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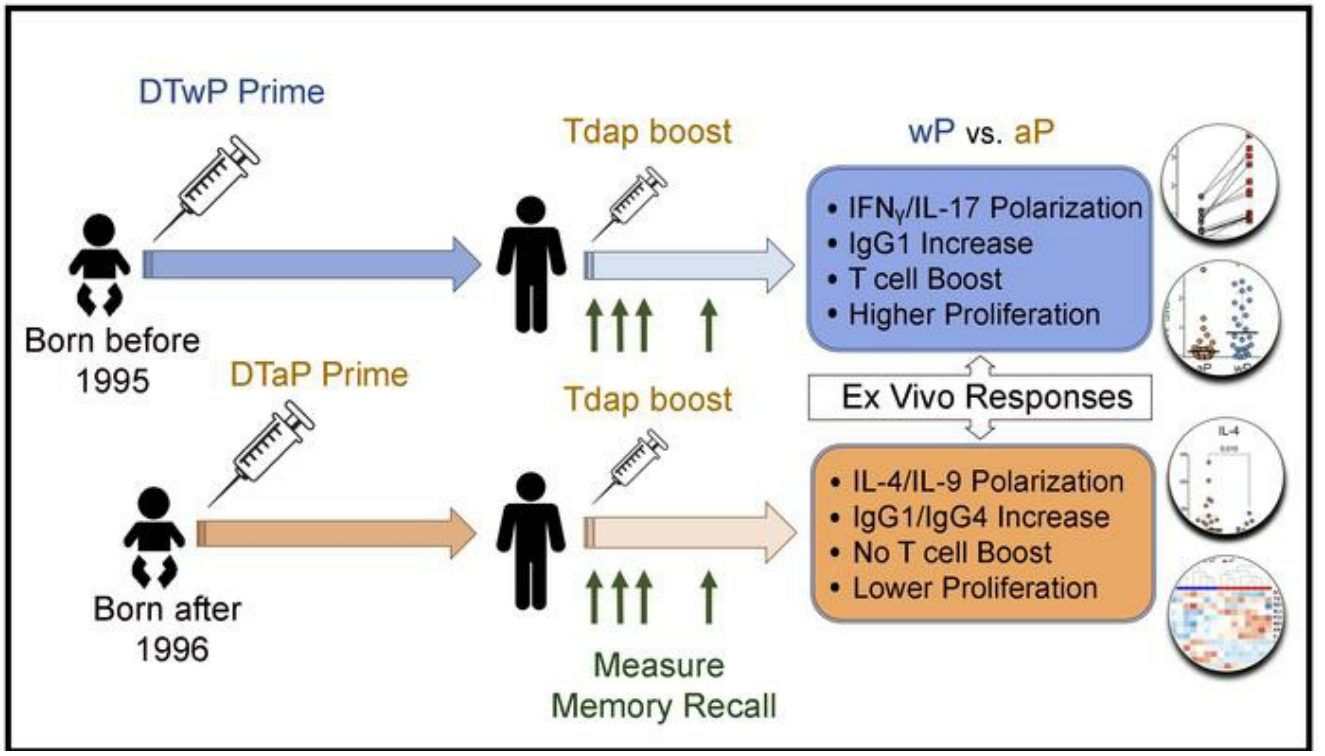
Graphical abstract



Th1/Th17-polarization persists following whole-cell pertussis vaccination despite repeated acellular boosters

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists

ABSTRACT

In the mid-1990s, whole-cell (wP) pertussis vaccines were associated with local and systemic adverse events, which prompted their replacement with acellular (aP) vaccines in many high-income countries. In the past decade rates of pertussis disease have increased in children receiving only acellular pertussis vaccines. We compared the immune responses to acellular pertussis boosters in children who received their initial doses with either wP or aP vaccines using activation-induced marker (AIM) assays. Specifically, we examined pertussis-specific memory CD4+ T cell responses ex vivo, highlighting a Type 2/Th2 versus Type 1/Th1 and Th17 differential polarization as a function of childhood vaccination. Remarkably, after a contemporary aP booster, cells from donors originally primed with aP were 1) associated with increased IL-4, IL-5, IL-13, IL-9 and TGF-β and decreased IFNγ and IL-17 production, 2) defective in their ex vivo capacity to expand memory cells and 3) less capable to proliferate in vitro. These differences appeared to be T cell-specific, since equivalent increases of antibody titers and plasmablasts after aP boost were seen in both groups. In conclusion, our data suggest that long lasting effects and differential polarization and proliferation exists between adults originally vaccinated with aP versus wP despite repeated acellular boosters.

INTRODUCTION

Before introduction of the pertussis vaccine, about 200,000 cases/year of whooping cough were reported in the USA alone (1). A licensed pertussis vaccine that was introduced in the mid-1940s substantially reduced disease in vaccinated infants (but not adults) and was credited for a gradual pertussis incidence decline that reached fewer than 3,000 cases per year during 1980-1990. The original vaccine, composed of whole, inactivated bacteria adjuvanted with aluminium salts (alum; wP) was phased out in the United States due to the association of prominent local and systemic adverse. However, wP is still used in many countries around the world. The wP was replaced by the acellular (aP) vaccine, a mixture of several different pertussis proteins and alum, which was universally adopted in the US in 1996 (2, 3). Additionally, pertussis vaccines have always been combined with diphtheria and tetanus toxoids since they were introduced for both the priming at infancy (DTwP or DTaP) or as booster vaccination henceforth (Tdap) (1). Recent years have seen a dramatic uptick in the incidence of disease in countries where aP vaccine is used exclusively despite effective initial protection (4-7). The reasons for this increase in disease incidence have not been fully elucidated and several potential explanations have been attributed. Indeed, evolutionary shifts favoring novel alleles for virulence factors, poor vaccination rates and/or vaccine refusals and detection bias due to enhanced diagnostic techniques have been proposed (8-11). Conversely, an emergent picture associated with differential mucosal immunity seems to offer a better explanation (9, 12-15), however human studies are lacking.

The effectiveness of pertussis vaccination and duration of immunity is thought to correlate with both antibody and T cell responses. Humoral responses to wP and aP have been characterized previously (16-21). Interestingly, protection against infection persists even after antibody titers have decreased (22-24), suggesting that a cellular component contributes to immunity to *B. pertussis*. Animal studies indicate that memory Th1 and Th17 CD4+ T cell responses to *B. pertussis* are required for long-lasting immunity, and significant responses in these subsets can be detected after wP vaccination and after infection (25-27). In humans, aP

vaccination was reported to induce a predominant Type 2/Th2 polarized response (28-31) and several studies have proposed qualitative differences in the phenotype of T cell responses, resulting in less effective and/or durable responses as immunological mechanisms to explain the decreasing efficacy of aP vaccination (32-35).

The recent resurgence of pertussis is particularly associated with children aged 6 to 11 years or adolescents and young adults, and has been linked to waning of the pertussis-specific immunity, despite the addition of a booster vaccination with the aP vaccine in this age bracket (15, 36-40). It would thus appear that some key difference(s) exist in pertussis immunity, as a function of the original childhood vaccination with aP compared to wP. Based on disease incidence, this difference is only revealed over one or two decades despite continued boosts of both populations with aP (4-5 additional aP vaccinations are routinely administered in childhood and adolescents, and emergency room tetanus vaccinations post-1996 in the US are often given with vaccines containing an aP component) (41, 42). This “waning immunity” is of great concern (37), and it is challenging to address because it manifests itself more than 15 years after the first immunization. Thus, it would be important to define the mechanisms associated with waning immunity, to guide modifications in vaccine composition, adjuvantation or schedules, and thus increase vaccine efficacy.

We recently characterized antibodies and CD4+ T cell responses to pertussis antigens in individuals originally vaccinated with either wP or aP using an in vitro and cross-sectional study approach (43). Notably, the differential Th polarization was maintained even in teenagers and adults. Since polarization is maintained for years after the original priming, even after identical boosting with aP (35, 43), data support the notion that wP priming enacts a differential molecular program in the vaccine-specific T cells, and that this imprinting is essentially life-long.

A head-to-head comparison of aP versus wP vaccination is not feasible in the USA because the wP vaccine formulations are no longer licensed. Here we compared T cell responses induced by original aP and wP priming (during the first years after birth), not immediately, but at

the time of waning, more than 15 years after the original priming, and following the additional aP boosts (administered from middle to high school). To specifically address any differences, (i.e., magnitude, polarization, memory compartments or capacity to respond to antigen stimulation), we measured responses ex vivo after aP booster to pertussis epitopes. A recent additional booster was taken as a “mimic” of how donors react when exposed to antigen in vivo. Importantly, these analyses revealed persistent differences at the level of T cell responses between individuals originally primed with aP versus wP.

RESULTS

Differential polarization of pertussis-specific memory CD4+ T cells as a function of childhood vaccination

In a first series of cross-sectional experiments, we examined whether the difference in polarization between aP and wP donors was still detected in adulthood. These donors were originally primed with either aP or wP, and were not, to the best of our knowledge, recently vaccinated at least in the last four years. The age, and other general characteristics of this first donor cohort are summarized in **Supplementary Table 1a.** By using a pertussis megapool stimulation together with activation-induced marker (AIM) and ICS assays, we were able to successfully detect and confirm, as previously reported (43), that Th polarization as a function of wP and aP childhood vaccination persists upon aP reimmunization in adolescents and adults (**Figure 1A**). Specifically, we measured IFNγ, IL-4 and IL-17 responses in the two donor cohorts 1-3 months after aP boosting, and calculated the number of total CD4+ T cells producing each cytokine. We observed a significant polarization toward IL-4 secreting cells in the aP vaccinated cohort and IFNγ and IL-17 in the wP vaccinated cohort. The separate determination of reactivity of pertussis epitopes derived from different antigens is of interest but was not performed, given the limited amount of cells available for analysis in several of the samples and that the separate testing would have required a fourfold higher number of cells. As expected, no difference in polarization of responses between the aP and wP cohorts was noted in the case of response to a megapool of epitopes derived from CMV and EBV, ubiquitous pathogens not included in the aP or wP vaccines (**Figure 1B**).

