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REVIEW

Clinical consequences of circulating CD28-negative T cells for solid organ transplantation

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Summary

CD28 is an important costimulatory molecule expressed on T cells, interacting with CD80 and CD86 on antigen-presenting cells. All naïve T cells express CD28, but memory T cells may become CD28 negative (CD28neg) as a result of repetitive cell divisions, the influence of TNF-alpha, and infection with cytomegalovirus. This results in accumulation of CD28neg T cells, which may constitute >50% of the total circulating T-cell population in the elderly. The frequency of CD28neg T cells is associated with diseases such as cancer, autoimmunity, and atherosclerosis in which they may act as either effector or suppressor cells. This functional heterogeneity probably underlies the finding that the CD8posCD28neg T-cell population harbors effector cells mediating acute allograft rejection, but also suppressor cells. The CD4posCD28neg T cells are predominantly considered to be a nonclassical risk factor for atherosclerotic disease, and their contribution to alloreactivity is less clear. CD28neg T cells depend on IL-15 for their proliferation, and they have increased the expression of specific costimulatory molecules, such as NK receptors, TNF-alpha receptor family members, and the adhesion/costimulatory molecule CD2. Targeting these costimulatory pathways presents potential therapeutic options to block allograft rejection mediated by CD28neg T cells which are resistant to belatacept.

Introduction

The CD28 molecule has been among one of the first costimulatory molecules recognized as pivotal for an optimal adaptive cellular immune response [1]. However, sizeable frequencies of CD28-negative (CD28neg) T cells are present in the circulation of humans and nonhuman primates [2,3]. In general, the loss of CD28 expression is considered a hall mark of T-cell development and aging as only memory T cells lose CD28 expression [4].

Over the years, our understanding of the (patho-)physiological role of CD28neg T cells has grown but is still incomplete, specifically in the field of solid organ transplantation, despite the development of therapeutic monoclonal antibodies that block the actions of CD28.

Antigen-driven proliferation of naïve T cells is essentially dependent on three signals. Signal 1 is interaction between T-cell receptor and the cognate antigen presented by the appropriate HLA molecule, signal 2 is activation of the costimulatory molecules, and signal 3 is delivered by cytokines that propagate cell activation and differentiation into different T-cell subsets [5,6]. In addition, "danger signals," which are usually signals derived from microorganisms and dying cells, stimulate so-called pattern recognition receptors which augment both costimulatory and cytokine signals [7,8]. Adequate costimulation is essential for the proliferation of naïve T cells, and a pivotal system in this respect is the CD28–CD80/86 axis [9,10]. Engagement of CD28 stabilizes the immunological synapse, enhances the proliferative response, and promotes T-cell survival. All naïve T cells express CD28 on their cell surface, but the frequency of CD28-positive T cells declines with progressive differentiation from central memory T cells (able to migrate into lymphoid tissue) to effector memory T cells (Tem) and the terminally differentiated Temra cells (re-expression of the naïve T-cell marker CD45RA) [11,12]. A substantial part of Tem and almost all Temra cells do not express CD28 [11,13]. This is not unique for

CD28 as for instance CD27 expression, which is another important costimulatory molecule on T cells, is also progressively lost upon T-cell differentiation. However, CD28 and CD27 do not completely overlap in their expression profiles and CD28 loss usually precedes the loss of CD27 with further T-cell differentiation [11]. Importantly, the type of antigenic stimulus, for example, the type of viral infection, is of importance in shaping the phenotype of the circulating effector memory T-cell compartment [14]. The loss of these important costimulatory molecules on memory T cells indicates that upon re-challenge of the immune system with the same pathogen, the T cells can rapidly proliferate and act within the tissues without the need for interaction with professional antigen-presenting cells in lymphoid tissues.

