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# Relevance of long-lived CD8$^+$ T effector memory cells for protective immunity elicited by heterologous prime-boost vaccination

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Owing to the importance of major histocompatibility complex class Ia-restricted CD8$^+$ T cells for host survival following viral, bacterial, fungal, or parasitic infection, it has become largely accepted that these cells should be considered in the design of a new generation of vaccines. For the past 20 years, solid evidence has been provided that the heterologous prime-boost regimen achieves the best results in terms of induction of long-lived protective CD8$^+$ T cells against a variety of experimental infections. Although this regimen has often been used experimentally, as is the case for many vaccines, the mechanism behind the efficacy of this vaccination regimen is still largely unknown. The main purpose of this review is to examine the characteristics of the protective CD8$^+$ T cells generated by this vaccination regimen. Part of its efficacy certainly relies on the generation and maintenance of large numbers of specific lymphocytes. Other specific characteristics may also be important, and studies on this direction have only recently been initiated. So far, the characterization of these protective, long-lived T cell populations suggests that there is a high frequency of polyfunctional T cells; these cells cover a large breadth and display a T effector memory (TEM) phenotype. These TEM cells are capable of proliferating after an infectious challenge and are highly refractory to apoptosis due to a control of the expression of pro-apoptotic receptors such as CD95. Also, they do not undergo significant long-term immunological erosion. Understanding the mechanisms that control the generation and maintenance of the protective activity of these long-lived TEM cells will certainly provide important insights into the physiology of CD8$^+$ T cells and pave the way for the design of new or improved vaccines.

**Keywords: memory, vaccines, CD8, adenovirus**

## GENETIC VACCINATION USING THE HETEROLOGOUS PRIME-BOOST REGIMEN

Genetic vaccination using naked DNA or recombinant viruses is being pursued as an alternative to traditional vaccines. This strategy could be particularly important in the case of intracellular pathogens and neoplastic cells, where the effectiveness relies heavily on the capacity of the vaccine to elicit specific immune responses mediated by cytotoxic CD8$^+$ T cells (reviewed in Rice et al., 2008; Lasaro and Ertl, 2009 Bassett et al., 2011; Gómez et al., 2011; Mudd and Watkins, 2011; Saade and Petrovsky, 2012).

Whereas the use of single-vector delivery for priming and boosting is usually the initial option, one of the most prolific areas of genetic vaccine development is the strategy known as the heterologous prime-boost regimen. This strategy uses two different vectors, each carrying a gene that encodes the same antigenic protein for priming and boosting immunizations. The utility and importance of this strategy was first proposed in the early 1990s using a combination of recombinant viruses (influenza and vaccinia) to induce protective immunity against malaria (Li et al., 1993; Rodrigues et al., 1994). Subsequently, this approach was extended and simplified by incorporating naked DNA for priming followed by a booster injection of a recombinant poxviral vector (i.e., modified vaccinia Ankara, MVA); this was also used to generate sterile protective immunity against rodent malaria (Schneider et al., 1998; Sedegah et al., 1998). Collectively, these studies demonstrated that the heterologous prime-boost regimen generated significantly higher immune responses and proved more effective than the use of any of these genetic vectors individually. The initial use of rodent malaria parasites as a model system may have delayed the development of the field, because malaria is not a conventional model system for developing antiviral or antibacterial vaccines. Nevertheless, this fortuitous fact established the important concept that the difficulty in generating immunity to malaria, and perhaps to other intracellular parasites, in many ways recapitulates the struggles encountered to elicit protective immunity against viruses and bacteria that case chronic infections such as HIV and tuberculosis (TB).

In subsequent years, the heterologous prime-boost vaccination regimen was adopted worldwide as a powerful means to elicit strong type 1 effector $CD8^+$ T cell-mediated immune responses (Tc1) against viral, parasitic, and neoplastic antigens in rodents and non-human primates (NHP; Irvine et al., 1997; Amara et al., 2001; McShane et al., 2001; Zavala et al., 2001; Moore and Hill, 2004; Ellenberger et al., 2006; Gilbert et al., 2006; Aidoo et al., 2007; Nigam et al., 2007; Martinon et al., 2008; Sadagopal et al., 2008). Based on pre-clinical studies, a number of human clinical trials have also been initiated. However, to our knowledge, heterologous prime-boost regimens using plasmid DNA and recombinant poxviruses have not yet provided meaningful protective immunity in humans (McConkey et al., 2003; Moorthy et al., 2004; Keating et al., 2005; Vuola et al., 2005; Cebere et al., 2006; Dunachie et al., 2006; Goonetilleke et al., 2006). The precise reason for such failures is not yet clear. It may be due to the target antigens chosen or to the possibility that the combination of vectors may elicit a type of effector $CD8^+$ T cells in humans that are not functionally and/or phenotypically related to mice, as discussed below.

