



Modulation of antigen-specific T-cells as immune therapy for chronic infectious diseases and cancer

Suling Li¹, Alistair L. J. Symonds², Tizong Miao², Ian Sanderson² and Ping Wang^{2*}

¹ Bioscience, Brunel University, London, UK

² Blizard Institute (BICMS), Barts and the London School of Medicine and Dentistry, London, UK

Edited by:

Fang-Ping Huang, Imperial College London, UK

Reviewed by:

Jianping Huang, University of Florida, USA

Alex Yee-Chen Huang, Case Western Reserve University School of Medicine, USA

*Correspondence:

Ping Wang, 4 Newark Street, London E1 2AT, UK
e-mail: p.wang@qmul.ac.uk

T-cell responses are induced by antigen presenting cells (APC) and signals from the microenvironment. Antigen persistence and inflammatory microenvironments in chronic infections and cancer can induce a tolerant state in T-cells resulting in hyporesponsiveness, loss of effector function, and weak biochemical signaling patterns in response to antigen stimulation. Although the mechanisms of T-cell tolerance induced in chronic infection and cancer may differ from those involved in tolerance to self-antigen, the impaired proliferation and production of IL-2 in response to antigen stimulation are hallmarks of all tolerant T cells. In this review, we will summarize the evidence that the immune responses change from non-self to “self”-like in chronic infection and cancer, and will provide an overview of strategies for re-balancing the immune response of antigen-specific T cells in chronic infection and cancer without affecting the homeostasis of the immune system.

Keywords: tolerance induction, antigen-specific T cells, bystander T-cells, nanoAPC, reverse tolerance

