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Pre-existing immunity against Ad vectors
Humoral, cellular, and innate response, what's important?

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Pre-existing immunity against human adenovirus (HAd) serotype 5 derived vector in the human population is widespread, thus hampering its clinical use. Various components of the immune system, including neutralizing antibodies (nAbs), Ad specific T cells and type I IFN activated NK cells, contribute to dampening the efficacy of Ad vectors in individuals with pre-existing Ad immunity. In order to circumvent pre-existing immunity to adenovirus, numerous strategies, such as developing alternative Ad serotypes, varying immunization routes and utilizing prime-boost regimens, are under pre-clinical or clinical phases of development. However, these strategies mainly focus on one arm of pre-existing immunity. Selection of alternative serotypes has been largely driven by the absence in the human population of nAbs against them with little attention paid to cross-reactive Ad specific T cells. Conversely, varying the route of immunization appears to mainly rely on avoiding Ad specific tissue-resident T cells. Finally, prime-boost regimens do not actually circumvent pre-existing immunity but instead generate immune responses of sufficient magnitude to confer protection despite pre-existing immunity. Combining the above strategies and thus taking into account all components regulating pre-existing Ad immunity will help further improve the development of Ad vectors for animal and human use.

Introduction

The great majority of commercially available vaccines, such as the tetanus, measles and yellow fever vaccines, are designed to induce robust humoral responses. Neutralization titers or index above 0.01, 200mIU/ml and 5 are indicative of protective immunity against tetanus,[1] measles[2] and yellow fever,[3] respectively, in vaccine recipients. However, for protection against numerous disease causing agents such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), *Plasmodium* parasites, *Mycobacterium tuberculosis*, Lassa virus, Ebolavirus and Marburg virus, induction of both humoral and cellular immune responses are desirable. As a result, considerable efforts are put into developing vectors capable of generating both cellular and antibody (Ab) responses against the target gene (transgene).

Adenovirus (Ad) derived vectors have been shown to successfully elicit strong cellular and humoral immune responses in rodents, non-human primates (NHP) as well as in humans after a single injection.[4-7] In preclinical studies, among all human Ad vectors tested, those derived from human adenovirus serotype 5 (HAd5) have emerged as the gold standard for immunization due to their superior immunogenicity. As a result, earlier clinical trials were almost exclusively performed using HAd5 vectors.[8] However, although HAd5 vectors were highly efficacious in pre-clinical studies, they did not perform as anticipated in clinical trials due to pre-existing HAd5 immunity in the participants from natural exposure. Adenoviruses are some of the pathogens that can cause the common cold and a significant proportion of the human population have antibodies against these viruses due to past infections naturally acquired in the community, notably during childhood.[9,10] These natural exposures can be responsible for long-lasting immunity (pre-existing immunity) that can interfere with HAd-based vaccines. In a phase I trial, individuals with pre-existing HAd5 immunity mounted lower immune responses compared with participants without pre-existing immunity.[5] Pre-existing immunity to HAd5 was even associated with undesirable effects beyond neutralization of the benefits intended from vaccination. The negative impact of pre-existing HAd5 immunity was illustrated in the infamous STEP trial during which a lower frequency of individuals with high HAd5 Ab titers developed a cellular response to the transgenes (HIV Gag, Pol and Nef). Furthermore, pre-existing HAd5 immunity was associated with an increase in HIV acquisition in vaccinated participants in comparison to the placebo.[7] Pre-existing HAd5 immunity was not taken into account in pre-clinical studies as animals used in these studies, such as mice and NHPs, are not naturally infected by HAd5 or other human Ads. In contrast, the percentage of the African, European and American population with neutralizing antibodies (nAbs) against HAd5 has been documented to be between 65–100, 61 and 37–70%, respectively.[11-14]

Since realizing the negative impact of pre-existing Ad immunity in early clinical trials, better understanding and ultimately circumventing pre-existing immunity has been a major focus in the development of new Ad based vaccines. In order to reach these goals, pre-existing immunity has been artificially generated

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in animal models by immunization with HAd vectors either lacking a foreign gene or encoding an irrelevant one. In addition, in order to determine the contribution of Ad specific T cells and Ab in pre-existing immunity, T cells or serum from Ad immune animals can be transferred in naïve ones. This review will first summarize the contributions of the different arms of the immune system toward pre-existing immunity against Ad vectors. Then, the mechanisms of evasion of the main strategies developed to circumvent pre-existing Ad immunity will be reviewed.

Components of pre-existing Ad vector immunity

Work on pre-existing Ad immunity revealed that nAbs, Ad specific T cells, as well as Ad induced inflammatory responses, all contribute to different extents to reducing Ad vector efficacy in pre-immune subjects.