A number of possible vector combinations that significantly improved cell-mediated immunity, particularly the generation of specific $CD8^+$ T cells, have been described in parallel. Among them, heterologous prime-boost vaccination using naked plasmid DNA for priming followed by a booster injection of recombinant replication-deficient human adenovirus 5 (AdHu5) has recently received significant attention. This strategy has proved successful in some relevant experimental models such as simian immunodeficiency virus (SIV), malaria, Marburg, and Ebola virus infection, and Chagas disease (American trypanosomiasis), providing a considerable degree of protective immunity (Gilbert et al., 2002; Casimiro et al., 2003, 2005; Santra et al., 2005; Acierno et al., 2006; Letvin et al., 2006; Mattapallil et al., 2006; Sun et al., 2006; Wilson et al., 2006; de Alencar et al., 2009; Geisbert et al., 2010; Hensley et al., 2010; Martins et al., 2010; Dominguez et al., 2011; Rigato et al., 2011). These relative successes obtained in pre-clinical experimental models fueled human phase I trials (Freel et al., 2010; Jaoko et al., 2010; Koup et al., 2010; Schooley et al., 2010; Churchyard et al., 2011; De Rosa et al., 2011).

Very recently, improved vector combinations have yielded results (measured in terms of protective immunity) that are slightly better than the results obtained by using plasmid DNA followed by replication-deficient AdHu5 viruses. These new strategies include (i) prime with plasmid DNA in the presence of cytokine genes such as IL-12 or GM-CSF (Lai et al., 2011; Winstone et al., 2011), (ii) genes encoding multimeric proteins (Lakhashe et al., 2011), (iii) boost of adenovirus-immunized animals with an optimized plasmid DNA (Hutnick et al., 2012), (iv) prime with rhesus cytomegalovirus (Hansen et al., 2011), and (v) prime with a different heterologous strain of adenovirus (Barouch et al., 2012).

The precise reason for the superior performance of the heterologous prime-boost vaccination compared to the sequential use of the same vector is still a matter of controversy. Some evidence indicates the possibility that the intense immunity to epitopes present on the priming vector prevents the boosting effect. For example, a recent study in humans shows that a second dose of a recombinant AdHu5 does not provide significant boosting. In parallel, recombinant AdHu5 boosting of DNA-primed individuals resulted in significantly higher immune responses (De Rosa et al., 2011). The anti-vector immunity can be either antibody mediated or independent (Cockburn et al., 2008; Schirmbeck et al., 2008; Frahm et al., 2012). Haut et al. (2011) found that in B cell-deficient mice transgene-specific $CD8^+$ T cell responses were significantly higher in systemic compartments. In contrast, recent studies in humans showed that neutralizing antibodies titers to AdHu5 did not correlate with the magnitude of specific $CD8^+$ T cell of priming after immunization with a recombinant AdHu5. In these experiments, the frequency of specific $CD4^+$ T cells negatively correlated with the intensity of specific $CD8^+$ T cells priming (Frahm et al., 2012).

In spite of the clear evidences that pre-existing immunity may interfere with the use of viral vectors, still, the heterologous prime-boost regimen of immunization is described as a possible solution to this problem. This can be achieved by strong priming with cytokine genes (for example, see Barouch et al., 2003).

Independent of the reasons why the heterologous prime-boost vaccination regimen is superior to the sequential use of the same vector, the main purpose of this review is to examine the characteristics of the protective $CD8^+$ T cells generated by this vaccination regimen.

## CHARACTERISTICS OF PROTECTIVE $CD8^+$ T CELLS ELICITED AFTER HETEROLOGOUS PRIME-BOOST VACCINATION

### HIGH FREQUENCIES OF SPECIFIC $CD8^+$ T CELLS

One hallmark of the heterologous prime-boost regimen is the elicitation of a higher frequency of epitope-specific $CD8^+$ T cells across multiple models. This high number of effector T cells was initially estimated by the presence of epitope-specific IFN-γ-producing cells using the *ex vivo* ELISPOT assay (Murata et al., 1996; Schneider et al., 1998; Sedegah et al., 1998; Bruña-Romero et al., 2001). Subsequently, the hypothesis was further validated by intracellular staining for IFN-γ (Pinto et al., 2003) and tetramer staining of epitope-specific $CD8^+$ T cells (Tao et al., 2005). More recently, intracellular staining for TNF, IL-2, MIP1-β, T cell surface mobilization of CD107a, and *in vivo* cytotoxicity provided extended evidence (for examples, see Masopust et al., 2006; Mattapallil et al., 2006; Cox et al., 2008; de Alencar et al., 2009; Freel et al., 2010; Reyes-Sandoval et al., 2010; Rigato et al., 2011).

Because most studies are performed with T cells collected from the spleen or peripheral blood lymphocytes (PBL) of mice or NHP, respectively, it is not clear whether these results reflect an overall increase in every tissue. The presence of a large number of epitope-specific T cells in several tissues has been documented in the case of mouse lung, liver, intraepithelial lymphocytes, and PBL (Masopust et al., 2006; Reyes-Sandoval et al., 2011); however, because parallel comparison was not performed with animals that were immunized with a single vector, it is not clear whether these levels were particularly higher than the other vaccination protocols. Conversely, the frequency of epitope-specific $CD8^+$ T cells seems to decrease in mouse lymph nodes (Masopust et al., 2006). This may be due to the lack of CD62L expression on the surface of these activated T cells, as discussed below. In addition, the pattern of circulation and recirculation of these lymphocytes has

been poorly explored. A single study, however, demonstrated that, after a recombinant plasmid DNA prime-AdHu5 boost, $CD8^+$ T cells need to recirculate in order to exert protective immunity against an infectious challenge with the protozoan parasite *Trypanosoma cruzi*. In these vaccinated mice, treatment with the drug FTY720 significantly reduced the efficacy of the protective immunity by interfering specifically with signaling through sphingosine-1-phosphate receptor-1, thereby inhibiting the egress of T cells from the lymph nodes (Dominguez et al., 2012). This observation is important because immunity to certain pathogens is not dependent on the recirculation of T lymphocytes (Pinschewer et al., 2000; Kursar et al., 2008; Jiang et al., 2012).

