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A push-pull vaccine strategy using Toll-like receptor ligands, IL-15, and blockade of negative regulation to improve the quality and quantity of T cell immune responses

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Abstract

We have developed a strategy to optimize the efficacy of vaccines to induce T-cell immunity against chronic viral infections and cancer based on a “push-pull” approach in which we first optimize the antigen structure by increasing the affinity of epitopes for major histocompatibility complex molecules (“epitope enhancement”), then push the response not only in magnitude but also in quality toward the desired response phenotype, using synergistic combinations of cytokines, Toll-like receptor ligands, and costimulatory molecules, and then pull the response by removing the brakes exerted by negative regulatory mechanisms, including regulatory cells, cell surface molecules, and cytokines. Components of this approach show promise in macaque models of AIDS virus infection and in murine models of cancer, and are being developed for human clinical trials.

Over the past decade, our lab has developed a vaccine strategy that we call a “push-pull approach” [1]. The basic skeleton of a vaccine is an antigen that is used to induce an immune response, but we need to improve on that to optimize the response, through several steps that will be reviewed here (Fig. 1).

First, we can improve the antigen itself, since viral antigens or tumor antigens did not evolve to be good vaccines, but rather to escape the immune system. Their epitopes are not necessarily optimally immunogenic. Focusing on T cell responses, we developed a process we call “epitope-enhancement,” in which amino acids in positions that interact with the major histocompatibility complex (MHC) molecule are substituted to increase the affinity of epitope peptides for MHC molecules [2, 3]. If we can do so without affecting the face of the peptide-MHC complex that is recognized by the T cell receptor (TCR), then the epitope-enhanced peptide should be more immunogenic because of its higher affinity for MHC molecules but still elicit T cells that react with the natural amino acid sequence, since that is necessary for efficacy. We have shown proof of principle for epitopes from HIV, hepatitis C virus, and cancer antigens [4–9]. One of these, an enhanced TARP peptide, is currently in a phase I clinical trial of a peptide vaccine for prostate cancer, and shows promising preliminary results.

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Second, we want to push the vaccine response, not only to increase the magnitude, but also to steer it in the appropriate direction, that is to improve the quality as well as the quantity of the response. This will be the primary focus of this review. To do so, we have used a series of molecular adjuvants, such as cytokines, costimulatory molecules, and Toll-like receptor (TLR) ligands. Our initial work in 1985 describing the first use of a cytokine as a vaccine adjuvant was using IL-2 in the adjuvant with the antigen to make low responder mice respond like high responders to myoglobin [10]. We then compared a series of cytokines delivered in a similar way in incomplete Freund's adjuvant with antigens, and found that GM-CSF induced the broadest increase in immune responses, whereas other cytokines such as IL-12 or IL-4 induced more selective types of responses to the same antigen [11]. We also discovered that GM-CSF and IL-12 synergized for induction of CD8⁺ cytotoxic T lymphocytes (CTL), and IL-12 and TNF synergized for interferon- γ production [11]. Mechanistically, GM-CSF increased antigen presentation while IL-12 plus TNF synergized by upregulating the IL-12 receptor [12, 13].

Most recently, we have focused on IL-15, as we found that this induced both high avidity CD8⁺ T cells [14] and also substituted for CD4⁺ T cell help to induce a long-lived CD8⁺ T cell response [15]. We had previously found that high functional avidity CD8⁺ T cells were more effective at clearing a virus infection in SCID mice that lacked T cells of their own, compared to lower avidity T cells specific for the same peptide-MHC complex [16]. This was confirmed for other viruses [17] and we and others extended the observation to lysis of tumor cells [18–20]. We therefore sought ways to selectively induce high functional avidity CTL with a vaccine. One approach we found was to use a combination of costimulatory molecules in the vaccine [21]. The second way was to use IL-15 as an adjuvant. IL-15 as an adjuvant promoted avidity maturation of CD8⁺ T cells, resulting in a higher avidity population [14].

