## Title

Memory and effector T cells modulate subsequently primed immune responses to unrelated antigens



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**The memory and effector T cells modulate subsequently primed immune responses to unrelated antigens**

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Running title: Regulation of different waves of immunity to unrelated Ags.

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**Abstract:**

Memory and effector T cells modulate subsequently primed T cell responses to the same antigen. However, little is known about the impact of pre-existing memory and effector T cell immunity on subsequently primed immune responses to unrelated antigens. Here, we showed that an antigen-primed first wave of Th1 and Th2 immunity enhanced or inhibited the subsequently primed T cell immunity to an unrelated antigen, depending on whether the second antigen was administered in the same or opposite type of adjuvant. The regulatory effects of the first wave of T cell immunity on the subsequent T cell responses to an unrelated antigen attenuated with time. Notably, following challenge with the second antigen, there was a mutual cross-regulation between the first and second wave of humoral responses to unrelated antigens. Thus, immunization with one antigen not only primes immune responses to that antigen, but also influences subsequently primed immune responses to unrelated antigens.

Keywords: Th1/Th2 cells; cytokines; memory; rodent.

## 1. Introduction:

Antigen-specific $CD4^+$ T cell responses have been divided into two subsets, Th1 and Th2, based on their effector function and their cytokine profiles [1-3]. The Th1/Th2 paradigm provides a useful framework for understanding the pathogenesis of certain infectious diseases as well as tissue-specific autoimmune diseases [4-9]. While Th1 responses are necessary to clear infectious virus and intracellular parasitic bacteria, Th2 responses predominantly promote humoral responses that can effectively prevent toxin-related bacterial infection, particularly in the mucosal surface [10-13]. Thus, the development of the appropriate type of T cell immunity is critical for successful vaccination and eradication of infectious organisms.

Much research has focused on developing adjuvants that guide immune responses toward a desired phenotype. For example, injection of antigen in complete Freund's adjuvant (CFA) usually induces a Th1 biased response, while administration of antigen in incomplete Freund's adjuvant (IFA) or Alum tends to promote a unipolar Th2 response [14,15]. Existing memory T cell responses usually enhance the same type of secondary immune responses after re-exposure to their cognate antigen, which are characterized by rapid and strong T cell recall proliferation [16-18]. However, the existing memory and effector T cell responses can also inhibit the subsequently primed T cell responses to the same antigen. Mice that were neonatally injected with antigen in IFA failed to generate proliferative, and IL-2 recall responses to that antigen when challenged in CFA later [19]. Thus, the existing memory T cell responses can enhance or down-regulate secondary responses to the same antigen, depending on the type of T cell response primed by antigen in the same or opposite type of adjuvant. However, little is known about the effect of pre-existing memory and effector Th1 or Th2 immunity on subsequently primed

immune responses to unrelated antigens. Furthermore, treatment with antigen in IFA to induce antigen-specific Th2 responses has been associated with inhibition of Th1 autoimmunity [20-23]. On the other hand, administration of antigen in modes that prime Th1 responses has been shown to inhibit Th2-mediated mucosal inflammation [24,25]. Whether the second wave of immune responses primed by challenging with an unrelated antigen modulates the existing memory and effector CD4+ T cell responses remains to be determined.

Notably, epidemiological studies have found that prior exposure to *tuberculosis* is associated with a reduction in incidence and severity of atopic disease [26]. These results suggest that pre-existing memory and effector Th1 responses to *tuberculosis* antigens have a long-term inhibitory affect on an individual's propensity to develop spontaneous Th2-type autoimmunity. If an individual's history of prior immune exposures shapes the quality or quantity of immune responses to subsequent challenges, it could have important implications for understanding susceptibility to autoimmune disease, the variations in human immune responses to pathogens, and may suggest improved protocols for effective vaccination.

To explore the consequence of the influence of different waves of immune responses to unrelated antigens, we immunized neonates with a prototypic antigen in IFA or CFA to establish the first wave of Th2 or Th1 responses, respectively. We then characterized immune responses following challenge with another antigen in the same or opposite type of adjuvant. We found that memory and effector T cells modulated subsequent immune responses to unrelated antigens. Thus, immunization with one antigen not only primes immune responses to that antigen, but also influences subsequent immune responses to other unrelated antigens. We discuss the implications of these findings for understanding the development of determinant spreading in autoimmune disease and vaccination strategies.

## 2. Materials and Methods

**2.1. Mice.** Balb/c$^{H\text{-}2d}$ and C57BL/6$^{H\text{-}2b}$ mice were obtained from Jackson Laboratory and bred under specific pathogen free conditions. Both female and male mice were used in this study.

**2.2. Antigens and immunization.** Hen eggwhite lysozyme (HEL), and chicken ovalbumin (OVA) were purchased from Sigma (St. Louis). IFA was purchased from Invitrogen (Grand Island, NY) and CFA was made by mixing *M. tuberculosis* H37 RA (Difco Laboratories, Detroit, MI) at 2.0 mg/ml of IFA. Individual antigen was mixed with the IFA or CFA at 2.0 mg/ml and emulsified. Individual mice were injected with 50 μl (100 μg) Antigen i.p or s.c., as specified.

**2.3. ELISPOT analysis.** Splenic mononuclear cells were isolated from antigen-treated mice at different time points post-immunization, and the frequency of antigen-specific T cells secreting IFNγ, IL-4, and IL-5 was determined using a modified ELISPOT assay, as previously described [23]. Briefly, $10^6$ splenic mononuclear cells were added per well (in duplicate) to an ELISPOT plate which had been coated with cytokine capture antibodies and incubated with antigen (100 μg/ml) at 24 hrs for IFNγ, or 40 hrs for IL-4 and IL-5 detection. After washing, biotinylated detection antibodies were added and the plates were incubated at 4°C overnight. Bound secondary antibodies were visualized using HRP-streptoavidin (DAKO Corp.) and 3-amino-9-ethylcarbazole. Antibodies R4-6A2/XMG 1.2-biotin, 11B11/BVD6-24G2-biotin and TRFK5/TRFK4-biotin (PharMingen) were used for capture and detection of IFNγ, IL-4, and IL-5, respectively.

**2.4. Intracytoplasmic cytokine staining**. Balb/c neonates were immunized with HEL/CFA or HEL/IFA, and three wks later they were challenged with OVA/IFA or OVA/CFA, respectively. Control groups of mice at 3 wks in age were immunized with OVA/CFA or OVA/IFA. Two wks after immunization with OVA, their splenic mononuclear cells were isolated and stimulated with OVA 100 μg/ml ($5x10^6$ cells/ml) in HL-1 media for 48 hours. The cells were harvested and $CD4^+$ T cells were purified by depleting $CD8^+$ T cell and APCs as our previously described [27]. The purified $CD4^+$ T cells were blocked by anti-CD16/32, and intracytoplasmic cytokines were stained using FITC-anti-IFNγ and biotinylated anti-IL-4 plus PE-streptoavidin as well as a Cytofix/Cytoperm Plus kit (PharMingen). FITC-rat IgG1 and biotinylated rat IgG1 were used as isotype controls. Antigen-specific cytokine-expressing $CD4^+$ T cells were characterized by FACS analysis.

**2.5. Adoptive transfer assay**. Balb/c neonates were immunized with HEL/CFA or HEL/IFA. Three wks later, splenic T cells or APCs from these mice or age-matched unmanipulated Balb/c mice were purified. Ten million T cells or APCs were transfused iv into 3 wks old Balb/c mice. The recipient mice were then immunized with OVA/IFA or OVA/CFA, respectively. Ten days after OVA immunization, their splenic T cell responses to OVA were characterized by the ELISPOT assay.

**2.6. ELISA analysis of antibodies.** Two weeks after the immunization, mouse blood was collected. and antibody levels were determined using indirect ELISA assay with HRP-conjugated goat-anti-mouse IgG, IgG1, or IgG2a (South Biotechnology, TX) [27]. The antibody levels were expressed as the average O.D. for each group of mice.

**2.5. Statistics.** A paired Student's t Test was used to analyze the data for statistical significance. The result was considered significant when $p<0.05$.

## 3. Results:

### 3.1. Existing memory and effector Th1 cells enhance subsequently primed Th1 responses to an unrelated antigen.

We tested two prototypic antigens, OVA and HEL, as well as two adjuvants, IFA and CFA, which are known to drive Th1 or Th2-biased $CD4^+$ T cell responses, respectively. We immunized neonatal Balb/c mice with OVA or HEL in CFA (OVA/CFA or HEL/CFA) i.p. to prime Th1 cell responses, and 4 wks later we challenged the mice with an unrelated antigen in CFA s.c. (HEL/CFA for OVA/CFA primed mice, and vice versa). Control groups received only one antigen immunization neonatally or at 4 wks of age. Two weeks after the challenge, we characterized T cell immunity to both antigens by ELISPOT assays. Balb/c mice that received one immunization with OVA/CFA or HEL/CFA neonatally or at 4 wks of age developed comparable levels of unipolar Th1 responses to that antigen (Fig. 1A and B), consistent with previous reports [15,28]. Interestingly, mice that were neonatally sensitized with one antigen in CFA, developed stronger Th1 responses to the unrelated antigen that they were challenged with at 4 wks of age. For example, the frequency of OVA-reactive IFNγ-secreting T cells in mice, that were neonatally sensitized with HEL/CFA and challenged with OVA/CFA at 4 wks of age, was significantly increased compared to that in control groups that received one OVA/CFA immunization at birth or at 4 wks of age ($p< 0.025$) (Fig. 1 A). Furthermore, mice neonatally immunized with CFA (containing mycobacterial antigens) alone and challenged with OVA/CFA

or HEL/CFA also developed stronger Th1 responses to the challenged antigen. These data indicate that the existing memory and effector Th1 immunity enhances subsequently primed Th1 responses to an unrelated antigen.