Overall these data highlighted a persistent polarization of T cell responses in adulthood decades after priming and despite continued aP boosting of both cohorts (from 1996 onwards as part of the recommend immunization schedule). The much stronger IL-17 polarization was

reported as being associated with protection and wP vaccination in mouse and baboon models, but had not been shown for humans (15, 26, 27, 44-46).

Original wP prime, but not aP prime, is associated with significantly higher number of pertussis specific CD4+ T cells after aP boost

Further studies focused on longitudinal analysis of T cell responses to aP booster in adolescents and adults originally vaccinated with either aP or wP. Specifically, we compared responses in 18-19 year olds originally primed with aP, to older individuals, originally primed with wP (**Supplementary Table 1b).** Ex vivo T cell responses were measured at baseline and 0.5-2 months after aP booster, to pertussis epitopes employing the AIM_{25} assay (CD25+OX40+) gating strategy (**Supplementary Figure 1**). The results in **Figure 2A** show that original wP prime, but not aP prime, was associated with significantly higher ex vivo CD4+ T cell responses after aP boost. Overall, for donors originally vaccinated 18 years (or more) earlier with wP, ex vivo responses were boosted by approximately 2.5-fold. In the case of the donors vaccinated with aP, no significant booster effect was noted at the level of T cell responses.

The responses of these donors were further analyzed over a 6-month period. This analysis demonstrated that the apparent lack of effective booster effect was not associated with differential kinetics of the responses (**Figure 2B**). In both cases, the booster effect already peaked at the 1-month time point, and waned afterwards (but is significant only for wP donors). These data could not determine whether the peak booster effect occurs before the 1-month time point, and thus the aP cohort might still be associated with a significant boost, but simply of brisker and more transient nature. To address this issue, we recruited a third cohort of donors, also originally aP or wP primed (**Supplementary Table 1c)**. In this cohort, responses were followed at days 1, 3, 7, 14, 30 and 60/120 post-boost. The results shown in **Figure 2C** indicate that the peak booster effect is indeed reached at the 1-week point for the wP donors, and that regardless, no significant boost is detected in the donors originally primed with aP.

Finally, we ruled out that the differences observed might be due to differences in age between the two cohorts, since the response in terms of booster effect tends to be inversely correlated with age in the wP cohort (opposite trend) (**Figure 2D**). It would be of interest to investigate in future studies whether the number of boosters or the timing of the boosters in relationship to the most recent booster makes a difference in the responses, since clinical studies indicate that the more boosters that are given, particularly in adolescence, the shorter duration of protection.

Memory subset composition of pertussis-specific T cells and their molecular markers as a function of aP vs. wP priming

To further characterize the responses, we analyzed the expression of CD45RA and CCR7 on the responding T cells. As expected, the response in both aP primed and wP primed donors, either before or after the aP boost, was mediated by memory T cells. A trend existed toward higher T central memory (TCM) composition in the case of the wP primed donors, especially after boost (**Figure 3A**). Further analysis demonstrated that indeed, the response to the boost observed in the case of the wP cohort was mediated by both T effector memory (TEM) and TCM, but more prominent in the case of the TCM subset (**Figure 3B**).

We reasoned that the differential responsiveness to the aP booster might be associated with differential expression of T cell markers commonly associated with T cell activation, apoptosis and exhaustion. To address this possibility, AIM responsive T cells were examined for the expression of the CD69, CD71, CD27 and CD28 activation markers. In terms of apoptosis markers, we utilized Bcl-2, and FasL. Finally, in terms of exhaustion and anergic markers we utilized PD-1, PD-L1, Tim3, CTLA-4, KLRG1 and BTLA. With the exception of CD69 (**Figure 3C** and data not shown), there were no significant differences in the responding T cells of the aP and wP cohorts at the level of activation, exhaustion and apoptotic pathways.

Original wP or aP prime are associated with equivalent increases of IgG antibody titers and plasmablasts but not IgG4 after aP boost

Antibody titers were measured at baseline and 1-3 months after aP booster, to the four pertussis antigens PT, PRN, FHA and FIM2/3 and represented as overall pertussis response (individual antibody responses to each pertussis antigen are shown in **Supplementary Figure 2** which follow similar trends). As shown in **Figure 4A-B** total pertussis antibody titers from both donors originally primed with wP or aP vaccine were generally similar before boost, were equally elevated after an aP boost and followed a similar kinetic pertussis antibody profile. Antibodies to non-aP antigens (i.e. ACT or measles), did not significantly increase after aP immunization (data not shown). Levels of pertussis-specific IgG secreting plasmablasts were also equivalent after an aP boost (**Figure 4C**). We then evaluated if the pertussis-specific IgG subclass distribution followed a similar trend to the Th polarization (**Figure 1**) after an aP boost by measuring IgG1 and IgG4 pertussis-specific Abs as an indicator for a Th1 or Th2 response, respectively. Both wP and aP primed individuals elicited increased pertussis-specific IgG1 but not IgG4 responses following aP immunization (**Figure 4D-E**). When assessing relative aP-specific IgG4 changes post , individuals in the aP cohort had significantly elevated aP-specific IgG4 antibodies compared to wP individuals (Figure 4F; 5.1 vs 0.7 median fold change pre- to post-aP boost, respectively).

The data above demonstrate that while total IgG antibody responses are boosted in both aP and wP cohorts and follows IgG subclass distribution corresponding to Th polarization, the T cell response is not effectively boosted in the originally primed aP donors (**Figure 2**). This suggests that waning immunity is a T cell specific and not B cell specific defect.

Transcriptomic profiles of pertussis-specific T cells from aP vs. wP vaccinated donors

To investigate the nature of the differences between aP and wP original priming, we determined transcriptomic profiles in T cells from wP and aP primed donors in response to pertussis megapool stimulation 2-3 months after Tdap boost vaccination. These donors were

randomly selected amongst donors for which sufficient cell numbers were available. AIM reactive T cells were sorted as a function of memory subsets. As a first step, we ran an unbiased principal component analysis (PCA; **(Figure 5A**). In the case of pertussis-reactive T cells, wP-TEM clearly separate from aP-TEM. Next, we determined the number of genes differentially expressed between aP-TEM and wP-TEM cells that responded to the pertussis megapool, which identified a total of 13 genes with a stringent Padj <0.05 (**Figure 5B**).

IL-9 was one notable gene upregulated in the aP primed donors. This gene is implicated in pleiotropic activities such as regulation of T cell immune responses in allergies and asthma as well as antitumor and antiworm immunity (47-52). The ANAPC2 and WDR4 genes were downregulated in wP primed donors. ANAPC2 is part of the anaphase-promoting complex, which modulates the progression through the different phases of the cell cycle (53, 54). WDR4 (AIP1) is also implicated in cell cycle progression and cell migration (55, 56). Also notable is the differential expression of TGIF2, which encodes a protein repressing transcription by recruiting histone deacetylases to TGF-β-responsive genes (57, 58). These results suggested, that aP vs. wP priming is associated with alterations in specific T cell subsets (as suggested by the differential polarization), and in cell proliferation (consistent with lack of in vivo boost in aP primed donors).

Enrichment and network analysis revealed two main alterations linked to differential priming

Further analysis revealed that the IL-5, IL-13 and TGF-β genes displayed a higher expression level for the aP cohort (**Figure 5C**). In addition, for genes encoding proteins differentially expressed in the previous experiments, similar trends at the transcriptomic level were found (**Figure 5D**). Conversely, the IL-9 difference originally detected by gene expression was also confirmed at the protein level (**Figure 5D**). We next analyzed the 500 genes that showed the largest difference in expression between aP and wP cohorts, using the Gene Set Enrichment Analysis (GSEA) algorithm. GSEA is a computational method that determines whether certain

biological functions are significantly represented in the input group of genes. We found (**Table 1**) that three different gene categories were significantly enriched below the 10^{-4} FDR threshold, the first two related to cell division (mitosis and cell cycle) and the third to immune system function genes. Next, the genes differentially expressed from three categories were subjected to Ingenuity Pathway Analysis (IPA) to obtain additional functional insights (**Figure 6**). We found that mitosis and cell cycle progression genes that promote cell division are increased in wP samples (**Figure 6A**). In addition, IPA analysis for the immune system category (**Table 1**) identified IRF3 and IFN-β as a potential upstream regulator of IL-17, and IL-9 (**Figure 6B-C**). Co-expression analysis also identified GFI-1 as the gene most highly correlated with IL-9 expression (data not shown). GFI-1 is of interest as it suppresses Th17 differentiation (and also inducible Tregs). IL-4 induces GFI-1 expression in Th2 cells, while TGF-β suppresses GFI-1 expression. Furthermore, GFI-1 transcription factor is required to maintain Th2 phenotype (59, 60).