We also found that IL-15 expressed in a vaccinia vector with antigen, so both antigen and IL-15 were taken up and expressed by the same antigen-presenting cell (APC), such as a dendritic cell (DC), induced longer-lived CTL [22]. Since helper T cells also induced longer lived CTL by activating DCs that were presenting antigen, and since activated DCs made IL-15, we hypothesized that one mechanism of CD4⁺ T cell help for CD8⁺ T cells was to induce the DC that was presenting antigen to also make IL-15, and we had mimicked that with our vaccine expressing both antigen and IL-15 in the same cell. A corollary of this hypothesis was that animals or people who were deficient in CD4⁺ T cell help, such as HIV-infected individuals or some cancer patients, might not be able to induce DCs to make IL-15 and thus would not make a long-lived CD8⁺ CTL response, impairing clearance of the virus or cancer. We tested this hypothesis by depleting mice of CD4⁺ T cells (or not) prior to immunization, and then following the CD8⁺ T cell response for a year [15]. The animals depleted of CD4⁺ T cells made an initial response only slightly lower than that of the control undepleted animals, but had no long-term memory response from 2 months to a year. However, when the vaccine vector expressed IL-15 along with the antigen, then even CD4⁺ cell-depleted mice had a long-term memory response almost as high as the control undepleted group from two months to a year [15]. Thus, IL-15 could substitute for CD4⁺ T cell help to induce a long-lived CD8⁺ T cell response. Like CD4⁺ T cell help, IL-15 expressed by the vaccine also overcame the “helpless” phenotype described by Janssen et al [23] leading to TRAIL-mediated apoptosis on restimulation with antigen [15]. Further, if the DCs presenting antigen were unable to make IL-15, then even normal helper cells delivered inadequate help. Thus, we concluded that IL-15 was both necessary and sufficient and was probably at least one key mediator of CD4⁺ T cell help for CD8⁺ T cells [15]. Note that these results reflect the effect of IL-15 made by the same cell that is presenting antigen at the time of priming, and persist for at least a year even though the vaccinia expressing IL-15 is cleared within 11 days, even in CD4-deficient mice. Thus, this effect is not due to

maintenance of the CD8⁺ T cells by IL-15 produced by the vector, even though it is well known that IL-15 promotes homeostatic proliferation and survival of CD8⁺ T cells [24, 25], but rather reflects programming that occurs at the time of priming. These results are also supported by other studies that have shown that IL-15 is an effective adjuvant to induce CD8⁺ T cell responses even in the absence of CD4⁺ T cell help [26, 27].

We have subsequently asked whether IL-15 can substitute for CD4⁺ T cell help in humans as well as mice. Although we could not do in vivo intervention studies in human volunteers, we could examine the induction of a primary in vitro CD8⁺ T cell response in culture. We looked at both recall responses and primary responses, but it is the primary response that is critical, to induce a new response in a naïve individual. When PBMC were depleted of CD4⁺ T cells, induction of a CD8⁺ T cell response to a recall antigen (such as influenza matrix peptide) was much lower than in the presence of CD4⁺ helper T cells. This deficiency could be overcome by providing exogenous IL-15 or IL-2 in the culture [28]. Likewise, when CD4-depleted PBMC were stimulated with an antigen to which the blood donor was naïve, such as a hepatitis C virus (HCV) core peptide presented by HLA-A2 in donors who are HCV negative, or a primary allogeneic response to allogeneic DCs, the depleted PBMC were unable to mount a substantial primary CD8⁺ T cell response to these antigens. This primary response could be reconstituted with exogenous IL-15 but not IL-2, in contrast to the recall antigen case in which both worked [28]. This was true over a wide range of concentrations of IL-15 and IL-2, and also when a phenotypically naïve population of T cells was isolated from naïve donor PBMC prior to the stimulation. Thus, we conclude that IL-15 but not IL-2 can substitute for help to induce a primary response whereas both work for a recall response [28]. This finding is consistent with earlier studies showing that DCs are needed for a primary de novo response, whereas other APCs such as macrophages were capable of a recall response by memory T cells [29, 30]. Moreover, we found that the quality of the T cells induced, in terms of polyfunctionality, when IL-15 was used to replace help, was similar to that found when CD4⁺ T cell help was present.