The regulation of CD28 expression is only partly understood, but in general the expression of CD28 on T cells is rather sharply delineated and either present or absent [13,15–17]. Transcription of the CD28 gene is controlled by two sequence motifs, sites alpha and beta, which are pivotal for the binding of the CD28-initiator protein complex allowing the gene to be active [18,19]. A temporary decrease in CD28 expression occurs after antigen-specific activation and proliferation of naïve T cells [20] but only after prolonged stimulation and cell divisions, the CD28 gene may become inactivated, giving rise to CD28-negative CD4 and CD8 T cells [17,21]. TNF-alpha has been shown to downregulate CD28 expression *in vitro*, which is initially reversible but becomes a stable phenotype after prolonged exposure to TNF-alpha, leading to inactivation of the CD28 gene [22,23]. Understanding the role and biology of CD28neg T cells is of great importance for transplantation medicine as these cells have been implicated in transplantation tolerance, rejection, and atherosclerotic vascular disease. In addition, the fusion protein belatacept (CTLA4-Ig), which inhibits the interaction of CD28 with CD80/86, has been introduced as an alternative to calcineurin inhibitor use after kidney transplantation. Although a sizeable minority of patients treated with belatacept experience acute rejection, which may have an unexpected severe character [24], it is actually intriguing why the majority of patients with large populations of circulating CD28neg T cell do not reject their kidney transplantation. This article critically reviews the existing data on the role of CD28neg T cells in solid organ transplantation in particular with respect to allograft rejection/tolerance and costimulatory pathways used when CD28 is lost.

CD28neg T cells in relation to age and CMV infection

At birth, hardly any CD28neg T cells are present in the circulation but they progressively increase during lifetime to more than 50% of all CD8-positive T cells in elderly individuals [2]. The relative increase is largely caused by the decrease in naïve CD8 T cells because in absolute numbers, the CD8CD28neg T-cell number remains stable in adult life [25]. This accumulation of CD28neg T cells in early life is consistent with the concept of exposure to several pathogens over the years, leading to the expansion of effector memory T cells [26]. The presence of expanded CD8CD28neg T-cell populations has been associated with age-related immune deficiencies such as a decreased vaccination response [27,28].

Another important driver of expanded CD28neg T cells is chronic stimulation of the immune system, as this may occur in chronic viral infections such as HIV and HCV infection, autoimmune diseases, and end-stage renal disease [7,29–31]. In the latter condition, a pro-inflammatory milieu is created by increased oxidative stress and activated immune cells [32]. A primary cytomegalovirus infection has the single greatest impact on the absolute number and differentiation of peripheral T cells. It leads to a doubling of CD28neg CD8 T cells and the emergence of CD4CD28neg T cells and highly differentiated Temra cells [15,25,33,34]. These changes remain present during life and are relevant to consider when analyzing peripheral T cells, as 60–80% of all adults are CMV seropositive [35].

CD8posCD28neg T cells: function and pathology

The majority of CD8CD28neg T cells are found within the terminally differentiated effector T cells, and in particular, the highly differentiated Temra cells are virtually all CD28 negative [36]. Typically, CD8CD28neg T cells produce large amounts of pro-inflammatory cytokines such as interferon-gamma and TNF-alpha, are cytotoxic, and contain short telomeres with low telomerase activity. In addition, CD28neg T cells can become CD57 positive [31]. CD57 has been identified as a marker of senescent T cells as the CD57-positive T cells have the poorest replicative capacity [37,38]. Senescence is frequently used as synonymous with terminal differentiation and/or aged T cells. However, the hallmark of cellular senescence is the permanent and irreversible cell cycle arrest, which cannot be overcome by known physiological stimuli [39,40]. Highly differentiated T cells may poorly proliferate upon antigen-specific stimulation *in vitro* but their full proliferation may require additional cytokine requirements such as Il-2 or IL-15, thereby excluding true senescence [17,41]. The subset of both CD27 and CD8CD28neg T cells has the shortest telomeres compared with all other memory T cells, impaired ability to restore telomerase activity, and decreased proliferative capacity [42]. This particular subset of CD28neg T cells may approach a state of true senescence.

