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Impact of epitope density on CD8$^+$ T cell development and function

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ABSTRACT

Effective immune responses against intracellular pathogens and tumors frequently rely upon CD8+ cytotoxic T lymphocytes (CTLs). In turn, CTL detection of foreign material from viruses and bacteria depends on antigen presentation by the MHC class I pathway. The underpinnings of antigen processing and presentation and, subsequent T cell activation and immunological memory development, have been extensively studied, leading to a better understanding of the balance between antigen dose, context, and, the T cell activation threshold. Still, the complexity of this process leads to apparent contradictions that hinder construction of rational strategies for generating optimal CD8 + T cell responses in a variety of settings. In this review we consolidate the current knowledge around the effects of peptide MHC I complex (pMHC) density and kinetics on CD8 + T cell responses and function during the acute phase of an infection.

1. Introduction

Pathogens not readily neutralized by innate barriers prompt the development of adaptive immunity (Dempsey et al., 2003), resulting in individualized primary responses and the establishment of memory cells poised to handle subsequent infections. These changes are achieved through selection and expansion of T cells and antibodies that recognize pathogen specific antigens (Baumgartner and Malherbe, 2011). In the case of cell mediated immunity, CD8 + T cell activation is generally initiated in the secondary lymphoid organs, the spleen and lymph nodes, where naïve CD8 + T cells encounter cognate antigens for the first time. Classical CD8 + T cells recognize short peptide fragments, termed epitopes, derived from foreign proteins presented by MHC molecules on the surfaces of the target cells (Nolz, 2015). Priming is performed by antigen presenting cells (APCs), most often dendritic cells (DCs), which possess special processing and presentation capabilities (Austyn, 2016). The newly differentiated and expanded T cells subsequently migrate to the site of infection where they carry out effector functions, cytotoxicity and antiviral cytokine release, on infected "target" cells (Wong and Pamer, 2003). Remarkably, even if many epitopes are theoretically processed and presented, the majority of the CD8 + T cell responses are directed against only a few antigenic peptides, a phenomenon known as immunodominance (Akram and Inman, 2012). Here, we review the role of epitope density in the selection and expansion of the T cell compartment taking into consideration the timing of both the priming and effector stages of a viral infection.

2. TCR-pMHC Molecular interactions at the immunological synapse

Communication between APCs and T cells is contact dependent. Thus, antigen recognition and subsequent T cell activation and cytotoxic function take place within the cell-cell interface known as the immunological synapse. Formation of the immunological synapse is induced by TCR-pMHC interactions of sufficient affinity, leading to the reorganization of membrane surface receptors. The synaptic interface typically consists of three concentric areas of supramolecular activation clusters (SMACs) although variations of this classical arrangement have been described in recent years (Grakoui et al., 1999; Monks et al., 1998; Hashimoto-Tane and Saito, 2016). The inner most layer, the central-SMAC, contains multiple TCR microclusters interacting with individual pMHC complexes (Monks et al., 1998). The second layer, the peripheral-SMAC, contains integrin receptor pairs facilitating cell-cell adhesion while the outermost layer, distal-SMAC, is composed of costimulatory molecules which are especially important in the priming phase (Monks et al., 1998). Synapses can last 8–10 h, as seen when naïve T cells arrest their migration in secondary lymphoid organs and interact with cognate pMHCs on the surfaces of priming DCs (Mempel et al., 2004). Transient interactions lasting only 5–10 min, sometimes referred to as kinapses, have also been observed and are due to unabated T cell

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motility (Dustin, 2008a, b; Thauland and Parker, 2010). While naïve T cells can form kinapses during the initial phase of scanning for cognate antigen bearing APCs, kinapses are typically formed by effector CTLs interacting with target cells in rapid succession (Mempel et al., 2004; Dustin, 2008a, b; Thauland and Parker, 2010).