The second immunization with an unrelated antigen had no distinguishable effect on the frequency of IFNγ-secreting T cells responding to the neonatally sensitized antigen. For example, following challenge with HEL/CFA, the frequency of IFNγ-secreting T cells responding to OVA in OVA/CFA neonatally sensitized mice was indistinguishable to that in controls that did not receive challenge. Similarly, mice that were neonatally sensitized with HEL/CFA developed comparable levels of Th1 responses to HEL regardless of whether or not they were challenged with OVA/CFA. These observations suggest that the pre-existing Th1 responses can up-regulate the subsequently primed Th1 immunity to an unrelated antigen, but the second wave of Th1 responses do not affect the frequency of pre-existing memory and effector Th1 cells.

### 3.2. Existing memory and effector Th2 cells up-regulate subsequently primed Th2 responses to an unrelated antigen.

Next, we tested whether pre-existing Th2 responses could modulate the subsequently primed Th2 responses to an unrelated antigen. We immunized neonates i.p. with OVA/IFA or HEL/IFA to prime Th2 effector and memory T cells, and challenged them s.c. with an unrelated antigen in IFA (OVA/IFA for HEL/IFA primed mice and vice versa) at 4 wks of age. Control groups of mice received IFA alone or only one antigen immunization at birth or at 4 wks of age. We characterized splenic T cell immunity to both antigens by ELISPOT assays when the mice were at 6 wks of age (Fig. 1 C and D). As expected, control mice that were immunized with

OVA/IFA or HEL/IFA at birth or at 4 wks of age displayed a similar magnitude of Th2 biased responses to the injected antigen. Following challenge with an unrelated antigen in IFA, the frequency of IL-5-secreting T cells responding to the antigen injected neonatally was similar to the frequency in mice that received only one antigen immunization. Therefore, the Th2 responses primed by second immunization with an unrelated antigen in IFA did not affect the frequency of pre-existing memory and effector Th2 cells. In contrast, mice with pre-existing memory and effector Th2 cells developed a stronger Th2 response to the unrelated antigen subsequently challenged in IFA. For example, the frequency of OVA-specific IL-5-secreting T cells in mice neonatally sensitized with HEL/IFA and subsequently challenged with OVA/IFA was increased by 35% ($p<0.02$) compared to the frequency in control mice that received one immunization with OVA/IFA at 4 wks of age. Notably, unlike CFA, neonatal immunization with IFA (which contains only lipid) alone had no effect on subsequently primed Th2 responses. Thus, the first wave of Th2 immunity enhances subsequently primed Th2 responses to an unrelated antigen.

### 3.3. The first wave of T cell immunity modulates the subsequent T cell responses primed by an unrelated antigen in an opposite type of adjuvant.

Next, we examined the impact of the first wave of Th2 immunity on the subsequent Th1 responses primed by an unrelated antigen in CFA. Newborn Balb/c or C57BL/6 mice were injected with OVA/IFA, HEL/IFA or IFA alone, and 4 wks later they were challenged s.c. with HEL/CFA, OVA/CFA, or CFA alone. Control groups of mice received only one immunization with a single antigen in CFA or IFA at birth or at 4 wks of age. Two weeks after challenge, their splenic T cell responses to both antigens were analyzed by ELISPOT assays (Fig. 2). Following immunization with OVA/CFA or HEL/CFA at 4 wks of age, mice neonatally sensitized with IFA

alone developed a unipolar Th1 response to the injected antigen, that was similar in magnitude to that in mice that received one immunization with antigen in CFA at birth or 4 wks of age (Fig. 1). Notably, the pre-existing memory and effector Th2 immunity down-regulated the subsequently primed Th1 responses to an unrelated antigen administered in CFA. For example, mice neonatally sensitized with HEL/IFA developed mixed Th1 and Th2 responses to OVA following challenge with OVA/CFA (Fig. 2 A and B). Although the primed OVA-specific Th1 responses were dominant, the frequency of OVA-specific IFNγ-secreting T cells was significantly reduced ($p<0.025$) compared to that of control mice that were neonatally immunized with IFA alone and challenged with OVA/CFA at 4 wks of age. Similarly, OVA/IFA sensitized mice that were subsequently challenged with HEL/CFA displayed both HEL-specific IFNγ-and IL-5-secreting T cells (Fig. 2 C and D). The frequency of HEL-reactive IFNγ-secreting Th1 cells was lower than that of control mice ($p< 0.025$). Thus, our data suggest that the first wave of Th2 responses down-regulates the subsequent development of Th1 immunity to an unrelated antigen administered in CFA and promotes some subsequent T cell responses towards a Th2 phenotype. Following challenge with the second antigen in CFA, the mice that were neonatally sensitized with the first antigen in IFA still maintained levels of unipolar Th2 responses to the first antigen comparable to those in control mice that did not receive the second antigen immunization. Thus, the primed second wave of Th1 responses did not appear to affect the frequency of pre-existing memory and effector Th2 cells that had been primed by neonatal immunization.

We further tested whether pre-existing Th1 immunity influences the subsequent T cell response to an unrelated antigen administered in IFA. Neonatal Balb/c or C57BL/6 mice were injected i.p. with HEL/CFA, OVA/CFA, or CFA, and the mice were challenged with an

unrelated antigen in IFA (such as OVA/IFA for HEL/CFA sensitized mice) at 4 wks of age. Control groups of mice received only one antigen immunization at birth or at 4 wks of age. Their splenic T cell responses to OVA and HEL were analyzed by ELISPOT assays at 6 wks of age. We found that the pre-existing memory and effector Th1 responses modulated subsequently primed T cell responses to an unrelated antigen when administered in IFA. As shown in Fig. 2 A and B, CFA or HEL/CFA sensitized mice that were challenged with OVA/IFA developed mixed Th1 and Th2 responses to OVA (with both IFNγ and IL-5 secreting T cells). Furthermore, mice neonatally sensitized with OVA/CFA or CFA displayed both Th1 and Th2 responses to HEL after challenging with HEL/IFA (Fig. 2 C and D). Although the challenge with the second antigen in IFA resulted in a predominant Th2 response to that antigen, the frequency of IL-5-secreting T cells responding to the second antigen was significantly decreased ($p<0.05$) compared to controls that received antigen in IFA once at birth or at 4 wks of age (Fig. 1 C and D). These data suggest that the first wave of Th1 immunity down-regulates the second wave of Th2 responses subsequently primed by an unrelated antigen in IFA. Following challenge with the second antigen in IFA, the mice still displayed unipolar Th1 responses to the first antigen, at a comparable level to those in controls that did not receive the second immunization. Thus, challenge with the second antigen in IFA had no discernable effect on the size of the existing memory and effector Th1 cell pool primed by the first antigen at birth.

In addition, we examined the regulatory effect of prior immunity on subsequent T cell responses to unrelated antigen by intracytoplasmic cytokine staining. We immunized Balb/c neonates with HEL/CFA or HEL/IFA and challenged them with OVA/IFA or OVA/CFA 3 wks later. Control groups of mice were immunized with OVA/CFA or OVA/IFA at 3 wks in age. Two wks after OVA immunization, their splenic mononuclear cells were isolated and stimulated

with OVA *in vitro.* The $CD4^+$ T cells were purified , and their intracytoplasmic IFNγ and IL-4 were analyzed by FACS scanning. Control mice immunized with OVA/CFA or OVA/IFA displayed unipolar Th1 or Th2 responses to OVA (Fig. 3 A and C). In contrast, mice sensitized with HEL/IFA or HEL/CFA and challenged with OVA/CFA or OVA/IFA developed both Th1 and Th2 responses to OVA (Fig. 3 B and D). Overall, our findings demonstrate that the first wave of immunity modulates subsequent T cell responses primed by an unrelated antigen in an opposite type of adjuvant.

### 3.4. Memory and effector T cells, but not APCs, mediate the regulatory effect of prior immunity on subsequent T cell immunity to unrelated antigens.

Antigen immunization with either type of adjuvant can activate APCs, leading to change in immune environments. To dissect whether memory and effector T cells, or APCs, mediate the regulatory effect of prior immunity on subsequent T cell responses to unrelated antigens, we immunized Balb/c neonates with HEL/CFA or HEL/IFA. Three wks later, we purified splenic T cells or APCs from HEL-immunized mice, as well as unmanipulated mice, and transfused them into age-matched Balb/c mice. We then immunized these mice with OVA/IFA or OVA/CFA, and ten days later, characterized T cell immunity to OVA by the ELISPOT assay (Fig. 4). Mice that received APCs from HEL-immunized mice and were challenged with OVA/IFA or OVA/CFA developed unipolar Th2 and Th1 responses to OVA, similar to that in mice that received APCs or T cells from unmanipulated mice, suggesting that APCs from the previously immunized mice had little effect on subsequent T cell immunity to unrelated antigens in their adoptive host. In contrast, the transfused memory and effector T cells modulated subsequent T cell immunity to unrelated antigens. Indeed, mice that received T cells from HEL-immunized

mice developed both Th1 and Th2 responses to OVA following challenge with OVA in an opposite type of adjuvant. Thus, these data demonstrate that memory and effector T cells, but not APCs, mediate the regulatory effect of prior immunity on subsequent T cell responses to unrelated antigens.