Humoral responses

The importance of nAbs in reducing Ad vector immunogenicity has been extensively demonstrated. Passive transfer of serum from Ad immune mice or purified nAbs against Ad decreases Ad transgene expression, as well as transgene specific cellular and humoral response in rodents.[15,16] Depletion of Ab against the three main Ad capsid proteins (fiber, penton, hexon) by affinity chromatography in Ad immune serum, greatly reduces the inhibitory effect of these sera on vaccine vector efficacy, as demonstrated by an almost complete restoration of transgene expression level.[15]

Although nAbs targeting the main 3 capsid proteins have been detected in vitro,[15,17-19] they do not equally contribute to Ad vector neutralization in vivo. The relative contribution of each subset of nAbs has been teased apart by vaccinating Ad pre-immuned animals with capsid chimeric Ad vectors. From these studies, nAbs targeting the hexon protein appears to play a dominant role in vivo.[18-22] Hexon specific nAbs are directed against exposed loops on the surface of the virus particle. These exposed loops are also known as hypervariable regions (HVR). Replacing the entire HAd5 hexon sequence[22] or simply the exposed epitopes of HAd5 hexon[21] with hexon or HVR from a different serotype, was sufficient to overcome pre-existing HAd5 immunity in rodents. In addition, a three amino acid substitution mutation in one of the HVR was able to greatly reduce in vitro neutralization by a NHP polyclonal serum raised against chimpanzee Ad serotype 68 (ChAd68) derived vector.[19] Conversely, addition of the hexon domain of simian Ad serotype 23 (SAd23/Pan6) into a SAd24/Pan7 vector rendered the chimeric vector susceptible to pre-existing SAd23/Pan6 immunity, as illustrated by reduce transgene expression in rodents.[18]

In contrast, nAbs against the Ad fiber only play a limited role in in vivo neutralization. NAbs generated after a single HAd7 injection can only weakly neutralize chimeric HAd5 vector expressing HAd7 fiber, indicating that most nAbs were generated against other capsid proteins. Conversely, nAbs generated after a single HAd5 injection can readily neutralize chimeric HAd5 vector expressing HAd7 fiber.[23] In vivo, pre-existing ChAd6 immunity inhibited expression of the transgene from chimeric SAd24/Pan7 vectors that possess the ChAd6 hexon protein but not those that possess the ChAd6 fiber protein.[18] Passive transfer of mouse serum containing Abs against a chimeric HAd35 expressing HAd5 fiber, did not impact cellular and humoral immune responses against the transgene generated after HAd5 injection.[20] One caveat of the above studies is that in vivo, nAbs targeting the fiber are less common after a single injection[23] but are more readily detectable after two or more immunizations with the same adenovirus.[15,17,20] However, in most animal studies, pre-existing immunity is generated by a single Ad injection. The induced Ab response is probably of limited breath and neutralizing titer compared with humoral responses generated in individuals after repeated natural infections with replication competent Ad. As a result, these studies may underestimate the role played by nAbs targeting Ad fiber; especially as nAbs against Ad fiber and penton can act synergistically.[17]

Although nAbs against Ad strongly reduce Ad vector immunogenicity, they are serotype specific with limited to no cross-neutralization of different Ad serotypes (**Fig. 1A**). The lack of cross-neutralization is due to high sequence heterogeneity of targeted epitopes such as hexon HVR or fiber knob.[19,21,23,24] However, non-neutralizing but cross-reactive Ab are also generated, especially after repeated Ad injections.[25] These non- neutralizing Abs can also reduce transgene expression, presumably using various Fc receptor dependent (such as antibody dependent cellular cytotoxicity, complement mediated lysis or opsonisation) and independent mechanisms. The impact of non-neutralizing Abs was elegantly illustrated by Pichla-Gollon and colleagues who demonstrated that passive transfer of an Ab able to bind, but not neutralize, could reduce transgene expression as well as immunogenicity of a modified Chad68 vector.[26] The inhibitory effect of cross-reactive but non neutralizing Ab is of particular concern, as these Abs seem to be readily inducible.[25]

Cellular responses

In addition to nAbs, Ad specific T cells also decrease vector efficacy. Despite the fact that passive transfer of either serum or T cells from HAd5 immune mice can significantly decrease cellular and humoral response against the insert after HAd5 immunization,[16] less attention has been given to the role of Ad specific T cells in pre-existing Ad vector immunity. Although, passive transfer of nAbs induced a greater inhibition of HAd5 induced immune response, they did not inhibit Ad immune stimulation to levels observed in Ad5 pre-immune mice,[16] pointing at the contribution of Ad specific T cells in dampening HAd immunogenicity in pre-immune subjects. Depletion of T cells in Ad immune animals would provide a more accurate evaluation of T cell contribution as both circulating and tissue-resident T cells will be taken into account.