Signal integration at the synaptic interface is highly complex and encompasses multiple forces such as the affinity and avidity of pMHC-TCR pairs and expression levels of adhesion or co-stimulatory receptors. Affinity is defined as the strength of the interaction between a single TCR and a pMHC complex (Dustin, 2014). For example, modifications to the primary amino acid sequence of a presented epitope that increase binding to the MHC molecule, may also increase the affinity of the TCR-pMHC pair (Chen et al., 2000, 2005; Madura et al., 2015; Luz et al., 2002). Avidity refers to the strength of interaction between multiple TCRs and pMHC molecules specific for the same epitope; in this context, the kinetics and density of epitope display can directly affect T cell development and function. Lastly, functional avidity determines T cell sensitivity to a range of antigen concentrations and is measured through a functional outcome, most commonly the production of the interferon gamma (IFNγ) cytokine or target cell killing as read out, for example, by chromium release (Dustin, 2014; Vigano et al., 2012). Overall, functional avidity encompasses the full spectrum of molecular forces operating at the immunological synapse, incorporating affinity, avidity and, the landscape of adhesion and co-stimulatory molecules (Vigano et al., 2012).

The T cell activation threshold at the synaptic interface represents the sum of multiple TCR-pMHC interactions within the cSMAC, but also of the signaling components activated through the pSMAC and dSMAC. For example, elevated levels of adhesion molecules compensate for weak TCR-pMHC engagement while strong TCR-pMHC signaling can still result in T cell activation under conditions of relatively low levels of adhesion. Even small differences in molecular interactions at this level can lead to large downstream effects in the emerging T cell population. One reason why tumor cells can be challenging to eliminate is the display of low affinity or low density epitopes in a molecular landscape where adhesion and costimulatory molecules are absent (Dustin, 2014; Hashimoto-Tane et al., 2016). Thus, while only one of many integrative signals taking place at the immunological synapse, pMHC density can significantly tip the balance and play a major role in shaping the T cell response, as the rest of this review will address.

3. Epitope density effects on naïve and antigen experienced T cells

While T cell receptors are internalized upon activation, pMHC complexes are stable at the cell surface for many hours, even after interacting with multiple TCRs (Alcover and Alarcon, 2000). Furthermore, early studies reported that an average of only three pMHC molecules are sufficient to stimulate T cell cytotoxic function *in vitro* (Sykulev et al., 1996). Thus, a small number of pMHC complexes can trigger, over time, over 100-fold more T cell receptors migrating to the immunological synapse interface (Valitutti et al., 1995a). Indeed, these observations led to the serial engagement model of T cell activation (Valitutti, 2012; Valitutti et al., 1995b; Valitutti and Lanzavecchia, 1997) which proposes that TCRs are organized at the plasma membrane in microclusters of 50–300 individual units, with each microcluster able to "trap" a single pMHC complex. Following engagement by the pMHC complex, a TCR is internalized and immediately replaced by another TCR due to high local density in the microcluster. The serial engagement model also explains the ability of the immunological synapse to maintain cell-cell contact and sustain continuous signaling for several hours, despite the half-life of a single TCR-pMHC interaction lasting only a few seconds. Interestingly, TCR numbers can be reduced to as low as ~5% of wild type levels *in vivo,* without penalty to proliferation or cytokine expression pattern of antigen-specific CD8 + T cells (Labrecque et al., 2001). In contrast, modulating antigen levels, even if very few pMHC complexes are theoretically required to stimulate a T cell response, can have a profound impact on the T cell response (Anikeeva et al., 2012). Thus, the limiting factor in the synaptic interaction appears to be the number of pMHC complexes on APCs rather than TCR levels.