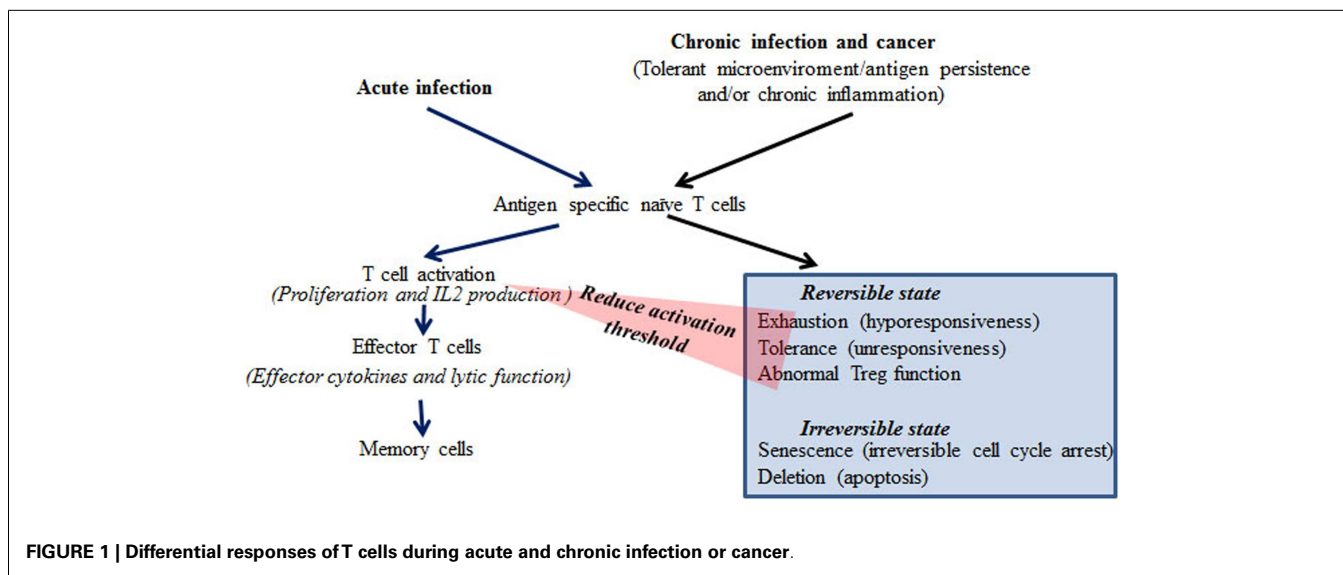
INTRODUCTION

T cells are essential for robust adaptive immune responses against pathogen invasion, as well as maintaining immune tolerance to self-antigens. In the tolerant state, T cells generally fail to proliferate and produce IL-2 in response to antigen stimulation (1, 2). Anergy and immune regulation are two interconnected mechanisms that maintain peripheral tolerance to self-antigens *in vivo*. In contrast to the biochemical events induced during effective responses to pathogenic antigens, in anergy the biochemical signaling pathways in T cells are only partially activated. Activation of the calcium/calcineurin/nuclear factor of activated T cells (NFAT) pathway, but not AP1 and NFκB pathways (1–3), in anergic conditions results in the expression of tolerance associated genes such as E3-ligases (1–3). This partial TCR signaling is largely due to the lack of additional signals such as costimulatory signals and activating cytokines such as IL-2, or due to direct regulation by Treg (2). Therefore, altered expression of costimulatory signals and/or activating cytokines, or defective Treg function, results in full activation of TCR signals in response to self-antigens and may induce autoimmune responses. Recent studies have uncovered hyporesponsive phenotypes with partial activation of biochemical events in virus specific T cells in chronic infectious diseases (4, 5) and models mimicking chronic infectious conditions (5, 6). These findings indicate that during chronic infection the T-cells switch from mounting robust non-self responses to a state similar to self-tolerance due to antigen persistence and/or changes in the microenvironment. Similar to the immunological milieu of chronic infection, the tumor microenvironment contains a multitude of suppressive mechanisms that allow tumors to escape immune surveillance (4, 7). Immune hyporesponsive states have been studied in many different models *in vitro* and *in vivo* and have been categorized based on the phenotypes discovered in each tolerant state (8).

This review will briefly summarize the extracellular signals that affect self-tolerance or effector function of antigen-specific T cells. We will describe the application of these signals in therapeutic intervention and focus on the recently developed nano-technologies that can reverse the tolerant state of viral specific T cells by delivering costimulatory or cytokine signals to antigen-specific T cells.

ALTERED T-CELL RESPONSES DURING CHRONIC VIRUS INFECTION AND CANCER

Chronic virus infections are associated with impaired anti-viral immunity, particularly in the infections caused by highly replicative viruses such as HIV, HBV, and HCV. In chronic infection, persistent viral antigen, and often chronic inflammation, renders T-cells dysfunctional. The mechanisms underlying dysfunctional immune responses in patients are largely unknown. Based on experimental systems studied *in vitro* and *in vivo*, different states of T-cell dysfunction have been discovered and are classified as exhaustion, tolerance, anergy, senescence, deletion, induced Treg, and ignorance based on the phenotypes, production of inhibitory cytokines such as IL-10 and TGFβ, impairment of T-cell receptor signaling molecules, and apoptosis of the T-cells in these models (Figure 1) (4–6, 8–10). These findings have been extensively reviewed (4–6, 8–10). Despite the differences in dysfunctional T-cells characterized in different model systems, the common feature is proliferative hyporesponsiveness, and impaired production of IL-2 following antigen stimulation *in vivo* or *in vitro* (4–6, 8–10). The chronic LCMV infection model resembles the observations from patients with chronic virus infections more closely than other models in terms of induction of dysfunctional T cells (4, 5). The phenotype of exhaustion of CD8 T cells in the chronic LCMV model is well-characterized, with hierarchical loss of effector cytokine production, including IL-2, TNFα, and IFNγ and



impaired proliferation in response to antigen receptor stimulation *in vitro* (4, 5). In addition to this hyporesponsive phenotype, increased expression of the inhibitory costimulatory molecule PD-1 and production of the repressive cytokine IL-10 are also found in T cells from chronic LCMV infected mice (9, 10). Notably, similar phenotypes have been found in T cells from HIV, HBV, and HCV patients (11–14). Under chronic infectious conditions, viral specific CD8 T cells often lose cytotoxic function (15, 16). At the late stages of exhaustion, viral specific CD8 T cells may be deleted (5, 6). However, in contrast to CD8 T cells, viral specific CD4 T cells can persist under chronic infectious conditions, but in a hyporesponsive state (17). Therefore, there is the potential to restore CD4 responses, which may thereafter help CD8 function. It has been reported that Treg cells are increased or induced in chronic infection (18, 19). The increased Treg cells can reduce chronic inflammation from persistent viral antigen stimulation, but may also contribute to the establishment of immune tolerance toward the virus (18, 19).