The presence and magnitude of the population of CD28neg T cells has studied in relation to cancer, chronic viral infections (see above), nonviral infections, pulmonary diseases, autoimmune diseases, and atherosclerosis. The role of CD8posCD28neg T cells has recently been reviewed extensively by Strioga *et al.* [31]. In general, there is an association between malignancy and increased numbers of CD8CD28neg T cells, both in the peripheral blood and in the microenvironment of the tumor. Analysis of tumor-infiltrating CD8CD28neg T cells showed the secretion of both regulatory and effector cytokines, likely reflecting the mixed composition of this T-cell population [43]. In several nonviral infections, for example, tuberculosis and toxoplasmosis, the CD8posCD57pos are increased, likely reflecting important antigen-specific T-cell activity combatting the ongoing infection [31]. In asthma and chronic obstructive pulmonary disease, both CD8posCD28neg and CD4posCD28neg T cells may be increased which probably reflects chronic inflammation and a subsequently dysregulated immune response [31].

A variety of autoimmune diseases (e.g., Graves' disease, ankylosing spondylitis, dermatomyositis, myositis, rheumatoid arthritis) are associated with the expansion of CD28neg T cells, which correlates with disease severity [31,44–46]. While this indicates the involvement of effector T cells in the disease, other autoimmune diseases such as type 1 diabetes mellitus [47], multiple sclerosis [48], and systemic lupus erythematosus [49] are associated with a decrease in CD28neg T cells. The latter has been interpreted as the loss of suppressor activity by CD8posCD28neg T cells, but formal proof of this concept by showing their suppressor function is lacking. Moreover, the local presence of the highly differentiated CD8 Temra cells is associated with delayed fracture healing [50] and CD28neg T cells are found in chronic infected bone [51], indicating that these cells may have a variety of functions in human pathologic conditions. Altogether, these findings illustrate that the CD8posCD28neg T-cell population is heterogeneous and may contain both effector and regulator cell populations that need to be carefully evaluated and dissected before the nature of their association with pathology can be ascertained.

CD4posCD28neg T cells: function and pathology

CD4CD28neg T cells comprise a small minority of the total CD4 memory population, usually not exceeding 5%. However, in a diversity of pro-inflammatory conditions, this cell population may significantly expand [44]. Most relevant, patients with chronic ESRD have on average a twofold expansion of circulating CD4CD28neg T cells, and in some patients, over 50% of the total circulating CD4 T-cell population is CD28neg [15]. Similar but even more strikingly, the expansion of CD4CD28neg T cells is strongly related to a previous infection with CMV [15,52].

Both the phenotype and function of CD4CD28neg T cells distinguish these cells from regular memory CD4 T cells. They express NK cell receptors and CX3CR1, which is a chemoreceptor for fractalkine, a chemokine secreted by endothelial cells. They typically secrete T helper 1 cytokines like interferon-gamma and TNF-alpha but also contain cytotoxic granule containing perforin and granzyme [15,44].

Similar to CD8 T cells, TNF-alpha promotes the loss of CD28 expression on the cell surface and anti-TNF treatment could reverse CD28 expression in RA patients [53]. IL-12-stimulated proliferation of CD4CD28neg T cells can reverse the CD28 phenotype in about half of the cell population, whereas in CD8 T cells, the loss of CD28 seems to constitute a stable trait [54].

The inflammatory milieu of ESRD is believed to be instrumental in the expansion of CD4CD28neg T cells, but relieve of inflammation and oxidative stress by kidney transplantation does not affect the size of this cell population [55].

