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
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., 2004; Gilbert et al., 2006; Aisoo 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; Moorothy et al., 2004; Keating et al., 2005; Vuda et al., 2005; Ceberio et al., 2006; Dunachie et al., 2006; Goonettekere 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; Acirno et al., 2006; Lertvin 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; Martinus 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; Iosko et al., 2010; Koop 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; Winstead et al., 2011), (ii) genes encoding multimeric proteins (Lahaashe et al., 2011), (iii) boost of adenovirus-immunized animals with an optimized plasmid DNA (Hurtink 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 regimen 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 anti-body 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; Schneid et al., 1998; Sedegah et al., 1996; Beuta-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, MP1-α, 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, intraarterital 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
be performed using genetically deficient mice, in the absence of either IFN-γ 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 immune 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; Choppa 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 AdHu5 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 (Domínguez 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 (Domínguez et al., 2011). These results unequivocally demonstrate the importance of the immune response 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 (Voipori 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 memory (TE), (ii) 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.

They have been 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 cell 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 naive 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, 2011).

**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 × 10^10φ) 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.
As mentioned above, after an intense immune response and CD95-induced apoptosis upregulate surface CD95 expression and are refractory to anti-PDL-1 (B7-H1) on antigen-activated CD8+ T cells. In contrast, increased expression of other receptors and TRAIL, might also play a role during the survival of specific TEM cells. In contrast, increased expression of other receptors that control T cell death, such as TNF receptor receptor and Fas, might be important for maintaining these long-lived CD8+ T 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 CCR5 and CCR3 (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+ T cells. After recombinant AdHu5 vaccination, CD8+ T 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 regime alone) or at the time of the infectious challenge. AdHu5 treatment modulated specifically the CD8+ T 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 protective receptor CD95 (Figure 2).
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 expanded largely and protected mice against an otherwise lethal infection (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-actin-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 different expression of surface molecules (death receptors such as CD95/FAS or apoptosis mediators (such as Bim)).

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 TEM 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 TEM cells without reducing the number of TEM cells. Fine tuning the TEM cell activation following prime-boost vaccination and infection is shown in Figure 3 (based on Vasconcelos et al., 2012).

**T Naive**

**Specific CD8\(^+\) T cells infected**

**Specific CD8\(^+\) T cells Immunized**

**CD11a Low**

**CD11a High**

**CD43 Low**

**CD43 High**

**CD69 Low**

**CD69 High**

**CD95 Low**

**CD95 High**

**KLRG1-Low**

**KLRG1-High**

**PD1 Low**

**PD1 High**

**CTLA4 Low**

**CTLA4 High**

**BTLA Low**

**BTLA High**

**Figure 3** | 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 i.c. with T. cruzi (100 blood stream trypomastigotes) or immunized i.m. with AdHu5 (2\(^{10}\)pfu) expressing the gene encoding the amastigote surface protein-2 of T. cruzi 19 days earlier.

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**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.

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.
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 × 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).

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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