Finally, we asked whether this finding could be translated to the CD8⁺ T cell response of HIV-infected individuals who are CD4-deficient [28]. We found that CD8⁺ T cells from PBMC from such HIV-infected individuals made a significantly lower proliferative response to allogeneic DCs than did CD8⁺ T cells from healthy controls. However, when IL-15 was provided in the cultures, the CD8⁺ T cell allogeneic response of HIV-infected individuals became comparable to that of healthy individuals. Thus, this functional deficiency in PBMC of HIV infected individuals could be overcome by IL-15 to replace inadequate CD4⁺ T cell help. Overall, we conclude that IL-15 can substitute for CD4⁺ T cell help to induce a functional CD8⁺ T cell response in humans as well as mice [28].

Another approach to activating DCs to achieve a similar effect is the use of Toll-like receptor (TLR) ligands. These are common molecular structures from pathogens that are recognized by pattern recognition receptors on DCs and other cells [31]. We reasoned that in nature, these microbial products often occur in combinations, so natural synergies may have evolved. We first compared different TLR ligands in combinations for synergy as vaccine adjuvants in combination with a synthetic peptide HIV vaccine in mice to induce a CD8⁺ T cell response [32]. We found that ligands for TLR2/6 (MALP-2) or for TLR9 (CpG oligodeoxynucleotides), synergized with ligands for TLR3 (Poly I:C), but TLR-2/6 and TLR9 did not synergize with each other. We also found that the synergy in vivo for inducing a CD8⁺ T cell response to a vaccine exactly paralleled the synergy with which these combinations of TLR ligands activated DCs, both in vivo and in vitro. Also, DCs activated by the synergistic combinations and then pulsed with peptide showed the same synergy as DC vaccines that we saw when we used the TLR ligands directly as adjuvants. We concluded that the synergy in vivo works through the effect of these ligands on DC

activation [32]. Why some combinations synergize and others do not was addressed by looking at the downstream signaling. We noted that TLR2 and 9 signal through MyD88 whereas TLR3 signals through TRIF. We showed that the synergy depended on both these molecules, using knockout mice. Thus, presumably, TLR2 and 9 do not synergize because they work through the same pathway, whereas both synergize with TLR3 because it works through a different pathway. Moreover, we found that the synergy correlated with DC function to make IL-12, which is induced by MyD88, and not upregulation of costimulatory molecules, controlled by TRIF. However, stimulation through TRIF synergized with the stimulation through MyD88 to upregulate IL-12 production. The synergy thus depended on a unilateral crosstalk between the pathways, in which TLR3 through TRIF amplified the signal through MyD88 to induce DC cytokine production [32]. These and other examples of TLR ligand synergy suggest that synergistic combinations of TLR ligands can function as potent immunological adjuvants [33–36].

When we compared a combination of all 3 TLR ligands with the synergistic pairs of cytokines, we found that there was no further increase in tetramer-staining antigen-specific CD8⁺ T cells with all 3 than with the best combinations of 2 ligands, as might have been predicted from the above analysis. However, it was a surprise, when we examined the protective efficacy of the vaccines against a viral challenge, that the triple combination gave substantially better protection than either of the synergistic double combinations [37]. Since there was no difference in the quantity of antigen-specific CD8⁺ T cells induced, we asked whether their quality was improved. Indeed, we found that the triple combination induced higher avidity T cell responses [37], accounting for the greater antiviral efficacy (consistent with our earlier studies on the importance of T cell functional avidity in viral clearance [16]). Nearly 100% of the specific CD8⁺ T cells induced by the triple combination were high avidity, as measured by ability to recognize target cells pulsed with antigen at nanomolar concentrations, whereas only 20% of the cells induced by the double combinations were high avidity, responding at such low concentrations. Moreover, this correlated with increased production of IL-15 by DCs [37], consistent with the findings above that IL-15 in a vaccine could increase CD8⁺ T cell avidity [14]. At a molecular level, TLR2 and TLR9 may not be completely redundant, because TLR2 signals also through TIRAP in a MyD88-independent fashion, in addition to signaling through MyD88. We showed using TIRAP KO mice that this was also necessary for the synergy, implying that TLR2 signaling through TIRAP may contribute to the synergistic combination. Overall, the triple combination of TLR ligands was most effective because it improved not only the quantity of the CD8⁺ T cell response but also its quality [37].