Original wP priming is associated with higher proliferative capacity

Based on the transcriptomic findings we hypothesized that the capacity of memory T cells to expand in response to antigenic stimulation might differ as a function of the original priming (aP vs. wP). Indeed, data from the literature highlighted higher proliferative capacity of aP primed donors relative to wP in 7 months and 4 year olds (31, 61), but equivalent capacity in 10 year olds (62). We tested this hypothesis using proliferation assays in which cells were labeled with CFSE at a final concentration of 10 μM. Analysis of cells immediately following labeling indicated a labeling efficiency higher than 99%. Rounds of cell division were determined by sequential halving of CFSE-fluorescence intensity. As expected the proliferating cells were derived from memory compartments (not shown). In the next series of experiments, we addressed whether the originally aP primed memory T cells, that could experience waning of immunity, were associated with lower proliferative capacity after Tdap boost vaccination. The results shown in **Figure 7A** show that this is indeed the case. In conclusion, these data validate, at the biological level, the hypothesis that

the original wP priming generates a T cell response associated with long lasting proliferative capacity, leading to the hypothesis that gradual loss of proliferative capacity might be associated with waning of pertussis-specific memory T cell responses. Further experiments suggested that these effects might be at least in part, linked to a regulatory cell population, since no difference in aP vs. wP donors was noted when purified T cell subpopulations were assayed, suggesting that a different population contained in the PBMC might be responsible for the effect (**Figure 7B**).

Some but not all alterations extend to TT specific responses

The data presented above demonstrate several alterations in pertussis specific responses, as a function of the original aP vs wP priming and implicate some alterations possibly related to APC function and upstream regulation of the T cell responses. In general, we also showed that the alterations detected did not extend to unrelated antigens like CMV, EBV or measles. The aP and wP vaccines however both contain TT. For this reason, we performed experiments to determine whether these alterations were limited to pertussis antigens, or more generally applicable to other components of the vaccine, such as TT. Strikingly, our results showed that TT-specific T cell responses were significantly boosted in wP but not aP donors, but total IgG antibody responses were significantly boosted in both cohorts (**Figure 8A-C**). Similar differences, albeit much less pronounced were also detected in terms of Th response polarization (**Figure 8D**) as well as no differences in TT-specific subclass distribution (**Supplementary Figure 3**). However, at the gene expression level, poor separation in the PCA was observed for TT responses (**Supplementary Figure 4**), and the genes differentially regulated in pertussis were not so in the case of TT responses. Finally, a similar trend in terms of proliferation of TT-specific T cells was noted between the aP and wP cohorts **(Figure 8E),** but this trend was not significant. These results suggest that the alterations seen in the case of pertussis responses also extend but only in part, to TT specific responses.

DISCUSSION

Our results demonstrate that priming in the first few months after birth with the aP or wP vaccines induces different T cell phenotypes. Ex vivo analysis of pertussis responses revealed that while original wP and aP prime are both initially capable of generating protective immunity, intrinsic differences in phenotype persist/evolve for >15 years. Pertussis responses remain polarized as a function of the original priming vaccine (which correlates with another report (35)), but not towards CMV/EBV. Polarization was associated with IL-4, IL-9 and TGF-β in aP and IFNγ and IL-17 in wP primed donors. We also observed pertussis-specific IgG4 antibodies were significantly elevated in aP compared to wP individuals (which correlates with other reports (21, 63)) and further supports the finding that pertussis-IgG subclass distribution is dictated based on the distinct Th polarization mediated by initial vaccine type. Interestingly, a few individuals predominantly in the aP cohort had PT antibody titers between 50-100IU/ml before their aP boost (**Supplementary Figure 2**), potentially indicating a recent *B. pertussis* exposure (64). Although beyond the scope of this study, it would be of interest to evaluate antibody, and T cell polarization and function in the context of a natural boost (i.e., exposure to *B. pertussis*) in both aP and wP primed cohorts.

Furthermore, over approximately 15 years and repeated aP boosts, T cells originally primed with aP become associated with diminished capacity to respond to a boost in vivo. Alterations in proliferative capacity were validated in CFSE proliferation assays and cell fractionation experiments suggest a potential regulatory effect. These observations are consistent with the known capacity of TGF-β to be either suppressive or to inhibit T cell proliferation (65). This report adds to a previous report from our group (43) that defined the epitopes recognized in aP and wP vaccination and reported a long-lasting polarization. In that study only cross-sectional cohorts were probed, while the present study performs longitudinal analyses, including ex vivo analysis of T cell reactivity, proliferation assays and transcriptomic profiling to investigate the

mechanisms of the differences involved in priming with the two different vaccines. The study design and major findings are highlighted in **Figure 9**.

As a result of the work described herein, we detected a key intrinsic difference in responses, associated with differential polarization. Besides the previous documented differential Th1 versus Th2 polarization (43) we show a novel differential IL-9/IL-17 polarization in adulthood after aP vs wP childhood vaccination. Indeed, stronger IL-17 polarization from wP vaccination has been reported to be associated with higher protection in baboon models (15, 26, 27, 44) and to be pivotal in the mediation of adaptive immunity by tissue-resident memory T cells after natural infection in mice (45, 66). Although IL-17 has been detected in supernatants of PBMCs stimulated with pertussis antigens (67, 68), to the best of our knowledge this is the first time that CD4+ T cells secreting both IL-9 and IL-17 have been shown to be associated with pertussis-specific responses after wP or aP vaccination regimens in humans.

Based on reports in the literature, TGF-β can work as a master-switch regulator (69) and promotes Th9, Th17 or Treg differentiation from naïve T cells upon presence of other cytokines (70, 71). Moreover, our network analysis suggests that IFN-β is an upstream regulator of both IL-9 and IL-17. It is thus possible that the IL-17/IL-9 polarization in pertussis responses results from differences in the priming and expansion of pertussis-specific T cells related to the production of TGF-β and the presence of mediators such as IL-1 and IFN-β.

Specifically, we speculate that priming with the aP vaccine results in production of IL-1 and IFN-β, while priming with wP results in lower levels of these mediators; we hypothesize that this will in turn modulate the effect and magnitude of TGF-β production which together with IL-9 production may block Th17 differentiation in aP donors and eventually induce Tregs that inhibit proliferation.

The functional data described above was also confirmed by transcriptomic analysis of T cells derived from the two cohorts of donors originally primed with aP or wP. This analysis identified additional potential candidates for further analyses, such as TGIF-2, a gene that

encodes a protein repressing transcription of TGF-β-responsive genes (57, 58), the transcriptional repressor GFI-1 whose gene expression has the highest correlation with IL-9 among all the genes in our set of data. GFI-1 has been shown to play pivotal roles in both Th2 cell expansion and negatively regulating Th17 differentiation (59, 60).

The other striking finding revealed by our study is that donors originally primed with aP have decreased capacity to respond to a booster immunization in vivo and diminished proliferative capacity in vitro. Differences in T-cell proliferative response have already been shown in children, and depict higher proliferative responses for aP vaccinated as compared to wP just after the 3 initial vaccinations or at the end of the primary series (5 vaccinations) (31, 61). However, and in stark contrast, our results and others (62) suggest that gradual aP loss of proliferative capacity occurs with increasing age and might contribute to impaired pertussis-specific memory T cell responses to boost vaccine and overall waning of immunity. The ANAPC2 and WDR4 genes differentially regulated in the wP vs. aP, are involved in cell cycle transitions (72, 73), and in cell proliferation and migration (55, 56), respectively.

Our data suggest that differential proliferative profiles might be linked to a regulatory cell population (either a pertussis-specific T cell subpopulation or a regulatory cell of different nature), since no difference in aP vs. wP donors was noted when purified T cell subpopulations were assayed. This suggests that a different population contained in the PBMC is responsible for the effect. It is possible that the effect is mediated by suppressive activity of classic Tregs (i.e. IL-10, TGF-β and CTLA-4) or IFN-β secreting APCs, although this inference is not strongly supported by the data available and will have to be addressed in further experiments. We consider it likely that any regulatory effect is mediated by antigen specific T cells, such as Th9 (this cell subset was not yet purified in the experiment shown in Figure 7), since it is unlikely that non-antigen specific T cells would develop over the course of over 10 years. Alternatively, a different interpretation of the results suggest that key intrinsic differences might exist at the level of memory T cells with differential susceptibility to being suppressed by the regulatory cells.

Another scenario is that the differences at the level of T cell phenotype might lead to different patterns of activation at the APC level, and as a feedback loop lead to a regulatory influence on T cell responses. Indeed, it is well recognized that dendritic cells enhance antigen presentation and elicit polyvalent immune responses in the vaccination against yellow fever (74, 75), Influenza (76, 77) or TB (78). In this context, pertussis antigens are also known to have adjuvant activity and activate APC. For this reason, in future studies it will be of interest to determine whether APC alterations are generally impacting responses to other components of the vaccines, such as TT.

A feedback loop involving APC "licensing" is an attractive possibility, based on the known adjuvant activity of wP, and the fact that certain features of differential pertussis responses appear to "spill over" to TT responses (since TT is also contained in the wP or aP vaccines). Concurringly, we have shown previously, that TT responses remained polarized despite the original priming having occurred decades before (79), suggesting enactment of specific molecular programs in memory T cells. More specifically we hypothesize that the differentially polarized T cells might impart, upon antigen encounter and stimulation, differential signaling to APC, resulting in the generation of a "suppressive" or "stimulatory" APC function for the IL-9 vs. IL-17 producing T cells, respectively.

