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Signaling Mechanisms that Balance Anti-viral, Auto-reactive, and Anti-tumor Potential of Low Affinity T cells

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Abstract

T cells protect us from a large number of infectious diseases. Several lines of evidence indicate that T cells can also eliminate malignant cells and alter the progression of tumors. These two types of immune responses were traditionally viewed to involve different types or qualities of T cells. Pathogen-specific immune responses were thought to be predominantly mediated by T cells bearing high affinity T cell receptors (TCRs) specific for microbial-derived antigens. In contrast, anti-tumor immunity or autoimmune diseases normally involve TCRs with intermediate-to-low affinity to self-antigens, and lower affinity T cells are believed to have severely reduced effector T cell potential. However, recent findings illustrate that the repertoire of pathogen-specific T cells is more diverse than previously considered and that significant numbers of differentiated and fully functional lower affinity effector T cells arise during infections. In this review, we will summarize our current understanding of the importance and the effector capacity of low affinity T cells during infection, autoimmunity and anti-tumor responses. We will discuss how T cell function is influenced by TCR affinity and TCR signal strength, and we will focus on how the expression of inhibitory and activating receptors impact the function of T cells with different antigen affinity. Manipulating T cell activity through engaging or blocking these pathways bears an enormous potential to alter the clinical outcome of malignant diseases, chronic infections, and autoimmune disorders.

Keywords: Cytotoxic T cells; pMHC; T cell receptors; T cell signaling; T cell inhibitory and activating receptors; Autoimmunity; Anti-tumor immune responses

The Naïve T cell Repertoire Contains Significant Numbers of Auto-Reactive T cells

T cells express antigen receptors that are randomly generated in the thymus through somatic gene-rearrangement [1]. This creates an enormously diverse receptor repertoire, but only cells bearing receptors that at least weakly interact with self-peptide presenting MHC molecules mature (positive selection) and are exported from the thymus [2]. At the same time, thymocytes or T cells that express receptors that respond strongly to self-peptide MHC molecules are forced to undergo apoptotic cell death in the thymus (negative selection) [1,3,4] and also in the periphery [5,6].

It has long been considered that the vast majority of T cells that are capable of mounting an effector T cell response to self-antigen are eliminated in the thymus. In contrast, several lines of evidence indicate that the elimination is incomplete and it is meanwhile well established that a large number of auto-reactive T cells escape negative selection without causing any pathology in the majority of individuals [7-10]. This incomplete elimination is also illustrated by the fact that immune tolerance is critically dependent on the presence of regulatory T cells, which control the auto-reactive potential of T cells that escaped negative selection [11]. The occurrence of T cell mediated organ-specific autoimmune diseases, for which in most cases no major impairments in negative selection have yet been reported, further indicates the failure of negative selection to eliminate all auto-reactive T cells.

The decision whether or not an auto-reactive T cell becomes eliminated depends on the strength of recognition with self-peptide MHC complexes. The most aggressive T cells, which strongly react to self-antigen [high affinity or avidity T cells, for simplicity reasons subsequently only referred to as high affinity T cells], are effectively eliminated, whereas T cells, which react with lower or intermediate affinity or avidity [referred to as low affinity T cells] are spared from elimination [10] and can be found in the periphery. Moreover, there seems to be a sharp affinity threshold, above which cells are eliminated and below which negative selection does not occur anymore [12-14] (Figure 1). Likely, most relevant for the development of autoimmunity are those cells, whose affinity for self-antigen is just below or at the threshold of negative selection (Figure 1). When T cells match the negative selection threshold, the decision whether or not a cell becomes deleted is a stochastic process and in this case a large fraction of cells escapes negative selection [15]. As discussed in detail in the following sections these escaping low affinity T cells bear the potential to cause tissue damage but there are a large number of peripheral restrictions in place that prevent them from getting activated and cause autoimmune pathology in healthy individuals.

Do We Benefit from the Presence of Low Affinity Auto-Reactive T cells?

The failure to eliminate all auto-reactive T cells poses the danger of developing autoimmunity. This raises the question why such leakiness in thymic selection has evolved and what is the evolutionary benefit

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from it? One argument has long been that these low affinity T cells could also be beneficial for immune responses against pathogens. In fact, even though the T cells weakly react with self-antigens, they might cross-react with high affinity to foreign-antigens (Figure 1, orange arrow). If these T cells would be rigorously eliminated by negative selection then the clonal diversity in the T cell repertoire might be strongly reduced. Such a scenario could have a negative impact on the immune system's ability to handle pathogen infections as this often critically relies on high clonal TCR diversity. In particular, for viruses, which can establish persistent infections, correlations have been made linking limited clonal diversity in the population of viral epitope specific T cells with the emergence of viral escape variants and the development of chronic infection [16-19]. Theoretical considerations also imply that a reduced clonal diversity would likely also restrict the number of possible epitopes that T cells could recognize. Thus, allowing thymic egress of T cells that weakly respond to self-antigen may enhance the ability of the immune system to better handle the diverse set of pathogens that we become exposed to, but probably, at the expense of developing autoimmunity. In other words, we normally assume that most T cells, which are activated by pathogen, respond with an affinity to self-antigen that is below the threshold for triggering an effector T cell response (blue arrow and cell in Figure 1). However, there are likely also cells responding to a foreign-antigen which show stronger reactivity to self-antigen and which resemble the pattern illustrated in orange in Figure 1. This scenario sounds very similar to what is understood under the concept of molecular mimicry [20] but there are subtle differences. Molecular mimicry proposes that structural similarities between self- and foreign-antigen may result in the activation of T cells that cross-react between these two types of antigen. However, T cells might also cross-react between self- and foreign-antigen in the absence of larger structural similarities in antigenic motifs. Thus, it could be that a T cell which has low affinity for a self-antigen may become activated because it responds with high affinity to a structurally unrelated pathogen derived antigen. As explained in more detail below, once activated by a foreign-antigen, these low affinity T cells can mount a self-destructive effector T cell response. However, the extent and magnitude at which autoimmune destruction triggered by these mechanisms might occur remains unknown. Nonetheless, the known phenomenon of bystander damage during infection and sometimes excessive tissue destructions might be related to the action of such auto-reactive T cells that get activated during infection. In the following sections, we will discuss the mechanisms that could counteract the autoimmune responses by such low affinity auto-reactive T cells.