The relation of CD4posCD28neg T cells and disease has focused strongly around their role in atherosclerosis and instability of atherosclerotic plaques and has been reviewed previously [44,45,56,57]. In short, expansion of this cell population is related to cardio- and cerebrovascular events and intima media thickness of the carotid artery and oligoclonal CD4posCD28neg T-cell populations are present in instable plaques. These cells are highly pro-inflammatory and are cytotoxic against endothelial cells and myofibroblasts, which accounts for their plaque-destabilizing activity as has been shown in a humanized mouse model [58]. Like the CD8posCD28neg T cells, the expansion of these cells has been associated with a variety of autoimmune diseases, but contrary to CD8 T cells, the CD4posCD28neg T cells do not seem to contain a subset of suppressor cells. Of interest is the notion that the differences between CD4pos and CD8pos T cells are disappearing at the stage of (very) late differentiation when CD28 expression is lost. For instance, both cell populations express a similar set of pro-inflammatory cytokines and cytotoxic molecules, express CD57 and natural killer receptors, and use preferentially IL-15 for proliferation [59–61]. In addition, recent microarray studies have suggested that transcriptomes of cytotoxic $CD4^+$ and $CD8^+$ T cells are conspicuously homologous [62].

CD28-negative CD8 T cells and organ transplantation

As the majority of CD8CD28neg T cells are pro-inflammatory and cytotoxic, they constitute a potentially serious

threat to an allogeneic transplanted organ. Given their high numbers in adult patients, it is surprising that a CD28–CD80/86 interaction-blocking agent such as belatacept (in combination with steroids and mycophenolate mofetil) can be effective in organ transplantation. As expected, belatacept does not reduce the proliferation of alloreactive CD8CD28neg T cells *in vitro* while, for example, mesenchymal stem cells can be suppressive [63,64]. Similarly, fully allogeneic skin allografts are rapidly rejected in mouse strains in which both CD28 and either CD4 or CD8 are knocked out, despite a significant decrease in the alloreactive T-cell clone sizes [65]. In humans, alloreactive T cells are present in the CD8CD28neg T-cell population [66]. Also, in a study by Lo *et al.* it was shown *in vitro* that alloreactive T cells within the CD8CD28neg fraction highly express CD2 [64]. These CD8CD28negCD2high cells contained the highest proportion of cells with polyfunctional cytokine and cytotoxic effector molecule expression capability.

Several observations, which are not mutually exclusive, may explain the lower than expected impact of allogeneic CD8CD28neg on the rate of acute rejection during belatacept treatment. First, *in vitro* proliferation of CD8CD28neg T cells in response to allogeneic APC is substantially lower than the CD8CD28pos T cells and the presence of IL-15 is needed to unlock the full potential of alloreactive proliferation. This observation indicates that CD8CD28pos T cells have different proliferation requirements but can mediate allograft rejection [61]. High levels of IL-15 are present locally during acute allograft rejection [67] and it is conceivable that CD8CD28neg T cells become pathogenic during rejection, which is initially initiated by CD28pos T cells. In support of this hypothesis, it has been shown that blocking of IL-15 (but not the CD80/CD86 pathway) is highly effective in preventing rejection in a CD4 T-cell-independent, CD8 T-cell-dependent islet transplantation model and substantially reduces intragraft accumulation of CD8 T cells [68,69].

Second, the expansion of CD8CD28neg T cells is in general oligoclonal in nature resulting in an overall loss of T-cell receptor diversity [31,70,71]. This will create "gaps" in the immunological repertoire in the T-cell population, which will be reflected in a decrease in the frequency of alloreactive CD28neg T cells. However, it will also create an unbalanced distribution of patients at risk for allograft rejection, as some recipients will have a clonal expansion of memory T cells after, for example, a viral infection or vaccination that are cross-reactive with certain allogeneic HLA molecules [72,73].

Third, the proliferation of CD8 effector T cells may be dependent on the support from CD4 T cells [74,75], of which most are CD28 positive and efficiently targeted by belatacept. However, above a critical CD4pos T-cell threshold precursor frequency, these cells can provide help even after blockade of the CD28 and CD154 costimulatory pathways, leading to graft rejection [76].

Lastly, clinical data from kidney, liver, and heart transplantation patient populations show a negative association between the number of circulating CD8CD28neg T cells and the risk of acute rejection [13,77–79]. A potential explanation for this association is the presence of suppressor cell populations within the CD8CD28neg T cells [49,80–84].