Comparable to chronic infection, high levels of tumor antigens and chronic inflammation can establish an immunosuppressive microenvironment. Tumor reactive T cells have been shown to respond to tumor antigens in a similar fashion to viral specific T cells in chronic infection with expression of high levels of inhibitory costimulatory molecules such as PD-1, CTLA-4, and LAG-3 and impaired production of effector cytokines including IFN γ , TNF α , and IL-2 (7, 20–22). It has been shown that advanced tumors with high loads of tumor antigens cause functional exhaustion and rapid elimination of tumor reactive T cells (23). However, in contrast to chronic viral infections, tumor antigens are generally poorly antigenic. Therefore, the frequency and avidity of tumor reactive T cells are low.

IMPAIRED TCR SIGNALING DURING CHRONIC VIRUS INFECTION

We have found that antigen persistence can impair TCR signaling resulting in hyporesponsiveness (24). This hyporesponsiveness is gradually induced during antigen persistence with reduction of

NF κ B and AP1 activation (2, 24). This characteristic phenotype of T-cell tolerance is similar to that observed in chronic HBV infection (25). Down-regulation of TCR proximal signaling molecules has been found in CD8 T cells from chronic HBV patients (25). The impaired TCR signaling in CD8 T cells from chronic HBV patients is partly due to the down-regulation of CD3 ζ (25). The reduced expression of CD3 ζ is associated with up-regulation of PD-1 and impaired production of IL-2, suggesting that it is part of the mechanism leading to exhaustion (25). Viral protein Nef of HIV and E2 and core protein of HCV directly modulate TCR signaling (26). HIV Nef protein interacts with a number of TCR signaling molecules including Lyn, Hck, and Lck (27). The interaction stimulates the TCR signaling pathways in the absence of antigens leading to maintenance of viral replication (26, 27). The altered TCR signaling induced by Nef negatively affects antigen-mediated TCR signaling (28). Similarly, viral proteins from HCV also modulate TCR signaling (29). E2 protein of HCV binds CD81 and promotes TCR signaling while the core protein inhibits JNK signaling and IL-2 expression (29). However, HCV does not infect T cells. Therefore, the altered TCR responses during chronic HCV infection are largely due to the persistence of viral antigens. Whether the persistent, but abnormal, TCR signaling induced by viral proteins causes the development of T-cell exhaustion is yet to be investigated.

It has been found that the tumor microenvironment impairs the formation of T-cell immunological synapses; supramolecular structures that assemble at the T cell-APC interface (30). Dysregulated synapse formation is associated with impaired activation of Rho-GTPases and can lead to partial activation or anergy of T cells.

INDUCTION OF NEGATIVE COSTIMULATORY MOLECULES

One of the important changes to the phenotype of CD8 T cells in chronic LCMV infection is the increased expression of the negative costimulatory molecules PD-1, 2B4, CTLA-4, and LAG-3 (5). A similar phenotype of increased negative costimulatory molecules has been discovered in CD4 and CD8 T cells from

chronic HBV and HIV patients (5, 15, 31–35). The function of these negative costimulatory molecules is important in the maintenance of immune tolerance toward self-antigens. Although the mechanisms underlying the induction of negative costimulatory molecules in T cells during chronic infection are not clear, it may be part of a physiological protection mechanism to reduce immunopathology induced by viral persistence and chronic inflammation. These negative costimulatory molecules are transiently up-regulated in activated effector T cells in the early stages of acute infection. However, the sustained expression of PD-1 on virus-specific CD8 T cells is associated with chronic infection, both in LCMV mouse models and in HBV patients (5). Co-expression of multiple inhibitory molecules correlates with increased functional deficits in anti-virus responses and decreased control of viral loads. Similarly, increased expression of PD-1 and CTLA-4 has been found on tumor infiltrating T cells (TIL), which can be associated with E3-ligase expression and increased Treg cells (7). Thus, the overexpression of inhibitory molecules results in shifting the balance of the immune responses from effective anti-virus or -cancer responses toward tolerance.