Besides that, the escape of low affinity T cells from negative selection is also beneficial for anti-tumor immune responses. This type of immune response targets tumor-associated antigens such as cancer-testis antigens (e.g. MAGEs or NY-ESO-1, expressed by several tumors) [21] or differentiation antigens (e.g. Melan-A/MART-1, gp100 or tyrosinase expressed in melanoma cells) [22,23]. Although the term tumor-associated antigen suggests that these epitopes are somehow special, most of them are normal self-antigens and an anti-tumor immune response therefore targets mostly non-mutated self-epitopes. As tumor-associated antigens appear all to be expressed in the thymus [24], the T cell repertoire is similarly deprived of high affinity tumor-antigen reactive T cells, as it is the case for any self-antigen. Therefore,

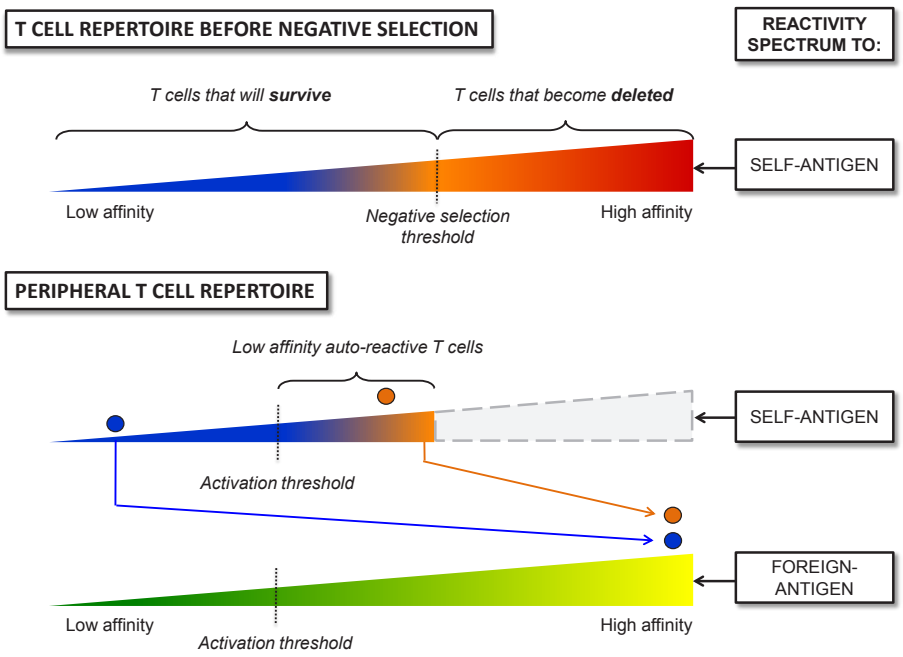

Figure 1: Comparison of the T cell repertoires available to respond to self- and foreign-antigen. Developing T cells generate T cell receptors that range from very weak to very strong self-reactivity. Negative selection eliminates thymocytes bearing a TCR that responds to self-antigen with an affinity that is above the negative selection threshold. The remaining fraction constitutes the peripheral repertoire. For most foreign-antigens, the peripheral repertoire contains naïve T cells that cover the entire affinity spectrum from very weak to very strong reactivity (indicated in green and yellow). The dashed line indicates the threshold for stimulating an effector response in peripheral T cells.

It is important to consider that peripheral T cells differ in their level of self-reactivity and that two T cell clones with similar reactivity to a foreign ligand could substantially differ in their auto-reactive potential, as depicted in orange and blue, for two examples of T cell clones. Thus, when a cell like the one shown in blue would become activated during an infection, it would not cause autoimmunity as its affinity for self is below the peripheral activation threshold. This is different for the orange cell which is above the threshold and whose activation can cause autoimmunity. Thus, the orange cell represents the low affinity auto-reactive T cells referred to in the text.

the leakiness in eliminating low affinity auto-reactive T cells in the thymus allows the presence of T cells, which may spontaneously or after vaccination respond to tumors.

Kinetic Aspects of Antigen Recognition by Low and High Affinity T cells

The dissociation constant, K_d describes the affinity with which two molecules bind each other and K_d is defined as the ratio of the dissociation and association rate. Despite the fact that we refer to cells as "high affinity" T cells, the strength with which the T cell receptor binds to peptide-MHC complexes is relatively low. "High and low affinity" T cells usually show a K_d in the range of 100 to 1 μM [3]. These have been determined for the monomeric interaction of the three molecules (peptide, MHC, TCR) by surface plasmon resonance, but the measured K_d value can significantly vary depending on the temperature and experimental system used to determine the K_d.

