Failure to Induce Neonatal Tolerance in Mice That Lack Both IL-4 and IL-13 but Not in Those That Lack IL-4 Alone

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Exposure of newborn animals to a foreign Ag often results in immunological tolerance to that Ag. This phenomenon, known as neonatal tolerance, was first described by Owen (1) in 1945 and was later demonstrated experimentally byBillingham et al. (2) in 1953. Owen observed that dizygotic bovine twins that shared placental blood supply during ontogeny tolerated each other’s RBC Ags as adults (1). In the classical experiments byBillingham et al. (2), newborn mice injected with lymphoid cells from a MHC-mismatched donor later accepted skin grafts from the same donor but rejected third-party skin transplants.

The mechanisms responsible for neonatal tolerance are controversial. Although it had been proposed that neonatal exposure to a foreign Ag induces clonal T cell deletion in the same manner in which self-Ags delete self-reactive T lymphocytes in the thymus (3, 4), more recent studies have provided evidence that immunoregulatory mechanisms play a dominant role in the induction and maintenance of neonatal tolerance (5–8). Specifically, neonatally tolerized mice were found to mount a vigorous Th2 immune response but failed to mount a Th1 response when rechallenged with the same Ag they encountered during the neonatal period (5–8), suggesting that Th2 immunity and not clonal T cell deletion underlies neonatal tolerance. Other investigators provided further support for this concept by demonstrating that IL-4-neutralizing Ab or recombinant IFN-γ, given at the time donor lymphoid cells are injected into neonatal mice, blocks Th2 while enhancing Th1 cytokine production and abrogates tolerance to donor skin grafts (9–11).

Current evidence suggests that neonatal tolerance to a foreign Ag is the consequence of IL-4-mediated Th2 immunity rather than the thymic deletion of Ag-specific T cells. Here, we addressed the role of IL-4 in neonatal tolerance by testing whether tolerance to a minor histocompatibility Ag can be induced in newborn mice that lack IL-4 (IL-4−/−). We found that IL-4 does not play a dominant role in the induction of neonatal tolerance as newborn female IL-4−/− mice could be readily tolerized to the H-Y male Ag. In contrast, mice that lack both IL-4 and IL-13 (IL-4−/−/IL-13−/−) were resistant to the induction of neonatal tolerance, and their splenocytes produced exaggerated amounts of IFN-γ on rechallenge with the same Ag encountered during the neonatal period. These findings argue against the view that IL-4 alone is critical for the induction of neonatal tolerance and suggest that the combined actions of both IL-4 and IL-13 are essential for this process. The Journal of Immunology, 2001, 167:1125–1128.

Although the IL-4 neutralization experiments (9–11) strongly suggest that a Th2 response is responsible for the induction of neonatal tolerance, it remains uncertain whether IL-4 plays a dominant role in the tolerance process because IL-4 is not the only mediator of Th2 immunity. One cytokine that could contribute to the development of a Th2 response is IL-13 because it shares many of the in vitro and in vivo biological actions of IL-4 (12). Characteristic markers of the Th2 response (eosinophil infiltration, IgE secretion, increased IL-5 production, and limited IFN-γ production) are present in parasite-infected IL-4 gene-knockout (IL-4−/−) and IL-13 gene-knockout (IL-13−/−) mice but are nearly absent only in mice that lack both IL-4 and IL-13 (IL-4−/−/IL-13−/−) (12–15), indicating that IL-4 and IL-13 cooperate in initiating Th2 immunity. In this article, we addressed the role of IL-4 and of Th2 immunity in the development of neonatal tolerance by testing whether tolerance to a minor histocompatibility Ag, the H-Y male Ag, can be induced in newborn mice that lack IL-4 alone (IL-4−/−) or both IL-4 and IL-13 (IL-4−/−/IL-13−/−).

Materials and Methods

Mice

Wild-type (wt) C57BL/6 (B6), wt BALB/c, IL-4−/− B6, and IL-4−/− BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-4 and IL-13 double gene-knockout (IL-4−/−/IL-13−/−) mice were generated as described (15) and bred onto a BALB/c background for at least eight generations.