The importance of recirculation is likely dependent on factors such as (i) the host, (ii) the vectors used for prime and/or boost, and (iii) the route of administration of each vector (for examples, see Mattapallil et al., 2006; Kaufman et al., 2010). These are important characteristics that will certainly influence the protective immunity, as pathogens can cause either tissue-specific or systemic infections.

The T cell protective immunity elicited by heterologous prime-boost regimen is not only significantly higher than the immunity elicited by the traditional vaccine regimen but is also longer-lived. Several experimental models have shown that the immunity lasts for significant periods of time (Amara et al., 2001; Bruña-Romero et al., 2001; de Alencar et al., 2009; Reyes-Sandoval et al., 2010; Rigato et al., 2011).

## POLYFUNCTIONALITY OF SPECIFIC $CD8^+$ T CELLS

Based on the assays described above, it became clear that the specific $CD8^+$ T cells elicited by the heterologous prime-boost regimen could exert different immunological functions, as measured by *ex vivo* or *in vivo* assays. Confirmation of the polyfunctionality of these cells was made possible through FACS analyses coupling intracellular cytokine staining and cell surface mobilization of the degranulation marker CD107a.

Accordingly, a number of studies confirmed that distinct heterologous prime-boost regimens elicited polyfunctional $CD8^+$ T cells as defined by the cells' capability to exert two or more functions at the same time. The most frequent example of this across different models is specific $CD8^+$ T cells producing IFN-$\gamma$ and TNF simultaneously. High frequencies of polyfunctional specific $CD8^+$ T cells were described in (i) mice (Masopust et al., 2006; Duke et al., 2007; de Alencar et al., 2009; Elvang et al., 2009; Reyes-Sandoval et al., 2010; Rigato et al., 2011; Rodríguez et al., 2012; Vijayan et al., 2012), (ii) NHP (Mattapallil et al., 2006; Sun et al., 2006; Cox et al., 2008; Liu et al., 2008; Magalhaes et al., 2008; Cayabyab et al., 2009; Wilks et al., 2010; Hutnick et al., 2012), and (iii) humans (Beveridge et al., 2007; Harari et al., 2008; Winstone et al., 2009; Freel et al., 2010; Jaoko et al., 2010; Koup et al., 2010; Schooley et al., 2010; Churchyard et al., 2011; De Rosa et al., 2011).

It is noteworthy that although these T cells have a high frequency of polyfunctionality, their ability to mediate multiple immunological functions has never been clearly linked to their protective capacity. It is possible that this characteristic aids in but is not critical for their effector functions, depending on the mechanism necessary for pathogen elimination. In a single study performed using genetically deficient mice, in the absence of either IFN-$\gamma$ or perforin, heterologous prime-boost vaccination failed to mediate protective immunity against infection with the intracellular parasite *T. cruzi*. In the case of perforin-deficient mice, the lack of protective immunity was associated mainly with a significant decrease in the induction of polyfunctional T cells (de Alencar et al., 2009). A second study correlated the presence of higher frequencies of polyfunctional T cells to protective immunity against liver stages of malaria parasite (Reyes-Sandoval et al., 2010). Although these studies may suggest a role for polyfunctional T cells during protective immunity, it is still too early to conclude that they are critical for the protective immunity exerted by $CD8^+$ T cells elicited following the heterologous prime-boost vaccination regimen.

## BREADTH OF SPECIFIC $CD8^+$ T CELLS

T cell immune responses are often restricted to a few immunodominant epitopes, a phenomenon termed immunodominance (Akram and Inman, 2012). The precise reason for such a restriction is not clear; however, it may have evolved to maximize the immune response, while at the same time reducing the risk of autoimmunity. For the purpose of vaccine development, having only a narrow number of recognized epitopes may be dangerous, as the pathogens will rapidly select for escape mutants to avoid effector immune responses (reviewed in Streeck and Nixon, 2010; Chopera et al., 2011).

