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Neonatal Tolerance in the Absence of Stat4- and Stat6-Dependent Th Cell Differentiation

Hua-Chen Chang, Shangming Zhang, and Mark H. Kaplan

Neonatal tolerance to specific Ag is achieved by nonimmunogenic exposure within the first day of life. The mechanism that regulates this tolerance may provide the basis for successful organ transplantation and has recently been thought to be immune deviation from the inflammatory Th1 response to a Th2 response. To test the importance of Th2 cells in the establishment of neonatal tolerance, we examined neonatal tolerance in Stat4- and Stat6-deficient mice, which have reduced Th1 and Th2 cell development, respectively. Neonatal tolerance of both the T and B cell compartments in Stat4- and Stat6-deficient mice was similar to that observed in wild-type mice. Cytokine production shifted from a Th1 to a Th2 response in wild-type mice tolerized as neonates. In contrast, tolerance was observed in Stat6-deficient mice despite maintenance of a Th1 cytokine profile. These results suggest that cells distinct from Stat6-dependent Th2 cells are required for the establishment of neonatal tolerance.

Materials and Methods
Mice
Generation of Stat4- and Stat6-deficient mice has been described (18, 31), and mice were backcrossed 10 generations to the BALB/c genetic background. Control (wild-type BALB/c) mice were purchased from Harlan Bioproducts (Indianapolis, IN). Experiments were performed following approval from the Indiana University animal care and use committee.

Tolerization or immunization
Neonatal mice were injected i.p. with 100 μg hen egg lysozyme (HEL; Sigma-Aldrich, St. Louis, MO) or PBS alone emulsified in IFA (Calbiochem, San Diego, CA) in a total volume of 0.1 ml using a 1-ml syringe and a 26-gauge needle. At 6 wk of age mice were immunized s.c. with 50 μg HEL in CFA (Calbiochem) in a total volume of 0.1 ml. Fourteen days after immunization, mice were sacrificed, and spleen cells, draining lymph node cells, and serum were collected from each mouse.

Proliferation assays
Lymph node and spleen cells (5 × 10^5/well) were stimulated in the absence or the presence of increasing doses of HEL (range, 62.5–500 μg/ml). After 72 h in culture, microtiter plates were pulsed with 0.8 μCi [3H]thymidine. Plates were harvested after 18 h and were counted in a scintillation counter. Cultures were stimulated with 2.5 μg/ml Con A as a control.

ELISA
For detection of Ag-specific Ab titers, ELISA plates were coated with 5 μg/ml HEL. Sera were diluted 1/10 in PBS and 2% BSA and were tested at serial 2-fold dilutions using isotype-specific Abs for detection (BD PharMingen, San Diego CA). Titers (arbitrary units) were calculated by multiplying the half-maximal OD by the dilution (20). Cytokines levels were assessed by [3H]thymidine incorporation. Stimulation indexes were calculated for each mouse. The results shown are pooled from six independent experiments, and stimulation indexes were calculated at one concentration (250 μg/ml) of HEL. Fig. 1A demonstrates that neonatal tolerance is established in all three genotypes. Mice that did not receive HEL as neonates had greater responses than neonatally tolerized mice. The reduction in stimulation index was statistically significant for all groups (p < 0.03). As has been seen in several other systems (5, 50), tolerance was only established in lymph nodes, and spleen cells remained equally responsive to HEL stimulation regardless of neonatal tolerance or genotype of the mice (Fig. 1B). To further demonstrate the level of tolerance, we determined the percentage of mice in each group that had a stimulation index >3 (high responders). Fig. 1C demonstrates that there was little difference in the percentage of high responders in spleen cells from all groups of mice or in the percentage of high responders from nontolerized lymph node cells. However, in lymph node cells there was a dramatic decrease in the percentage of high responders in the tolerized groups compared with nontolerized groups of all three genotypes of mice. As a control for the ability of T cells to proliferate, we stimulated lymph node cells from all three genotypes with Con A. Wild-type, Stat4-deficient, and Stat6-deficient lymph node cells had similar levels of Con A-induced proliferation regardless of the induction of tolerance (data not shown). Thus, T cells maintain the ability to proliferate in response to a polyclonal stimulus.

To determine the level of B cell tolerance we assayed the level of Ag-specific Abs in the serum of mice treated as described.
above. Wild-type mice not given HEL as neonates generated both IgG1 and IgG2a Ag-specific Abs following immunization (Fig. 2). However, the HEL-tolerized group generated low titers of HEL-specific Abs of both isotypes. Stat4-deficient mice mounted a predominantly IgG1 response to HEL following immunization, with little Ag-specific IgG2a produced, correlating with low IFN-γ levels in vivo and as seen previously. By contrast, Stat6-deficient mice had almost exclusively IgG2a anti-HEL titers. This correlates with decreased class switching to IgG2a in Stat6-deficient B cells (49). Importantly, both Stat4- and Stat6-deficient mice were unable to generate significant titers of anti-HEL when they were neonatally tolerated, similar to wild-type mice.