In conclusion, our data suggest that long lasting effects and differences exist between adults originally vaccinated with aP versus wP. In this context, it must be noted that adverse reactions to the original wP vaccine although already substantial from the first administration, increased with each subsequent dose in the primary series and specifically upon booster doses (80-82). Also, albeit globally less reactogenic, the aP vaccine's elevated production costs limits its worldwide use, especially in developing countries (83). Thus, a vaccination regimen where only the initial round of vaccination would be administered utilizing the wP vaccine, and followed by aP boosters could be considered. Alternatively, the addition of a Th1-polarizing adjuvant to the existing aP vaccine may be a way forward. Our data clearly illustrate that at least some of the

immunological features imparted by the wP prime will be maintained despite middle and high school booster vaccinations with aP. This in turn parallels the epidemiologic observations that individuals originally vaccinated with wP versus aP have more durable protection from disease, regardless of aP boosters during adolescence (1, 15, 83).

METHODS

Study subjects

We recruited 114 healthy adults from San Diego, USA (**Supplementary Table 1**). All participants provided written informed consent for participation and clinical medical history was collected and evaluated. Clinical data for each patient was collected by multiple approaches. Whenever possible vaccination records were collected from study participants or parents/custodian as appropriate. For some donors, the original clinical vaccine record was not available or incomplete, in which cases information was collected by the clinical coordinators through questionnaires, recording dates and numbers of vaccination, including the information that no boost was administered at least in the previous four years prior to this study. All donors were from the San Diego area, and to the best of our knowledge followed the recommended vaccination regimen (which is also necessary for enrollment in the California school system), which entails five DTaP doses for children under 7 years old (three doses at 2, 4 and 6 months and then two doses between 15-18 months and 4-6 years) and a Tdap booster immunization at 11-12 years and then every 10 years. Individuals who had been diagnosed with *B. pertussis* infection at any given time in their life were excluded. Other exclusion criteria were pregnancy at the start of the study; present severe disease or medical treatment that might interfere with study results; any vaccination in the last month and/or antibiotic use or fever (>100.4F (38 C). In all groups, male and female subjects were included equally. From the remaining participants, a subset of these donors, originally vaccinated with either DTwP or DTaP in infancy, received a booster vaccination with Tdap and donated blood 1, 2, 3 or ≥ 4 months after the boost. Alternatively, in a second subset of aP- or wP-primed patients, blood was collected at days 1, 3, 7, 14, 30 and 60/120 after Tdap boost. This study was performed with approvals from the Institutional Review Board at La Jolla Institute for Allergy and Immunology.

Booster Vaccination

For booster vaccinations participants received a booster vaccine (Adacel®, Sanofi Pasteur, Lyon, France) with Tetanus Toxoid, Reduced Diphtheria Toxoid and Acellular Pertussis Vaccine Adsorbed (Tdap). Each dose of Adacel vaccine (0.5mL) contains the following active ingredients: Detoxified Pertussis Toxin (PT), 2.5µg; Filamentous Hemagglutinin (FHA), 5µg; Pertactin (PRN), 3µg; Fimbriae Types 2 and 3 (FIM); 5µg Tetanus Toxoid (TT); 5Lf; Diphtheria Toxoid (DT), 2Lf. Other ingredients include 1,5mg aluminum phosphate (0.33mg of aluminium) as the adjuvant besides residual formaldehyde, glutaraldehyde and phenoxyethanol.

Peptides

Peptides were derived from *Bordetella pertussis* antigens included in the aP vaccines (FHA, Fim2/3, PRN, and PT) from the Tohama I and 18323 strains. Of 785, 16-mers overlapping by eight residues, the top epitopes recognized by >5% donors corresponding to 132 peptides were chosen (43). Epitopes from *Clostridium tetani* toxin (TT), were also selected as recently described (79). A total of 122 previously defined epitopes of EBV/CMV were also used as a control for antigens not contained in the vaccine (84). Peptides were synthesized as crude material on a small (1 mg) scale by A and A (San Diego, California). Individual peptides were resuspended in DMSO and equal amounts of each peptide were pooled to construct peptide pools. To minimize DMSO concentrations in the assays, pools of more than 100 peptides ("megapools") were generated as described (84). According to this procedure, each individual lyophilized peptide is dissolved in 100% DMSO at 20 mg/mL (for 0.5 mg of peptide, this corresponds to a volume of 50 µL). Then equal amounts of each peptide are mixed well; for 100 peptides, this corresponds to 50X100 = 5 mL; the total peptide concentration is still 20 mg/mL, but the concentration of each peptide is now 0.2 mg/mL, all in 100% DMSO. The resulting 5 mL are then lyophilized, adding water if required. The resulting “lyocake”, or megapool, is next carefully dissolved in the smallest amount of DMSO feasible. Usually a megapool easily dissolves in 100 µL of 100% DMSO. This

corresponds to 10 mg/mL of each peptide in 100% DMSO, and a final concentration of 2.5 µl/mL in an assay corresponds to 0.05% DMSO. This approach has been used to develop megapools specific for timothy grass, TB, DENV, pertussis and tetanus (43, 79, 85-87). All the peptides used in this study are listed in **Supplementary Table 2.**

PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood or leukapheresis by density gradient centrifugation according to the manufacturer's instructions (Ficoll-Paque Plus, Amersham Biosciences, Uppsala, Sweden) as previously described (88). Cells were cryopreserved in liquid nitrogen suspended in FBS containing 10% (vol/vol) DMSO.

Multiplexed Luminex Immunoassays

Antigen-specific antibody responses were measured through a modified multiplexed Luminex® assay (89). Pertussis and tetanus proteins (Pertussis Toxin Mutant; PT, Pertactin; PRN, Filamentous Hemagglutinin; FHA, Fimbriae2/3; FIM2/3, Adenylate Cyclase Toxin; ACT and Tetanus toxoid; TT from List Biological Laboratory, Campbell, CA and Sigma-Aldrich St. Louis, MO), inactivated Rubeola antigen as an internal vaccine control (Edmonston strain from Meridian Life Science, Inc., Memphis, TN) and an irrelevant protein (PD1) as an internal negative control were coupled to distinct fluorescent-barcoded MagPlex® microspheres (Luminex Corporation). Serum from each individual, WHO *B. pertussis* human serum reference standard (NIBSC 06/140 Hertfordshire, UK) or TT antibody standards were mixed with an equimolar mixture of each conjugated microsphere. The microspheres were then washed with a PBS-tween 20 buffer to release non-specific antibodies and bound antibodies were detected via anti-human IgG phycoerythrin (PE; clone JDC-10, Southern Biotech, AL), anti-human IgG1-PE (clone 4E3, Abcam, MA) or anti-human IgG4-PE (clone HP6025, Abcam, MA) to measure total or subclass

antigen-specific antibodies. Samples were subsequently analyzed on a Luminex® FLEXMAP 3D instrument (Luminex Corporation). PT-, PRN- and FHA –specific IgG positive beads were calculated as IU/mL based on the WHO reference serum and TT-specific IgG positive beads calculated as standardized international units based on diluted TT antibody standards. Other antigen-specific IgGs that had no reference standard or antigen-specific IgG1 or IgG4 -positive beads are reported as arbitrary units (AU) /mL and were calculated as the median fluorescent intensity (MFI) over total IgG, IgG1 or IgG4 as previously reported (21). For each sample, total serum IgG, IgE, IgG1 and IgG4 were measured via ELISA through ThermoFisher's Ready-set-go kits for each respective antibody isotype and subclass.

B-cell ELISpot assay

Freshly collected PBMCs were assayed for memory B-cell responses at day 7 after Tdap boost as previously described (90). Briefly, multiscreen 96-well plates were antigen coated with 10 µg/well of all pertussis antigens combined and anti-IgG and the coating antibody were diluted in PBS. PBMCs were then added in triplicates to wells ($2x10^5$ cells/well) and incubated at 37°C, 5%CO_2 for 16-20 h. After washing with PBS-Tween20, anti-IgG alkaline phosphatase was added and incubated for 4 h at room temperature. After washing, alkaline phosphatase substrate was prepared and added to the plate allowing spots to develop. The reaction was stopped by washing with tap water.

AIM and ICS assays

The Activation Induced Marker (AIM) assay was previously described (91). This assay detects cells that are activated as a result of antigen-specific stimulation by staining antigen-experienced $CD4^+$ T cells for TCR-dependent upregulation of OX40 and CD25 (AIM_{25}) and/or PD-L1 (AIM_{PD}) after an optimal time of 18–24 h of culture. Briefly, cryopreserved PBMCs were thawed and $1x10^6$ cells/condition were immediately cultured together with TT peptide pools (2 µg/mL),

pertussis peptide pools (2 μg/mL), or PHA (10 μg/mL; Roche) as a positive control in 5% human serum (Gemini Bioproducts) for 24 h. To determine the memory phenotype of responding T cells, staining for CD45RA and CCR7 markers were performed. For intracellular cytokine staining (ICS), PBMCs were incubated with pertussis, TT or CMV/EBV peptide pools for 24 h. After 20 h, BFA (1ug/mL; BD Bioscience, San Diego, CA) was added for an additional 4 h. Cells were then washed, stained for extracellular markers for 30 min, and then washed, fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin (Sigma) and stained for intracellular IL-4, IL-9, IL-17 and IFN-γ. A combination of PHA or PMA and Ionomycin (1 μg/mL) was used as positive control. Samples for both AIM_{25} and combined AIM_{25} and ICS assays were acquired on a BD LSRII Flow Cytometer and analyzed using FlowJo X Software. Phenotypic characterization of responding T cells, including activation, apoptosis, exhaustion and anergic markers was performed by similar protocols. All flow cytometry mAb reagents for surface or intracelullar staining are listed in **Supplementary Table 3.**

Cellular Proliferation

PBMCs were thawed and cells ($1x10^7$ cells/mL) were labeled with CFSE cell proliferation kit (Thermo Scientific, Waltham, MA) at a final concentration of 10 uM and cultured for 6-7 days in in RPMI media (Omega Scientific) supplemented with 5% human AB serum (Gemini Bioscience), GlutaMAX (Gibco), and penicillin/streptomycin (Omega Scientific) and stimulated with TT peptide pools (2 μg/mL), pertussis peptide pools (2 μg/mL), or PHA (10 μg/mL). Rounds of cell division were determined by sequential halving of CFSE-fluorescence intensity after additional surface phenotypic staining was performed (**Supplementary Table 3)**.