Although it has been possible to increase the frequency of T cells specific for immunodominant epitopes, it is still a challenge to broaden the vaccine-induced $CD8^+$ T cell response to a number of subdominant T cell epitopes. There is evidence that heterologous rAdHu5 boosting improved not only the magnitude but also the breadth of specific $CD8^+$ cells (Liu et al., 2008). However, the impact of this response on protective immunity is not clear. Two recent studies indicated that immunity to subdominant epitopes might participate in vaccine-induced protective immunity following a DNA prime-AdHu5 boost vaccination regimen. The first study provided a correlation between the breadth of the immune response and the protective immunity observed in individual rhesus monkeys vaccinated with SIV genes (Martins et al., 2010). A second study formally demonstrated that heterologous prime-boost vaccination with plasmid DNA followed by recombinant AdHu5 elicited strong immune response to two subdominant epitopes that were not recognized during infection (Dominguez et al., 2011). Based on these observations, mutant genes were generated in which the dominant epitope was removed. Heterologous prime-boost vaccination with these mutant genes-induced $CD8^+$ T cell immune responses only to the subdominant epitopes. Most importantly, strong $CD8^+$ T cell-mediated immunity was still observed (Dominguez et al., 2011). These results unequivocally demonstrate the importance of immunity to the subdominant epitopes and the ability of the heterologous prime-boost to elicit them. Nevertheless, other groups still have difficulty improving the immune response to subdominant epitopes following heterologous prime-boost vaccination of NHP (Vojnov et al., 2011). Therefore, new strategies to improve the breadth of the immune response might be developed in order to potentiate vaccine formulation.

## TEM PHENOTYPE OF SPECIFIC LONG-LIVED CD8$^+$ T CELLS

The current immunological paradigm divides antigen-experienced CD8$^+$ T cells into three main types: (i) T effector (TE), (ii) T effector memory (TEM), and (iii) T central memory (TCM). These populations of T cells can be distinguished by the presence of activation markers, as well as by differences observed in their localization and recirculation patterns and their ability to proliferate and present certain effector functions/molecules (Angelosanto and Wherry, 2010; Cui and Kaech, 2010; Ahmed and Akondy, 2011; Sheridan and Lefrançois, 2011). More recently, other subpopulations of CD8$^+$ T cells have also been described (Wakim et al., 2010; Sheridan and Lefrançois, 2011; Jiang et al., 2012). The concept of TE/TEM/TCM was primarily established by experimental infections with self-curing pathogens. In these cases, TE are short-lived effector cells (CD44$^{High}$ CD11a$^{High}$ CD62L$^{Low}$ CD127$^{Low}$ KLRG1$^{High}$); TCM are long-lived memory cells (CD44$^{High}$ CD11a$^{High}$ CD62L$^{High}$ CD127$^{High}$ KLRG1$^{High}$); and TEM are transitional cells that exist for a short period of time and have distinct surface marker expression (CD44$^{High}$ CD11a$^{High}$ CD62L$^{Low}$ CD127$^{High}$ KLRG1$^{High}$). However, even by using models of self-curing infections, the existence of other memory T cell subpopulations that might be more relevant for long-term T cell immunity have been proposed (Hikono et al., 2007).

In addition, very recently, considerable interest has been placed on tissue-resident memory T (TRM) cells. These cells are potent long-lived effector cells present in a variety of peripheral tissues including the skin and sensory ganglia, gut, brain, lung, etc. (Wakim et al., 2008; Gebhardt et al., 2009; Masopust et al., 2010; Purwar et al., 2011). They mediate protective immunity against brain infection with vesicular stomatitis virus or skin infection with vaccinia or herpes simplex virus (Wakim et al., 2010; Jiang et al., 2012; Mackay et al., 2012). Their phenotype is CD44$^{High}$, CD62L$^{Low}$, CD103$^{High}$, CD69$^{High}$, CD127$^{Low}$, and PD-1$^{Low}$. The relationship of TRM cells with TEM or TCM is yet to be determined. Some authors propose that these cells constitute an independent lineage primed within the tissue (Wakim et al., 2010, 2012).

Very recently, the development of a methodology to perform transcriptional profiling at the single cell level has detected further differentiation profiles among TEM and TCM. The analysis of specific splenic CD8$^+$ T cells from mice immunized with distinct vaccination protocols yields significant differences among these subpopulations (Flatz et al., 2011). For example, while 74% of the specific TEM elicited by the heterologous DNA prime-recombinant AdHu5 boost vaccination were Klrk1$^-$ Klrg1$^-$ Ccr5$^-$, only 20% of the cells generated by HuAd5-prime-recombinant LCMV boost had a similar phenotype (Flatz et al., 2011). This type of analysis further highlights the heterogeneity still being uncovered within these memory cells.

It has been observed on multiple occasions that after heterologous prime-boost vaccination, the boosting immunization drives not only an expansion of the T cells but also a different phenotype in the long-lived memory T cells. Different doses of the vaccine also caused a significant increase in the frequency of specific CD8$^+$ T cells with a TEM phenotype (CD44$^{High}$ CD11a$^{High}$ CD62L$^{Low}$ CD127$^{High}$ KLRG1$^{High}$; Masopust et al., 2006). TEM cells were described initially as highly protective against certain viral and bacterial infections (Bachmann et al., 2005a,b; Huster et al., 2006). Likewise, protective immunity afforded by the different heterologous prime-boost vaccination protocols has been associated with the presence of this type of T cell (Hansen et al., 2011; Reyes-Sandoval et al., 2011; Rigato et al., 2011; Xiao et al., 2011; Barouch et al., 2012; Yamamoto et al., 2012).