For positive selection, even K_d values higher than 100 μM may apply [25]. The threshold for negative selection is considered to be a K_d of 6 μM (at 37°C and at the surface of T cells) or a half-life of interaction between the TCR with pMHC of about 2 s [13]. Thus, when we speak about low affinity T cells in the context of tumor-or autoimmunity, we are referring to cells that are below the threshold of negative selection (Figure 1) and likely have a K_d higher than 6 μM or $t_{1/2}$ times shorter than 2s.

What remains still unknown is the peripheral affinity margin below which the cells do not respond anymore during an infection. We recently observed that there is a larger mismatch between the threshold of negative selection and peripheral T cell activation (as illustrated in Figure 1). Accordingly, peptide-MHC ligands that are far weaker in stimulating T cells than the thymic threshold still effectively activate T cells in the periphery during an infection and support effector function and the formation of memory T cells [15,26].

Largely puzzling questions are how T cells sense the differences in the affinity with which a TCR interacts with different peptide-MHC complexes but also what characterizes an optimal TCR ligand? High affinity binding (low K_d) can result from a very slow dissociation rate. In case of T cells, such interaction would allow a long time of interaction between the TCR and the peptide-MHC ligand (dwell time). The *kinetic proofreading* of TCR activation predicts that T cells sense how long this interaction lasts and that a minimum dwell time is needed before the cell receives an activating signal. Moreover, it is thought that the dwell time needs to be long enough to allow the recruitment of the co-receptor towards the TCR. Insufficient co-receptor recruitment signals the cell that the TCR had encountered a lower affinity ligand [14]. This model is supported by several *in vitro* experimental systems [27-29]. In its simplest form, it predicts that there is no upper affinity limit for TCR stimulation. According to this model, even very strongly binding ligands would efficiently activate T cells [30].

Alternatively, the *productive hit rate model* stipulates that multiple bindings and serial triggering of the TCR are required to activate T cells [31,32]. This model integrates the following TCR-pMHC binding characteristics: (i) TCR-pMHC interactions must be long enough to initiate productive TCR signaling and (ii) TCR-pMHC bonds must be released quickly enough to enable serial triggering, that is multiple engagement of a single pMHC to different TCRs [33,34]. TCR with fast association rates for pMHC would be able to rebind rapidly to the same pMHC after dissociation, extending the effective half-life or confinement time of the interaction [35]. Combined, this led to the prediction that extremely short or long interaction half-lives would reduce the activation potential [36], and that cumulative effects of individual productive TCR-pMHC interactions would predict functional T cell outcome rather than the absolute duration of TCR interaction. As higher affinity pMHC binding by the TCR goes usually along with longer $t_{1/2}$ times and thus with a lower extent of receptor re-engagements per time, the *productive hit rate model* proposes the existence of an affinity optimum and when this is exceeded, T cell activation would decline to levels reached with lower affinity ligands.

Therefore, the critical question which of these two models might most suitably describe the requirements for T cell activation depends on whether or not there is an optimum affinity range for T cell activation. Evidence has been provided which questioned the existence of such an upper limit [30], but newer observations suggest that it indeed exists. It has been reported that a superagonist may result in suboptimal stimulation of $CD4^+$ T cells [37] and the same applies to $CD8^+$ T cells [38]. We used a panel of $CD8^+$ T cells equipped with engineered TCRs of incremental affinities for an NY-ESO-1 derived peptide presented in the context of HLA-A2. We saw that T lymphocytes expressing highly supraphysiological TCR affinities responded less well to antigen stimulation than the natural lower affinity TCR [38], (MH and NR, unpublished observations). This observation argues in favor of the *productive hit rate model* but it needs to be said that this notion does not allow concluding that there is an absolute need to serially stimulate the TCR when activating a T cell. Instead, it could also be that too long lasting interactions simply over stimulate T cells and that this leads to a suboptimum response.

Moreover, there are *in vivo* observations that cannot be explained by both models. This includes the very efficient *in vivo* activation of T cells by very low affinity TCR ligands (which will be discussed in detail below). Interestingly, those initially induce a similar rapid proliferation and similar differentiation as very high affinity ligands [26] though the pMHC binding kinetics are largely different from high affinity ligands.

The Proximal TCR signaling Complex - a Sensor and Regulator of T cell Function to Low and High Affinity Antigen

During an infection, the T cell signaling machinery needs to be able to precisely discriminate between the overwhelming amount of TCR and self-peptide MHC engagement and related signaling background noise and the rare number of foreign-antigens that are presented among the many self-antigens. In other words, T cells need to ignore in the periphery those self- peptide-MHC complexes that stimulate T cells during postive selection in the thymus or during lymphopenia driven proliferation [39]. This illustrates the large need of the TCR signaling apparatus to adapt to different stimulation conditions and we have just begun to understand how those are achieved.

The binding of the TCR to the peptide-MHC complex triggers the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) in the TCR-associated CD3 subunits, which is mediated by the Src family kinases Lck and Fyn [40]. How ligand engagement by the TCR facilitates this phosphorylation is still not clear. So far, clustering of the TCR, the induction of a conformational change in the TCR [41] along with the recruitment of the $CD4^+$ or $CD8^+$ T cell co-receptor, to bring Lck in closer proximity to the TCR, are thought to be responsible for initiating TCR mediated T cell activation. Besides strong support that TCR clustering is required for T cell activation *in vitro*, this mode of activation is somewhat challenged by observations that the presence

of a single or very few peptide-MHC ligands on the surface of a cell is sufficient to activate a T cell [42-45].