Ad specific T cell inhibition of vector efficacy is important due to their cross-reactivity. Indeed, unlike nAbs, Ad specific T cells are able to recognize a broad range of HAd from different subgroups (**Fig. 1B**). Several groups have shown that human CD4 and CD8 T cells predominantly recognize conserved epitopes in the adenovirus hexon, especially in the C-terminal region,[27-31]

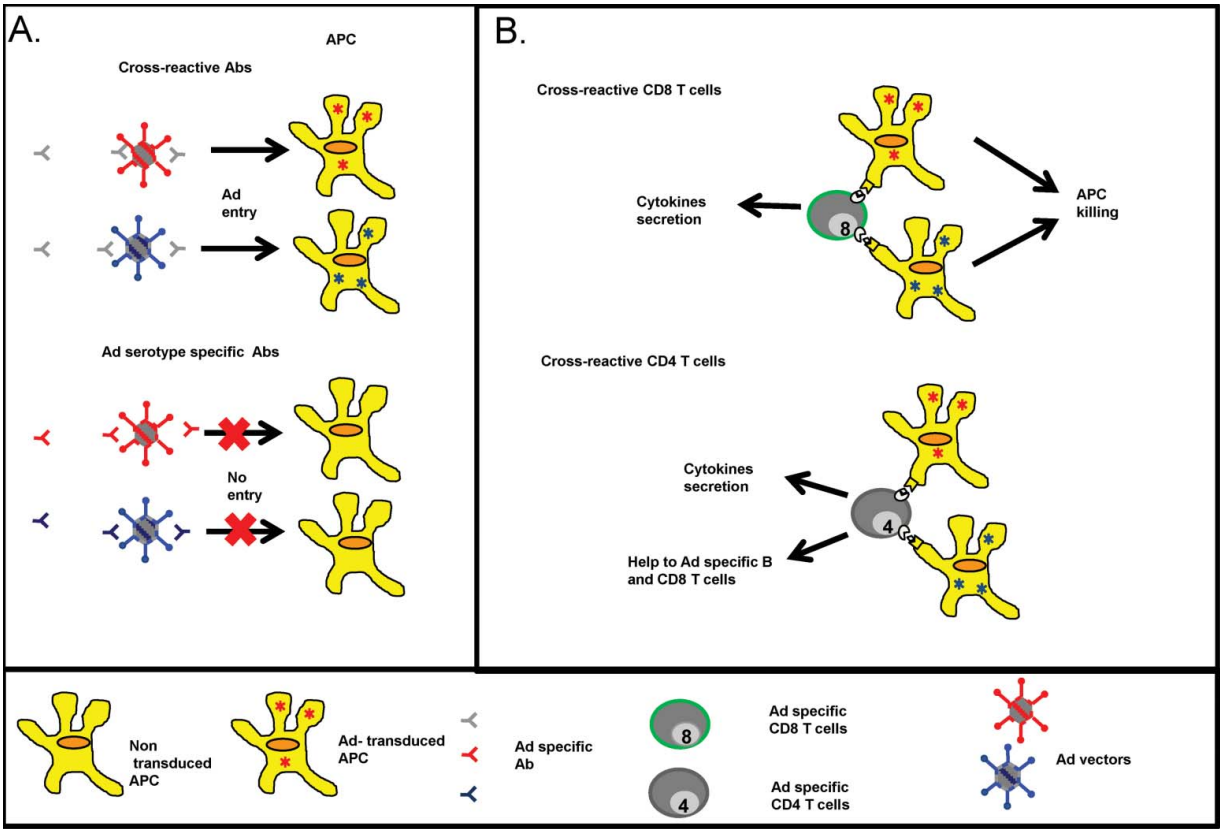

Figure 1. Humoral and cellular recognition of Ad vector. (**A**) Cross-reactive Ad specific Ab (gray) can bind to conserved regions (depicted in gray) within various serotypes but they lack neutralizing ability. In contrast, neutralizing Ab (red, blue) against Ad vector pre-dominantly target highly variable regions (depicted in red and blue) in the hexon and to a lower extent in the fiber. These nAbs are unable to neutralize Ad from different serotypes. (**B**) Cross-reactive (gray) Ad specific CD8 (top) and CD4 (bottom) T cells are generated upon Ad vector immunization. CD8 T cells mediate killing of Ad transduced APC and secrete inflammatory cytokines, while Ad specific CD4 T cell secrete various cytokines and provide help to Ad specific CD8 and B cells.

with a limited number of T cells targeting the fiber and penton region.[29,30] In addition to being widely cross-reactive, Ad specific T cells are detected in the great majority of tested individuals. Ad5 reactive CD4 or CD8 T cells were detected in 80% to 100% of subjects in Amsterdam, Netherlands[30,32] and in all subjects in Philadelphia, USA.[29,33,34] It is worth noting that these individuals were not screened for pre-existing Ad immunity using nAbs.