To determine the level of immune deviation of these mice, we examined supernatants of Ag-stimulated lymph node cells from mice treated as described above. IL-4 levels were low or undetectable in many samples; thus, IL-5 was used as a marker for Th2 responses, and IFN-γ was used as a marker for Th1 cells. Wild-type mice that did not receive HEL as neonates had high IFN-γ and low IL-5 following immunizations and thus a high IFN-γ/IL-5 ratio, indicating the Th1/Th2 ratio (Fig. 3). The induction of tolerance in wild-type mice resulted in a significant increase in IL-5 secretion, although with no significant change in IFN-γ levels, indicating a decrease in the Th1/Th2 ratio (p = 0.01). In contrast, Stat4-deficient mice had a low ratio, indicating higher Th2 activity than Th1, although there were relatively low levels of both IFN-γ and IL-5 production (Fig. 3). Tolerance had no significant effect on the cytokine production in Stat4-deficient cultures (p > 0.05). Stat6-deficient mice had high IFN-γ and low IL-5 production, indicating low Th2 activity. The establishment of tolerance in Stat6-deficient mice did not alter this pattern of cytokine secretion (p > 0.05). Thus, tolerance induction was not accompanied by significant immune deviation in either the Stat4- or Stat6-deficient mice. Therefore, neonatal tolerance does not require Stat4- or Stat6-dependent immune deviation.

Discussion

In the 5 decades that the phenomenon of neonatal tolerance has been recognized, little of the actual mechanism has been elucidated. In the past decade a correlation between the establishment of neonatal tolerance and immune deviation (a shift from Th1 to Th2 responses) has been observed (4, 5, 7, 14). However, whether the resulting immune deviation is a cause or an effect of neonatal tolerance was not clear. In this report we have used Stat4-deficient
and Stat6-deficient mice, which have defects in Th1 and Th2 development, respectively, to test the importance of immune deviation in tolerance establishment. We found that tolerance is efficiently established in wild-type, Stat4-deficient, and Stat6-deficient mice regardless of immune deviation. Thus, activation of cells other than Stat6-dependent Th2 cells is responsible for the tolerant state when neonates are exposed to Ag.

Previous studies have noted that administration of IL-12, IFN-γ, or anti-IL-4 at the time of neonatal exposure to alloantigen abrogates the tolerant state (10–13). This was taken to support the concept of immune deviation in neonatal tolerance. Indeed, recent evidence suggests that mice deficient in IL-4 and IL-13, but not IL-4 alone, cannot develop tolerance (51). This report did not examine T cell proliferative or Ab responses, but demonstrated graft rejection in IL-4/IL-13 double-deficient mice that had been tolerized as neonates. Importantly, both IL-4 and IL-13 may activate signaling pathways other than Stat6. These distinct pathways may be important for tolerance and account for the difference between the phenotype of cytokine-deficient and Stat6-deficient mice. Furthermore, IL-4 and IFN-γ may have reciprocal effects on many T cell subsets. Indeed, IFN-γ inhibits, and IL-4 enhances, the development of TGF-β-secreting Th3 cells (52). Thus, the presence or the absence of Th1 and Th2-polarizing cytokines during the induction of neonatal tolerance may have profound effects on the immune system distinct from a shift from Th1 to Th2 responses.

The cell populations that are responsible for neonatal tolerance are still elusive. It is still possible that Stat6-independent Th2 cells may play some role in this process. Furthermore, several other T cell subsets have been implicated in immunoregulatory responses. We have examined the in vitro differentiation of Tr1 cells (53, 54) and found that Stat6-deficient T cell cultures have greatly decreased numbers of IL-10-secreting cells compared with wild-type or Stat4-deficient T cell cultures (our unpublished observations). Thus, Tr1 cells seem unlikely candidates for regulating this response. We have also examined the development of Th3 cells in vitro and found that while all three genotypes can develop T cells secreting TGF-β, levels are lower (by ~50%) in Stat6-deficient cultures. This correlates with the reported ability of IL-4 to promote TGF-β secretion and observed decreased TGF-β in T. cruzi-infected, Stat6-deficient mice (22, 52). The biological significance of this decrease is unclear, since both Stat4- and Stat6-deficient mice can be orally tolerized (55). Whether these T cell subsets or other functional subsets are required for neonatal tolerance will require further examination.

One somewhat surprising aspect of our results was the lack of an Ag-specific IgG1 response in tolerized mice. Forsthuber et al. (5) demonstrate increased levels of anti-HEL IgG1 and decreased IgG2a in mice that were tolerized as neonates. However, the adult challenge to examine Ab levels in that study used HEL in saline, not CFA as in our study. Maverakis et al. (50) observed unchanged levels of anti-HEL IgG1 with decreased IgG2a in a similar model of neonatal tolerance. The adult challenge in that study used HEL emulsified in CFA as we did, but their challenge was by footpad injection, while ours was by s.c. injection. Thus, it is not clear whether the site of administration or the presence or the absence of adjuvant during challenge may explain these differing results. However, it is clear that there is no consensus on the effects of neonatal tolerance on Ag-specific IgG1 levels.

STAT proteins have become intriguing targets for drug discovery because they play critical roles in many responses. The established roles of Stat4 in inflammatory disease and of Stat6 in allergic disease suggest these pathways as important mediators of in vivo immunity. However, Stat4 and Stat6 have been shown to have relatively minor roles in oral tolerance (55) and, as we have shown here, neonatal tolerance. Thus, these studies alter the paradigm of elements required for neonatal tolerance and provide a starting point for further dissection of the components necessary for the establishment of tolerance.

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References