In response to *in vitro* stimulation, a special organization of surface and intracellular signaling molecules, known as the immunological synapse, can typically be observed. A characteristic feature of the synapse is the special separation of the TCR, the co-receptor, Lck and Fyn and the phosphatase CD45. So far, it remained unknown if the spatial exclusion of CD45 is a consequence of TCR stimulation and cell activation or a requirement for transducing TCR stimulation into a cellular response. A recent report elegantly shows that the latter appears to be the case and that the special separation of CD45 is needed to shift the kinase-phosphatase balance in close proximity to the TCR and to activate the signaling cascade downstream of the TCR. Moreover, the binding energy of TCR-pMHC interaction is sufficient to generate the exclusion force needed to segregate the rather large CD45 transmembrane protein away from the TCR [46]. After the initial phosphorylation of ITAMs in the CD3 chains ZAP-70 is recruited and it phosphorylates and activates LAT and SLP-76. This leads to the assembly of several proteins that propagate the initial signal resulting in calcium flux seconds after TCR engagement and eventually full activation of the T cell [40,47].

The important question is how T cells can adapt these signaling pathways to different stimulation conditions and different requirements for antigen sensitivity? As mentioned above, the first event after TCR triggering is the phosphorylation of CD3 ITAM by Lck. The activity of Lck is controlled by a balance of kinases and phosphatases. It is negatively regulated by Csk and positively regulated by TSAD and CD45. However, CD45 can also limit the extent of Lck activity by dephosphorylating an activatory tyrosine residue [47]. The activation state of Lck is thought to act as a sensor for the strength of TCR engagement. Weak binding of the TCR triggers Lck-dependent activation and recruitment of SHP-1, which in a classical feedback loop inactivates Lck and downregulates TCR signaling. In contrast, stronger TCR activation induces an Erk-dependent Lck phosphorylation that impairs the inhibitory SHP-1 recruitment and thus reinforces TCR signaling [48]. Lower activity of SHP-1 decreases the threshold for activating T cells and converts antagonists into partial agonists [48]. Interestingly, using the above-mentioned normal and supraphysiological TCRs that are HLA-A2/NY-ESO-1 specific, we saw an upregulation of SHP-1 phosphatase level in T cells with the supraphysiological TCRs and an impaired T cell function [38], and (MH and NR, unpublished observations). This suggests that SHP-1 may play a dual role and restricts not only T cell signaling at the low but also at the very high range of TCR stimulation.

There are further phosphatases that act on the proximal TCR signaling such as Lyp, a PTPN22 encoded phosphatase. This phosphatase was shown to act together with Csk to inhibit T cell activation likely through dephosphorylation of the activating tyrosine on Lck and ZAP-70 [49]. The importance of PTPN22 is highlighted by the fact that PTPN22 deficient mice have augmented TCR-induced phosphorylation and activation [50]. Moreover, a point mutation in PTPN22 is associated with several autoimmune diseases [51]. The exact role of PTPN22 in T cell activation is not known and there is contradictory data on the effect of the polymorphism found in autoimmune patients and whether or not it causes a loss or gain of function [52].

These TCR affinity-dependent feedback mechanisms are likely part of a tunable instrument that enables T cells to adapt their reactivity to different conditions but the question is how can this be achieved? At the stage when thymocytes become positively selected, they are known to be very sensitive to antigen, which enables them to effectively respond to the very weak peptide-MHC interactions involved in positive selection [53]. The increased sensitivity to low affinity ligands is thought to be caused by a decrease in the negative regulation of TCR signaling. miR-181a – a micro RNA that is highly expressed in double-positive (DP) thymocytes and which decreases in later stages of T cell development – is considered to mediate this incerase in antigen sensitivity in developing thymocytes [54]. In the thymus, the expression of miR-181a has been shown to decrease the amount of PTPN22, SHP-2, DUSP5, and DUSP6 phosphatases. This results in an elevated steady-state level of phosphorylated proteins of the TCR signaling cascade and therefore a reduction in the TCR signaling threshold [54,55]. Furthermore, the mRNA levels of the negative regulator SHP-1 are lower in DP thymocytes compared to mature T cells [40,56]. Whether or not enforcing miR-181a expression can be used to modify the sensitivity of peripheral T cells needs to be determined.

Moreover, SHP-1 and SHP-2 can be recruited by multiple inhibitory surface receptors in T cells, and inhibit TCR signaling through dephosphorylation of proximal targets including Lck and ZAP-70 [57]. For instance, Yokosuka et al. recently showed that upon PD-L1 binding, ITIM-containing PD-1 could directly inhibit TCR-mediated signaling by recruiting SHP-2 phosphatase in a TCR stimulation strength-dependent manner [58].

How Do Differences in Affinity Impact the T cell Response and How Effective are Low Affinity T cells?