Due to the wide distribution of Ad reactive T cells, pre-existing Ad immunity might be more widespread than previously thought. Indeed, most studies solely rely on the presence of nAbs against Ad to assess seroprevalence. However in a few studies, both nAbs and cellular response against Ad were assessed and a higher proportion of individuals possessed T cell responses compare with nAbs against Ad. For example, in a study by Calcedo and colleagues, only 28% of tested individuals had neutralizing Ab against HAd5 whereas all of these individuals had Ad specific T cell responses as measured by Enzyme-Linked ImmunoSpot (ELISPOT).[33] Similarly, a higher proportion of individuals had Ad specific T cell responses (64%) compared with neutralizing Ab against HAd5 (55%) in another study.[35]

In addition to being widely distributed and cross-reactive, Ad specific T cells were mainly effector memory T cells that could readily be reactivated in vitro[28] and were polyfunctional.[33,34] This high potency may result from frequent reactivation by infection with different Ad serotypes.[28,33,34] Taken together, due to their ability to cross-react with Ad from different sub-groups, their wide-distribution in the human population and their high potency, the impact of Ad specific T cells cannot be overlooked when developing future Ad vectors.

Innate immune responses

In addition to Ad specific T and B cells, innate immune responses to Ad vectors can also dampen Ad efficacy, especially in the context of pre-existing immunity. The innate immune response does not possess immunological memory, with the exception of NK cells when previously activated by haptens[36] or cytokines.[37] However, despite this lack of "conventional" memory, Ad induced innate immune responses can regulate Ad immunogenicity.

Ad vectors, like other viral vectors can activate the innate immune response upon injection. Various pattern recognition receptors (PRR) including retinoic acid-inducible gene 1 (RIG-I), nucleotide oligomerization domain (NOD)-like receptors (NLR), factor X, and Toll-like receptors (TLR) 4 and 9 are

involved in Ad recognition. In addition, Ad binding to the Coxsackievirus and Adenovirus receptor (CAR), as well as integrins, are also involved in this process.[38-41] Upon activation, the above PRR molecules trigger signaling pathways such as NF-KB, which can induce the production of numerous pro-inflammatory mediators including cytokines (interferon (IFN) α/ β, interleukin (IL)-1α/ β, tumor necrosis factor (TNF)a, and IL-6) and chemokines (IL-8, macrophage inflammatory protein (MIP)-1α/β, IP-10, RANTES, and monocyte chemoattractant protein (MCP)-1).[38-40] Among these pro-inflammatory molecules, IL-1 and its receptor IL-1R1, as well as type I IFN, play a central role as they induce positive feed-back loops that in turn, produce additional pro-inflammatory mediators.[42-44]

The role of innate immunity on antigen expression and vector efficacy after Ad immunization was revealed by comparing different HAd of various immunogenicities. It was first observed that when compared with the benchmark HAd5, both in vitro and in vivo, HAd vectors with lower immunogenicity such HAd35, HAd26 and HAd48,[12,45,46] induce greater inflammatory responses, as illustrated by higher levels of inflammatory cytokines (IFNα, IFNγ, IL-6) and chemokines (IP-10, I-TAC, MIP1α/β).[47,48] In addition, HAd35 and HAd28 also induced stronger NK cells activation. Stronger IFNα dependent NK cells activation can lead to lower transgene expression and persistence in vitro, due to NK killing of Ad transduced monocytes.[48]

The inverse relation between NK cell activation, type I IFN production and transgene expression were confirmed in vivo. For example, in rodents, high type I IFN levels were associated with lower transgene expression. Indeed, compared with HAd5, ChAd68 induces a higher level of type I IFN but lower transgene expression in vivo.[49,50] Furthermore, higher transgene expression was detected after ChAd68 and HAd5 injection in IFN-α/β-R knockout mice when compared with the same vectors in wild type mice.[42,50,51] Similarly, NK depletion prior to HAd5 immunization increases transgene expression level while NK transplantation had the opposite effect.[51] The above studies indicate that Ad vectors inducing higher inflammation and NK activation have lower transgene expression and persistence. Of note, type I IFN and NK cells are also able to control the expressed level of antigen, which influences the T cell response in mice models of mouse cytomegalovirus (MCMV) infection.[52,53] In individuals or animals with pre-existing Ad immunity, high inflammatory responses will further reduce transgene expression and persistence due to Ad specific CD8 T cell recruitment to the site of inflammation and subsequent killing of Ad transduced antigen presenting cells (APC) (**Fig. 2**).