### 3.5. Mutual cross-regulation between the first and second wave of humoral responses to unrelated antigens.

Activated and effector Th1 and Th2 cells can help B cells produce different subclasses of antibodies. Given our observations that pre-existing memory T cell responses affect the phenotype and magnitude of subsequent T cell responses to unrelated antigens, we hypothesized that they would also modulate the development of humoral responses. Neonatal Balb/c or C57BL/6 mice were sensitized with OVA or HEL in IFA or CFA and then challenged with an unrelated antigen (OVA for HEL sensitized mice, and vice versa) in the same or opposite type of adjuvant at 4 wks of age. Control groups of mice received only one immunization with antigen in IFA or CFA at birth or at 4 wks of age. When the mice reached 6 wks of age, their humoral responses to both antigens were analyzed by ELISA assays. Control mice that only received one immunization with antigen in IFA developed high levels of IgG1 antibodies against the injected antigen (Fig. 5 A and B). Other groups of mice immunized with one antigen in CFA displayed medium levels of IgG2a and low levels of IgG1 (Fig. 5 C and D). We observed that when mice were challenged with a second unrelated antigen in the same type of adjuvant their humoral responses to both antigens were enhanced. For example, mice that were neonatally sensitized with HEL/IFA and were challenged with OVA/IFA developed higher levels of IgG1 antibodies against both OVA and HEL compared to those in control mice that only received one

immunization with antigen in IFA ($p<0.02$ for OVA and $p<0.035$ for HEL, Fig. 5 A and B). A similar pattern of enhanced humoral responses was observed in mice neonatally sensitized with HEL/CFA and challenged with OVA/CFA and mice pre-immunized with OVA and challenged with HEL (Fig. 5).

Furthermore, we found that the pre-existing immune responses influenced the development of humoral responses to challenged antigen when the second antigen was administered in an opposite type of adjuvant. Mice that were neonatally sensitized with CFA or HEL/CFA and challenged with OVA/IFA not only developed IgG1, but also produced significantly increased levels of IgG2a antibodies against OVA compared to control groups that were only immunized with OVA/IFA (Fig. 6 A, $p<0.001$). Moreover, neonatal injection with HEL/IFA and challenge with OVA/CFA promoted higher levels of IgG1 antibodies against OVA compared to those in mice injected with OVA/CFA only (Fig. 6 C, $p<0.045$). Similarly, the pre-existing immune responses to OVA also modulated subsequently primed humoral responses to HEL when administered in an opposite type of adjuvant (Fig. 6 B and D). Finally, challenge with an unrelated antigen in an opposite type of adjuvant modulated the isotype of humoral responses to antigen that the animal had been previously sensitized to. For example, while mice that were immunized with OVA/IFA developed unipolar IgG1 antibodies against OVA (Fig. 6 A), mice that were neonatally sensitized with OVA/IFA and challenged with HEL/CFA displayed both IgG1 and increased levels of IgG2a antibodies against OVA (Fig. 6 A, $p<0.001$). Challenge with HEL/IFA caused mice that were neonatally sensitized with OVA/CFA to produce higher levels of IgG1 antibodies against OVA (Fig 6 C, $p<0.02$). A similar change in the pattern of humoral responses to HEL was observed in HEL neonatally sensitized mice following challenge with OVA in an opposite type of adjuvant (Fig. 6 B and D). These observations indicate that

challenge with an unrelated antigen in an opposite type of adjuvant modulates the humoral responses to the neonatally sensitized Antigen. Therefore, our data suggest that the first wave of immune responses enhances the subsequently primed humoral responses, and that the second wave of immune responses also up-regulates the pre-existing first wave of humoral responses.

### 3.6. The impact of the first wave of T cell immunity on the subsequently primed T cell responses to unrelated antigens attenuates over time.

We further examined the longitudinal effect of the first wave of T cell immunity on subsequently primed T cell responses to an unrelated antigen. We first determined the capacities of Th1 and Th2 responses primed by neonatal immunization to sustain over time. Balb/c, or C57BL/6 mice were injected with OVA or HEL in IFA or CFA at birth only, and control groups of mice received one immunization with OVA or HEL in IFA or CFA at 4, 8 or 12 wks of age. We characterized T cell immunity to the injected antigen by ELISPOT assays at 6, 10, or 14 wks of age (Fig. 7 A and B). Control mice that received one immunization with OVA/IFA or OVA/CFA developed comparable levels of polarized Th2 or Th1 cell responses to OVA, respectively (Fig. 7 A). The neonatally primed Th2 responses to OVA appeared to be well maintained as the frequency of IL-5 secreting T cells only slightly reduced when tested at 14 wks of age (Fig. 7 B). However, the frequency of IFNγ-secreting T cells primed by neonatal immunization with OVA/CFA dramatically decreased by about 50% when tested at 10 wks of age and further reduced at 14 wks of age (Fig. 7 B) compared to that in mice primed at those ages (Fig. 7 A). Immunization with HEL induced a similar pattern of T cell responses in both strains of mice (data not shown). Thus, the neonatally primed Th1 and Th2 responses appeared to have different capacities to sustain over time.

The attenuation of primed T cell responses over time should also diminish their impact on subsequent T cell responses primed by an unrelated antigen. To test this contention, we neonatally injected Balb/c, or C57BL/6 mice with one antigen in one type of adjuvant and then challenged them with a second antigen in the opposite type of adjuvant at 4, 8, or 12 wks of age. Two wks after challenge with the second antigen, we characterized T cell immunity to both antigens by ELISPOT assays. As previously described, the second wave of T cell immunity had no impact on the unipolar T cell responses induced by neonatal immunization. Mice that were sensitized with HEL and challenged with OVA at 4, 8, or 12 wks of age still displayed unipolar T cell immunity to HEL. We found that the levels of T cell responses to HEL gradually attenuated over time (data not shown), similar to that in control groups of mice that did not receive the challenge (Fig. 7 B). Notably, the impact of pre-existing Th1 immunity on subsequent T cell responses to an unrelated antigen primed by antigen in IFA attenuated over time. For example, challenge with OVA/IFA at 4 wks of age promoted not only Th2, but also Th1 responses to OVA in mice that were neonatally sensitized with HEL/CFA when tested at 6 wks of age (Fig. 7 C). However, when challenged at 8 or 12 wks of age and tested at 10 or 14 wks of age, T cell responses to OVA gradually became more Th2-polarized. Indeed, by 14 wks of age the magnitude of unipolar Th2 responses was similar to the control group that received one immunization with OVA/IFA (Fig. 7 A). Thus, over time, the first wave of Th1 immunity no longer impacted the second wave of T cell responses.

Similarly, the influence of the first wave of Th2 responses on subsequently primed T cell immunity to an unrelated antigen primed by antigen in CFA also decreased as the interval of time between immunizations increased. For example, mice neonatally sensitized with HEL/IFA and then challenged with OVA/CFA at 4 wks of age displayed mixed Th1 and Th2 responses to

OVA when tested at 6 wks of age. When challenged at 8 or 12 wks of age and tested at 10 or 14 wks of age respectively, the mice progressively developed more Th1-biased responses to OVA (Fig. 7 D). A similar pattern of T cell immunity to HEL developed in mice neonatally sensitized with OVA following challenge with HEL in an opposite type of adjuvant at different ages (data not shown). Thus, although many memory and effector Th2 or Th1 cells responding to the first antigen still existed *in vivo* they had little effect on the subsequent T cell response when primed by an unrelated antigen at 12 wks of age. Our data indicate that the interference of the first wave of T cell immunity with the subsequently primed T cell responses to an unrelated antigen attenuated over time.

**4. Discussion:**

We studied the regulation of different waves of immune responses to unrelated antigens and found that the pre-existing memory T cell responses enhanced the subsequently primed T cell response to a second antigen administered in the same type of adjuvant. The pre-existing memory and effector T cells, but not APCs, also modulated the phenotype and magnitude of subsequent T cell responses to an unrelated antigen administered in an opposite type of adjuvant. Conceivably, the persisting memory and effector T cells release Th1 (or Th2) cytokines, that affect subsequently primed immune responses to an unrelated antigen by directly regulating the activation and differentiation of antigen-specific naïve T cells and/or indirectly modulating APCs [3,29,30]. Importantly, humans are exposed to many viruses, bacteria, and parasites, may create a similar Th1 or Th2 bias that influences immune responses to subsequently infected microbials, especially in the case of persisting infections.

We observed that immune responses primed by an unrelated antigen have little effect on regulating the frequency of existing memory and effector T cells regardless of what type of adjuvant is used that contrasts with the original antigenic sin [31-33]. One possible explanation is that there is no cross-activity between the antigen-reactive T cells in our model system. Alternatively, if the second wave of T cell immunity does modulate the pre-existing memory and effector T cell responses it might be via an antigen-independent cytokine-mediated homeostatic cell division [34-36]. Indeed, memory T cells undergoing a series of cell divisions may lead to a decrease in cell number via IFNγ-induced apoptosis of memory T cells, particularly for Th1 cells [37]. Recent reports have demonstrated that sequential viral infection results in the attrition of antigen-specific memory $CD8^+$ T cells [35]. Accordingly, if the cell division and death of the first wave of T cells are balanced it will not affect the frequency of pre-existing memory and effector T cells.