We translated these findings into a non-human primate study of molecular adjuvants in a peptide and viral vector intrarectal vaccine in rhesus macaques against SIV intrarectal challenge [38]. Using Mamu-A*01 positive macaques so that we could use peptides presented by this MHC molecule and also monitor the T cell response, we immunized mice with a mixture of peptides and boosted with recombinant modified vaccinia Ankara (MVA), an attenuated replication incompetent vaccinia virus, expressing SIV env, gag, pol, nef, tat and rev. With each intrarectal peptide vaccination and each intrarectal MVA boost, we delivered either IL-15, the triple combination of TLR ligands, or both or neither. After 3 doses of peptide at 3 week intervals and two boosts with MVA, we biopsied the mesenteric lymph nodes and colon, and then 3 weeks later, challenged intrarectally with 10 animal infectious doses of SIVmac251, a virulent strain of SIV. We then monitored viral load and CD4 count for about 6 months, and then necropsied the animals. Since this dose of the same batch of SIVmac251 had been shown to infect 30/30 naïve controls, we included an adjuvant control group instead, with both types of adjuvant. Most of the animals in the groups getting vaccine alone or combined with either adjuvant alone showed high peak viral load and high set point, around 10⁵ to 10⁷ copies per ml, but a few dropped into the 10⁴ range. However,

in the group that received both types of adjuvants, 3/5 animals showed some protection. One never developed viremia; one showed a blunted peak response and then the viral load went below detection after about 6 weeks; and a third went below detection intermittently, with blips up to 10^3 particles/ml. This result suggested that the combination of both types of adjuvants was synergistic to control viral load [38]. However, the adjuvant-only control group also had one animal that never showed viremia, and a second that reduced its viral load to 10^3 particles/ml by 6 months. This finding suggested that the adjuvants were also inducing an innate immune response that could result in some level of protection [38].

We first examined the adaptive immune response. Surprisingly, although there were strong pre-challenge $CD8^+$ T cell responses in the colon lamina propria to all the epitope peptides in the vaccine as measured by tetramer staining, there was no correlation with protective efficacy. This was also true when central and effector memory pools were examined separately. However, there was a strong correlation between protection and the polyfunctional $CD8^+$ T cell response, as measured by intracellular staining for interferon-gamma, TNF-alpha, and IL-2 in response to peptide stimulation [38]. The group that got both types of adjuvants combined showed the highest polyfunctional responses, and in particular, the three animals which showed protection had the highest level of these responses. Moreover, there was a significant inverse correlation between set point viral load and the log of the polyfunctional T cell response. However, this was not a linear inverse correlation, but rather a classic inhibition curve, showing a threshold effect [38]. The threshold effect is of interest because it means that below about 2% polyfunctional T cells, no correlation was apparent, as the viral load was on a plateau, whereas above the threshold of 2%, the viral load fell steeply with increasing numbers of polyfunctional T cells. This threshold effect may account for the finding that some vaccine studies did not show a correlation of viral load with T cell response and others did.

Among the innate immune responses measured, we found that APOBEC3G mRNA in the mesenteric lymph nodes prior to challenge was the only one that correlated inversely with set point viral load after challenge [38]. First, the group that received both adjuvants and in particular the animals who had the lowest viral load had the highest APOBEC3G levels. Second, the animal in the adjuvant control group that remained uninfected had the highest APOBEC3G level of any animal, a strong outlier, and the animal that showed partial protection had the next highest level. In addition, there was a strongly significant linear inverse correlation between set point viral load after challenge and log (APOBEC3G) levels before challenge. It should be noted that the challenge took place 7 weeks after the last boost, so this innate response must have lasted at least 7 weeks. Also, we showed in vitro that the combination of IL-15 and the triple TLR ligand combination upregulated APOBEC3G levels, accounting for the effect [38]. Overall, we concluded that molecular adjuvants are valuable not only to increase the adaptive immune response, but also to trigger an innate immune response that can provide some immediate protection that allows the adaptive immune response time to develop.