It is worth noting that although excessive innate immune responses are detrimental to Ad vector immunogenicity, inflammatory responses are still required to trigger a robust adaptive response and therefore a good vaccine-derived immune response. The requirement for inflammatory responses was illustrated with IFN-α/β-R knockout mice. In these studies, depending on the immunogenicity of the transgene, knockout mice failed to mount cellular responses[42,51] or mounted lower responses after Ad5 immunization.[49,50]

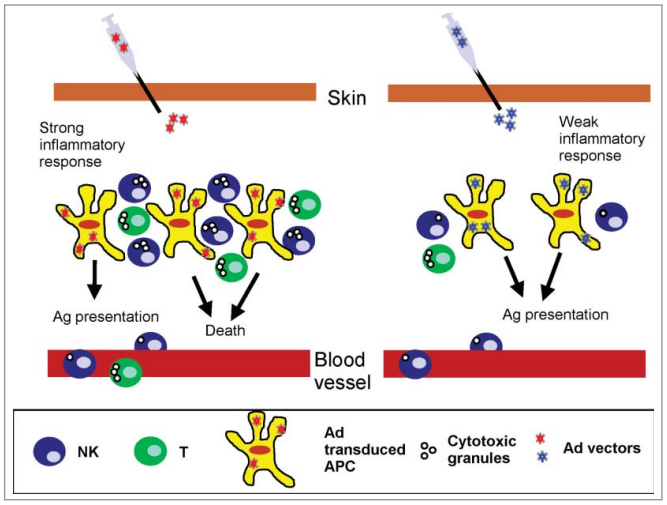

Figure 2. Strong innate immunity. Strong Ad induced inflammatory responses result in higher levels of chemokines that in turn recruit APC, NK cells, and CD8 T cells, among other lymphocytes. Recruited NK cells are activated by the cytokine milieu. Activated NK cells, as well as Ad specific CD8 T cells, mediate lysis of Ad transduced APCs leading to lower and shorter transgene expression as well as lower cellular and humoral response against the transgene. Milder inflammatory responses result in lower chemokine levels and lower NK cell recruitment and activation. Concomitantly, lower numbers of Ad specific CD8 T cells are recruited. As a result, Ad transduced APCs are not killed, transgene expression is higher and persists for longer period of time, resulting in higher cellular and humoral response against the transgene.

Solutions to overcome pre-existing HAd immunity

Ad vectors based on alternative adenoviruses

Low seroprevalence human Ad

Due to the importance of nAbs in dampening Ad vector efficacy, Ad vectors were developed using human Ad with low seroprevalence in the human population. Among these vectors, both HAd35 and HAd26 vectors have been tested and shown to be safe in phase I clinical trials.[54-56] However, compared with HAd5, human serotype vectors based on low seroprevalence, including Ad35 and Ad26, have shown lower immunogenicity[12,46,57-59] and protection efficacy in the non-human primate (NHP) model of Ebola virus infection,[45] therefore questioning their clinical utility. In addition to lower immunogenicity, the protection efficacy of vectors based on low seroprevalence serotypes will probably also suffer from pre-existing Ad specific T cells. Although HAd5 specific neutralizing Ab do not cross-react with HAd35,[60] HAd2 specific T cells, which are common in the human population, were able to cross-react with HAd35. Furthermore, HAd35 cross-reactive T cells were detected in individuals that lack nAbs against HAd35.[61]

Animal derived adenoviruses

Due to the lower immunogenicity of low seroprevalence HAd derived vectors, attention shifted toward the development of vectors based on animal Ad. Among these, chimpanzee derived Ad

vector (ChAd) are the most studied. NAbs against simian Ad vectors are relatively rare. For example, nAbs against ChAd7 are detected in less than 15% of American, European, Chinese and African populations. The frequency of nAbs against ChAd6 are similar in these populations except in Africa where it peaks below 40%.[14,46,62] Furthermore, unlike rare human Ad serotypes, ChAd have demonstrated great efficacy in pre-clinical studies including ChAd7 in a mouse model of EBOV infection,[63] ChAd68 (SAd-24) in a NHP model of rabies,[64,65] ChAd63, ChAd7 or ChAd9 in combination with modified vaccinia Ankara (MVA) boost in a malaria mouse model,[66,67] and ChAdOx1 in a rodent model of Rift Valley Fever (RVF).[68] In addition, in Phase I clinical trials against Malaria and HCV, ChAd63[46] and ChAd3,[69] respectively, were found to be safe and able to generate robust, broad and polyfunctional T cell responses against the transgene.