RNA-sequencing and software analysis

RNA sequencing (RNA-seq) were performed as described in (92) using a HiSeq2500 (Illumina) to obtain 50-bp single end reads. The resulting RNA-seq reads were aligned to the hg19

reference using TopHat (v 1.4.1, library-type fr-secondstrand-C) (93). Genes were annotated according to RefSeq (obtained from UCSC Genome Browser). The HTSeq-count (-m union -s yes -t exon -i gene_id) program was used to determine the sequencing read coverage per gene. Differentially expressed genes between aP- and wP-primed donors were identified using the Bioconductor package DESeq2 (Cooks filter cutoff) (94). Genes were considered differentially expressed between aP and wP groups when the DESeq2 analysis resulted in a Benjamini-Hochberg–adjusted p value of < 0.05. A more relaxed cutoff was established considering the 500 genes with the highest difference in expression between the two cohorts. Principal component analysis (PCA) was performed using the plotPCA method from the DESeq2 package and considering the top 1000 most variable genes.

Identification of overrepresented pathways for the differentially expressed gene set was performed using the web tool GeneSetEnrichmentAnalysis (GSEA)(http://software.broadinstitute.org/gsea/index.jsp) (95). To determine possible relationships among the genes represented in the "GSEA enriched pathways" the core analysis from the 'Integrated Pathway Analysis' (IPA) software was performed (www.ingenuity.com).

Data availability

The RNA-sequencing data have been deposited in Gene Expression Omnibus (GEO) under the accession code GSE113891 and ImmPort under the study number SDY787.

Statistics

Comparisons between groups were made using the nonparametric Two-tailed Unpaired Mann-Whitney U or Paired Wilcoxon test. Prism 5.0 (GraphPad) was used for all these calculations. All data in all figure parts in which error bars are shown is presented as median ± interquartile range. A p value <0.05 was considered statistically significant.

Study approval

This study was performed with approvals from the Institutional Review Board at La Jolla Institute for Allergy and Immunology (protocols; VD-101-0513 and VD-059-0813). All participants provided written informed consent for participation and clinical medical history was collected and evaluated.

AUTHOR CONTRIBUTIONS

RdSA, BPe, BPu and AS participated in the design and planning of the study. RdSA, MB, CC, NK, AM, CP and LP performed and analyzed experiments. GS and PV prepared RNA-seq libraries and coordinated RNA-sequencing. MC gave technical advice. MB conducted bioinformatics analyses. SC gave critical advice. RdSA, MB, CP, LP and AS wrote the article. All authors have read, edited, and approved the manuscript.

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FIGURES AND FIGURE LEGENDS

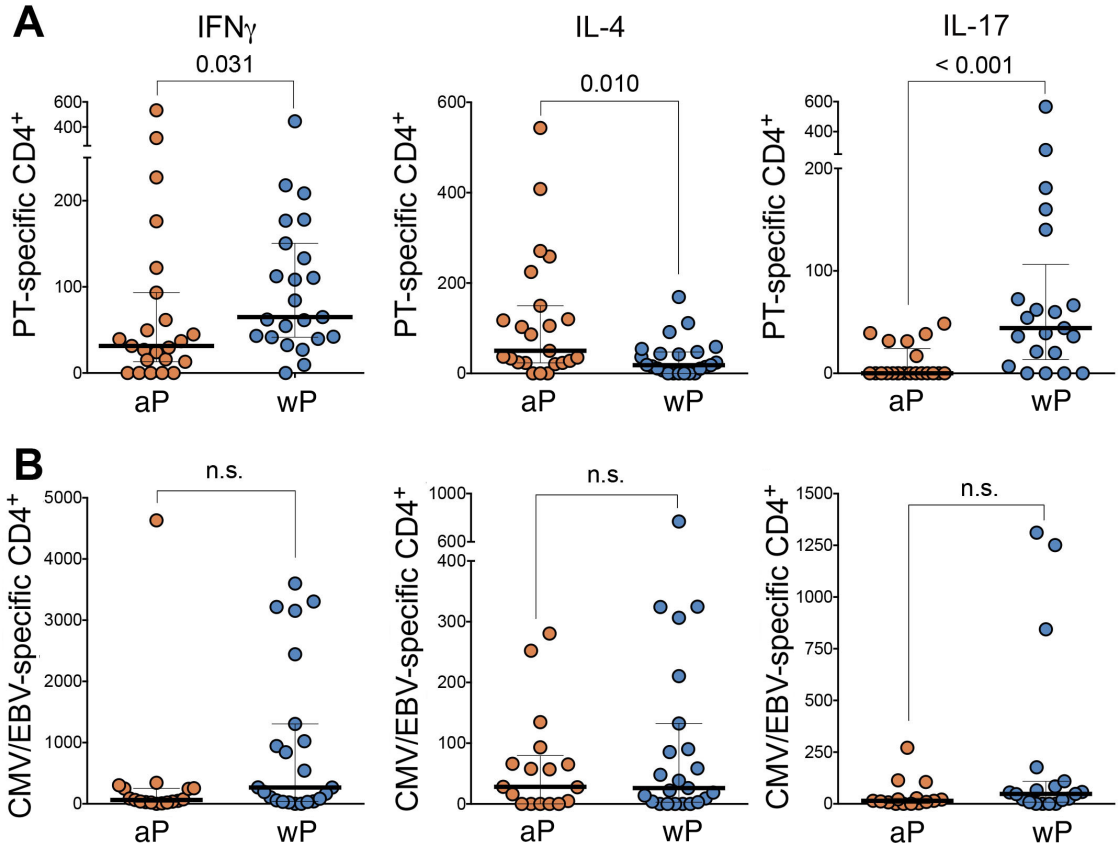

Figure 1. Differential polarization of PT-specific CD4$^+$ T cells as a function of childhood vaccination. IFNy, IL-4 and IL-17 secreting cells was measured by ICS staining in PT (A) or CMV/EBV (B) specific CD4$^+$ T cells by AIM_{25} assay. Responses represent the cohorts of donors originally DTaP or DTwP vaccinated, 1-3 months after Tdap boosting vaccination. Each dot represents the number of total CD4$^+$ T cell that was to be ascribed to each cytokine. For all panels data are expressed as median ± the interquartile range for each cohort and each data point represent a single donor (n=23 for each cohort). p value is shown as statistically significant accordingly with two-tailed Mann-Whitney test. n.s.- not statistically significant.