Initial cognate APC-naïve CD8 + T cell contact initiates an "autopilot" program of multiple cell divisions which is not dependent on further antigen input for the first seven to eight rounds of expansion (Kaech and Ahmed, 2001; van Stipdonk et al., 2001; Prlic et al., 2006). This proliferative burst can be initiated at very low epitope levels, in line with the exceptional antigen presentation capabilities of dendritic cells (Kroger et al., 2008). Nevertheless, the magnitude of the final effector population at later stages of the infection can be influenced by the conditions present at initial engagement. According to a stochastic model of T cell activation, more naïve T cells have an opportunity to cross the proliferative threshold when higher levels of pMHC complexes are available (Au-Yeung et al., 2014). In the case of low ligand densities, proliferation and entry into cell cycle is delayed due to a slower rate of signal-based internalization of TCRs (Balyan et al., 2017). In other words, fewer surface pMHC molecules will take longer to trigger the required number of TCRs needed to cross the proliferation threshold, although the same number of pre-programed rounds of cell division will take place. At the same time, fewer naïve T cells are activated, decreasing the magnitude of the effector T cell compartment at later stages of the response. For example, in a *Listeria monocytogenes* infection model, low- and high-affinity clones were observed to undergo a similar proliferative burst at days 3–5, yet by day 7 the higher affinity clones became 30 fold more abundant (Zehn et al., 2009). Thus, small differences at the priming stages may be compounded at the effector level, especially if the antigen is limiting and pMHC complexes are degraded before DCs are able to stimulate additional T cells.

While naïve T cells can be activated by epitopes expressed at very low levels due to serial receptor engagement within a long-lived synapse, activated T cells will require additional processes to compensate for short lived kinapse interactions with target cells, that theoretically do not allow sufficient time for signal integration to reach activation threshold. Fittingly, *in vitro* experiments indicate that naive T cells do require higher peptide concentrations for activation compared to their antigen-experienced effector counterparts and furthermore, differentiation into effector CTLs has been shown to lower the signaling threshold requirements through a process termed avidity maturation (Fahmy et al., 2001; Leggatt, 2014; Slifka and Whitton, 2001; von Essen et al., 2012). Avidity maturation occurs without somatic hypermutation or changes in the native TCR affinity, as is typically seen in B cells (Fahmy et al., 2001; Slifka and Whitton, 2001). Rather, at the individual cell level, the molecular mechanisms include increased concentration of signaling molecules, rearrangement of TCR microclusters, and lowered requirements for signal 3 cytokine stimulation (von Essen et al., 2012). At resting state, TCRs are present in both monovalent and nanocluster forms, which later coalesce to form microclusters upon activation by cognate pMHC and immunological synapse initiation (Hashimoto-Tane and Saito, 2016), allowing for faster detection of rare pMHC complexes (Kumar et al., 2011). This TCR cluster oligomerization is both ligand dependent and independent but the resulting density of pre-formed nanoclusters becomes an inherent property of the T cell (Crites et al., 2014). Low epitope densities typically lead to a single pMHC complex interacting with a TCR microcluster in the serial engagement fashion, but high levels of presented epitopes can also trigger monovalent TCRs increasing the total signaling strength at the synapse and decreasing the total time required for activation (Schamel et al., 2005; Gonzalez et al., 2005). Alternatively, there are studies (Brameshuber et al., 2018; Rossboth et al., 2018) that call into question the requirement for TCR oligomerization—both pre-existing and ligand induced—and, provide support for monomeric TCRs as the main drivers of signaling in response to antigen recognition. This recent work is based on novel non-invasive microscopy techniques which can more accurately discern molecular events at the immunological synapse. As

these studies focused on the TCR-pMHC II dynamic, it would be interesting to see how this applies to CD8 + T cells, given inherent differences in co-receptor usage and synapse function and formation. Also, future experiments directly comparing naïve and antigen experienced T cells would provide exciting new insights into the mechanisms governing avidity maturation.

