.

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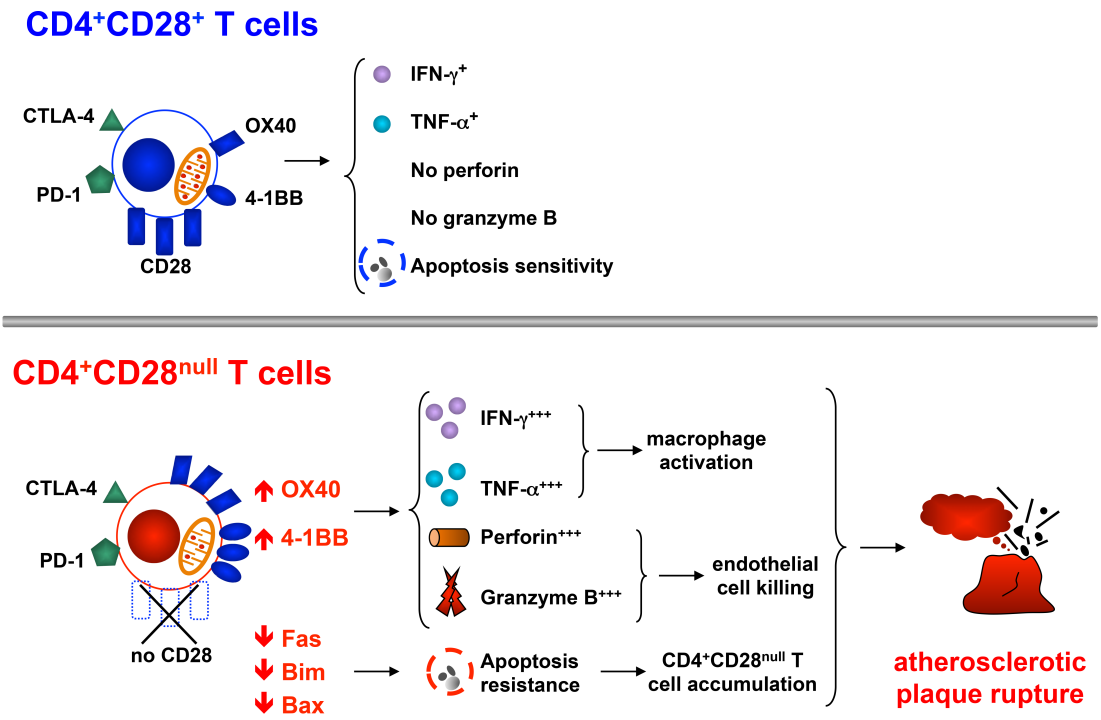
Figure 1


CD4+CD28+ T cells
CTLA-4
OX40
PD-1
4-1BB
CD28
IFN-γ+
TNF-α+
No perforin
No granzyme B
Apoptosis sensitivity
CD4+CD28null T cells
CTLA-4
OX40
PD-1
4-1BB
no CD28
Fas
Bim
Bax
IFN-γ+++
TNF-α+++
macrophage activation
Perforin+++
Granzyme B+++
endothelial cell killing
Apoptosis resistance
CD4+CD28null T cell accumulation
atherosclerotic plaque rupture