Several studies have shown that both the quality and the quantity of agonist pMHC engagement by the TCR impacts T cell activation [37,59] and it has long ago been recognized that high affinity T cells are superior in executing effector function than low affinity T cells [60,61]. Despite of this, we know that even low affinity auto-reactive T cells are able to eliminate tumors and mediate autoimmunity [8-10,62]. The escape of low affinity T cells from negative selection and their ability to cause autoimmunity have been shown in experimental systems in which pathogen derived or model antigen are expressed as neo self-antigen such as the nucleoprotein from the Lymphocytic choriomeningitis virus (LCMV) [9] and later also ovalbumin [10]. The advantage of expressing these neo self-antigens is that one can directly compare which affinities of T cells can be found in the presence or absence of the self-antigen. Nevertheless, it remained rather difficult to judge how effectively these T cells can execute effector T cell function. Obviously, their effector capacity is below that of higher affinity T cells, but by how much?

In cancer patients, self/tumor-antigen specific $CD8^+$ T cells can undergo considerable clonal expansion, persist during several years at relatively high frequencies, and differentiate to memory and effector cells, and they are in principle able to lyse tumor cells [21,63]. In parallel to these findings with human self-specific T cells, we recently intensively studied T cells responding with low affinity to pathogen-derived antigen. We achieved this by using an approach in which TCR transgenic OT-1 T cells are stimulated during an infection by ligands that gradually differ in the strength of binding to the OT-1 TCR. Thus, with this system we can mimic high, intermediate or low affinity stimulation, as it would be the case with polyclonal T cells of which some respond with high and others with low affinity to pathogen-derived antigen [26].

To our large surprise, we saw that the OT-1 T cells initially responded

similar to peptide-MHC complexes that very differently stimulate the OT-1 TCR. In fact, even very low affinity complexes induce the same initial rapid proliferation as high affinity ones. Low affinity stimulated OT-1 T cells were early on phenotypically indistinguishable from cells stimulated by high affinity complexes. They even expressed effector molecules such as granzyme B and were able to execute effector T cell functions and form memory cells [10,64]. We could recently show that very low affinity stimulated T cells support pathogen elimination (SO and DZ, unpublished observations). That low affinity T cells can eliminate pathogens was also observed in mice which express Ova as a neo self-antigen and in which Ova-specific high affinity T cells were eliminated by tolerance enforcing mechanisms. In these mice protection against Ova-expressing pathogen was mediated by low affinity T cells [7]. Overall, our observations indicate that lower affinity T cells fully participate in the immune response. A major difference between low and high affinity T cells is that weaker stimulated T cells undergo fewer rounds of division and decline in numbers faster than high affinity stimulated T cells. This results in lower absolute numbers of low affinity primed effector T cells, with the consequence that high affinity T cells dominate in numbers at the peak of the T cell expansion phase [26].

In particular, the large numbers of high affinity T cells at the peak of the immune response have so far distracted us from exploring the relevance of low affinity T cells during infection and, obviously, one could question their importance given their lower numbers. Nonetheless, there are several kinetic aspects that need to be considered and which in our opinion indicate that low affinity T cells are more important than previously appreciated. High affinity T cell clones specific to any given foreign-antigen are rare in the naïve T cell repertoire. In contrast, theoretical considerations imply that there are likely more low than high affinity T cell clones. Given that low and high affinity clones expand equally at the beginning, there should be a larger number of low than high affinity effector T cells in the early phase of the T cell response, which is what we observed in our experimental systems. The above-mentioned dominance of high affinity T cells develops later, and only because these cells overgrow the lower affinity T cells in the late T cell expansion phase [26]. Most importantly, we noticed that low affinity T cells leave secondary lymphoid organs earlier than high affinity T cells, suggesting that the earliest wave of effector T cells that enter peripheral organs is predominately composed of low affinity T cells. Thus, the critical early containment of a pathogen infection could be to a large extent involve immune responses mediated by low affinity T cells [26].

Moreover, there are possibly also foreign epitopes against which only low affinity T cells respond and which we normally ignore when analyzing a T cell response. Importantly, while the number of low affinity T cells responding to one of such epitopes might be low, there could be many of these epitopes which cumulatively might result in a reasonably sized population of low affinity effector T cells. These observations and considerations are suggesting that low affinity T cells play a more important role during infection than previously anticipated, and that their effector potential has been underestimated so far. Their participation in pathogen responses and their full differentiation into effector T cells also strongly support the notion that low affinity self-reactive T cells can effectively mount an anti-tumor immune response.

Despite of these observations, we need to be aware that there are several challenges or difficulties associated with activating low affinity T cells. Low affinity T cells require higher numbers of presented peptide-MHC complexes than high affinity T cells before they become activated and for mounting an effector T cell response. It also likely takes more excessive DC interactions before they receive sufficient TCR triggering and co-stimulation to undergo proliferation. Another limitation is that following stimulation, lower affinity T cells divide less vigorously than high affinity T cells and therefore lower numbers of cells will be obtained after vaccination. Given these limitations, we need to find better ways to more effectively activate these T cells, which may enhance their functionality, and selectively interfere with any mechanism that prevents them from interacting with tumors.