We also found that the pre-existing memory and effector immune responses up-regulated the subsequent humoral responses to an unrelated antigen administered in the same type of adjuvant. However, the first wave of immune responses not only deviated, but also enhanced, humoral responses to the second antigen when it was administered in an opposite type of adjuvant. We observed that immunization with antigen in CFA or IFA always primed IgG1 antibodies regardless of the genetic background of mouse strain, suggesting that the γ1 gene may be preferably selected during effector B cell development [38]. Interestingly, although the second wave of immune responses primed by an unrelated antigen did not affect the frequency of pre-existing memory and effector T cells responding to the first antigen, it did modulate humoral responses to the first antigen independent of whether the second antigen was administered in the same or opposite type of adjuvant. As memory B cells are like to have completed their isotype

switching, the influence of the second wave of humoral responses on the first wave may be affecting the activation and differentiation of the B cells responding to the first antigen. Indeed, bone marrow continues to produce naïve B cells, which can respond to the first antigen and differentiate into effector plasma cells in the presence of antigen-specific helper T cells and bystander helper from the second wave of T cell immunity. This, together with the propensity of memory B cells to respond many different cytokines by proliferation, may contribute to the ability of the second wave of immunity to regulate the first wave of humoral responses.

Notably, neonatally primed memory and effector Th1 and Th2 responses have different capacities to sustain over time. Antigen-primed Th2 responses were only slightly reduced while antigen-primed Th1 responses dramatically declined over time. The rapid decline in memory and effector Th1 responses may stem from pro-inflammatory Th1 cells undergoing apoptosis. Indeed, effector Th1 cells, but not Th2 cells, are highly sensitive to apoptotic induction [39]. Furthermore, CFA which is co-injected with antigen contains mycobacteria and can strongly activate APCs, particularly dendritic cells, creating in a “dangerous’ environment [40]. The activated dendritic cells can produce free radicals and NO, which may trigger effector Th1 cell apoptosis [41].

Importantly, we found that the regulatory effect of the first wave of Th2 (or Th1) responses on the second wave of primed Th1 (or Th2) responses gradually decreased as the time between immunizations increased. By 14 weeks of age, mice that had been challenged with an unrelated antigen in an opposite type of adjuvant displayed unipolar Th2 or Th1 responses to the challenged antigen. This was surprising given that memory Th2 (or Th1) cells from the first immunization persist at that time point. If cytokines produced by memory and effector T cells mediate the regulatory effect they may no longer produce these cytokines *in vivo,* and become

resting memory T cells. Alternatively, cytokines produced by pre-existing memory T cells may be insufficient to regulate the activation and differentiation of naïve T cells responding to the second antigen.

Effective vaccination for prevention of microorganism-mediated diseases often depends on the type of primed immune responses. Immunization to induce neutralizing antibodies can effectively prevent toxin-producing bacteria-mediated diseases, such as diphtheria, tetanus, cholera, and anthrax [10]. In contrast, induction of Th1 immunity can protect from many virus- or intracellular parasite-mediated diseases, such as typhoid fever, tuberculosis, hepatitis, and *Leishmania* [11,12]. In the clinic, children regularly receive sequential vaccinations against many bacteria and viruses. While immunization with the purified protein (like toxoid) in Alum usually induces Th2-like responses, immunization with attenuated bacteria and virus often promotes Th1-like responses. If the first primed immune responses interfere with subsequently primed immune responses, like we have observed in mice, sequential immunizations with mixed types of vaccines might reduce the efficacy of vaccination. Thus, our findings about the interference between waves of immune responses may aid in designing improved strategies for vaccination.

Epidemiological studies have revealed that there is an inverse association between tuberculosis infection and asthma incidence in humans [25,26]. Our findings that the interference of different waves of immune responses to unrelated antigens support the notion that tuberculin-primed memory and effector Th1 cells modulate allergen-induced immune responses, and inhibit atopic diseases in humans. Moreover, our findings may provide new insights into the mechanism underlying the development of determinant spreading and therapeutic effect of treatment with autoantigen in a mode to induce Th2 responses that inhibit tissue-specific autoimmune diseases, as experimental autoimmune encephalomyelitis (EAE) and Type 1 diabetes (T1D) [6,21,23,42-

44]. As in our model system, the first wave of Th1 responses may act to enhance the magnitude of subsequent autoimmune T cell responses to target tissue autoantigens, creating a positive feedback loop that promotes determinant spreading. Immunotherapy induced effector and memory Th2 cells may inhibit the subsequent activation of self-antigen specific pathogenic Th1 cells, and deviate them towards regulatory and protective T cell responses. Thus, our data may explain why there is inverse correlation between tuberculosis rates and asthma incidence, and provide new insights into the mechanism(s) underlying the development of determinant spreading and antigen-based immunotherapies for autoimmune diseases.

In summary, we have examined the interaction between different waves of immune responses to unrelated antigens. We observed that the first wave of T cell immunity enhanced subsequently primed T cell responses to unrelated antigens when administered in the same type of adjuvant, but down-regulated them when antigen was challenged with the opposite type of adjuvant. However, the second wave of T cell responses does not significantly affect the frequency of effector and memory T cells. The regulatory effects of the first wave of T cell immunity on the subsequent T cell responses to unrelated antigens attenuated over time. Moreover, there was a cross-regulation between the first and second waves of humoral responses. The wave interference of immune responses to unrelated antigens may shed light on the mechanism(s) underlying determinant spreading and antigen-based immunotherapies, and may help explain why tuberculosis infection is inversely correlated to lower asthma incidence. In addition, our findings may aid in designing improved strategies for vaccinations.

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**References:**


1. Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A. and Coffman, R.L. (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*, **136**, 2348-57.
2. Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol*, **7**, 145-73.
3. Abbas, A.K., Murphy, K.M. and Sher, A. (1996) Functional diversity of helper T lymphocytes. *Nature*, **383**, 787-93.
4. Powrie, F. and Coffman, R.L. (1993) Cytokine regulation of T-cell function: potential for therapeutic intervention. *Immunol Today*, **14**, 270-4.
5. O'Garra, A. and Murphy, K. (1994) Role of cytokines in determining T-lymphocyte function. *Curr Opin Immunol*, **6**, 458-66.
6. Kuchroo, V.K., Das, M.P., Brown, J.A., Ranger, A.M., Zamvil, S.S., Sobel, R.A., Weiner, H.L., Nabavi, N. and Glimcher, L.H. (1995) B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell*, **80**, 707-18.
7. Liblau, R.S., Singer, S.M. and McDevitt, H.O. (1995) Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. *Immunol Today*, **16**, 34-8.
8. Allen, J.E. and Maizels, R.M. (1997) Th1-Th2: Reliable Paradigm or Dangerous Dogma? *Immunology Today*, **18**, 387-92.
9. Locksley, R.M. and Scott, P. (1991) Helper T-cell subsets in mouse leishmaniasis: induction, expansion and effector function. *Immunol Today*, **12**, A58-61.

10. Kovarik, J. and Siegrist, C.A. (1998) Optimization of vaccine responses in early life: the role of delivery systems and immunomodulators. *Immunol Cell Biol*, **76**, 222-36.

11. Bretsche, P.A., Ismail, N., Menon, J.N., Power, C.A., Uzonna, J. and Wei, G. (2001) Vaccination against and treatment of tuberculosis, the leishmaniases and AIDS: perspectives from basic immunology and immunity to chronic intracellular infections. *Cell Mol Life Sci*, **58**, 1879-96.

12. Fletcher, M.A. and Saliou, P. (2000) Vaccines and infectious disease. *Exs*, **89**, 69-88.

13. von Herrath, M.G., Berger, D.P., Homann, D., Tishon, T., Sette, A. and Oldstone, M.B. (2000) Vaccination to treat persistent viral infection. *Virology*, **268**, 411-9.

14. Power, C.A., Wei, G. and Bretscher, P.A. (1998) Mycobacterial dose defines the Th1/Th2 nature of the immune response independently of whether immunization is administered by the intravenous, subcutaneous, or intradermal route. *Infect Immun*, **66**, 5743-50.

15. Forsthuber, T., Yip, H.C. and Lehmann, P.V. (1996) Induction of TH1 and TH2 immunity in neonatal mice. *Science*, **271**, 1728-30.

16. Sprent, J. (1997) Immunological memory. *Curr Opin Immunol*, **9**, 371-9.

17. Ahmed, R. and Gray, D. (1996) Immunological memory and protective immunity: understanding their relation. *Science*, **272**, 54-60.

18. Croft, M., Bradley, L.M. and Swain, S.L. (1994) Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. *J Immunol*, **152**, 2675-85.

19. Gammon, G., Dunn, K., Shastri, N., Oki, A., Wilbur, S. and Sercarz, E.E. (1986) Neonatal T-cell tolerance to minimal immunogenic peptides is caused by clonal inactivation. *Nature*, **319**, 413-5.

20. Lenschow, D.J., Herold, K.C., Rhee, L., Patel, B., Koons, A., Qin, H.Y., Fuchs, E., Singh, B., Thompson, C.B. and Bluestone, J.A. (1996) CD28/B7 regulation of Th1 and Th2 subsets in the development of autoimmune diabetes [published erratum appears in Immunity 1997 Feb;6(2):following 215]. *Immunity*, **5**, 285-93.

21. Hafler, D.A. and Weiner, H.L. (1995) Antigen-specific therapies for the treatment of autoimmune diseases. *Springer Semin Immunopathol*, **17**, 61-76.

22. Adorini, L., Guéry, J.C. and Trembleau, S. (1996) Manipulation of the Th1/Th2 cell balance: an approach to treat human autoimmune diseases? *Autoimmunity*, **23**, 53-68.

23. Tian, J., Clare-Salzler, M., Herschenfeld, A., Middleton, B., Newman, D., Mueller, R., Arita, S., Evans, C., Atkinson, M.A., Mullen, Y., Sarvetnick, N., Tobin, A.J., Lehmann, P.V. and Kaufman, D.L. (1996) Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice [see comments]. *Nat Med*, **2**, 1348-53.