ALTERED CYTOKINE PRODUCTION IN T CELLS

One of the most pronounced changes in T cells in chronic infectious conditions is the altered production of cytokines (4–6). In contrast to acute infection, antigen-specific T cells from chronic infectious diseases fail to produce IL-2 and TNF α , but express the regulatory cytokine IL-10 (4–6). We have discovered that antigen-specific CD4 T cells gradually alter their cytokine profile in response to antigen persistence *in vivo* (24). Initial antigen stimulation effectively induces IL-2 production in antigen-specific CD4 T cells *in vivo*, while repeated exposure to the same antigen yields CD4 T cells that produce both IL-2 and IL-10 (24). Antigen persistence can finally switch off the expression of IL-2 in T cells, but these cells still produce high levels of IL-10 (24). This altered cytokine profile is associated with impaired proliferative responses and reduced AP1 and NF κ B activation in response to antigen stimulation *in vivo* (24). Impaired production of effector cytokines such as IL-2, TNF α , and IFN γ is also associated with the defective activation of TCR signaling pathways and effector function of viral-specific CD4 and CD8 T cells in chronic HBV, HCV, and HIV infections (5, 6, 25). The up-regulation of inhibitory molecules, especially PD-1, is closely associated with the production of IL-10 and/or TGF β (5, 6). Thus, virus persistence skews the T-cell response from activation and differentiation into effector cells toward antigen-specific immune tolerance. However, the mechanisms whereby IL-10 and/or TGF β result in tolerance in chronic infections are still undefined. In the LCMV model, the lack of IL-10 or a defect in IL-10 signaling improves CD8 T-cell responses and drastically enhances the control of the infection (36, 37). TIL also display an altered cytokine profile, which is similar to that seen in chronic infections. High levels of IL-10 producing Treg cells have been found in TILs, which is associated with impaired production of IL-2, TNF α and IFN γ (7, 38).

THERAPEUTIC INTERVENTIONS TO REVERSE IMMUNE TOLERANCE IN CHRONIC INFECTION AND CANCER

Therapeutic interventions for chronic viral infection and cancer aim to counter the effects of the immunosuppressive

microenvironment and skew responses toward antigenic determinants that are highly immunogenic. Various approaches have been tried to increase antigen presentation quality via immunization with selected antigenic peptides, using methods such as recombinant vaccinia vaccines, DNA vaccines, peptide vaccines, and DC vaccines, to boost the anti-viral and -tumor responses (39). So far these therapeutic vaccines have not been successful. One of the possible explanations is that the hyporesponsiveness of T cells is not due to the lack of antigens, but to aspects of the chronic disease such as antigen persistence and chronic inflammation, which increase the activation threshold of T cells in response to antigen. Therefore, to overcome the high activation threshold of antigen-specific T cells in these conditions, immune therapy has to consider the antagonizing tolerogenic environment. Thus, therapeutic vaccines in combination with targeted immune modulation have been proposed as a more effective strategy to reverse the hyporesponsive state of T cells in chronic infections and cancer. In ovarian cancer, improved anti-tumor immune responses were observed after blockade of PD-1 (40). Similarly, in the LCMV model, immunization with LCMV GP33 encoding vaccinia virus coupled with administration of anti-PD-L1 blocking antibody significantly improved viral-specific CD8 T-cell responses and reduced viral load (41). Moreover, in chronic LCMV, combined therapy with a DNA vaccine and IL-10 neutralizing antibody effectively reversed viral specific CD8 T-cell tolerance (42). Immune tolerance induced by virus persistence is due to a network with multiple suppressive components. Blockade of multiple inhibitory receptors including PD-1, LAG-3, and CTLA-4 or combined blockade of inhibitory receptors and immunosuppressive cytokines achieves greater efficacy than blockade of a single inhibitory molecule in chronic LCMV models (43, 44). Although the increased T-cell function and concomitant decrease in viral load in these interventions are transient, these data support the hypothesis that reversing immune tolerance to the virus or tumor is the key for successful immunotherapy. While blockade of PD-1 and IL-10 resulted in restoration of viral specific CD8 T-cell function in a mouse model (45), the mechanisms underlying this recovery of effector function are still unknown. As many of these interventions do not specifically target the virus- or tumor-specific T cells and these pathways are important for maintenance of peripheral tolerance, it is essential to control the balance between restoration of anti-viral or -tumor responses and prevention of autoimmune diseases (5, 7). The ideal intervention will be to specifically reverse the tolerance of viral or tumor specific T cells, while maintaining the overall self-tolerance of the immune system.