The third component of our vaccine strategy is the “pull” part of the approach, in which we remove the brakes to allow the push to have its maximal effect. There are a number of regulatory cells and molecules that dampen the immune response, including T-regulatory (Treg) cells, myeloid-derived suppressor cells, M2 macrophages, regulatory DCs, and type II NKT cells, and also cell surface molecules on T cells like PD-1 and CTLA-4 as well as soluble molecules like TGF-beta and IL-13. We were especially interested in type II NKT cells because our lab, with a number of collaborating labs using four different tumor models [39], had found that these negatively regulated tumor immunity [39–43], and such negative regulation of tumor immunity by type II NKT cells was then observed in other tumor models by other labs as well [44, 45]. Moreover, we found that type I and type II NKT cells cross-

regulated each other, defining a new immunoregulatory axis [43], like the Th1-Th2 axis, that may have profound implications because NKT cells are among the first responders and can set the stage for subsequent adaptive immune responses [46–51]. We focused on blocking TGF-beta, because we had previously found that it is the downstream effector molecule induced by type II NKT cells that suppress tumor immunity [41], and is also known to be important for induction of inducible Treg cells and for maintenance of Treg activity [52]. TGF-beta can also be produced by tumors to directly inhibit the response against them. We found that blockade of TGF-beta with a monoclonal antibody could protect against some cancers in mice as a single agent [41]. However, in two other mouse cancer models in two strains of mice where it was not effective alone, anti-TGF-beta synergized with a cancer vaccine to significantly improve vaccine efficacy, slow tumor growth, and prolong survival [53, 54]. Thus, we can further improve vaccine efficacy by blocking negative regulators like TGF-beta at the time of vaccine delivery. We now have completed a phase I clinical trial of a human monoclonal antibody (GC1008) against TGF-beta in melanoma and renal cell cancer patients with promising results [55]. Based on the mouse studies, we would like to translate our findings of synergy to combine a human monoclonal anti-TGF-beta with a cancer vaccine, such as the TARP peptide prostate cancer vaccine that uses epitope enhanced peptides described above.

Overall, therefore, we conclude that this type of push-pull approach can improve vaccine efficacy to induce T cell responses and protect against viruses or cancers. We first try to optimize the antigen, for example by epitope enhancement, and then improve the quality as well as quantity of the T cell response using selective combinations of cytokines, like IL-15, and TLR ligands, as well as costimulatory molecules, and then block negative regulation to allow the vaccine response to achieve its full potential (Fig. 1). In the case of HIV, molecular adjuvants like cytokines and TLR ligands will need to be used cautiously, either in a prophylactic setting in the absence of virus, or in the case of therapeutic vaccines, delivered locally, not systemically, and only under cover of antiretroviral therapy, to avoid activating more target cells for HIV to infect. Nevertheless, we hope that this strategy of using defined molecular adjuvants and immunomodulators will prove successful in vaccines for chronic diseases like HIV infection and cancer, in which traditional vaccine strategies have so far had limited efficacy.

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PUSH-PULL Approach to Optimizing Vaccine-induced T-cell Immunity

Figure 1.

Push-pull model to optimize immune responses to vaccines. The skeleton of a vaccine is an antigen used to induce an immune response. In this strategy, we 2) first improve the structure of the antigen by “epitope enhancement,” then 2) push the response toward not only higher but also higher quality immune responses, steering it in the right direction, using synergistic combinations of cytokines, Toll-like receptor (TLR) ligands and costimulatory molecules, and then 3) “pull” the response by removing the braking mechanisms to allow it to achieve its full potential. (Drawing courtesy of Dr. Masaki Terabe.) Modified from Sui and Berzofsky [56], with permission.