The CD8CD28neg FOXP3pos suppressor cells have been described by the group of Suciu-Foca [85–89] and are CD57 negative. They induce the overexpression of the immunoglobulin-like inhibitory receptors ILT3 and ILT4, which downregulates the transcription of CD80 and CD86 and leads to tolerogenic ILT3/ILT4 APC. This model of suppression is bidirectional and creates antigen-specific infectious tolerance.

CD8CD28neg FOXP3neg suppressor cells can be generated *in vitro* in the presence of IL-2 and IL-10 [43,81,90]. Secretion of IL-10 mediates the inhibition of APC function and T-cell proliferation. Their role in transplantation is not known.

A French group showed a strong cytotoxicity-associated CD8posCD28neg signature in patients with chronic rejection of their kidney allograft as compared to drug-free tolerant patients and healthy individuals [91]. In a recent study, they extended these findings by showing in a longitudinal study with a median follow-up of 15 years, which increased levels of circulating CD8pos Temra cells are associated with a twofold higher risk of long-term allograft dysfunction [92]. As the numbers of CD28neg T cells remain stable after kidney transplantation, this offers a potential early marker to identify patients at risk for graft dysfunction [55].

CD4CD28neg T cells in transplantation

There are two potential pathways by which CD4CD28neg are important in relation to transplantation. First, cytotoxic CD4CD28neg T cells may be substantially expanded in patients with ESRD [15]. Expansion of these cells is associated with the prevalence of atherosclerotic disease in these patients and therefore constitutes a nonclassical risk factor [15,35,93]. In accordance with this finding, it was shown that high numbers of these cells pretransplantation are associated with a higher incidence of early post-transplantation cardiovascular events [94].

Second, CD4CD28neg T cells may play a role in alloreactivity against the graft. Similar to CD8CD28neg T cells, alloreactive effector CD4 T cells can be identified by the expression of CD154 or CD137 after short-term culture with allogeneic APC [66,95]. In addition, there are some

reports that indicate an association between the presence of CD4CD28neg T cells and the risk for acute rejection after T-cell-depleting therapy [96,97]. These cells are among the first to repopulate within the circulation, which may indicate an increased resistance to T-cell-depleting therapy. The use of low-dose CNI seems to be able to control these cells [97]. Another report indicated that the pretransplant values of circulating CD4CD28neg T cells, as part of a senescent immune system [32,98], were negatively associated with acute rejection after kidney transplantation [99]. On the other hand, a recent publication indicated that CD4CD28neg T cells could proliferate in response to allogeneic tubular epithelial cells, which could not be suppressed by tacrolimus or everolimus [100].

In summary, cytotoxic CD4CD28neg T cells are expanded in patients with ESRD under the influence of a previous CMV infection and a persistent inflammatory uremic environment. These cells constitute a nonclassical risk factor for atherosclerotic disease before and after kidney transplantation, but their role in rejection/tolerance of the graft remains to be established.

Expression of costimulatory molecules including NK receptor expression

Despite the loss of the important CD28/B7 costimulatory pathway, there are other costimulatory pathways still in place or uniquely gained by CD28neg T cells, which may offer important therapeutic targets to control alloreactive CD28neg T cells. The latter is exemplified by the gain of expression of natural killer receptors (NKRs) which are predominantly found in the CD28neg T-cell population [101]. The NKR family (reviewed in [102]) encompasses both inhibitory and stimulatory receptors, of which most are MHC class I restricted. The activating NKR can act as costimulatory molecules augmenting TCR signaling or mediate TCR-independent cytotoxicity. The presence of inhibitory NKRs on CD8pos T cells is associated with decreased susceptibility for apoptosis, less TCR-mediated activation, and loss of effector functions. Therefore, the expression of NKRs on highly differentiated T cells broadens the spectrum of effector functions as it bridges innate and adaptive immunity. Both activating and inhibitory NKRs may be expressed by CD8 T cells and will modulate the effector functions. In general, the expression of inhibitory NKRs, specifically the killer-cell lectin receptor G1 (KLRG1), is associated with late-stage differentiation of T cells. This probably reflects an adaptive response to a chronic antigenic stimulation and results in limited effector functions as well as preventing apoptosis. For this reason, some NKRs (e.g., KLRG1, CD57, and CD244) are also considered as exhaustion markers for T cells [103]. Similar to the concept of cellular senescence, this can represent a biased interpretation as these cells may be functionally active and proliferate after appropriate stimulation. The CD4posCD28neg T cells express several NKRs (e.g., activating NK2GD), but their functional significance is less well studied. NKG2D expression might act as a costimulatory molecule in the absence of CD27 and CD28 [57]. Of note, CD57 is a glycoepitope expressed by NK cells and neuronal cells [31]. However, it is also expressed by a subset of effector CD4pos and CD8pos T cells and presumably has a function on cell adhesion molecules.