Although ChAd vectors appear very promising, cross-reacting pre-existing cellular immunity against Ad should not be overlooked. Indeed, in vitro, HAd specific T cells cross-react with ChAd vectors, such as ChAd6 and 7.[34] Similarly, Ad specific T cells from a human cohort recognize HAd5, HAd4 as well as simian derived Ad (SAd-24/ChAd68 and SAd-32) to a similar extent.[33] This could be expected due to the close phylogenetic proximity between human and chimpanzee Ad,[46,70] more specifically to the highly conserved hexon C-terminal sequences that are recognized by Ad cross-reactive T cells.[27-30,33] The inhibitory role of Ad specific T cells on ChAd vectors was confirmed in animal models. In rare occasion, such as in the mouse model of malaria infection, pre-existing immunity to HAd5 reduced protection mediated by ChAd7 or ChAd 9 immunization.[66] However, in most animal models, although ChAd induced protection was not impacted by pre-existing HAd5 immunity,[46,63,65,71] ChAd induced immune responses tend to be lower in the presence of pre-existing immunity to HAd5. It is worth noting that the animal models mentioned above might underestimate the importance of cellular immunity. In pre-clinical studies, pre-existing immunity is usually generated by a single injection with a replication deficient Ad vector.[46,63,65,71] This artificial pre-existing immunity may not recapitulate the potent Ad specific T cell response observed in humans following repeated exposure to replication competent Ad that initiate productive infections. Indeed, in primates, multiple exposures of replication competent Ad over time gives rise to polyfunctional and highly potent T cells.

In addition to chimpanzee Ad vectors, bovine, porcine, ovine, canine and fowl derived Ad vectors are currently in preclinical stages of development.[72] NAbs against these vectors, including porcine Ad serotype 3 (PAd3), bovine Ad serotype 3 (BAd3) and Ovine Ad serotype 7 (OAd7), have not been detected in the human population.[73,74] Similar to simian Ad vectors, cross-nAbs against PAd3 and BAd3 were not generated by mice immunized with HAd5.[25] Furthermore, little to no CD4 and CD8 T cells cross-reactive against PAd3 and BAd3 were detected in HAd5 immunized mice.[75] Finally, immunization with either BAd3 or Pad3-based vaccines protected both naïve and HAd5 pre-immune mice against influenza virus challenge. Both humoral and cellular immune responses were not impacted by pre-existing HAd5 immunity.[76,77] Similarly, transgene expression and cellular responses were not affected in HAd5 pre-immune mice after OAd7 and OAdV injection, respectively.[73,78] However, although promising results were obtained in rodents using non-primate Ad-derived vectors including BAd, PAd and OAd, their usefulness needs to be ultimately confirmed in humans. Whether Ad cross-reactive T cells can react with BAd and Pad still needs to be assessed as the cellular immune response is heavily influenced by MHC alleles, which are different in mice and humans.[75]

Varying immunization routes

In addition to using alternative Ad serotypes, pre-existing immunity has been overcome by varying the route of immunization. Indeed, mice with pre-existing HAd5 immunity generated by intranasal or intramuscular immunization did not experience reduced humoral responses upon subsequent oral immunization.[79] Similarly, in the NHP model of Ebola virus, HAd5 mediated protection after intranasal/intratracheal immunization was not impacted by pre-existing HAd5 immunity generated by intramuscular (IM) injection of an irrelevant HAd5 vector.[80] The lack of impact despite pre-existing immunity may be due to evasion from tissue resident Ad specific T cells.

Tissue resident memory T cells (T_{RM}) are memory CD8 T cells which, unlike effector memory CD8 T (T_{EM}) cells that circulate between blood and extra-lymphoid organs, do not circulate and remain confined to the tissue where the original infection occurred (For reviews see refs.[81-83]). T_{RM} have been shown to induce better protection compared with T_{EM}. Using parabiotic mice, skin resident T cells were shown to confer better protection against vaccinia virus skin infection compared with circulating memory T cells.[84] Similarly, T_{RM} induced a greater viral load reduction after skin and vaginal infection with Herpes simplex virus (HSV) compared with $T_{EM.}$[85] In addition, T_{RM} were still detectable in tissues after effector memory T cells were no longer visible in circulation.[86]

Tissue specificity of T_{RM} is mainly determined during priming by tissue derived migratory dendritic cells (DC). Migratory DCs from different tissues induce T_{RM} with different homing molecules and therefore different tissue specificity.[83] Various immunization routes result in antigen (Ag) presentation by distinct tissue derived migratory DC and therefore, generation of T_{RM} with different tissue specificity (**Fig. 3**). In the Richardson and colleagues study, IM injection probably did not generate T_{RM} in the lung. Subsequent airway immunizations (intranasal/intratracheal) would be affected by the previously generated Ad specific T_{EM} in the circulation but not by $T_{RM,}$ which would be absent from the lung. The ability to detect Ad specific T cells in human colon biopsies as well as in NHP gut (Ileum, colon and rectum) is supporting the existence of Ad specific T_{RM}.[33]