Based on the relatively poor knowledge of the surface activation markers present on long-lived specific TEM CD8$^+$ T cells, we performed a detailed analysis of the different T cells markers following intramuscular DNA prime-adenovirus boost immunization. We identified transgenic epitope-specific T cells in the spleen of immunized mice 14 or 98 days after the boost vaccination. **Figure 1** summarizes the surface marker phenotype of these epitope-specific T cells compared to the phenotype of the naïve cells.

## PROLIFERATIVE CAPACITY OF SPECIFIC CD8$^+$ T CELLS

The proliferative capacity of specific T cells elicited by heterologous prime-boost vaccination has not been thoroughly studied to date. In general, after resolution of experimental self-curing infections, the frequency of total CD8$^+$ T cells declines to less than 10% of the maximal number of specific T cells observed during the peak of the immune response; this is known as the contraction phase. The decrease in the number of specific T cells occurs mainly among the short-lived effector cells (Angelosanto and Wherry, 2010; Cui and Kaech, 2010; Ahmed and Akondy, 2011; Sheridan and Lefrançois,

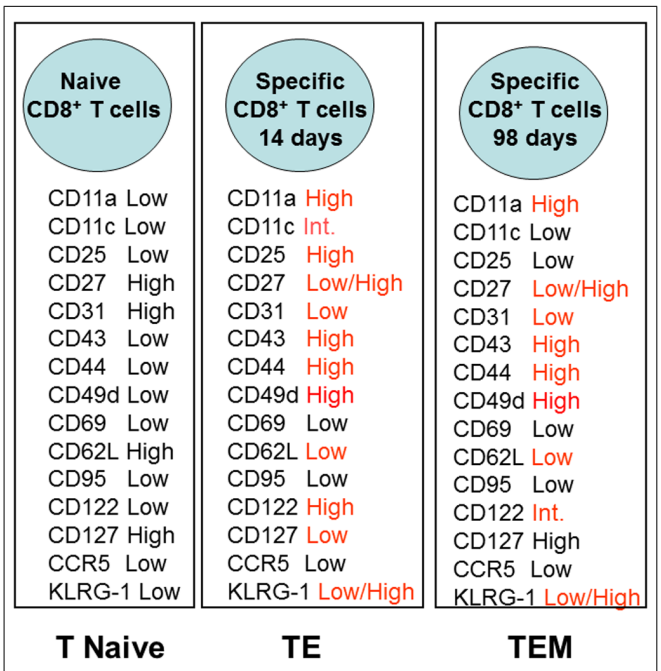


**FIGURE 1 | Phenotype of specific CD8$^+$ T cells elicited by heterologous prime-boost vaccination using recombinant plasmid DNA and AdHu5.** Prime-boost regimen was performed as detailed described by Rigato et al. (2011). Mice were primed i.m. with plasmid DNA (100 μg) and boosted 21 days later with AdHu5 ($2 \times 10^8$) both expressing the gene encoding the amastigote surface protein-2 of *T. cruzi*. Expression of distinct adhesion/activation receptors on the surface of splenic CD8$^+$ specific T cells is shown at day 14 or day 98 after the boost immunization.

2011). However, this does not seem to be the case for the TE cells elicited by the heterologous prime-boost vaccination regimen (Jameson and Masopust, 2009). In this case, the cells expand and are maintained at a high frequency in the spleen for a long period of time (Masopust et al., 2006; Vezys et al., 2009; Rigato et al., 2011). This is a somewhat unexpected finding and might be of great relevance for the success of this vaccination protocol. Because this expansion and maintenance contrasts with the retraction observed after self-curing infections, it opens a number of questions that should be studied in depth: (i) How can cells expand out of control to reach frequencies higher than the primary immune response? (ii) How do T cells avoid the apoptotic process observed for the short-lived effector cells during the contraction phase? (iii) How are T cells maintained for these long periods? These questions are keys to understanding the physiology of $CD8^+$ T cells and developing better T cell vaccines.

After an infectious challenge, specific T cells proliferate. However, it is still unknown whether this proliferative response occurs among the antigen-primed TE(M) cells, rare TCM or naïve T cells. To address this question, it is now possible to use the gzm-BCreERT2/ROSA26EYFP transgenic mouse line (Bannard et al., 2009). Specific TE $CD8^+$ T lymphocytes can be indelibly labeled with enhanced yellow fluorescent protein (EYFP). Following an infectious challenge, it will be possible to follow the expansion of EYFP-labeled TE cells. This result will confirm or not whether the cells that expand after the infectious challenge are indeed mainly antigen-experienced TE. Also, by the adoptive transference of green-fluorescent naïve specific T cells prior to the infectious challenge it will be possible to determine whether these cells proliferate or not together with the specific TE cells. Whether this is true for all different protocols of heterologous prime-boost vaccination strategies is still unknown and might be relevant for the development of vaccines to be used in individuals primed with conventional vaccines such as Bacillus Calmette–Guérin (BCG; Hoft et al., 2012).