24. Hogan, S.P., Foster, P.S., Charlton, B. and Slattery, R.M. (1998) Prevention of Th2-mediated murine allergic airways disease by soluble antigen administration in the neonate. *Proc Natl Acad Sci U S A*, **95**, 2441-5.

25. Tang, C., Inman, M.D., van Rooijen, N., Yang, P., Shen, H., Matsumoto, K. and O'Byrne, P.M. (2001) Th type 1-stimulating activity of lung macrophages inhibits Th2-mediated allergic airway inflammation by an IFN-gamma-dependent mechanism. *J Immunol*, **166**, 1471-81.

26. Shirakawa, T., Enomoto, T., Shimazu, S. and Hopkin, J.M. (1997) The inverse association between tuberculin responses and atopic disorder. *Science*, **275**, 77-9.

27. Tian, J., Atkinson, M.A., Clare-Salzler, M., Herschenfeld, A., Forsthuber, T., Lehmann, P.V. and Kaufman, D.L. (1996) Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes. *J Exp Med*, **183**, 1561-7.

28. Singh, R.R., Hahn, B.H. and Sercarz, E.E. (1996) Neonatal peptide exposure can prime T cells and, upon subsequent immunization, induce their immune deviation: implications for antibody vs. T cell-mediated autoimmunity. *J Exp Med*, **183**, 1613-21.

29. Lehmann, P.V., Sercarz, E.E., Forsthuber, T., Dayan, C.M. and Gammon, G. (1993) Determinant spreading and the dynamics of the autoimmune T-cell repertoire. *Immunol Today*, **14**, 203-8.

30. Curtsinger, J.M., Schmidt, C.S., Mondino, A., Lins, D.C., Kedl, R.M., Jenkins, M.K. and Mescher, M.F. (1999) Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J Immunol*, **162**, 3256-62.

31. Webster, R.G. (1966) Original antigenic sin in ferrets: the response to sequential infections with influenza viruses. *J Immunol*, **97**, 177-83.

32. Fazekas de St, G. and Webster, R.G. (1966) Disquisitions of Original Antigenic Sin. I. Evidence in man. *J Exp Med*, **124**, 331-45.

33. Klenerman, P. and Zinkernagel, R.M. (1998) Original antigenic sin impairs cytotoxic T lymphocyte responses to viruses bearing variant epitopes. *Nature*, **394**, 482-5.

34. Selin, L.K., Varga, S.M., Wong, I.C. and Welsh, R.M. (1998) Protective heterologous antiviral immunity and enhanced immunopathogenesis mediated by memory T cell populations. *J Exp Med*, **188**, 1705-15.

35. Selin, L.K., Lin, M.Y., Kraemer, K.A., Pardoll, D.M., Schneck, J.P., Varga, S.M., Santolucito, P.A., Pinto, A.K. and Welsh, R.M. (1999) Attrition of T cell memory: selective loss of LCMV epitope-specific memory CD8 T cells following infections with heterologous viruses. *Immunity*, **11**, 733-42.

36. Kim, S.K., Brehm, M.A., Welsh, R.M. and Selin, L.K. (2002) Dynamics of memory T cell proliferation under conditions of heterologous immunity and bystander stimulation. *J Immunol*, **169**, 90-8.

37. McNally, J.M., Zarozinski, C.C., Lin, M.Y., Brehm, M.A., Chen, H.D. and Welsh, R.M. (2001) Attrition of bystander CD8 T cells during virus-induced T-cell and interferon responses. *J Virol*, **75**, 5965-76.

38. Lin, S.C., Wortis, H.H. and Stavnezer, J. (1998) The ability of CD40L, but not lipopolysaccharide, to initiate immunoglobulin switching to immunoglobulin G1 is explained by differential induction of NF-kappaB/Rel proteins. *Mol Cell Biol*, **18**, 5523-32.

39. Zhang, X., Brunner, T., Carter, L., Dutton, R.W., Rogers, P., Bradley, L., Sato, T., Reed, J.C., Green, D. and Swain, S.L. (1997) Unequal death in T helper cell (Th)1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid Fas/FasL-mediated apoptosis. *J Exp Med*, **185**, 1837-49.

40. Ridge, J.P., Fuchs, E.J. and Matzinger, P. (1996) Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. *Science*, **271**, 1723-6.

41. Downing, J.E., Virag, L. and Perry, M.E. (1998) Nitrergic mechanism of DC-mediated T-cell elimination. *Immunol Today*, **19**, 190-1.

42. Tisch, R., Wang, B. and Serreze, D.V. (1999) Induction of glutamic acid decarboxylase 65-specific Th2 cells and suppression of autoimmune diabetes at late stages of disease is epitope dependent. *J Immunol*, **163**, 1178-87.

43. Fathman, C.G., Costa, G.L. and Seroogy, C.M. (2000) Gene therapy for autoimmune disease. *Clin Immunol*, **95**, S39-43.

44. Tian, J., Lehmann, P.V. and Kaufman, D.L. (1997) Determinant spreading of T helper cell 2 (Th2) responses to pancreatic islet autoantigens. *J Exp Med*, **186**, 2039-43.

**Legends**:

**Fig. 1. Existing immunity up-regulates subsequent T cell response to an unrelated antigen administered in the same type of adjuvant**. Neonatal Balb/c were immunized with OVA or HEL in CFA or CFA alone (panel A and B) or antigen in IFA or IFA alone (panel C and D), and the mice were challenged with the second antigen (such as HEL/CFA for OVA/CFA-immunized mice) at 4 wks of age. Control groups of mice received one immunization with a single antigen in CFA or IFA at birth or at 4 wks of age. T cell immunity to both antigens was characterized by the ELISPOT assay at 6 wks of age. Data are presented as the mean number of spot-forming colonies (SFC) per $10^6$ splenic cells ± S.E.M. Mice from experimental and control groups (n=4-6 mice) were tested simultaneously in two independent experiments. Mice immunized with antigen in CFA did not display detectable IL-4 and IL-5 responses to both antigens and mice injected with antigen in IFA failed to develop IFNγ responses (data not shown). A similar pattern of T cell responses to both antigens developed in C57BL/6 mice following immunization with antigen in CFA or IFA (data not shown)

**Fig. 2. The first wave of immunity modulates the subsequent T cell responses to an unrelated antigen when administered in an opposite type of adjuvant.** Neonatal Balb/c and C57BL/6 mice were immunized with OVA/IFA, HEL/IFA, or IFA alone, and were challenged with HEL/CFA or OVA/CFA (HEL for OVA sensitized mice and vice versa) at 4 wks of age. Additional mice were neonatally immunized with OVA/CFA, HEL/CFA, or CFA alone and challenged with HEL/IFA or OVA/IFA at 4 wks of age. Control groups of mice received one immunization with a single antigen in IFA or CFA at birth or at 4 wks of age. The frequency of antigen-specific IFNγ-, IL-4-, and IL-5-secreting T cells responding to OVA (panel A and B) or

to HEL (panel C and D) was determined by the ELISPOT assay at 2 wks post challenge. Mice from experimental and control groups (n=4-6 mice) were tested simultaneously in three independent experiments. Data are shown as the mean number of SFC per $10^6$ splenic cells ± S.E.M. The pattern of IL-4 responses was similar to those shown for IL-5 in all groups of mice (data not shown). Mice immunized neonatally or at 4 wks of age (with antigen in CFA or IFA) developed similar magnitude T cell responses to the antigen (data not shown). The OVA-I, HEL-I, OVA-C, or HEL-C represents OVA/IFA, HEL/IFA, OVA/CFA, or HEL/CFA, respectively, in this figure as well as in Fig. 3-6.

**Fig. 3. Mixed Th1/Th2 responses develop in mice sensitized with one antigen and challenged with the second antigen in an opposite type of adjuvant.** Balb/c neonates were immunized with HEL/IFA or HEL/CFA, and 3 wks later they were challenged with OVA/CFA (panel B) or OVA/IFA (panel D). Control groups of mice received one immunization with OVA/CFA (panel A) or OVA/IFA (panel C) at 3 wks in age. Two wks after OVA immunization, splenic mononuclear cells from all mouse groups were isolated and stimulated with OVA *in vitro*. The $CD4^+$ T cells were purified and their intracytoplasmic cytokines (IFNγ and IL-4) were characterized by FACS analysis. Data shown is a representative of two independent experiments (n=3 per group).

**Fig. 4. Adoptively transferable memory and effector T cells, but not APCs, mediate the regulatory effects.** Balb/c neonates were immunized with HEL/CFA or HEL/IFA, and splenic T cells or APCs from HEL-immunized mice, as well as from unmanipulated mice (for naïve T cells and APCs), were purified. The T cells, or APCs, were transfused into Balb/c mice, which were

then immunized with OVA/CFA or OVA/IFA, and ten days later their T cell immunity to OVA was characterized by the ELISPOT assay (panel A and B, respectively). Mice from experimental and control groups were tested simultaneously in two independent experiments (n=4 per group). The pattern of IL-4 responses was similar to those shown for IL-5 in all groups of mice (data not shown).

**Fig. 5. The first and second wave of humoral responses to unrelated antigens are mutually up-regulated.** Neonatal mice were immunized with OVA or HEL in IFA or CFA and challenged with an unrelated antigen in the same type of adjuvant at 4 wks of age. Control groups of mice received one immunization at birth or 4 wks of age. At six weeks of age, their antibodies (IgG, IgG1, and IgG2a) against both antigens were characterized by the ELISA assay. Data are represented as the mean O.D. (405-490) ± SEM of Balb/c mice (1:500 dilution for mouse sera and n=4-5 per group). Panel A and C showed antibodies against OVA, and panel B and D reflected HEL-specific humoral responses. Negative control wells showed about 0.056-0.084 (O.D.). A similar pattern of humoral responses to both antigens developed in C57BL/6 mice (data not shown).