Manipulation of TCR Binding Kinetics to Enhance T cell Responses to Tumor-Antigen

Since tumor-associated antigens are essentially self-antigens, the T cell repertoire becomes in both the thymus and the periphery deprived of high affinity tumor-antigen reactive T cells. Given this situation it is for experimental and/or therapeutic purposes appealing to improve the function of tumor-reactive T cells by modulating the kinetics of TCR-pMHC binding and/or by manipulating the signaling cascades downstream of the TCR. Adoptive transfer of T lymphocytes (ATC) into patients with metastatic cancer is a promising therapeutic approach which generates objective responses in late-stage melanoma patients [65]. Nevertheless, there is a strong need to increase the efficacy of these treatments. As mentioned above, the reduced affinity of tumor antigen-reactive T cells is a major limitation in ATC therapy. This problem could be bypassed by engineering T cells to express TCRs with increased affinity for tumor-antigens. This approach turned out to augment the functional and protective capacity of tumor-antigen reactive T cells [66-72]. However, TCR engineering also bears the risk that the normal tissue could be harmed. It has been shown that T cells, whose TCR binds to peptide-MHC complexes with a K_d < 1 nM, can become cross-reactive [30,71,73] and could mount harmful cytotoxic immune responses *in vivo*. As such, TCR optimization through affinity alteration has to include the evaluation of optimal T cell responsiveness and lack of cross-reactivity. Yet, it needs to be highlighted that unexpected auto-reactive responses cannot be completely excluded. Nonetheless, the high therapeutic potential and the severity of malignant diseases justify in our opinion the use of such therapies.

As mentioned above, we recently generated a panel of human $CD8^+$ T cells expressing engineered TCRs of optimized affinities against the tumor-antigen NY-ESO-1. These were obtained through structure-based rational predictions [74,75]. We characterized the functional potential of these T cells [38,76] and found that T cells expressing TCRs with affinities in the upper natural limit (K_d from 5 to 1 μM) displayed greater biological responses when compared to those expressing intermediate wild-type TCR (K_d at 21.4 μM) or very low affinity (K_d > 100 μM). Largely unexpected, we noticed that T cells which express TCR with supraphysiological affinity (K_d from 1 μM to 15 nM) showed a severe decline in their functionality, but retained their antigen specificity without broad cross-reactivity as observed in other studies [30]. Similarly, other *in vitro* and *in vivo* studies also reported maximal T cell responses at an optimal TCR-pMHC off rate (k_{off}) or K_d while attenuation of intracellular signal transmission, impaired expansion potential and responsiveness were observed when kinetic parameters extended above the natural range [36,37,77-80].

In summary, these observations show that maximum T cell responses occur at intermediate TCR/pMHC binding parameters. This supports the *productive hit rate model* of T cell activation [31,32]. Moreover, these observations are corroborated by mathematical

models showing that the existence of an optimal response (E_{max}) as a function of the dissociation rate (k_{off}) or K_d at a fixed antigen dose is predicted only by the productive hit rate model, independently of whether a parameter of TCR internalization was included or not [81].

Role of Activating and Inhibitory Surface Receptors in Modulating T cell Immune Responses

T cell responses are under the influence of several surface molecules that either positively or negatively impact T cell responses. T cell co-stimulation is the oldest known mechanism that prevents T cell anergy, a state of unresponsiveness that is induced after stimulation of T cell only through their TCRs. Co-stimulation of T cells through interaction of CD28 with its ligands B7.1 (CD80) and B7.2 (CD86) expressed on an antigen presenting cell (APC) has also been shown to lower the threshold for T cell activation, thus allowing increased IL-2 production and promoting cell proliferation and survival [82]. At the molecular level, CD28 ligation stimulates T cell function by activating phosphoinositol-3-kinase (PI3K) and protein kinase C theta (PKCθ) and the downstream Akt, mTOR, and Ras signaling pathways, which eventually synergize with TCR signaling [47]. In parallel, T cell activation induces surface expression of CTLA-4, which outcompetes CD28 in binding B7.1 and B7.2 due to its much higher binding affinity for these ligands [83]. In addition, it has been reported that CTLA-4 directly triggers inhibitory signaling by interacting with SHP-1, SHP-2 and PP2A phosphatases, resulting in downregulation of the TCR signaling pathway [84]. Other mechanisms of CTLA-4 inhibition may also occur indirectly via retro-signaling through B7.1 and B7.2 and production of IDO in APCs [85]. Interestingly, accumulation of CTLA-4 at the immunological synapse was shown to depend on the strength of TCR triggering, suggesting that CTLA-4 preferentially inhibits T cell responses under conditions of more potent TCR signaling [86].

Similar to CTLA-4, PD-1 (programmed death-1) is also highly upregulated in T cells following TCR stimulation. However, unlike CTLA-4, PD-1 expression is not solely restricted to T cells, suggesting a broader role in immune regulation [87]. PD-1 interacts with at least two ligands, PD-L1 and PD-L2, which are expressed non-redundantly in different tissues and cell types. Due to the differential expression of CTLA-4 and PD-1 ligands, it was proposed that CTLA-4 plays a preferential role in limiting T cell function early during thymocyte development and in secondary lymphoid structures. In contrast, PD-1-associated inhibition would be critical later in maintaining long-term peripheral tolerance to self-antigens by preventing activation of self-reactive T cells that have escaped negative selection [88]. Accordingly, PD-1-deficient mice spontaneously develop milder forms of autoimmune diseases [89], while CTLA-4–deficient mice have lymphoproliferative disorders and early fatal multi-organ tissue destruction [90,91]. In humans, a regulatory polymorphism in PD-1 is associated with susceptibility to systemic lupus erythematosus and multiple sclerosis [92,93], while polymorphisms of the CTLA-4 have been linked to multiple autoimmune diseases including asthma, systemic lupus erythematosus, Graves' disease, and autoimmune thyroid diseases [94]. Finally, induction of PD-L1 ligand expression has been described in various tumor cells as a mechanism of cancer immune evasion [95], while a specific polymorphism of CTLA-4, which is protective for autoimmune disease, is associated with risk of multiple type of cancers [96].