Thus, due to avidity maturation and the low number of pMHC complexes initially thought to be required for cytotoxic responses of activated cells, CTLs have been viewed as highly efficient serial killers. That being said, serial killing may be less extensive than generally imagined due to experiments primarily performed *in vitro*, where various technical limitations may not accurately depict *in vivo* interactions. Planar lipid bilayers used to mimic the surface of APCs have been instrumental in understanding the inner workings of the immunological synapse yet, the use of high concentrations of adhesion molecules such as integrins may overcompensate for the TCR-pMHC signaling component, artificially inflating the sensitivity of effector CD8 + T cells. Indeed, integrins are heavily utilized in stable long-term synapses during naïve T cells-DCs interactions (Liu et al., 2009). In contrast, effector CTLs primarily engage in short lived kinapses in order to maintain mobility, and do not engage integrins in this type of synaptic interaction (Halle et al., 2016). Under physiological conditions, whereby infections take place in peripheral tissues, T cell killing efficiencies are reported to be significantly more restricted (Halle et al., 2016). Garcia et. al have demonstrated that CD8 + T cell killing efficiency is reduced tenfold in an *in vivo* killing assay when target cells are pulsed with lower peptide concentrations that more accurately approximate presentation levels during natural infections (Garcia et al., 2015). High presentation levels of the model epitope $OVA_{257–264}$ did result in target elimination by a single effector CD8 + T cell however, when the antigen density was greatly reduced, multiple T cells interacting with the same target cell were required for target cell elimination (Halle et al., 2016). Interestingly, this limitation can be reduced by increasing the numbers of circulating antigen specific CD8s. In conclusion, *in vivo* experiments suggest that in a natural infection setting, the epitope density threshold required for cytotoxic function remains high compared to previous inferences from *in vitro* studies, and high levels of antigen presentation, along with robust numbers of effector T cells, remain beneficial for target cell clearance.

4. Epitope density shapes the magnitude, avidity and functionality of the effector T cell population

Multiple groups have established a strong link between antigen dose and the resulting magnitude and functional avidity of the T cell response (Kroger et al., 2008; Bullock et al., 2000, 2003; Wherry et al., 1999, 2002; Leachman et al., 2002; Tobery and Siliciano, 1997; Townsend et al., 1988; Wong et al., 2004; Plesa et al., 2008), both parameters playing important roles in disease control. Early studies report that increasing antigen presentation levels can lead to a hierarchical induction of functions: cytotoxic granule release, followed by antiviral cytokine production and lastly, proliferation (Betts et al., 2004; Valitutti et al., 1996). Furthermore, differences in T cell receptor signaling determined by antigen concentration can also result in distinct cell fates such as altered effector:memory ratios (Smith-Garvin et al., 2010). Regarding the magnitude of T cell responses in relation to epitope levels, most studies in which the priming dose of antigen is varied, report that increased levels of presentation lead, correspondingly, to an increase in the number of responding CD8 + T cells *in vivo* (Bullock et al., 2000, 2003; Wherry et al., 2002; Spencer et al., 2014). Such studies are in accordance with the stochastic model of naïve T cell proliferation described above. Reports of enhanced presentation levels leading to no change in the magnitude of the T cell response might be explained by attainment of maximal T cell induction at the lowest antigen doses that were utilized (Wong et al., 2004; Altenburg et al., 2016; Schliehe et al., 2012). At the opposite end of the spectrum, excessively high levels of presentation, can sometimes decrease the magnitude of responding CD8 + T cells in both primary and recall responses (Wherry et al., 1999, 2002; Plesa et al., 2008; Schliehe et al., 2012). One issue is that responding CTLs are often identified by a single functional characteristic, IFNγ production, as opposed to tetramer staining. Thus, the differences observed in these studies might also reflect changes in the functional avidity of the responding T cells.

High avidity T cells have been shown to perform better in adoptive transfer experiments against tumors and chronic viruses (Alexander-Miller et al., 1996; Yee et al., 1999; Zeh et al., 1999; Foley et al., 2014; Almeida et al., 2007; Almeida et al., 2009; Derby et al., 2001). Interestingly, *in vitro,* high avidity cells are generated with low peptide concentrations, while low avidity cells are primed at high levels of ligand, and this finding holds true for both monoclonal as well as oligoclonal T cell populations (Kroger and Alexander-Miller, 2007). However, *in vivo* studies show that the initial naïve T cell encounter with a DC bearing either low or high levels of pMHC complexes results in a similar, moderately high avidity to a particular epitope (Kroger et al., 2008). In subsequent cell divisions driven by varying epitope presentation levels on non-professional APCs encountered in peripheral tissues, the final average functional avidity is adjusted according to the same inverse relationship with antigen dose that is observed *in vitro* (Kroger et al., 2008). Finally, epitope density can also influence the resulting effector:memory T cell ratio; both low and high epitope densities on priming DCs in the lymph nodes induce naïve T cell proliferation and effector differentiation, but only DCs bearing high levels of epitopes also induced memory T cell development (Henrickson et al., 2013; Ozga et al., 2016). This could be due to the fact that when antigen is presented at high levels, naïve T cells engage more readily and productively in long-lived immunological synapses (Henrickson et al., 2013; Ozga et al., 2016).