**Fig. 6. Humoral responses to unrelated Antigens are modulated following challenge with antigen in the opposite type of adjuvant.** Neonatal mice were immunized with OVA or HEL in IFA or CFA and challenged with an unrelated antigen in the opposite type of adjuvant at 4 wks of age. Control groups of mice received one immunization at birth or 4 wks of age. At six wks of age, their antibodies (IgG, IgG1, and IgG2a) against both antigens were characterized by the ELISA assay. Data are represented as the mean O.D. (405-490) ± SEM of Balb/c mice (1:500

dilution for mouse sera and n=4-5 per group). Panel A and C represented antibody responses against OVA, and panel B and D reflected HEL-specific humoral responses. Negative control wells showed about 0.056-0.084 (O.D.). A similar pattern of humoral responses to both antigens developed in C57BL/6 mice (data not shown).

**Fig. 7. The regulatory effects of the first wave of T cell immunity on the second wave of T cell responses to an unrelated antigen attenuate over time.** (A). Mice that received one immunization with OVA/IFA or OVA/CFA at different ages (4, 8, or 12 wks of age) and tested at 6, 10, or 14 wks of age, respectively, developed comparable levels of unipolar T cell responses. (B). Mice that were neonatally immunized with OVA/IFA or OVA/CFA were characterized for IL-5 and IFNγ responses to OVA at 6, 10, or 14 wks of age. Mice neonatally immunized with HEL/IFA or HEL/CFA showed a similar pattern of memory T cells to sustain over time (data not shown). (C and D). Interference of the first wave of T immunity with subsequent T responses to an unrelated antigen gradually attenuates over time. Mice were neonatally immunized with HEL/CFA and then challenged with OVA/IFA at 4, 8, or 12 wks later, respectively. Their T cell immunity to OVA was characterized at 2 wks post-challenge (panel C). Mice were neonatally sensitized with HEL/IFA and challenged with OVA/CFA at 4, 8 or 12 wks of age, respectively. T cell responses to OVA were tested at two weeks post-challenge (panel D). Data are presented as the mean number of SFC per $10^6$ splenic cells over background from medium alone (n=4 for each time point of each group) in two independent experiments. The intra-group variation was less than 15% of the mean number of SFC. A similar pattern of T cell responses to HEL was observed in mice neonatally sensitized with OVA and challenged with HEL (data not shown). C57BL/6 mice displayed a similar pattern of T cell immunity

following neonatal immunization with one antigen and challenge with an unrelated antigen in the opposite type of adjuvant at different ages (data not shown).

**Fig. 1**

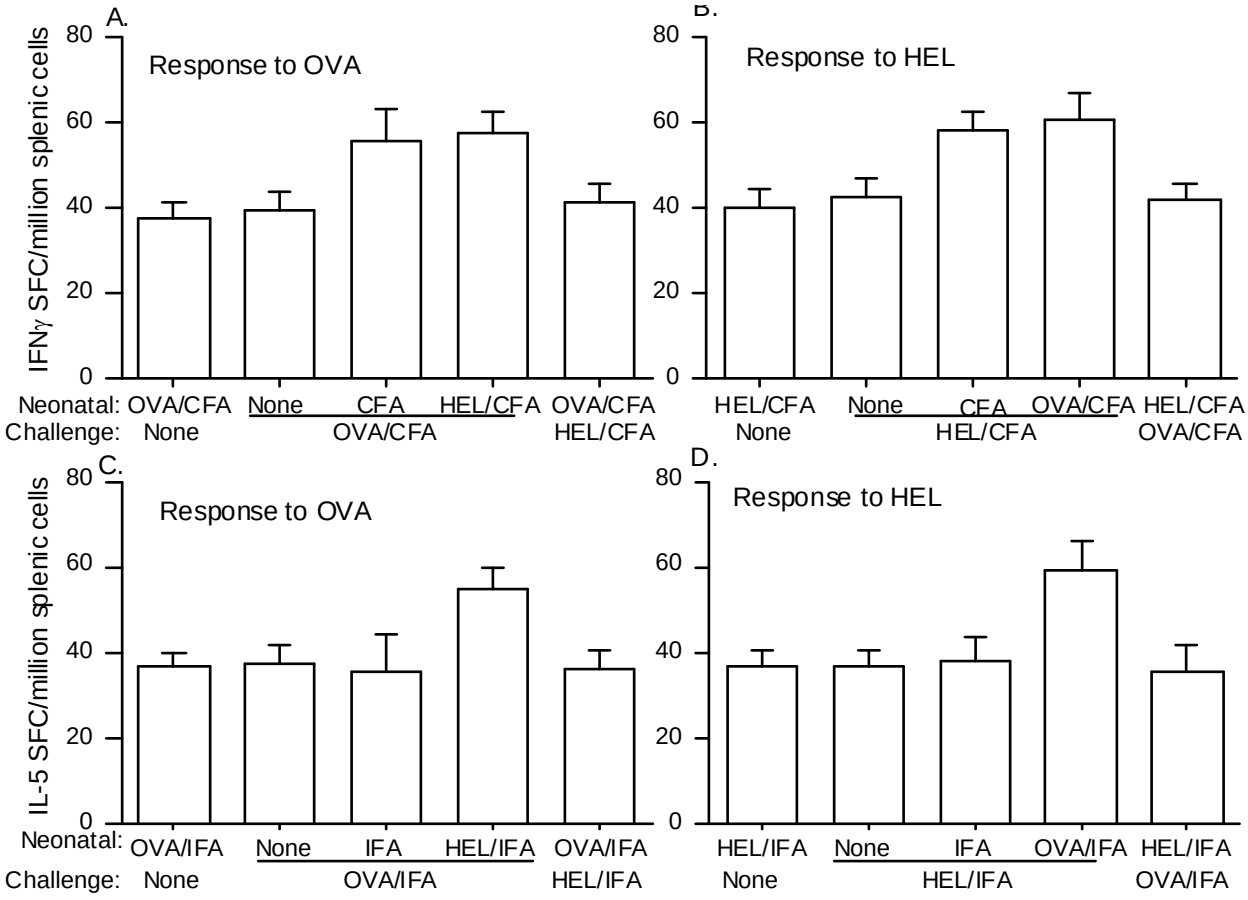

A.
Response to OVA
IFNγ SFC/million splenic cells
80
60
40
20
0
Neonatal: OVA/CFA None CFA HEL/CFA OVA/CFA
Challenge: None OVA/CFA HEL/CFA
B.
Response to HEL
HEL/CFA None CFA OVA/CFA HEL/CFA
None HEL/CFA OVA/CFA
C.
Response to OVA
IL-5 SFC/million splenic cells
Neonatal: OVA/IFA None IFA HEL/IFA OVA/IFA
Challenge: None OVA/IFA HEL/IFA
D.
Response to HEL
HEL/IFA None IFA OVA/IFA HEL/IFA
None HEL/IFA OVA/IFA