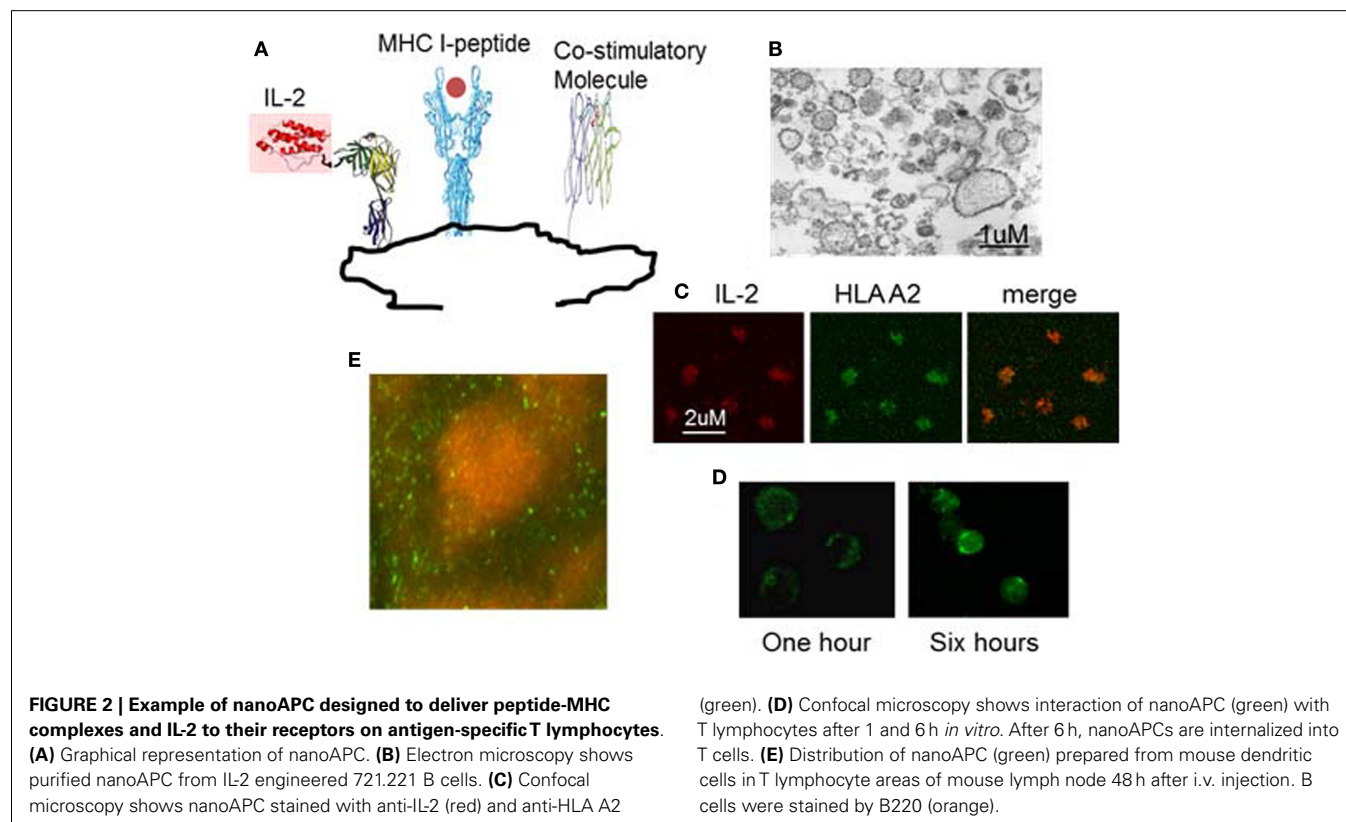
RESTORING NON-SELF-RESPONSES OF VIRAL SPECIFIC T CELLS, WHILE MAINTAINING THE SELF-TOLERANCE OF BYSTANDER T CELLS IN CHRONIC INFECTION