Neonatal tolerance protocol

To induce neonatal tolerance to the H-Y minor histocompatibility Ag, 3-day-old female wt and IL-4−/− B6 or BALB/c mice were injected i.p with 5 × 10^7 male wt and IL-4−/− B6 or BALB/c splenocytes, respectively. Similarly, 3-day-old female wt, IL-4−/−, and IL-4−/−/IL-13−/− BALB/c mice were injected i.p. with 5 × 10^7 male wt, IL-4−/−, and interleukin-4−/−/IL-13−/− BALB/c splenocytes, respectively. Control mice did not receive any neonatal injections. After 4 wk, full-thickness, syngeneic, male trunk skin grafts were transplanted to the upper flanks of these mice. Rejection was defined as >90% graft necrosis. Tolerance was deemed to be present if neonatally injected female mice accepted the first syngeneic male skin graft (>80-day survival), failed to reject a second syngeneic male skin graft but rejected third-party skin, and failed to mount a CTL response on rechallenge with syngeneic male splenocytes.

Abbreviation used in this paper: wt, wild type.
Mixed lymphocyte culture

Female B6 and BALB/c mice were rechallenged with $1 \times 10^7$ syngeneic (i.e., sharing the same strain background and gene-knockout status) male splenocytes i.p. 90 days after placing the first or second skin transplant. After 1 wk, splenocytes were isolated and cultured at $4 \times 10^6$ cells/ml in the presence of $2 \times 10^5$ cells/ml mitomycin-treated, syngeneic male splenocytes in complete DMEM (10% heat-inactivated FCS, 2 mM L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 10 mM HEPES buffer, 50 µM 2-ME, 100 U/ml penicillin, and 100 µg/ml streptomycin) at 37°C and 5% CO₂. Control wells included cultures of either responder or stimulator splenocytes alone. MLC were harvested 4 days later for CTL activity determination. Culture supernatants collected at 48 and 72 h were stored at ~80°C for cytokine measurements.

CTL assay

Female splenocytes, stimulated in MLC as described above, were assayed for CTL activity by incubating with syngeneic male B6 target splenocytes. Target splenocytes were stimulated for 3 days with Con A (2 µg/ml) in complete RPMI and loaded with calcein acetoxymethyl ester (Molecular Probes, Eugene, OR) before the CTL assay (16). Calcein release, quantitated in a LSS50 luminescence spectrometer (Perkin-Elmer, Norwalk, CT), was used to measure target cell lysis. Experiments in which spontaneous calcein release was >30% of maximum release were rejected. Allospecific cytotoxic activity was calculated as % specific lysis = 100 × [(test release − spontaneous release)/(maximum release − spontaneous release)].

Cytokine measurements

Cytokine concentrations in supernatants of mixed lymphocyte cultures were measured by mouse cytokine ELISA according to the manufacturer’s instructions (R&D, Minneapolis, MN). Lower limits of cytokine detection by these ELISA systems were: IL-4, 5 pg/ml; IL-5, 7 pg/ml; IL-10, 4 pg/ml; IL-13, 10 pg/ml; and IFN-γ, 4 pg/ml.

Results

IL-4 does not play a dominant role in the induction of neonatal tolerance

To determine whether IL-4 is crucial for the induction of neonatal tolerance, we injected newborn female wt and IL-4−/− B6 mice with syngeneic male splenocytes. Control mice did not receive any neonatal injections. After 4 wk, all mice were transplanted with syngeneic male skin grafts to test whether tolerance to the male H-Y Ag was achieved. As shown in Fig. 1A, control mice (no injection) uniformly rejected their skin grafts within 28 days of transplantation. In contrast, both wt and IL-4−/− mice that had received neonatal injection of syngeneic male splenocytes accepted their skin grafts for >80 days. Moreover, these mice did not mount a CTL response on restimulation with syngeneic male splenocytes (Fig. 1B), confirming that immunological unresponsiveness to the H-Y Ag was achieved. To test for the presence of specific immunological unresponsiveness, we then transplanted neonatally tolerized mice with second skin grafts from either syngeneic male B6 (first-party) or female BALB/c (third-party) donors. As shown in Fig. 1C, third-party skin grafts were uniformly rejected whereas all first-party skin grafts were accepted for greater than 80 days in both wt and IL-4−/− recipients. Similarly, neonatally tolerized mice did not mount a CTL response to the H-Y Ag but generated a normal CTL response to third-party (female BALB/c) Ags (Fig. 1D). Taken together, the data demonstrate that IL-4 does not play a dominant role in the induction of neonatal tolerance.