However, there are limitations in using different immunization routes to circumvent Ad pre-existing immunity. There are limited routes of immunization including intradermal, oral, intranasal/intratracheal, IM, sublingual and intravenous (IV). The latter may not be the best route for Ad vector immunization as one fatality has been reported in a gene therapy clinical trial.[87]

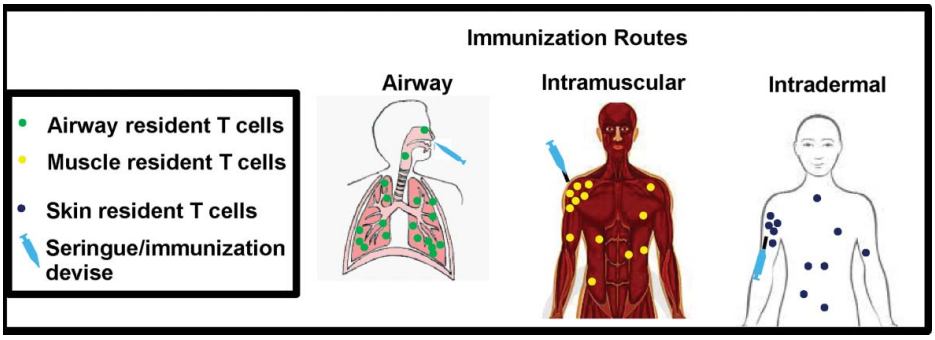

Figure 3. Model of tissue resident T cells induction by various vaccination routes. The majority of vector and insert specific tissue resident T cells will reside close to the site of immunization, in the tissue where the antigen was first encountered. Some of these resident T cells migrate away from the injection site while remaining within the same tissue. Depending on the immunization routes, the generated tissue resident T cells will reside in different tissues. Of note, circulating T cells are not depicted.

Furthermore, the route of natural Ad infections has to be considered in order to determine where putative Ad specific T_{RM} are to be located in humans. Natural Ad infections mainly target the respiratory tract, and to lower extend the eye, gut and urinary tract of infected individuals.[9,40] Therefore, Ad specific T_{RM} may be present in the lung and gastro-intestinal tract of Ad immune individuals, which may affect intranasal/intratracheal and oral immunizations, respectively. This will need to be evaluated experimentally in the future.

After infection or vaccination, Abs can readily migrate throughout the body, as demonstrated by systemic administration of monoclonal Ab to treat various solid cancers that are located in different tissues such as breast, colorectal, gastric and kidney.[88,89] However, Abs do not persist in all organs. Interestingly, nAbs against HAd5 could not be detected in the airway of a cohort of 51 volunteers naturally exposed to HAd, including those that possessed high levels of circulating HAd5 specific nAbs in the blood.[90] This is likely due to the rapid turnover of immune cells and IgA type antibodies in the airway, making Ad-mediated vaccination at this site promising.

Prime-boost regimens

Prime-boost regimens may also help to overcome pre-existing Ad immunity. Prime-boost regimens do not bypass pre-existing Ad immunity per se. Immune responses induced by prime-boost regimens are still decreased by pre-existing immunity; however they are of sufficient magnitude to still confer protection. Indeed, more robust immune responses are obtained after heterologous prime-boost regimens compared with single vaccination or homologous prime-boost immunizations.[91,92] This rule also holds true for heterologous Ad prime/boost regimens.[12,45,93-96]

The usefulness of prime-boost regimens in overcoming pre-existing Ad immunity was demonstrated more than a decade ago using several DNA primes followed by a single Ad boost. Yang and colleagues demonstrated that cellular responses generated by DNA primes and HAd boost (DNA/HAd5) were not affected by pre-existing HAd5 immunity. In addition, although generated humoral responses were significantly reduced by pre-existing immunity, the antibody titers obtained were similar to those generated in naïve mice immunized with homologous HAd5 prime-boost.[97] The ability of a DNA prime to circumvent pre-existing immunity was further suggested in a clinical trial. In tested individuals, pre-existing HAd5 immunity did not affect T cell responses induced by a DNA prime, HAd5 boost regimen, while pre-existing HAd5 immunity decreases cellular responses generated after a single HAd5 injection.[98]