Initially, at the population level, high antigen display levels appear to come with a tradeoff: increased numbers of responding T cells, including both effector and memory cell fates, typically arise with high epitope densities. In contrast, low levels of antigen lead to higher functional avidity T cells but a paucity of memory cells. This discrepancy might arise from the fact that frequency and functional avidity of the resulting CTLs are rarely investigated in the same study, except for cases where increased presentation compromises magnitude of the T cell compartment. This phenomenon is reminiscent of many chronic infections where even moderate antigen doses sustained over a long timeframe produce non-responsive, exhausted cells characterized by low functional avidity. However, more characterization is needed to understand the underlying mechanisms and whether other T cell functions like cytotoxicity are equally affected (Lichterfeld et al., 2007). In the experimental models where reduction in T cell responses has been explored, high avidity functional clones are either absent or, are driven to a dysfunctional state where they do not produce cytokines (and thus cannot be readily detected) as evidenced by increased expression of the inhibitory receptor PD1 (Vigano et al., 2012; Harari et al., 2007). By the same token, high epitope density is beneficial in generating memory T cells, but only in the very early priming stages of an infection, and these findings have not been extended temporally beyond this point (Henrickson et al., 2013). Standardizing the identification of antigen-specific T cells in a dual approach of monitoring both tetramer staining and cytokine production could be a more effective way of investigating the impact of antigen dose on the resulting effector population and effectiveness in viral clearance (Plesa et al., 2008).

5. Epitope density effects on immunodominance

A common feature of many infections is that the majority of responding CD8 + T cells are specific to only a handful of immunodominant epitopes (Yewdell, 2006). Weaker, but still detectable responses are elicited by another class of epitopes termed subdominant

and finally, immunorecessive epitopes do not induce any apparent CD8 + T cell responses unless the dominant and subdominant epitopes are eliminated from the immunogen (Tanaka et al., 1989). A similar relationship has been observed many times between dominant and subdominant epitopes (Thomas et al., 2007; Weidt et al., 1998; Steffensen et al., 2016; Rodriguez et al., 2002). For example, the lymphocytic choriomeningitis virus (LCMV) induces responses to minor epitopes such as g283 only when the immunodominant epitope n118 is eliminated (Weidt et al., 1998). Immunodominance is a complex phenomenon and multiple factors contribute to establishing an epitope hierarchy, including pMHC binding affinity, T cell precursor frequency, antigen availability or T cell competition (Yewdell, 2006).

As detailed in the previous sections, increased levels of antigen presentation generally result in a higher frequency of responding T cells. Indeed, increased epitope density, arising through a plethora of molecular mechanisms, has been proposed as one of the main factors influencing immunodominance hierarchy yet most evidence is based on modulation of a single epitope (Wherry et al., 1999; Yewdell, 2006; Yewdell and Bennink, 1999). In addition, epitopes expressed during early stages of infection have been shown to be disproportionately immunodominant and drivers of protective responses (Derby et al., 2001; Zinkernagel and Althage, 1977; van Baalen et al., 2002; Ehl et al., 1997). Paradoxically, recent studies show that some immunodominant epitopes, such as the Epstein Barr Virus-specific RRIYDLIEL(Crotzer et al., 2000) or the West Nile Virus-specific SVG9 (Kaabinejadian et al., 2016), are expressed at later times and presented at lower levels in comparison to subdominant or immunorecessive epitopes from the same pathogen. Finally, an in-depth mass spectrometry and liquid chromatography study simultaneously analyzed 8 epitopes from *Vaccinia* Virus in terms of both expression levels and kinetics of antigen presentation within the first 12 h post infection (Croft et al., 2013). The study found no correlation between epitope abundance as detected in an *in vitro* system, and the immunodominance hierarchy of the epitopes studied. It remains to be seen whether these findings reflect conditions *in vivo*, where direct and cross presentation are performed by specialized APCs. Another possibility is that immunodominant responding T cells are of high functional avidity, which, as mentioned above, are typically induced by low levels of epitope presentation.