## REFRACTORINESS TO APOPTOSIS OF SPECIFIC $CD8^+$ T CELLS

As mentioned above, after an intense immune response and pathogen elimination, the number of specific $CD8^+$ T cell is drastically reduced during a period called the contraction phase. In that period, TE or short-lived effector cells are eliminated by mechanisms that involve apoptosis mediated by BCL-2-interacting mediator of cell death (Bim) and CD95 (also known as FAS; Green, 2008; Hughes et al., 2008; Weant et al., 2008; Bouillet and O'Reilly, 2009). We observed that following prime-boost immunization with recombinant plasmid DNA and AdHu5, TEM cells do not upregulate surface CD95 expression and are refractory to anti-CD95-induced apoptosis *in vitro* (Rigato et al., 2011). Because CD95 is an important initiator of the intrinsic pathway of apoptosis in T lymphocytes, low levels of expression may protect T cells from apoptotic death.

Decreased levels of expression or resistance to activation of other receptors that control T cell death, such as TNF receptor and TRAIL, might also play a role during the survival of specific TEM cells. In contrast, increased expression of other receptors may act to protect these specific T cells. Recently, expression of PDL-1 (B7-H1) on antigen-activated $CD8^+$ T cells made these cells more resistant to $Ca^{2+}$-dependent and Fas ligand-dependent killing by cytotoxic T lymphocytes (Pulko et al., 2011). However, more details are necessary to understand the expression levels of many of these molecules controlling T cell survival.

It is plausible that Bim is poorly activated, or not activated at all, on these cells. Because Bim is activated in response to the lack of certain external stimuli, these activation signals might be maintained for long-term. One such mechanism is antigen presentation by dendritic cells. Using a replication-deficient adenoviral vector, Tatsis et al. (2007) demonstrated that the transgene product remains available for antigen presentation for as long as 16 weeks after a single immunizing dose. The importance of continuous transgene expression in the maintenance of the specific $CD8^+$ T cells after AdHu5 vaccination was also demonstrated by a causal relationship between both using an inducible system to turn off the transgene expression (Finn et al., 2009). Bassett et al. (2011) showed that both hematopoietic and non-hematopoietic antigen-presenting cells are necessary for the maintenance of $CD8^+$ T cells following immunization with recombinant adenovirus. However, it is not clear whether antigen persistence is also observed with other vectors.

We tested whether the IFN-γ or IL12/IL23 signaling pathways might be important for maintaining these long-lived $CD8^+$ TEM cells. The absence of either pathway individually made little difference in the generation of specific $CD8^+$ T cells. The absence of the IL12/IL23 pathway, but not the IFN-γ pathway, was important for the long-term survival of these cells (Rigato et al., 2011). Further details regarding the relevance of these signaling pathways in maintaining a high frequency of $CD8^+$ T cells are unknown. In addition, little is known about the impact of the lack of the IL12/IL23 pathway in the maintenance of specific $CD8^+$ T cells after other heterologous prime-boost vaccination regimens. We consider this area of critical importance in understanding how T cells can be maintained at high frequencies for long periods of time. A possible explanation that remains to be tested is whether the lack of contraction could be due to the fact that many specific long-lived $CD8^+$ T cells express low levels of the chemokine receptors CXCR3 and CCR5 (Flatz et al., 2011). This possibility is supported by recent observations that genetically deficient $CD8^+$ T cells that do not express both these receptors were refractory to contraction and accumulated in higher numbers in the spleen (Kohlmeier et al., 2011).

Recently, we described a new and potentially very important aspect of $CD8^+$ TE cells. After recombinant AdHu5 vaccination, $CD8^+$ TE cells do not undergo apoptosis, as well as prevent the development of a pro-apoptotic phenotype that occurs during experimental infection with the protozoan parasite *T. cruzi*. This phenomenon was observed when the administration of the recombinant AdHu5 vaccine was provided before (as part of a prime-boost regimen or alone) or at the time of the infectious challenge. AdHu5 treatment modulated specifically the $CD8^+$ TE cells to express lower levels of CD95 (FAS) and become resistant to CD95-induced apoptosis. The determination of the distinct adhesion/activation receptors on the surface of the $CD8^+$-specific T cells elicited by either infection or recombinant AdHu5 immunization showed very limited differences that were almost exclusively confined to the apoptotic receptor CD95 (**Figure 2**).