The tumor necrosis factor receptor (TNFR) superfamily members represent another important group of co-stimulators, which mediate survival signals in T cells subsequently to the initial effects of CD28-B7 interaction [97]. Among TNFR/TNF ligand pairs, multiple members have been shown to directly impact T cell function following TCR activation, namely CD27/CD70, OX40/OX40L, 4-1BB/4-1BBL GITR/GITRL, and CD30/CD30L [98]. TNFR and their ligands are expressed on a variety of immune and non immune cells and are inducible and non-ubiquitous, suggesting that they are involved in modulating and coordinating global immune responses [99]. As such, they have also become the focus of intense translational and clinical research that aim to modulate T cell function in pathological settings such as autoimmunity and cancer (Figure 2). Ligation of TNFR by its ligand induces a series of bi-directional activating signaling pathways in both the APC and the T cell. In effector $CD4^+$ and $CD8^+$ T cells, recruitment of TNFR-associated factors (TRAF) activate the NF-κB signaling pathway and increase the expression of anti-apoptotic molecules, thus promoting cell survival [99]. Like CD28, TNFR signaling can also synergize with the TCR pathway to promote cell cycle progression and cytokine production. Of note, ligation of OX40 and 4-1BB has been shown to concomitantly block the generation of inducible regulatory T cells (Tregs), as well as to inhibit their suppressive activity [100].

HVEM is a particularly unique and interesting member of the TNFR superfamily. In addition to binding to its TNFR ligands LIGHT and lymphotoxin Lτα3, which are predominantly co-stimulatory and pro-inflammatory in T cells, HVEM also binds to BTLA and CD160, which are structurally similar to PD-1 and CTLA-4 and transduce inhibitory signals, in part through recruitment of SHP-1 and SHP-2 phosphatases [101,102]. The individual effects of HVEM interaction with its different ligands are particularly complex to elucidate since both receptor and ligands can be expressed on the same T cell, as well as on other immune and epithelial cell types [103]. *In vitro* experiments using *Hvem*$^{-/-}$ and *Btla*$^{-/-}$ T cells showed that they were hyper-responsive to TCR stimulation, while *in vivo, Hvem*$^{-/-}$ and *Btla*$^{-/-}$ knockout mice had enhanced susceptibility to autoimmune diseases, suggesting a predominant inhibitory role in T cells during inflammatory conditions [101,104]. Furthermore, BTLA was found to inhibit tumor-antigen specific cytotoxic T cells in melanoma patients [105]. However, it was also shown that HVEM can interact in *cis* with BTLA, which is believed to interfere with HVEM activation by other ligands [106]. Thus, the ability of HVEM to mediate immune stimulation or inhibition in a switch-like, bi-directional and context-dependent mode suggests that the HVEM/LIGHT/BTLA/CD160 might represent an important regulatory network for controlling immune responses.

Collectively, TCR triggering and co-stimulation through CD28 and TNFRs primes the system for regulation by simultaneously inducing the expression of multiple negative regulators, like CTLA-4, PD-1, and BTLA. This balancing act highlights the intricate regulatory network in place to control the immune system during steady-state and after pathogen encounter, and represents mechanisms that might be exploited therapeutically in various immuno-pathological settings [107,108].

Therapeutic Targeting of T cells for Regulating Activating or Inhibitory Signaling as a Strategy to Treat Autoimmune Diseases and Cancer

Enhancing T cell function in cancer patients is a major therapeutic aim, given the promising ability of cytotoxic $CD8^+$ T cells and T-helper type 1 [Th1] cells in eliminating cancer cells and in mediating long-term protection from disease [109]. Over the last years, basic immunology revealed a number of interesting pathways, including many of the aforementioned, that could be targeted to enhance the performance of