The picture is further complicated by the observation that immunodominance hierarchies can change upon secondary exposures. For example, primary influenza infection generates two immunodominant CD8 + T cell populations specific for $NP_{366–374}$ and $PA_{224–233}$ epitopes with similar effector T cell frequencies. However, upon re-exposure, only the $NP_{366–374}$ epitope maintains immunodominance. This is explained by the fact that in the spleen and lymph nodes, classical DCs are able to present both epitopes to naïve T cells (Crowe et al., 2003) while at the site of infection, where the composition of antigen presenting cells is different, only the $NP_{366–374}$ epitope is processed and presented, in this case by monocyte derived DCs (Crowe et al., 2003; Cruz et al., 2017). Thus, in a secondary infection, the memory CD8 + T cells specific for $NP_{366–374}$ are selectively expanded, causing the shift in immunodominance due to a large proliferative advantage over naïve cells of both NP and PA specificities. Furthermore, ablation of the NP epitope in the vaccination vector, resulting in only PA-specific immunodominant responses, resulted in compromised protection from a secondary influenza challenge (Crowe et al., 2003). Similarly, both F85-93 and F249-258 epitopes arising from the fusion protein of the respiratory syncytial virus (RSV) are immunodominant in a primary infection, but only F85-93 maintains this status in a secondary response (Johnstone et al., 2004). Thus, the effect of epitope levels on the immunodominance hierarchy may be apparent only with multiple pathogen encounters.

6. Conclusions and future considerations

CD8 + T cell functionality and development through naïve, effector and, memory stages have been studied extensively in hopes of directing the cytotoxic potential of this population towards tumors and chronic infections. Here, we have reviewed the influences of epitope presentation levels in shaping CD8 + T cell responses. Several contributions have been discussed in an attempt to reconcile and unify current understanding. One recurring theme is that antigen presentation differences *in vitro* do not necessarily predict effective T cell responses *in vivo*. Future studies could focus on the development of tools such as antibodies that detect specific pMHC complexes, which would enable direct assessment of pMHC numbers along with presentation kinetics *in vivo*. Assessing the optimal range of expressed epitopes in escalating antigen dose models, followed by investigating the biological relevance in vaccine-challenge settings, would help validate and reconcile previous *in vitro* antigen presentation findings.

Another avenue of future study is elucidation of how epitope density impacts function of CD4 + T cells, which exhibit subtle developmental and functional differences compared to CD8 + T cells. Unlike CTLs, CD4 + T cells do not release cytotoxic granules, and exhibit differences in immunological synapse organization and timing (Xie et al., 2013; Ueda et al., 2011). Naïve CD4s do not follow an “autopilot” model of pre-programed division, and require antigen re-stimulation and longer engagement time for each cell division (Foulds and Shen, 2006; Zehn et al., 2012). At the same time, because MHC class II is not as ubiquitously expressed as MHC class I, the contribution of initial priming events compared to encounters with APCs in the effector phase may be yet another point of distinction, particularly as it pertains to immunodominance.

Finally, it is important to delineate the physical context and timing in which epitope density is a potential modulator of T cell responses since multiple antigenic exposures can affect the final memory population as well as the immunodominance hierarchy. To this end, static or non-replicating priming methods such as peptide pulsed DC’s or DNA vaccine vectors may not fully recapitulate the temporal dynamics of epitope presentation in a live infection. On the other hand, such strategies could be viable for inducing high T cell numbers while preserving high avidity by restricting the CD8 + T cell expansion to the initial autopilot proliferative burst and could be achieved through prime-boost regimens with low doses of antigen. Thus, thorough understanding of how epitope density impacts CD8 + T cell expansion and functionality might be harnessed for the development of more effective T cell-targeting vaccines.

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