**FIGURE 1.** Induction of donor-specific neonatal tolerance in wt and IL-4−/− mice. A, Survival of first skin transplants in control (no injection) and neonatally tolerized (neonatal injection) mice. Neonatal tolerization was performed by injecting three-day old female wt and IL-4−/− B6 mice i.p with male wt and IL-4−/− B6 splenocytes, respectively. After 4 wk, syngeneic, male trunk skin grafts were transplanted to these mice and observed for 80 days. Rejection was defined as >90% graft necrosis. B, Donor-specific CTL response in control and neonatally tolerized mice. Control and neonatally injected female wt and IL-4−/− B6 mice were rechallenged with syngeneic male B6 splenocytes 90 days after placing the first skin transplant. After 1 wk, splenocytes were harvested and restimulated in a MLC for 4 days before measuring their CTL activity against syngeneic male B6 target splenocytes. C, Survival of second skin transplants in neonatally tolerized mice. Ninety days after placing the first skin transplant, second skin grafts from either syngeneic male B6 (first-party) or female BALB/c (third-party) donors were transplanted to neonatally tolerized wt and IL-4−/− mice and observed for 80 days. D, Donor-specific and third-party CTL responses in neonatally tolerized mice. Ninety days after placing either a syngeneic male B6 or a female BALB/c second skin transplant, neonatally tolerized wt and IL-4−/− mice were rechallenged with either syngeneic male B6 or female BALB/c splenocytes, respectively. After 1 wk, splenocytes were harvested and restimulated in a mixed lymphocyte culture for 4 days before measuring their CTL activity against syngeneic male B6 (first-party) or female BALB/c (third-party) target splenocytes.
Failure to induce neonatal tolerance in the absence of both IL-4 and IL-13

Successful induction of neonatal tolerance in IL-4−/− mice could have resulted from the presence of other cytokines, such as IL-13, that contribute to the development of a Th2 immune response (12–15). Therefore, we tested whether neonatal tolerance to the H-Y Ag is abrogated in mice that lack both IL-4 and IL-13 (15). As shown in Fig. 2A, female IL-4−/−/IL-13−/− BALB/c mice, injected with syngeneic, littermate, male spleen cells during the neonatal period, failed to accept syngeneic male skin transplants as adults. Instead, all skin grafts were rejected within 28 days of transplantation. In contrast, neonatal tolerance to male skin was successfully induced in female wt and IL-4−/− BALB/c mice (Fig. 2A). Neonatally injected IL-4−/−/IL-13−/− mice mounted a normal CTL response to male target cells (Fig. 2B), providing further evidence that neonatal tolerance was not achieved in the absence of both IL-4 and IL-13.

Failure to suppress IFN-γ production in the absence of both IL-4 and IL-13

To test whether failure to induce neonatal tolerance in mice that lack both IL-4 and IL-13 resulted from altered cytokine production, we measured Th2 (IL-5 and IL-10)- and Th1 (IFN-γ)-type cytokine production in neonatally injected and control (no injection) IL-4−/− mice. All mice were rechallenged in vivo with syngeneic male spleen cells. One week later, their splenocytes were restimulated in vitro (MLC), and cytokine production was quantitated by ELISA after 72 h. Splenocytes from neonatally injected wt mice produced significantly greater amounts of IL-5 and IL-10 than their control counterparts and, at the same time, made significantly less IFN-γ (Fig. 3). Likewise, splenocytes from neonatally injected IL-4−/− mice displayed increased IL-5 and IL-10 production, albeit more modest than that observed in the wt group, in the face of markedly suppressed IFN-γ production. In sharp contrast, splenocytes from neonatally injected IL-4−/−/IL-13−/− mice produced more IL-5 and IL-10 but, paradoxically, also made much more IFN-γ than their control counterparts. Thus, the absence of tolerance in mice that lack both

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**FIGURE 2.** Failure to induce neonatal tolerance in IL-4−/−/IL-13−/− mice. A, Survival of first skin transplants in control (no injection) and neonatally injected IL-4−/−/IL-13−/−, wt, and IL-4−/− BALB/c mice. Neonatal tolerization was attempted by injecting 3-day-old female mice i.p with syngeneic male splenocytes (obtained from littermates in the IL-4−/−/IL-13−/− group). After 4 wk, syngeneic, male trunk skin grafts were transplanted to these mice and observed for 80 days. Rejection was defined as >90% graft necrosis. B, Donor-specific CTL response in control and neonatally injected IL-4−/−/IL-13−/− mice. Control and neonatally injected female IL-4−/−/IL-13−/− BALB/c mice were rechallenged with syngeneic male splenocytes 1 wk after they rejected their skin grafts. After 1 wk, splenocytes were harvested and restimulated in a MLC for 4 days before measuring their CTL activity against syngeneic male BALB/c target splenocytes.