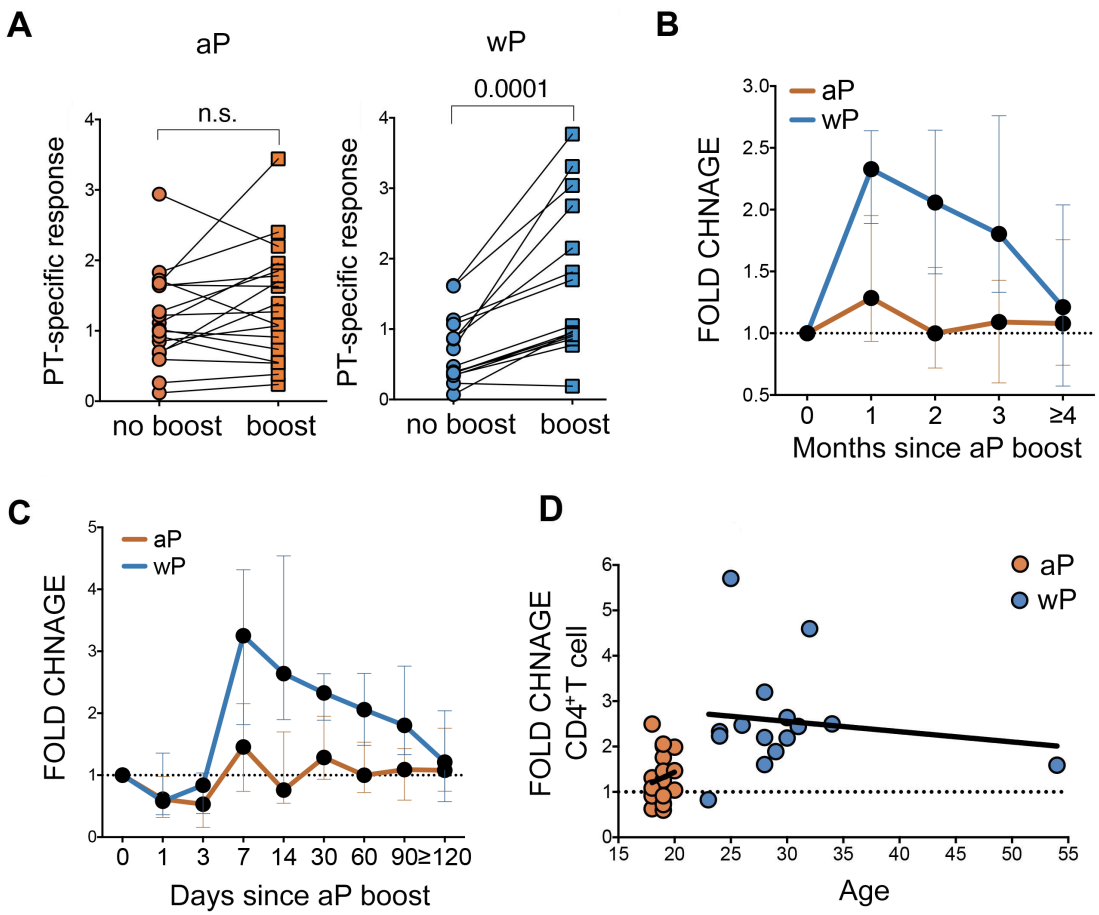

Figure 2. Original wP prime but not aP prime is associated with significantly higher CD4$^+$ T cells after aP boost. (A) % of PT-specific CD4+ memory T cells by AIM$_{25}$ assay for donors originally primed with wP or aP vaccine, before or after the earliest boost time for each individual (0.5-2 months range). Each dot represents a single donor determination followed longitudinally (n=18 for aP and n=15 for wP cohorts). n.s. -no significant difference by Wilcoxon paired t-test. (B-C) longitudinal kinetics of PT-specific CD4$^+$ T cell responses after boost represented as fold increase of the % of AIM$_{25}^+$ cells to non-boost responses for aP or wP primed cohorts. Data are expressed as median ± the interquartile range for each cohort. (Panel B n=18 for aP and n=17 for wP). Panel C n=12 for aP and n=12 for wP). (D) PT-specific CD4$^+$ T cell responses after boost represented as function of age for aP or wP cohorts. Each data point represents the fold increase to non-boost response from each donor (n=18 for aP and n=15 for wP cohorts). The best fit of each data set is represented by linear regression lines (black).

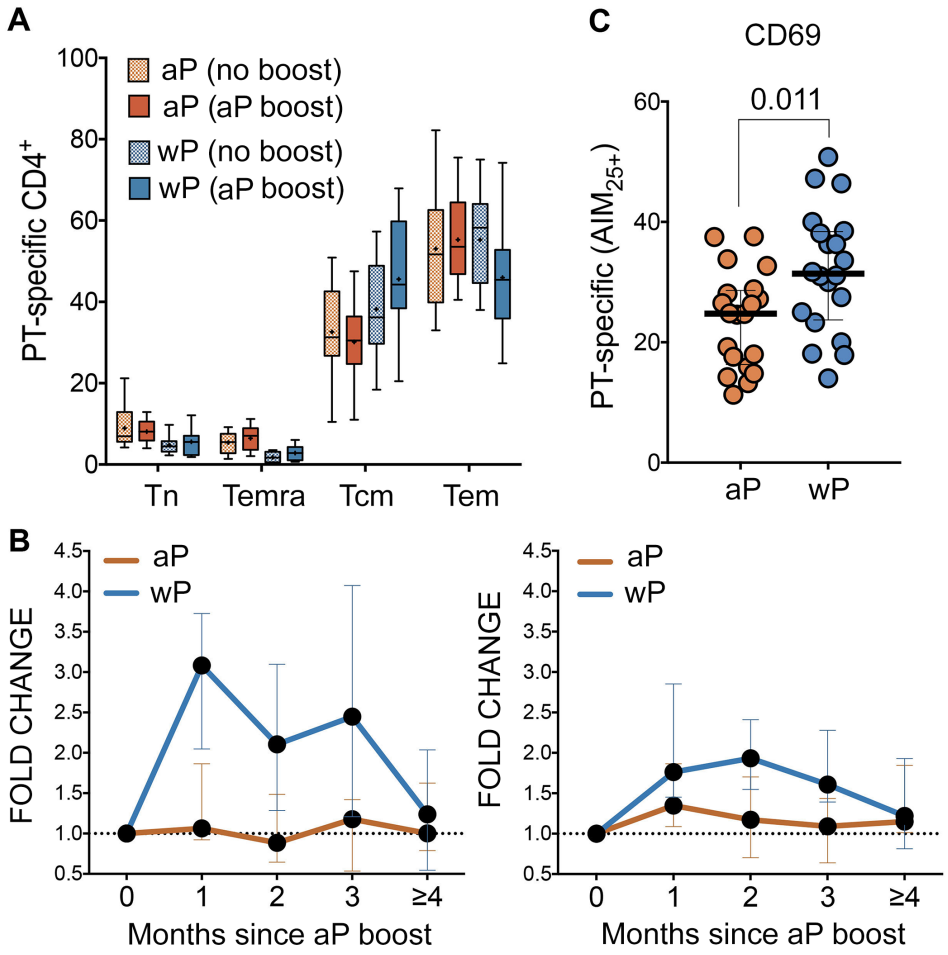

Figure 3. Memory subset composition of PT-specific responses. (A) % of PT-specific $CD4^+$ T cells subsets followed longitudinally (Tn: $CD45RA^+$ $CCR7^+$, Temra: $CD45RA^+$ $CCR7^-$, Tcm: $CD45RA^-$ $CCR7^+$ and Tem: $CD45RA^-$ $CCR7^-$) gated in AIM_{25}^+ cells. Median (*) and min and max error bars are shown (aP boost: 0.5-2 months range; n=16 for aP and n=14 for wP cohorts). (B) longitudinal kinetics of PT-specific $CD4^+$ responses from Tcm (left panel) or Tcm (right panel) subsets after aP boost. Data are expressed as median ± the interquartile range for each cohort. (n=18 for aP and n=17 for wP in both panels) (C) expression of CD69 in aP vs wP cohorts as the % of AIM_{25}^+ cells after aP boost (1-3 months range). Differences between cohorts analyzed via two-tailed Mann-Whitney test. Each data point represents a single donor determination n=20 for each cohort).

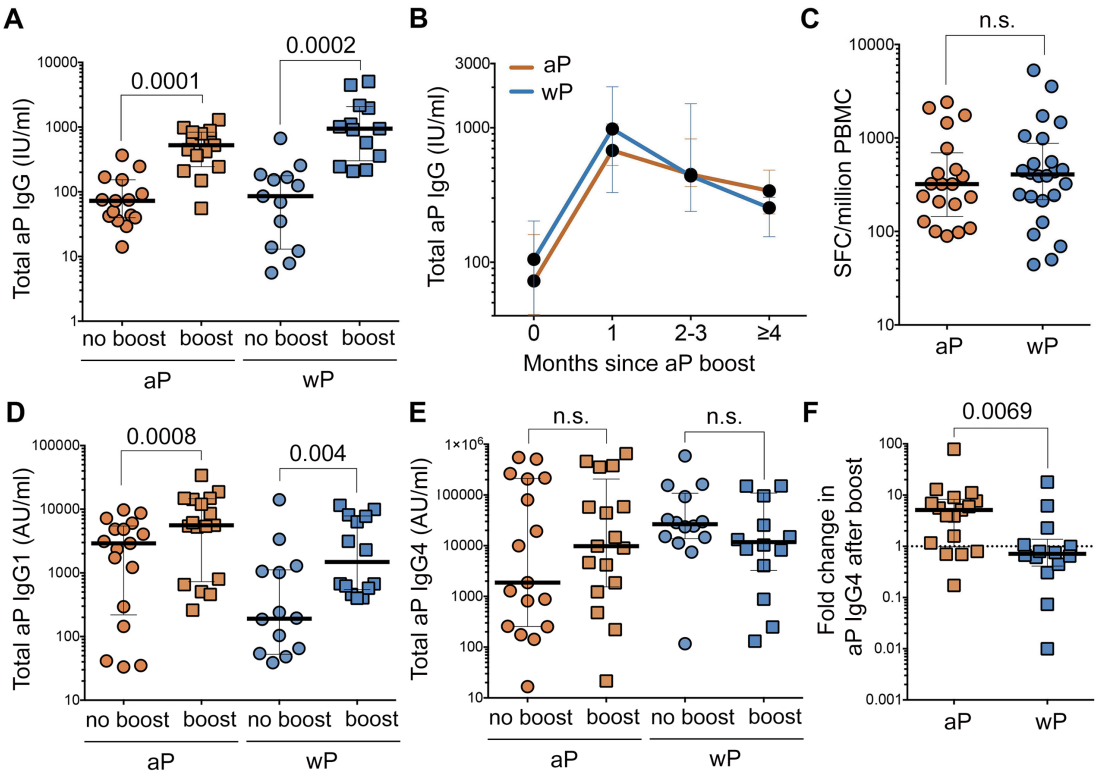

Figure 4. wP and aP primed donors elicit elevated pertussis-specific IgG and IgG1 but not IgG4 titers after aP boost. (A) Sum of IgG antibody titers for aP antigens (FHA, PT and PRN) in respective cohorts. Pre- and post-response analyzed via Wilcoxon paired t-test. (B) Kinetic representation of antibody titers. (C) Analysis of plasmablast memory B-cell responses at day 7 after Tdap boost. Data represent overall Ab secretion against aP antigens as measured by ELISPOT. (D) Sum of pertussis (FHA, PT, PRN and FIM2/3) IgG1 and (E) IgG4 levels as representative responses to aP for each cohort. Pre- and post-response analyzed via Wilcoxon paired t-test. (F) Fold change in aP IgG4 levels after aP boost for each cohort. Data represent the average fold change of all aP antigens (PT, FHA, PRN and FIM2/3) for each individual. Comparison between aP and wP fold change analyzed via Mann-Whitney unpaired t-test. For all panels data are expressed as median ± the interquartile range for each cohort and each data point represents a single donor (n=19 for aP and n=14 for wP cohorts except for panel C in which n= 20 for aP and n=24 for wP).