The differential responses of antigen-specific T cells result from biochemical signals induced in T cells following interaction with antigen-MHC complexes, costimulatory molecules, and cytokines. When the mitogenic biochemical signals break the activation threshold, the T cell will enter into the cell cycle and produce growth cytokines such as IL-2 to promote clonal expansion. Due to the persistence of viral antigen, the chronic inflammatory environment and the increased production of inhibitory molecules,

the activation threshold of viral specific T cells is increased and the T cells are unable to enter the cell cycle following antigen stimulation (5, 24). However, chronic infection normally does not induce tolerance in T cells responding to antigens other than those derived from the virus itself. Therefore, systemic intervention may reverse the tolerance of viral-specific T cells, but also break the self-tolerance of bystander T cells potentially resulting in autoimmunity (5). Therefore, the ideal strategy is to reverse tolerance via modulations that increase positive and/or dampen negative costimulatory signals thereby breaking the activation threshold and driving clonal expansion of virus responding T cells, but importantly, without affecting bystander T cells. Cytokine modified and viral antigen pulsed DCs have been used to deliver antigen and positive costimulatory signals to viral specific T cells in chronic infection (46–48). However, DCs are unstable and very heterogeneous in terms of population and function. It is therefore difficult to target and deliver additional positive signals to antigen-specific T cells (49). We found that an increase in the amount of antigen presented by activated dendritic cells (DC) cannot reverse tolerance (50, 51). Although exogenous IL-2 can effectively overcome tolerance and restore the full activation of tolerant T cells in response to antigen stimulation in animal models and HBV specific CD8 T cells, systemic administration of high doses of IL-2 not only induces severe side effects, such as cardiovascular, pulmonary, hematological, hepatic, neurological, endocrine, renal, and/or dermatological complications (52), but may also promote Treg function, which can further increase the activation threshold of antigen-specific T cells (53).

In order to use IL-2 and/or anti-PD1 to overcome the hyporesponsiveness of viral specific T cells induced in chronic

HBV infection while avoiding the side effects of systemic administration, we have developed a novel therapeutic vaccine (nanoAPC). These nanoAPC are derived from an APC line; the human B cell line 721.221. This cell line is MHC deficient, but expresses high levels of costimulatory molecules (51). The nanoAPC are prepared from the endoplasmic reticulum (ER) membranes of 721.221 cells (51), that are genetically engineered to express ER retained MHC class I alleles and membrane-bound IL-2. Therefore, MHC and IL-2 are synthesized physiologically in 721.221 human B cells and immobilized on ER-membranes (Figure 2) (51). After assembly with HBV antigenic peptide *in vitro*, the nanoAPC contain peptide-MHC complexes, costimulatory molecules, and IL-2. Unlike therapeutic DCs, the nanoAPC are homogeneous, stable, and can be stored at -80°C (50, 51). Equipped with defined viral-peptide-MHC complexes, the administered nanoAPC can directly interact with antigen-specific T cells *in vivo* (51). Due to the native structure of their membranes, nanoAPC effectively induce immune synapses and expression of the high affinity IL-2 receptor on T cells (Figure 2) (51). The IL-2 delivered by nanoAPC enhanced antigen-specific T-cell responses and effector function, but did not affect bystander T cells or Treg cells. When assembled with a pool of HLA A2 associated HBV peptides and HBV peptides associated with HLA DR and DP, IL-2-nanoAPC induced strong CD4 and CD8 T-cell responses in peripheral lymphocytes from chronic HBV patients (51). We demonstrated that IL-2 on nanoAPC is able to enhance TCR signaling and downregulate PD-1 expression on virus responding CD8 T cells from chronic HBV patients, which could effectively reverse tolerance as demonstrated by induction of IFN γ producing CD8 T cells in lymphocytes from chronic HBV patients (51). In