In addition to DNA/HAd, heterologous prime-boost regimens involving Ad from different serotypes may also prove protective in HAd5 immune subjects. Pre-clinical studies involving ChAd68/ChAd1 prime-boost immunizations have shown encouraging signs. Indeed, although compare with naïve animals immune responses generated after ChAd68/ChAd1 prime-boost immunization were decreased in HAd5 pre-immune NHPs, stronger humoral responses were obtained compared with HAd5 homologous prime-boost.[99] Finally, it will be a great interest to determine whether prime-boost regimens including Ad vectors in combination with other vaccine platforms such as modified vaccinia virus ankara (MVA)are also able to provide protection despite pre-existing immunity. Indeed in pre-clinical studies, regimens involving MVA and Ad encoding *plasmodium berghei* CS protein or severe acute respiratory syndrome (SARS) S protein were able to induce very robust T cell and Ab responses of higher magnitude than Ad/DNA regimens expressing the same insert.[93,100] ChAd63/MVA prime-boost are even being tested in various stages of clinical trials against Malaria, HIV and HCV.[101] Results from these trials will be crucial in terms of determining the ability of ChAd/MVA prime-boost regimens to overcome pre-existing HAd immunity. It is worth noting that prime-boost immunization has also been used to overcome pre-existing MVA immunity.[102]

One attractive feature of developing prime-boost regimens to circumvent pre-existing immunity is that it does not require the development of additional Ad vectors. Existing Ad vectors, especially those with robust immunogenicity can be used. However, determining which of the numerous vaccine platforms available, including DNA, VSV, AAV, and MVA, are best suited for priming or boosting of Ad vectors will be crucial. In addition, prime-boost regimens are time consuming thus precluding the rapid induction of protective immunity, which can be desirable in emergency vaccination situations, for example against pathogens that emerge or re-emerge sporadically in unpredictable locations.

Conclusions

Tremendous effort has been made in circumventing pre-existing Ad immunity in the human population. An important focus

has been on the development of new animal based Ad vectors, especially Ad vectors of simian origin. While most of these vectors are in pre-clinical phases of development, some, including ChAd3 and ChAd63, have reached clinical trials. Selection of alternative Ad serotypes has been mainly driven by the absence of neutralizing Ab against these serotypes in the human population. However, the impact of Ad specific T cells on the efficacy of these vectors has been mainly overlooked. This is in part due to the complex nature of T cell assays that are designed to detect anti-Ad T cell responses. Compared with nAbs assays, T cell assays require complex stimulatory conditions and/or multi-parametric flow cytometry. The study of Ad specific T cell responses are further complicated by the presence of T_{RM} that do not circulate in the blood. Isolation of these T cells usually requires mechanical and/or enzymatic disruption of the tissue of interest. These isolation procedures can affect T cell viability and function, which further complicates analysis of T_{RM} cells. In addition, for obvious ethical reasons, isolation of T_{RM} cells is restricted to animal models for most tissues except the skin and gut. Although harder to obtain, information regarding both Ad specific circulating and tissue resident T cells is necessary for a full understanding of pre-existing immunity against Ad vectors and to select the most appropriate Ad vector for clinical use. Therefore, the ability of Ad specific T cells in the target population to cross-react with the Ad vector being developed (chimpanzee, porcine, bovine, ovine) should be systematically investigated.

In pre-clinical studies, alternating the immunization route has bypassed pre-existing Ad immunity. Immunization through the airway holds promise and may turn out to be a delivery method of choice for inducing potent and long lasting protective immune responses from an Ad vector. While the airway may be a viable solution to bypass pre-existing immunity, several remaining questions will need to be addressed. Despite the relatively rapid turnover of resident immune cells and IgA in the lungs, the effect of immune stimulation following natural Ad exposure or vaccination will be critical to determine whether the same or other Ad vectors can be re-administer in the airway, at what frequency, and according to what regimen (single dose vs. prime-boost). This knowledge should ultimately inform on the number of independent vaccines one antigenic Ad backbone could be used for. Ultimately, this will have to be determined in clinical studies.

In addition, heterologous prime-boost regimens involving Ad vectors should be further optimized. The immune response generated by prime-boost regimens may be sufficient to induce full protection despite pre-existing Ad immunity. Developing prime-boost regimens using Ad vectors where limited to no nAbs or cross-reactive T cells in the target population should further increase the chance of success. However, optimization of prime-boost regimens will be labor intensive due to the increasing number of available vaccine platforms. Useful data regarding the ability of prime-boost regimens to overcome pre-existing HAd immunity will certainly be generated by clinical trials involving ChAd/MVA prime-boost. Careful analysis of these trials should provide crucial protection information in the presence or absence of pre-existing Ad immunity. In addition, future clinical trials involving Ad vectors should also determine whether Ad-based vaccine regimens impact HIV acquisition. Although HAd5 immunization increased HIV acquisition in participants with pre-existing HAd5 immunity in the STEP trial, whether this trend holds true for other Ad vectors expressing different inserts needs to be systematically evaluated. Upcoming clinical trials performed in populations at high risk of HIV infection will address this critical issue.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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