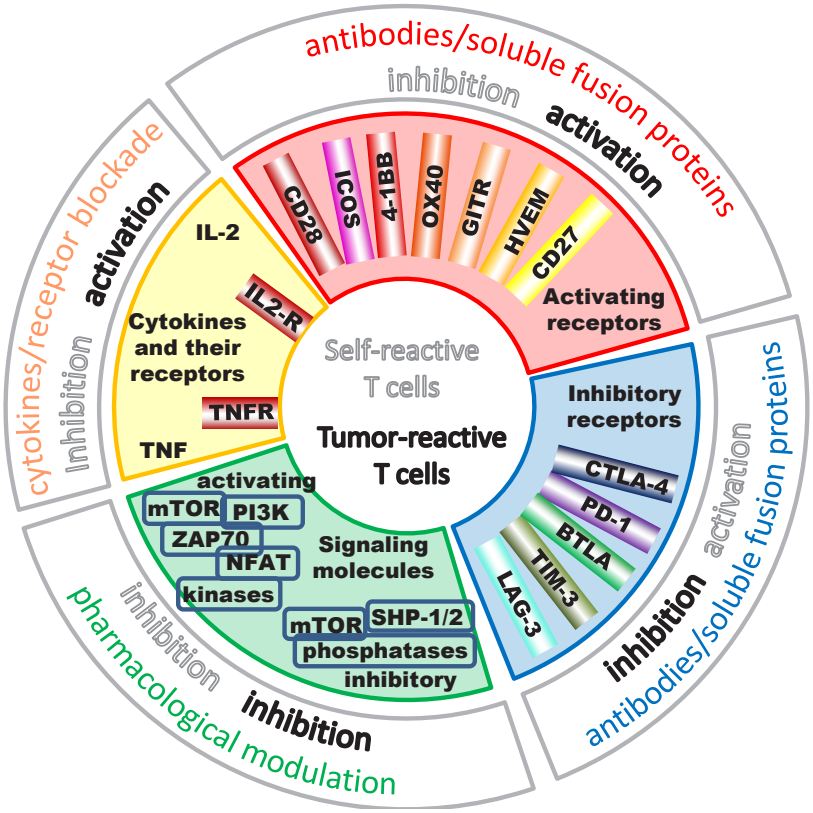

Figure 2: Overview of mechanisms and potential molecular targets to alter the function of self- and tumor-antigen reactive T cells.

T cell response to self-antigen during autoimmunity or to tumor-antigen in immunotherapy could be inhibited or augmented via manipulating similar mechanisms. These include receptors that positively or negatively regulate T cell function, cytokines and their receptors, but also intracellular signaling pathways. The figure illustrates such possible intervention points.

tumor-specific T cells. Most of them still need to be tested in clinical trials. Nonetheless, several immune based therapies are already used for cancer patients. For solid tumors, the currently most efficient therapy is by adoptive transfer of autologous tumor-antigen specific T cells [110]. This approach also permits molecular modification of T cells before transfer. The clinical usefulness of inserting TCRs [110] or chimeric antigen receptors [111] has been suggested by several small-scale clinical studies. Currently, techniques are being improved [112-115], which should soon allow applying cell transfer therapy for larger numbers of patients.

Besides antigen receptors, lymphocytes can be targeted at the level of their co-receptors (Figure 2). Inhibitory receptors are particularly attractive, because blocking those can enhance T cell activity. Ipilimumab (Yervoy®) is a recently approved drug that follows this principle and that powerfully blocks the inhibitory receptor CTLA-4. It enhances numbers and function of tumor-specific T cells and improves the clinical outcome of melanoma patients [116]. Very recently, treatment with antibodies against PD-1 [117] or its ligand PD-L1 [118] provided remarkable benefit for patients with advanced kidney cancer, non–small-cell lung cancer and melanoma [119]. Furthermore, antibodies that block LAG-3, TIM-3, B7-H3, or B7-H4 are under development [120]. Most likely, such novel approaches will continue to change the clinical oncology landscape during the next few years.

While blocking T cell inhibition is practical for promoting anti-tumor immunity, blocking co-stimulation is also feasible for reducing autoimmunity. Indeed, novel reagents have been developed e.g. for treating multiple sclerosis. Theoretically, several co-stimulatory molecules can be targeted [121]. CD28 and CD40 are the two pathways that are the most clinically validated (Figure 2). Prominently, hybrid CTLA4-Fc molecules have been generated, with or without enhanced affinity to CD80 and CD86. They are potent reagents blocking co-stimulation via CD28, and have provided evidence for clinical activity in multiple sclerosis, systemic lupus erythematosus, and psoriatic arthritis [122,123]. Manipulating T cell function via injecting blocking or stimulating antibodies need to be established with enormous precaution as such treatments may cause severe and largely unexpected effects that might not be observed in animal models. The latter happened when anti-CD28 antibodies were injected into healthy humans [124].

Besides cell surface receptors, intracellular mechanisms may be targeted. There are many options for intervening with the complex signaling network. TCR related signals might be targeted via several different E3 ligases [125] (Figure 2). The SHP protein tyrosine phosphatases have been proposed as therapeutic targets [126]. The tyrosine phosphatase inhibitor-1 (TPI-1) is a member of a new class of SHP-1 inhibitors. TPI-1 has been shown to inhibit the growth of transplanted tumor cells in mice, due to immune mechanisms involving cytokine producing T cells [127]. However, SHP-1 and many other signal transducers are widely expressed which challenges

optimal targeting. SHP-1 may suppress e.g. hematopoietic tumors [128] and thus blocking of SHP-1 may not be suitable in these diseases. Currently, more specific targets need to be characterized, with the aim to enhance targeting to particular cell populations. This strategy has proven useful e.g. for tyrosine kinase inhibitors, a class of drugs that are now widely used to treat cancer [129-131]. Finally, novel treatments aim at modifying the functions of further immune cells (e.g. B cells), adhesion- and homing-receptors, or cytokines (e.g. IL-6, TNFα, interferons), as reviewed elsewhere [132-134].

The examples provided in this review illustrate recent progress in specifically manipulating T cell functions, but today we are just at the beginning of understanding the enormous potential that is associated with such treatments. Immunotherapy of cancer has made significant progress, with the introduction of anti-CTLA, anti-PD-1, and anti-PD-L1 mAb treatments in cancer patients, and the presently developed personalized therapy options such as ATC. Besides the need to improve and further validate the different therapies, we need to be able to predict which new therapy option would be most suitable for which patient. In the field of tumor immunology, we need to develop algorithms that can predict which treatment option is the most suitable, based on the frequencies of tumor-reactive T cells, their ability to migrate to tumor sites, their affinity for antigen recognition, status of effector function and presence of inhibitory regulatory circuits. Unfortunately the novel therapies are very expensive and a large focus should be on developing effective lower cost anti-cancer therapies.

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