Fig. 2

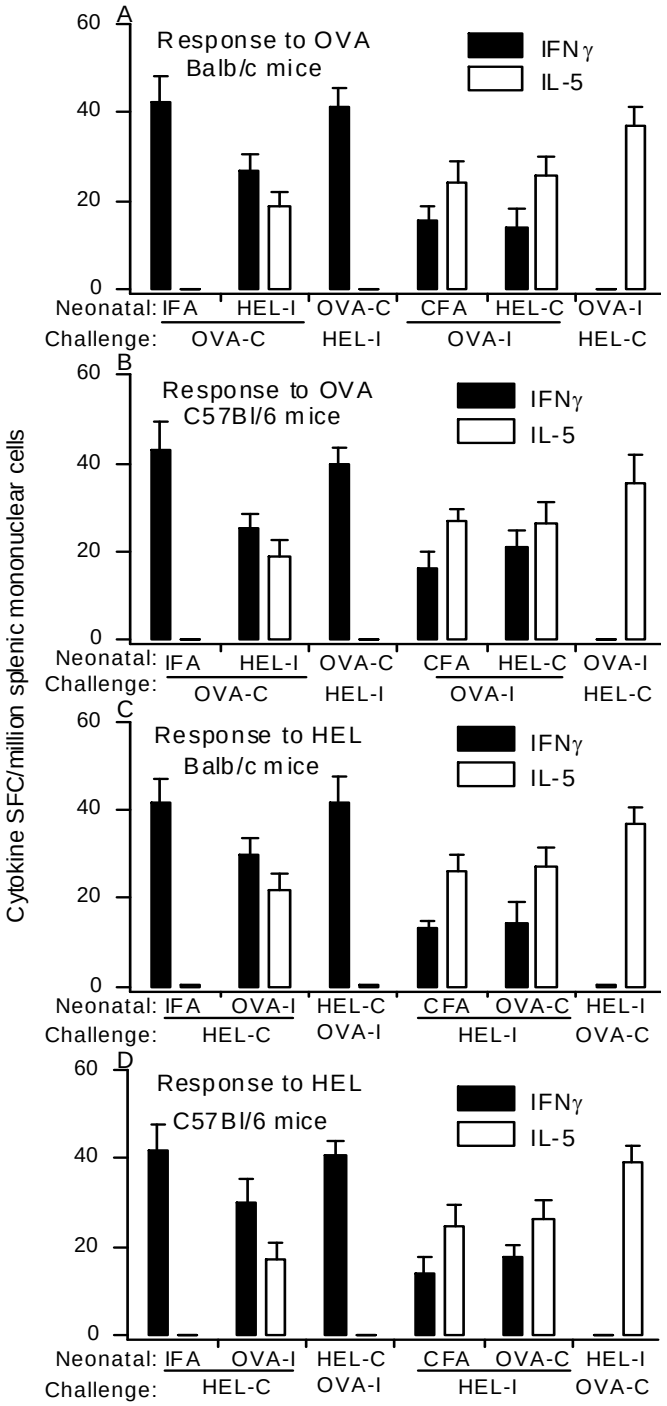

A
Response to OVA
Balb/c mice
IFNγ
IL-5
60
40
20
0
Neonatal: IFA HEL-I OVA-C CFA HEL-C OVA-I
Challenge: OVA-C HEL-I OVA-I HEL-C
B
Response to OVA
C57Bl/6 mice
IFNγ
IL-5
Neonatal: IFA HEL-I OVA-C CFA HEL-C OVA-I
Challenge: OVA-C HEL-I OVA-I HEL-C
C
Response to HEL
Balb/c mice
IFNγ
IL-5
Neonatal: IFA OVA-I HEL-C CFA OVA-C HEL-I
Challenge: HEL-C OVA-I HEL-I OVA-C
D
Response to HEL
C57Bl/6 mice
IFNγ
IL-5
Neonatal: IFA OVA-I HEL-C CFA OVA-C HEL-I
Challenge: HEL-C OVA-I HEL-I OVA-C
Cytokine SFC/million splenic mononuclear cells

Fig. 3

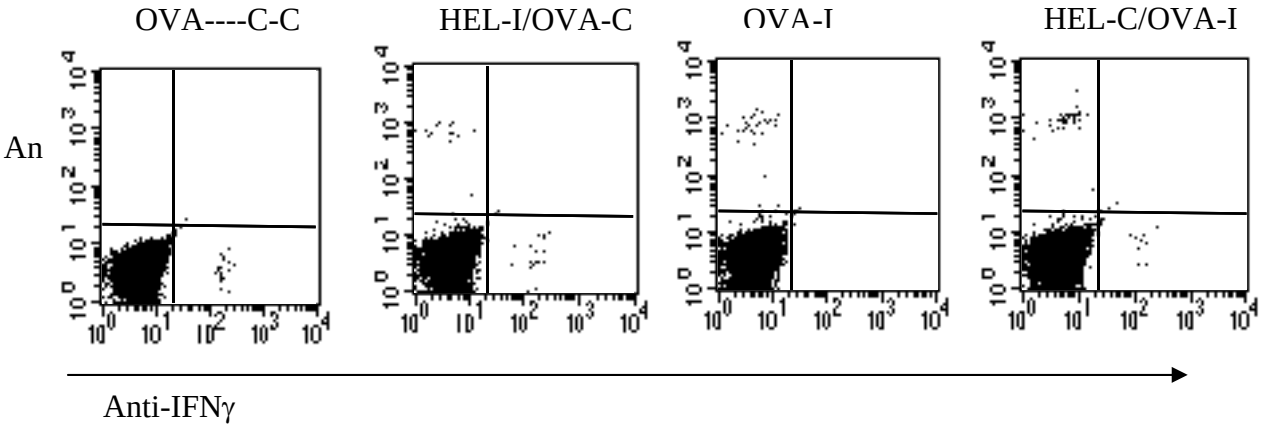


Fig. 4

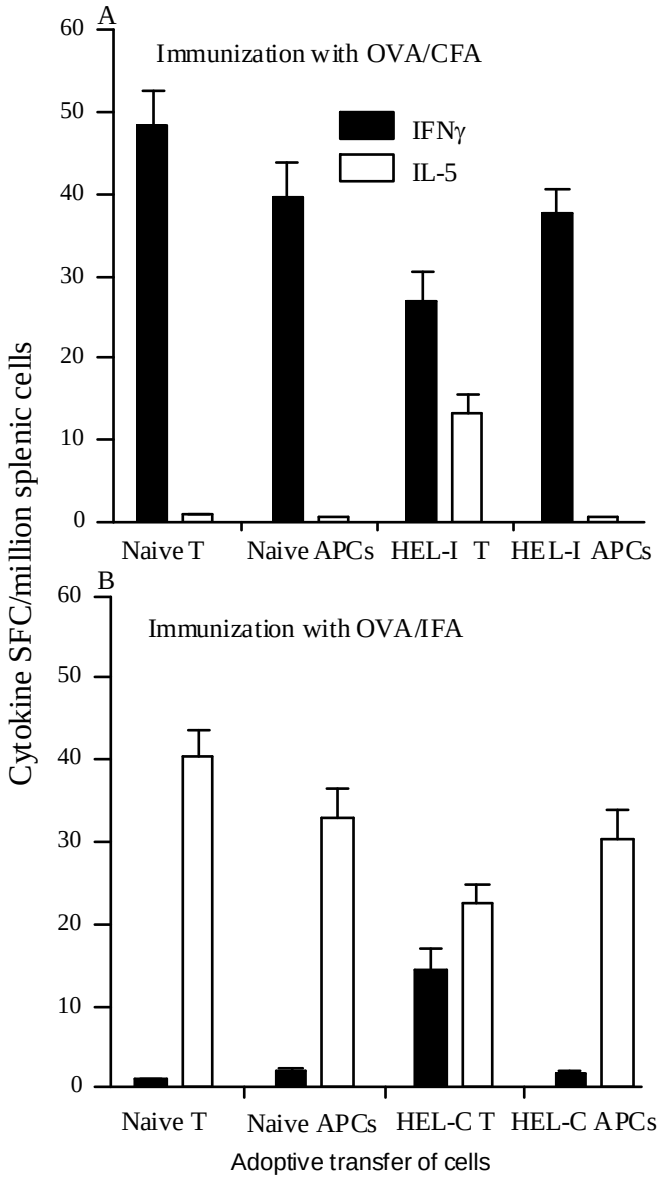

A
Immunization with OVA/CFA
IFNγ
IL-5
60
50
40
30
20
10
0
Naive T
Naive APCs
HEL-I T
HEL-I APCs
B
Immunization with OVA/IFA
60
50
40
30
20
10
0
Naive T
Naive APCs
HEL-C T
HEL-C APCs
Cytokine SFC/million splenic cells
Adoptive transfer of cells

Fig. 5

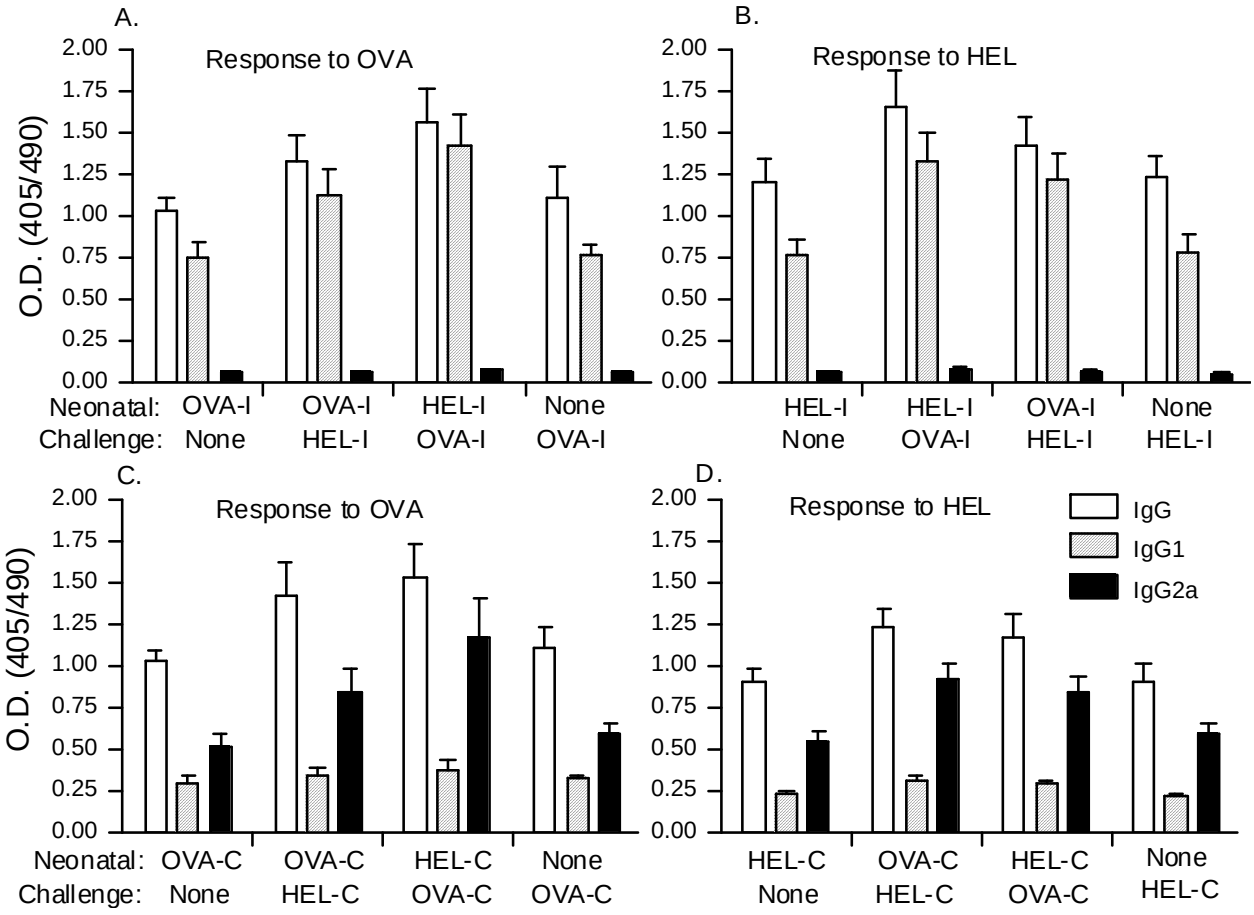

A.
Response to OVA
O.D. (405/490)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Neonatal: OVA-I OVA-I HEL-I None
Challenge: None HEL-I OVA-I OVA-I
B.
Response to HEL
HEL-I HEL-I OVA-I None
None OVA-I HEL-I HEL-I
C.
Response to OVA
Neonatal: OVA-C OVA-C HEL-C None
Challenge: None HEL-C OVA-C OVA-C
D.
Response to HEL
IgG
IgG1
IgG2a
HEL-C OVA-C HEL-C None
None HEL-C OVA-C HEL-C