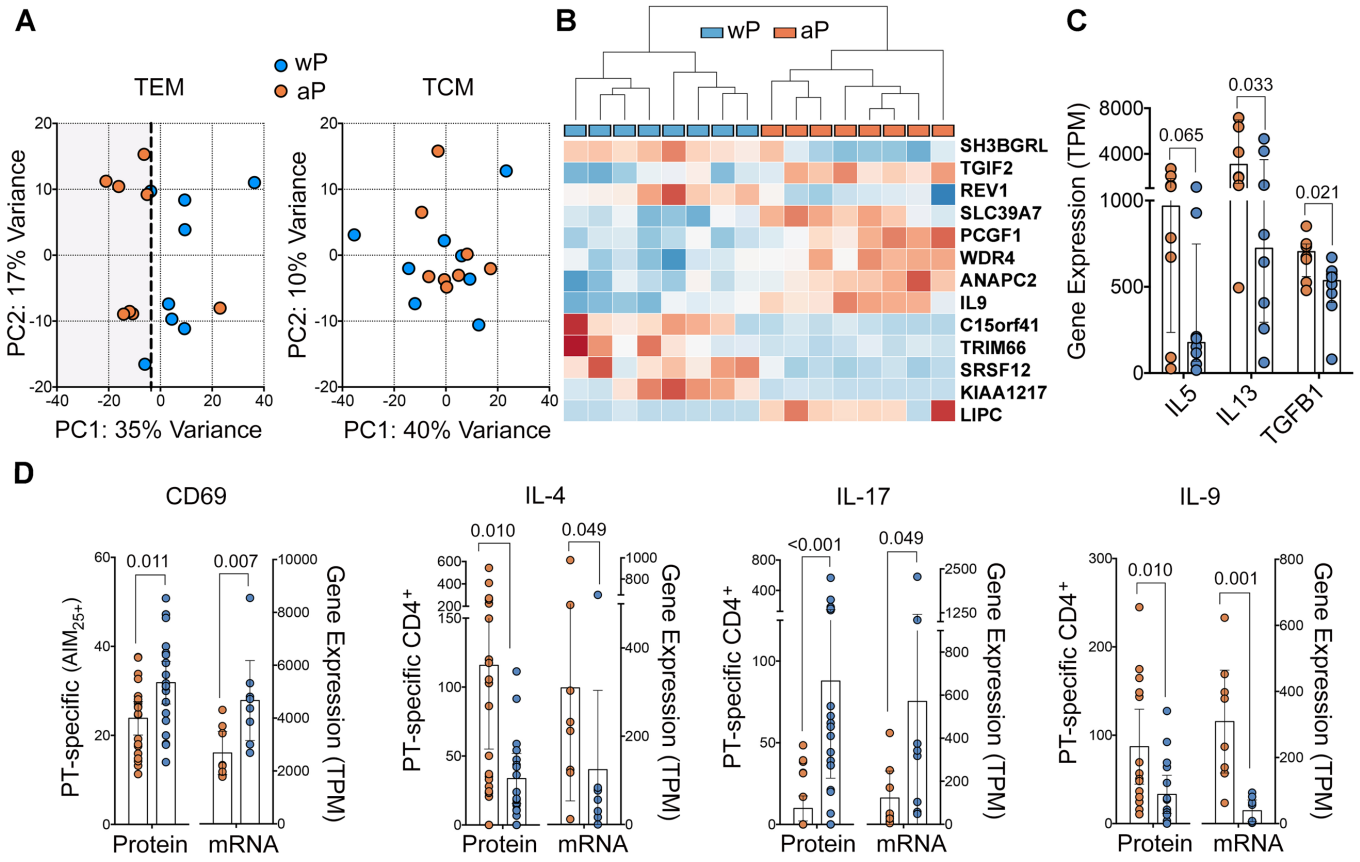

Figure 5. Comparison of gene expression profiles of PT stimulated T cells for aP and wP vaccinated donors after boost. PT-specific CD4+ T cells were isolated by AIM25 assay, sorted as a function of memory Tem and Tcm subsets and RNA-seq was performed. (A) Unbiased principal component analysis (PCA) of Tem and Tcm subsets from aP and wP donors based on the top 1000 variable genes. Each data point represents a single donor n=8 for each cohort). (B) Tem cells from aP and wP donors (n=8) were clustered based on the 13 differentially expressed genes (Padj < 0.05) (C-D) Expression of the indicated cytokines and CD69 at the mRNA (n=8 for each cohort) or protein level. Protein data are represented as the % of CD69 in AIM_{25}^{+} cells (n=20 for each cohort) or the number of IL-4 and IL-17 (n=23 for each cohort) or IL-9 (n=15 for each cohort) secreting cells measured in ICS staining by Pertussis-specific AIM_{25} assay. mRNA data are represented as the number of Transcripts Per Million (TPM) after RNA-Seq normalization for the respective gene. Results are presented as median ± interquartile range. All determinations were performed in recently boosted donors (1-3 months range) Each dot represents one donor from aP (orange) vs wP (blue). Differences analyzed via two-tailed Mann-Whitney test.

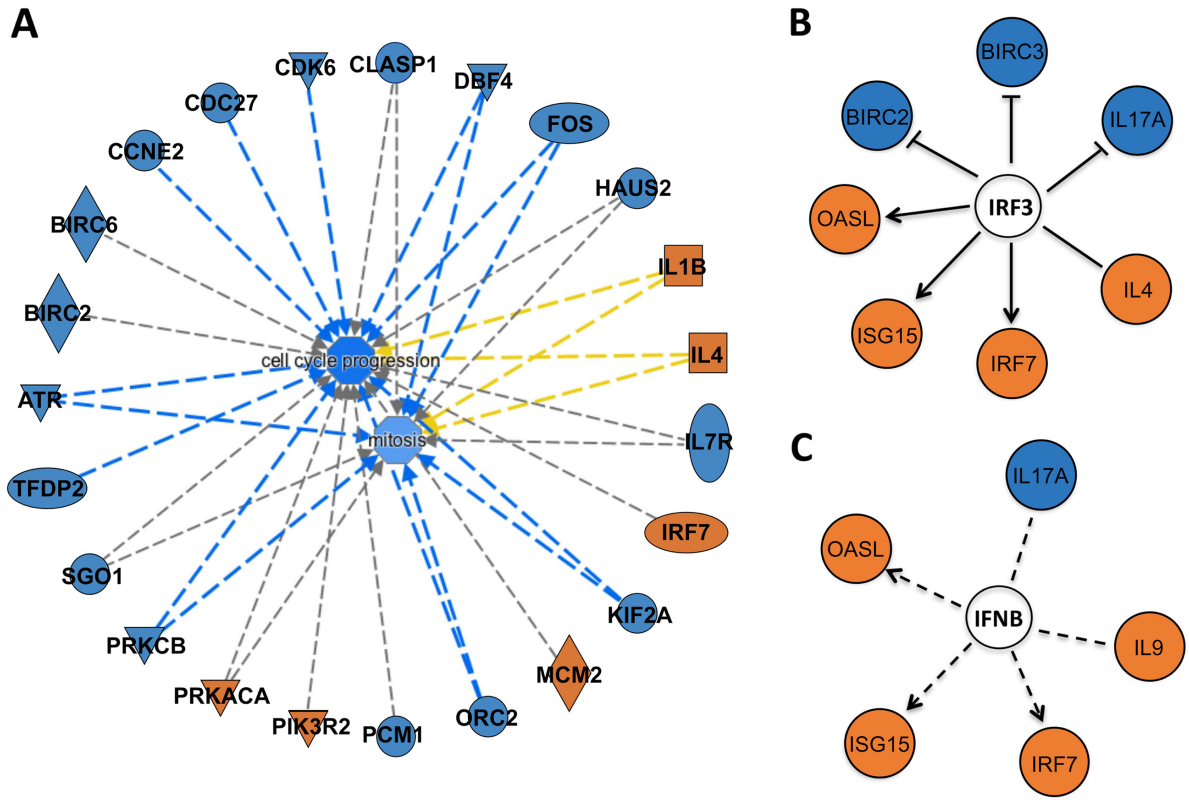

Figure 6. Modular transcriptomic and pathway analysis reveals alterations linked to differential priming. (A) Network analysis for gene functions involved in mitosis and cell cycle progression. These functions were significantly enriched for the 500 genes with the highest value of fold-change between aP and wP donors. Lines indicate relationships between genes and functions. Blue lines indicate activation effects and grey lines indicate genes known to be involved in function but with unknown effect. Red and green nodes indicate up-regulated in aP and wP, respectively. Yellow lines indicate that the relationships are inconsistent with the state of the node. (B) IRF3 and (C) IFNB were identified as upstream regulators. Solid and broken lines denote a direct or indirect effect, respectively. Line with an arrowhead indicates activation, while a flat end indicates inhibitory effect.