**FIGURE 3.** Cytokine production by splenocytes of control (no neonatal injection) and neonatally injected wt, IL-4−/−, and IL-4−/−/IL-13−/− mice. Female BALB/c mice that had already undergone skin grafting were rechallenged in vivo with syngeneic male BALB/c spleen cells. After 1 wk, their splenocytes were restimulated in vitro (MLC), and IL-5, IL-10, and IFN-γ production was measured after 72 h by ELISA. Data are mean ± SD of three experiments.
IL-4 and IL-13 is associated with distinct failure to suppress IFN-γ production after neonatal immunization.

Discussion
We have addressed here the roles of IL-4 and of Th2 immunity in the minor histocompatibility Ags can be induced in newborn mice that lack either IL-4 alone or both IL-4 and IL-13. We found that IL-4 does not play a dominant role in the induction of neonatal tolerance as demonstrated in various strains of newborn female IL-4−/−/IL-13−/− mice (C57BL/6 and BALB/c) could be readily tolerated to the H-Y male Ag. In contrast, IL-4−/−/IL-13−/− mice, in which IFN-γ production could not be suppressed, were resistant to the induction of neonatal tolerance. These findings indicate that IL-4 alone is not critical for the induction of neonatal tolerance and suggest that the combined actions of both IL-4 and IL-13 are essential for this process.

Our observation that neonatal tolerance can be successfully achieved in IL-4-deficient mice disagrees with published studies in which IL-4-neutralizing Abs were found to block the induction of neonatal tolerance in wt mice (9–11). In these studies, monoclonal anti-IL-4 Ab, given at the time semiallogeneic donor splenocytes were injected into newborn mice, prevented the subsequent acceptance of donor skin grafts. However, donor-specific CTL activity was not restored (9), indicating that in vivo IL-4 neutralization did not completely block tolerance induction. Moreover, T cells from Ab-treated mice produced normal levels of IL-4 (9, 11), suggesting that IL-4 is not the only mediator of neonatally induced Th2 immunity. An alternative explanation is that anti-IL-4 Ab treatment did not completely neutralize IL-4 activity in vivo, raising the possibility that Ab injection precipitated skin rejection in neonatally tolerated mice by exerting nonspecific effects on the immune system. By binding to Fc receptors, for example, an Ab could activate NK cells and macrophages leading to the rejection of skin allografts. It is also important to note that previous studies addressing the role of IL-4 in neonatal tolerance (9, 11) dealt with skin grafts. It is also important to note that previous studies addressing the role of IL-4 in neonatal tolerance (9, 11) dealt with skin grafts. However, only IL-4−/−/IL-13−/− mice default to a Th1 response (exaggerated IFN-γ production) in these experiments. Because suppression of Th1 responses is critical for achieving neonatal tolerance (our data and those of Ref. 11), it is likely that newborn IL-13−/− mice, like IL-4−/− mice will be amenable to tolerance induction. This possibility will be tested once IL-13−/− mice that have been backcrossed onto a pure MHC background become available.

Our results do not rule out the possibility that IL-13 alone could play a critical role in the induction of neonatal tolerance. Although IL-4 and IL-13 cooperate in the development of Th2 immunity, these cytokines have divergent in vivo functions. Studies comparing the host response to parasitic infection among IL-4−/−, IL-13−/−, and IL-4−/−/IL-13−/− mice have shown that IL-13 is primarily involved in the expulsion of N. brasiliensis-infected worms and in the in vivo fibrosis caused by Schistosoma mansoni (15, 17). However, only IL-4−/−/IL-13−/− mice default to a Th1 response (exaggerated IFN-γ production) in these experiments. Because suppression of Th1 responses is critical for achieving neonatal tolerance (our data and those of Ref. 11), it is likely that newborn IL-13−/− mice, like IL-4−/− mice will be amenable to tolerance induction. This possibility will be tested once IL-13−/− mice that have been backcrossed onto a pure MHC background become available.

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