The potential importance of NKR expression on CD28neg T cells for allograft rejection was recently shown in a study by D'Addio al. In this study, a novel CD16Ig fusion protein (CD160 belongs to the immunoglobulin-like family NKR) to inhibit CD160–CD160L interactions prolonged graft survival significantly in a heart allograft model in $CD28^{-/-}$ hosts or with the administration of CTLA4-Ig, demonstrating that this pathway is particularly relevant in the absence of CD28 signaling cells. In addition, the NKR CD244 (2B4) has a role for co-inhibitory signaling on graft-specific $CD8^{+}$ T cells that functions to further attenuate responses in the absence of CD28 signaling [104].

There are numerous recent reviews discussing the potential of costimulatory signal blocking in transplantation (e.g., [105–107]). Some costimulatory pathways in particular may be important for CD28neg T cells. Classification by structure divides costimulatory molecules into four distinct groups: (i) immunoglobulin (Ig) superfamily members (e.g., activating CD28 and ICOS and inhibitory CTLA4 and PD-1); (ii) tumor necrosis factor receptor (TNFR) family members (e.g., CD40, CD134, and CD137); (iii) cell adhesion molecules or integrins (e.g., LFA-1, CD2/CD52); and (iv) T-cell immunoglobulin domain and mucin domain (TIM) molecules. Some, like ICOS, are upregulated in activated T cells although their expression is decreasing with increased T-cell differentiation. In a NHP model, it was shown that CD8CD28neg T cells are predominantly found in the rejecting kidney allograft under belatacept treatment, but these cells did not express ICOS. Probably, for this reason blocking ICOS with a monoclonal antibody was not effective in preventing rejection in this study as well in a CD28 knockout mouse model [65].

CD154 (CD40L) expression is low on CD8pos T cells and diminished on CD4CD28neg T cells [108,109]. This may account for the lack of efficacy of blocking the CD40L–CD40 pathway in CD8posCD28neg-mediated rejection [110]. Other costimulatory molecules to consider are CD134, CD137, and CD2 on CD28neg T cells, as these molecules are expressed at higher levels [64,111–113]. For instance, CD2/CD58 has recently been identified as the predominant costimulatory pathway for the proliferation of allogeneic CD8posCD28neg T cells [114]. Targeting this pathway with alefacept may be of value in preventing

allograft rejection, depending on the model used [115–118]. In addition, blocking integrins, for example, with efalizumab (anti-LFA-1) or the CD134 and CD137 pathway may be of interest to control the alloreactive CD28neg T cells [111,119]. Unfortunately, further development of efalizumab in transplantation was halted when the drug was withdrawn from the market after three of ~40 000 treated patients (0.008%) presented with progressive multifocal leukoencephalopathy [119]. An interesting perspective is the use of a combination of costimulatory pathway inhibitors (e.g., belatacept with anti-CD40 or anti-LFA) to control the whole alloreactive T-cell potential with the additional benefit of preventing anti-HLA antibody formation [119,120].