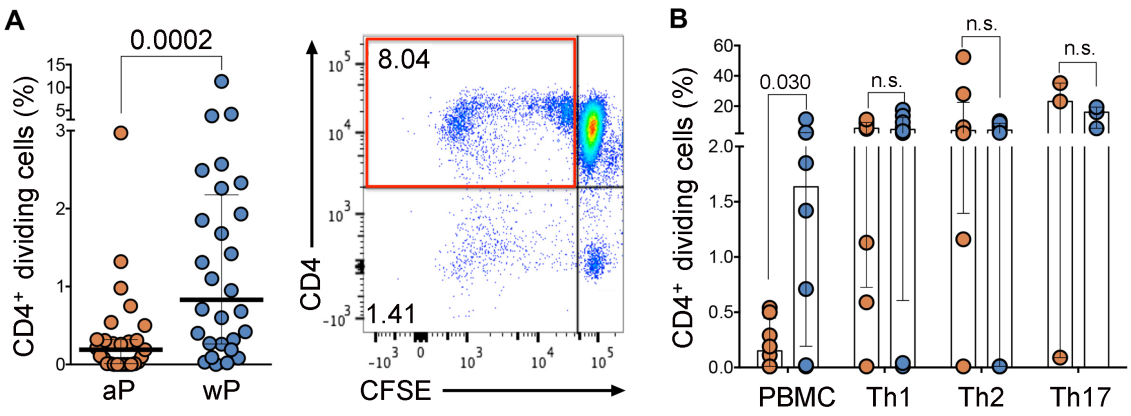

Figure 7. Original wP priming is associated with higher proliferative capacity. The proliferative capacity of PT-specific cells was assessed by CFSE assay after 6 days of stimulation. (A) % of dividing $CD4^+$ T cells by CFSE quenching in both cohorts. Results are presented as median ± interquartile range for each cohort and each data point represents a single recently boosted donor (1-3 months range; n=27 for aP and n=28 for wP cohorts). Dot plot shows double labeling of CD4 vs CFSE for a representative wP primed donor. (B) % of dividing $CD4^+$ T cells in purified T cell subsets from aP (red) vs wP (blue). Differences between cohorts analyzed via two-tailed Mann-Whitney test. Donors samples obtained from leukapheresis after Tdap boost (1-12 months range); n=8 donors. n.s.- no significant difference.

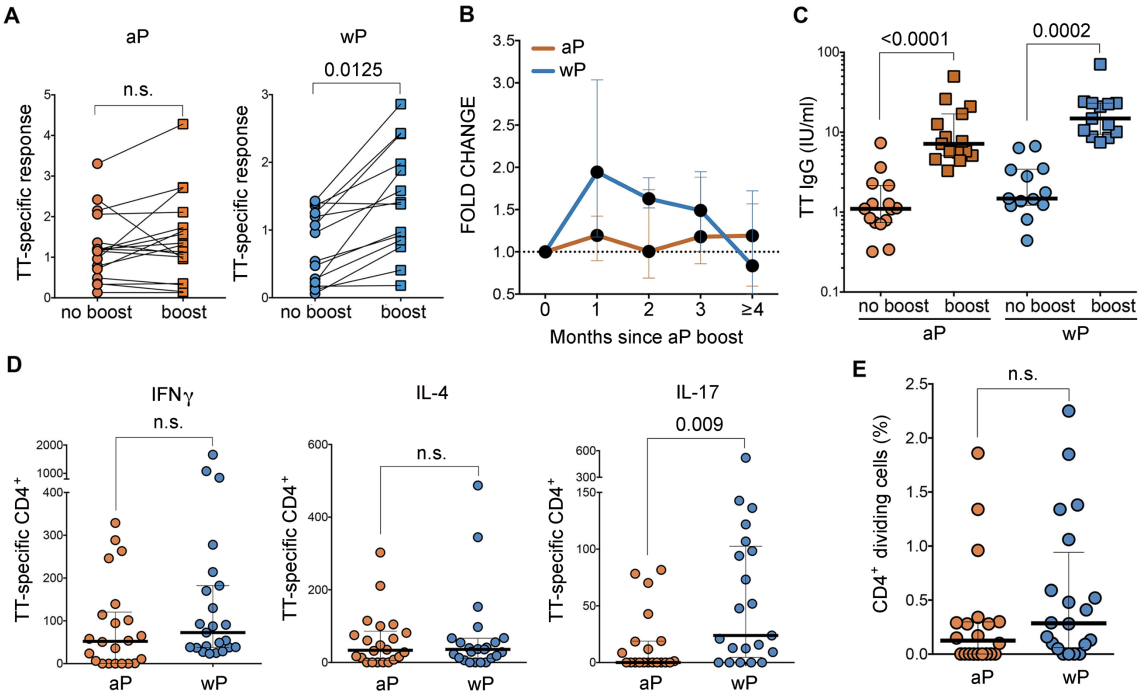

Figure 8. Some but not all alterations extend to TT specific responses (A) % of TT-specific $CD4^+$ T cells subsets gated in AIM_{25}^+ cells before or following boost. Each dot represents one donor followed longitudinally (n=17 for aP and n=14 for wP cohorts). n.s. - no significant difference by Wilcoxon paired t-test. (B) longitudinal kinetics of PT-specific $CD4^+$ T cell responses after boost represented as fold increase of the % of AIM_{25}^+ cells to non-boost responses for aP or wP primed cohorts. Data are expressed as median ± the interquartile range for each cohort (n=18 for aP and n=17 for wP cohorts). (C). Ab titers for TT toxoid antigen in respective cohorts (n=19 for aP and n=14 for wP cohorts). Pre- and post-response analyzed via Wilcoxon paired t-test. (D) Total TT-specific $CD4^+$ T cell number response after AIM_{25} assay to each indicated cytokine. Each dot represents one donor (n=23 for each cohort). p value is shown as statistically significant accordingly with two-tailed Mann-Whitney test. (E) % of TT-specific $CD4^+$ T after 6 days of CFSE assay. Results are presented as median ± interquartile range (n=20 for each cohort). n.s.- not statistically significant.

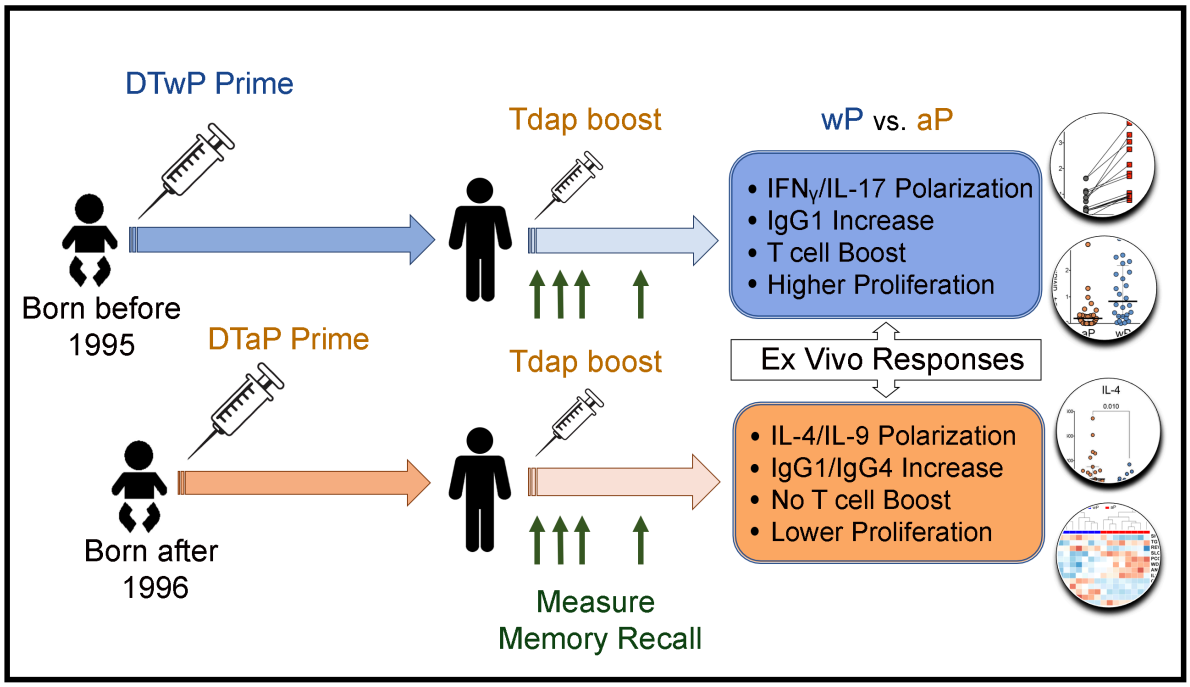

Figure 9. Experimental design and major findings of the study. Primary vaccination with 5 doses (three doses at 2, 4 and 6 months and then two doses between 15-18 months and 4-6 years) of whole-cell (DTwP) or acellular vaccine (DTaP) occurred during the first years of life. A contemporary acellular vaccine (Tdap) boost was administered >15 years later and memory recall response measured using ex vivo analysis of T cell or B cell reactivity, proliferation assays and transcriptomic profiling. The major immunological differences for each cohort (wP vs. aP) are depicted in the boxes (blue and orange respectively).

Table 1. Summary of gene categories analyzed by GSEA

Categories	# Genes in Gene Set (k)	# Genes in Overlap (k)	p-value	FDR q-value
Mitosis	325	18	1.34E-08	1.78E-05
Cell Cycle Progression	421	20	2.74E-08	1.82E-05
Immune System Function	933	30	7.69E-08	3.41E-05