Fig. 6

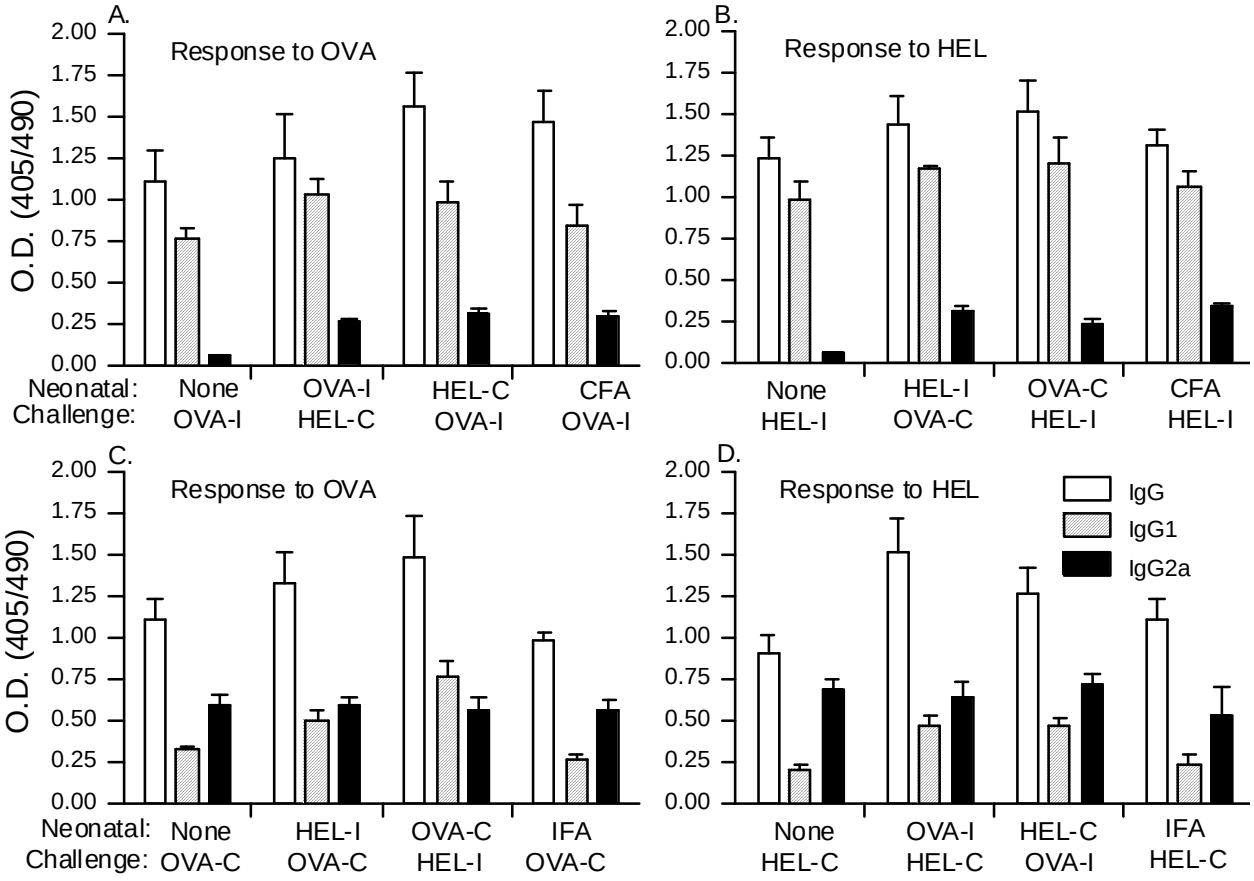
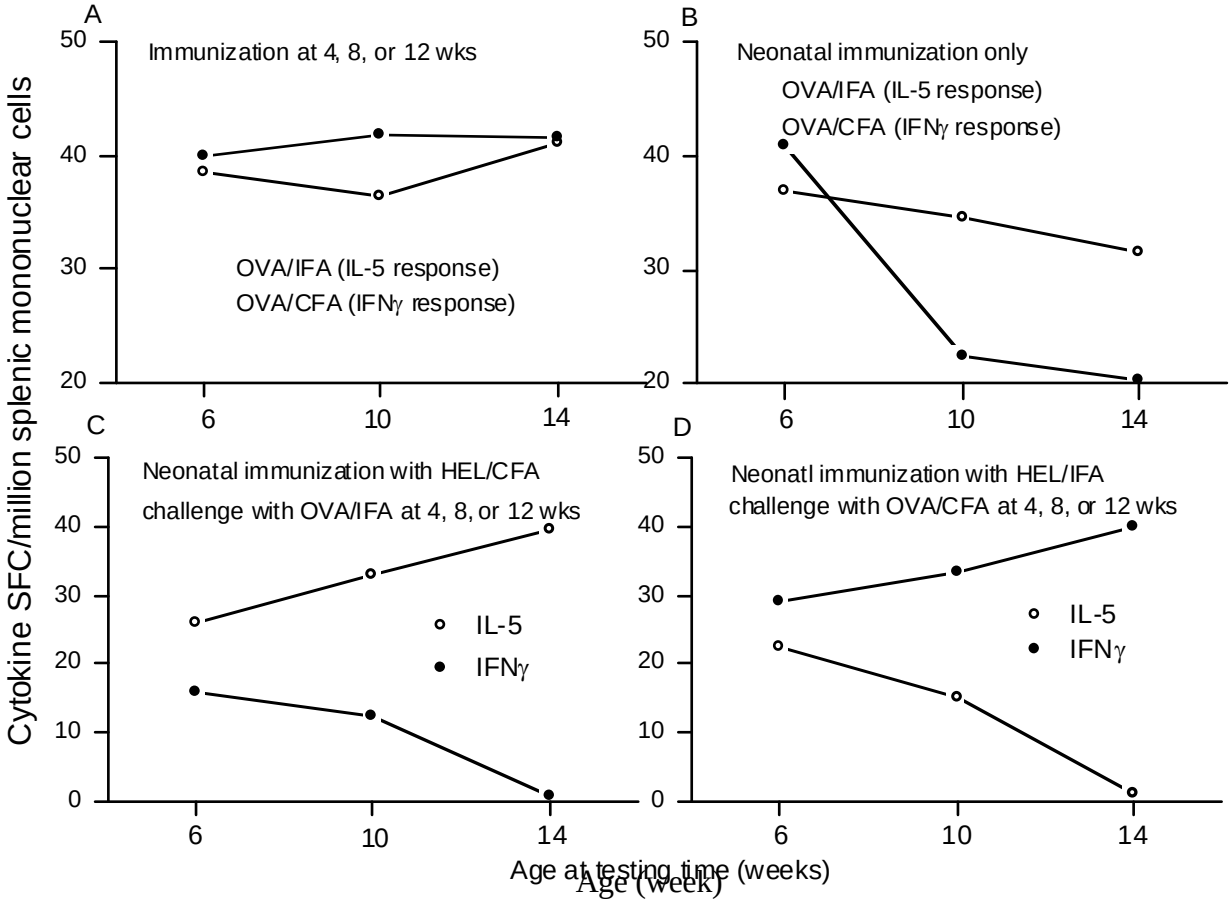

A.
Response to OVA
O.D. (405/490)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Neonatal:
Challenge:
None
OVA-I
OVA-I
HEL-C
HEL-C
OVA-I
CFA
OVA-I
B.
Response to HEL
None
HEL-I
HEL-I
OVA-C
OVA-C
HEL-I
CFA
HEL-I
C.
Response to OVA
None
OVA-C
HEL-I
OVA-C
OVA-C
HEL-I
IFA
OVA-C
D.
Response to HEL
IgG
IgG1
IgG2a
None
HEL-C
OVA-I
HEL-C
HEL-C
OVA-I
IFA
HEL-C

Fig. 7


A
Immunization at 4, 8, or 12 wks
OVA/IFA (IL-5 response)
OVA/CFA (IFNγ response)
B
Neonatal immunization only
OVA/IFA (IL-5 response)
OVA/CFA (IFNγ response)
C
Neonatal immunization with HEL/CFA
challenge with OVA/IFA at 4, 8, or 12 wks
IL-5
IFNγ
D
Neonatl immunization with HEL/IFA
challenge with OVA/CFA at 4, 8, or 12 wks
IL-5
IFNγ
Cytokine SFC/million splenic mononuclear cells
Age at testing time (weeks)
Age (week)