addition to TCR signaling, MAPK activation can result directly from IL-2R signaling (53). It has been found that the activation of MAPK and PI3K through Shc recruited by the IL-2R is independent of STAT5 signaling in effector T cells, which differs from that in Treg cells, and is important for the expansion of activated CD8 T cells (54). We have demonstrated that nanoAPC can induce CD25 expression and immune synapse formation, which not only enables the induction of T-cell activation but also brings engineered bio-adjuvants such as IL-2 stably into signalsomes of effector T cells (51). The increased expression of CD25 on CMV antigen-specific CD8 T cells by IL-2-CMV_{nlv}A2-nanoAPC is consistent with the well-known observation that IL-2 can induce CD25 expression on pre-activated CD8 T cells (51). Thus, together with peptide-MHC complexes and costimulatory molecules, the selective delivery of IL-2 is important in inducing activation of HBV responding T cells in chronic HBV patients. As the overall pharmacological dose remains low, the IL-2-nanoAPC do not activate Treg cells indicating that this approach can be adapted for use with other bio-adjuvants. Our results demonstrate that IL-2-nanoAPC, which deliver both antigen and IL-2 to antigen-responding T cells, can significantly increase functional anti-viral responses, thereby overcoming the immune tolerance induced by persistent viral load.

Nano-particles prepared from synthetic materials or genetically engineered microbes have been used to deliver antigens to DC for induction of anti-viral or -cancer immune responses (55). In contrast to these particles, nanoAPC are prepared from the ER-membranes of bio-engineered APC. Therefore, they are not only more biocompatible than synthetic nano-particles or microbes, but also deliver therapeutic molecules that are physiologically synthesized by APC seed cells. Thus, the IL-2 on IL-2-nanoAPC is more stable than free IL-2 *in vivo*, and maintains its physiological conformation allowing optimal interaction with the IL-2 receptor (data not shown). Unlike other nano-particle based vaccines, we have demonstrated that nanoAPC can directly activate T cells. NanoAPC are derived from APC cells and contain high levels of costimulatory molecules (51). Therefore, the nanoAPC mimic live DC to induce lipid raft clustering on T cells and formation of an immunological synapse, which is essential for T-cell activation. Furthermore, using HLA I negative 721.221 cells as seed cells allows us to specifically express selected HLA alleles allowing construction of HLA allele matched nanoAPC for individual patient populations.

Previously, we observed nanoAPC homing to T-cell areas of peripheral lymphoid organs, largely due to the expression of homing receptors by the cells from which the nanoAPC are derived (50). We have now further demonstrated that nanoAPC are not efficiently endocytosed by DC *in vivo* (50, 51). This is important as it allows the nanoAPC to remain as free-particles in peripheral lymphoid organs. The absence of endocytosis may be due to the lack of molecules on nanoAPC recognized by DC pattern recognition molecules (56). Thus, nanoAPC effectively target viral specific T cells and deliver immune modulation to reverse their tolerant state.

SUMMARY

In chronic infection and cancer, T cells are continuously confronted with moderate to high levels of antigens, which, in

combination with the induced immunosuppressive microenvironment resulting from high antigen load and dysregulated immune responses, leads to increased activation thresholds and, subsequently, a reduction in effector function resulting in a tolerant state. This tolerant state can be reversed by positive regulatory molecules such as IL-2, IL-7, and/or blockade of PD-1 and CTLA-4. However, systemic administration of positive regulatory cytokines, or blocking antibodies, may cause autoimmunity. Therefore, one of the major challenges for immunotherapy against chronic infectious diseases and cancer is to reverse the tolerance of antigen-specific T cells, without affecting bystander T cells, thereby maintaining immune homeostasis to self-antigens. The development of delivery vehicles targeting antigen-specific T cells allows the provision of not only antigen but also engineered bio-adjuvant(s), which can restore effector function.

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