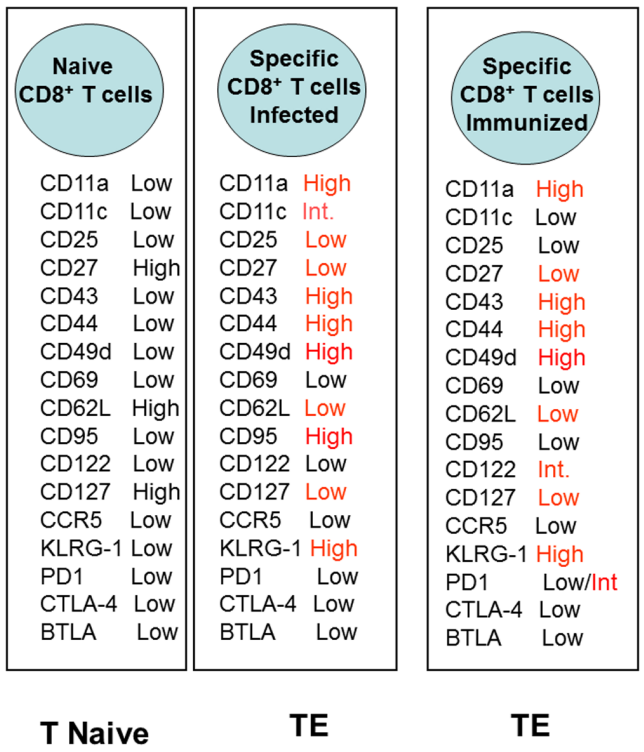


**FIGURE 2 | Expression of distinct adhesion/activation receptors on the surface of specific CD8$^+$ T cells elicited by either *T. cruzi* infection or recombinant AdHu5 immunization (Vasconcelos et al., 2012).** Mice were infected s.c. with *T. cruzi* (150 blood stream trypomastigotes) or immunized i.m. with AdHu5 ($2 \times 10^8$ pfu) expressing the gene encoding the amastigote surface protein-2 of *T. cruzi* 19 days earlier.

Despite the above results, we have not ruled out that other pro-apoptotic signaling pathways might also be altered. In these immunized and challenged mice, the CD8$^+$ T cell population expanded largely and protected mice against an otherwise lethal infection (Vasconcelos et al., 2012). An important mechanism that controls the expression of CD95 on the surface of specific CD8$^+$ T cells has just been described during development of immune responses to viral infections. TCR activation led to an increase of the RIG-I-like receptor LGP2 expression which down-regulated CD95 expression. In KO mice, the absence of this molecule significantly increased the apoptosis reducing the effectiveness of the immune response and resistance to the viral infection (Suthar et al., 2012). We consider this a promising area of study because it suggests that perhaps the control of T cell survival might be the key element for the development of new or improved vaccines. The proposed pathway of specific CD8$^+$ T cell activation following prime-boost vaccination and infection is shown in **Figure 3** (based on Vasconcelos et al., 2012).

### REDUCED IMMUNOLOGICAL EROSION OF SPECIFIC CD8$^+$ T CELLS

Memory CD8$^+$ T cells are not a stable pool; subsequent infections may cause attrition and erosion of the memory T cell pool (Selin et al., 1996, 1999; Dudani et al., 2008; Huster et al., 2009; Schmidt and Harty, 2011). However, different heterologous prime-boost regimens generated a pool of CD8$^+$ T cells that was not eroded by subsequent viral infections (Vezys et al., 2009; Rigato et al., 2011). After a vaccination regimen consisting of recombinant plasmid DNA prime-AdHu5 boost, we observed that viral infections had limited impact on the number or quality of the TEM cells as measured by different functional immunological assays. Most importantly, protective immunity mediated by these CD8$^+$ TEM cells was not altered in these mice (Rigato et al., 2011).

In contrast, using a different prime-boost vaccination protocol consisting of dendritic cells coated with circumsporozoite protein peptide and a booster immunization with recombinant *actA–inlB*-deficient *Listeria monocytogenes* expressing the same epitope elicited an immune response that could be eroded by multiple subsequent infections (Schmidt and Harty, 2011). The discrepant results highlight the importance of determining the characteristic of each TEM elicited by the distinct regimen of vaccination, as suggested earlier (Flatz et al., 2011).

The resistance to immunological erosion is one more interesting characteristic of these long-lived TEM cells that has been poorly explored and may have an important impact in the development of efficient vaccines. As mentioned above, this high-resistance immunological erosion can be linked to the differential expression of surface molecules (death receptors such as CD95/FAS) or apoptosis mediators (such as Bim).

### UNKNOWN FATE OF THE SPECIFIC CD8$^+$ TEM CELLS

Although some studies described that theses TEM can be long-lived in some mouse models, it is not clear what will be the fate of these cells on extended periods of time. Will they become TCM by upregulating CD62L? Will they simple die? Can they be boosted? Perhaps these questions can be addressed by using the gzmBCreERT2/ROSA26EYFP transgenic mouse line (Bannard et al., 2009). Labeling specific TE CD8$^+$ T lymphocytes will allow us to follow theses cells for longer periods of time.

Also, their long-term fate in NHP or humans is even more important for the purpose of development of practical vaccines. Due to the obvious constrains, few studies so far have addressed this issue.

## CONCLUDING REMARKS AND PERSPECTIVES

Genetic vaccination using heterologous prime-boost regimen protocols has the potential to serve as the basis for the development of new vaccines against many pathogens. In spite of the progress over the past 20 years, much work is required to improve the relevance of vaccine research, considering that human immune response is still at least one order of magnitude lower than that observed in mouse or even NHP models.