Concluding remarks

CD28neg T cells are highly differentiated cytotoxic effector T cells, which accumulate with aging in both the circulating CD4 and CD8 T-cell populations. Repetitive cell divisions, the presence of an inflammatory milieu, and a previous infection with cytomegalovirus are driving the expansion of the CD28neg T-cell population (Fig. 1), which may exceed over 50% of all circulating T cells. Both CD8pos and CD4posCD28neg T cells are involved in different pathologic conditions, specifically autoimmune diseases. The CD4posCD28neg T cells are considered to be a nonclassical risk factor for cardio- and cerebrovascular disease as they can destabilize atherosclerotic plaques. Their role in solid organ transplantation is only partly elucidated, but specifically, the presence of CD8posCD28neg T cells poses a risk for allograft rejection under belatacept treatment. There are several potential, but still hypothetical explanations for the relative efficacy of costimulatory blockade with belatacept as an alternative to calcineurin inhibitors. An important notion is that the population of CD8CD28neg T cells is heterogeneous and contains cells with highly different functionality, for example, cytotoxic cells, regulatory cells, and senescent cells. Preventing belatacept-resistent allograft rejection is still a valid clinical goal and may be achieved by

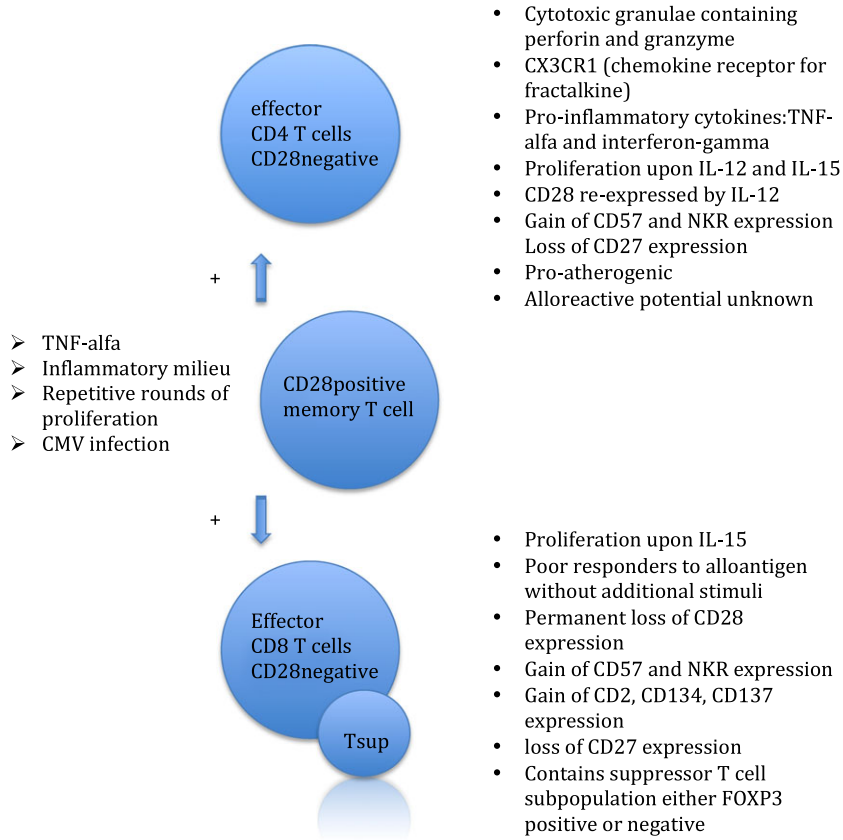

Figure 1 CD28-positive (CD28pos) memory T cells may become CD28 negative (CD28neg) under the influence of repetitive cell proliferation and cytomegalovirus (CMV) infection, which is further promoted by an inflammatory milieu and tumor necrosis factor-alpha (TNF-alpha). The defining characteristics of the CD4 and CD8 CD28neg T cells are shown on the right side. The CD8 CD28neg T cells contain suppressor cells (Tsup). NKR: natural killer receptor.

complementary inhibition of other costimulatory pathways. CD28neg T cells specifically express NKRs and increased levels of costimulatory molecules such as CD137 and CD2 compared with the CD28pos T cells. Future studies are needed to delineate which combination of costimulation pathway blockers is most effective.

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