Several areas can be pursued for this matter. So far, protective immunity is clearly associated with the presence of a high number of long-lived specific polyfunctional TEM cells. Nevertheless, several points should be considered. First, TEM may vary from one protocol to the other. It is also important to improve the number of specific TCM cells, for example, by using pharmacological modulators (Li et al., 2012; Takai et al., 2012). However, it will be a challenge to increase the number of TCM cells without reducing the number of TEM cells. Fine tuning the TEM

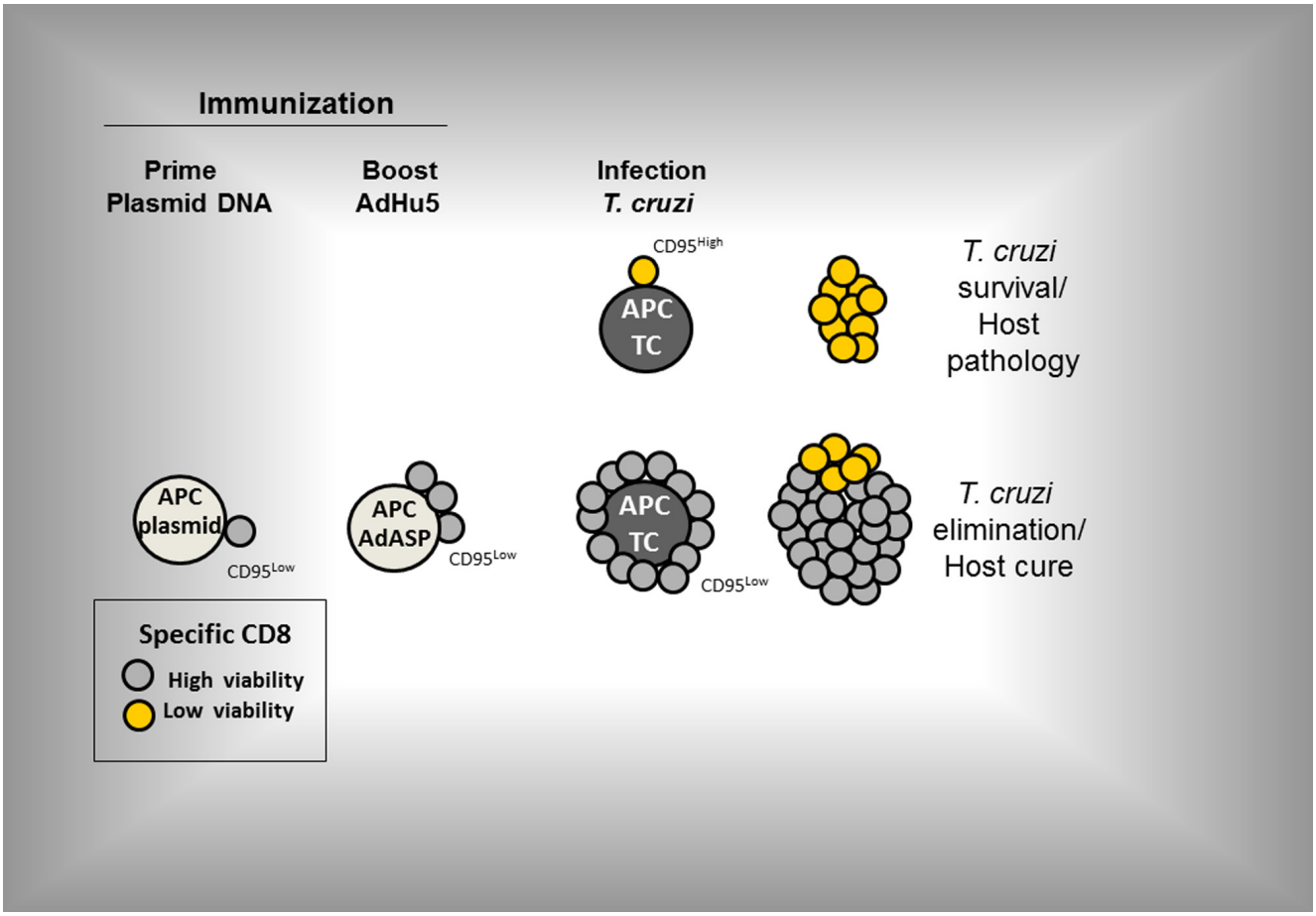


**FIGURE 3 | The proposed pathway of activation of specific CD8$^+$ T cells following prime-boost vaccination and infection (based on Vasconcelos et al., 2012).** Prime-boost regimen was performed as detailed described by Rigato et al. (2011). Mice were primed i.m. with plasmid DNA (100 μg) and boosted 21 days later with AdHu5 ($2 \times 10^8$) both expressing the gene encoding the amastigote surface protein-2 of *T. cruzi*. Mice were infected s.c. with *T. cruzi* (150 blood stream trypomastigotes).

subpopulations and increasing the number of TCM cells could further improve and prolong protective immunity beyond the levels currently achieved. Furthermore, new strategies to broaden the epitopes recognized by protective T cells would also improve both the quantity and the quality of the immune response. Finally, the circulation of these cells should be further studied for the purpose of personalizing the vaccination regimen for different types of infections, considering that the site of entry of each pathogen will vary, as will the localization of the T cells at the time of the infection.

In summary, the study of the control of memory T cell generation, maintenance, quality, and recirculation after distinct heterologous prime-boost vaccination regimens will provide important clues regarding the physiology of lymphocytes and the immune system, with potential applications in public health.

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**Conflict of Interest Statement:** Mauricio M. Rodrigues and Oscar Bruna-Romero are named inventors on patent applications covering *Trypanosoma cruzi* vectored vaccines and immunization regimens. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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