Neonatal Immunity Develops in a Transgenic TCR Transfer Model and Reveals a Requirement for Elevated Cell Input to Achieve Organ-Specific Responses

Lequn Li, Kevin L. Legge, Booki Min, J. Jeremiah Bell, Randal Gregg, Jacque Caprio and Habib Zaghouani

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Habib Zaghouani

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Knoxville, TN 37996. E-mail address: hzagh@utk.edu

fectious Diseases, National Institutes of Health, Building 10, Room 11N314, Be-

Virginia, 400 Lane Road, MR-4 Building, P.O. Box 801386, Charlottesville, VA

Address correspondence and reprint requests to Dr. Habib Zaghouani, Department

of Microbiology, University of Tennessee, M409 Walters Life Sciences Building,

of Immunology, National Institute of Arthritis and In-

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2 Current address: Beirne B. Carter Center for Immunology Research, University of

Virginia, 400 Lane Road, MR-4 Building, P.O. Box 801386, Charlottesville, VA

25908-1386.

3 Current address: Laboratory of Immunology, National Institute of Arthritis and In-

fectious Diseases, National Institutes of Health, Building 10, Room 11N314, Bel-

thesda, MD 20892-1892.

4 Address correspondence and reprint requests to Dr. Habib Zaghouani, Department

of Microbiology, University of Tennessee, M409 Walters Life Sciences Building,

Knoxville, TN 37996. E-mail address: hzagh@utk.edu

A

half-century ago, it was shown that neonatal exposure to an

alloantigen induced a form of tolerance permissive for

grafting tissues that otherwise would be rejected (1).

Since then, significant progress has been made to show that injection

of Ag during the neonatal stage induces rather than suppresses

immunity (2–6), but such responses are biased and lead to toler-

ance of Ag during a subsequent encounter (7). In most cases, neo-

nata exposure to Ag changes the phenotype of the T cells from

Th1 to Th2 (3, 4, 6) and diverts the response to develop in the

spleen instead of the lymph node (3, 4). Indeed, mice given soluble

proteins or peptides in IFA on the day of birth and challenged with

the same Ag in CFA later at an adult age develop Th2 instead of

Th1 T cell responses, and such responses arise in the spleen instead

of the lymph node (3, 4). Although the exact mechanism for the

apparent selective maintenance of the Th2 cells is unknown, it has

been suggested that the primary Th1 cells that arise upon neonatal

exposure to Ag are more vulnerable to apoptosis (8). Also, recent

investigations have envisioned the involvement of regulatory (9)

and CD4⁺Thy1⁻ (10) T cells to maintain such biased neonatal

immunity. Furthermore, although neonatal tolerance has proved

effective in conferring resistance against autoimmunity (11–14),

the mechanism initiating and perpetuating the biased neonatal im-

munity is still undefined. Recent investigations reported that fac-

tors such as the type of APCs (5, 15); the adjuvant into which the

Ag is emulsified (4, 14); and the dose (6, 14), form (16, 17), in vivo

availability (18), and continuous supply (19) of Ag control the

induction of neonatal tolerance. Our own investigations using Igs

as a vehicle for peptide delivery revealed yet another bias in neo-

nata immunity, namely, development of organ-specific responses

in the lymph node vs the spleen (7, 13, 14) as opposed to spleen

alone in free peptide systems (3, 4, 12). Indeed, Ig-PLP1 (20, 21)

a chimera harboring the proteolipid protein (PLP)⁵ 139–151 se-

quence, or PLP1, given to SJL mice in saline on the day of birth,

induced an organ-specific regulation of T cells characterized by a

deviation in the lymph node and a novel form of IFN-γ-dependent

anergy in the spleen (7, 13, 14). Specifically, mice given Ig-PLP1

on the day of birth and challenged with PLP1 peptide at 7 wk of

age developed PLP1-specific T cells in the lymph node that pro-

duced IL-4 instead of IL-2, and in the spleen, the cells, although

nonproliferative and unable to produce IFN-γ, secreted significant

amounts of IL-2. Furthermore, when supplied with IFN-γ or the

IFN-γ inducer IL-12, these splenic cells regained proliferative and

IFN-γ responsiveness (13, 22). This form of neonatal immunity,

which circumvents the use of adjuvant, confers protection against

autoimmunity and allows resistance to the induction of experi-

mental allergic encephalomyelitis (EAE) (7, 13). However, free PLP1

was unable to restore Th1 responses in the spleen. This form of

spleenic unresponsiveness referred to as IFN-γ-dependent anergy

required a transfer of a high number of neonatal DO11.10/SCID T cells to develop. Thus, the frequency of neonatal T cell

precursors rather than repertoire diversity exerts control on the development of organ-specific neonatal immunity. The Journal of


In recent years, it has become clear that neonatal exposure to Ag induces rather than ablates T cell immunity. Moreover,

rechallenge with the Ag at adult age can trigger secondary responses that are distinct in the lymph node vs the spleen. The question

addressed in this report is whether organ-specific secondary responses occur as a result of the diversity of the T cell repertoire or could

they arise with homogeneous TCR-transgenic T cells. To test this premise, we used the OVA-specific DO11.10 TCR-transgenic T cells and

established a neonatal T cell transfer system suitable for these investigations. In this system, neonatal T cells transferred from 1-day-old DO11.10/SCID mice into newborn (1-day-old) BALB/c mice migrate to the host’s spleen and maintain stable frequency. The newborn BALB/c hosts were then given Ig-OVA, an Ig molecule carrying the OVA peptide, and challenged with the OVA peptide in CFA at the age of 7 wk; then their secondary responses were analyzed. The findings show that the lymph node T cells were deviated IL-4 instead of IFN-γ and the splenic T cells, although unable to proliferate or produce IFN-γ, secreted a significant level of IL-2. Supply of exogenous IL-12 during Ag stimulation restores both proliferation and IFN-γ production by the splenic T cells. This restorable form of splenic unresponsiveness referred to as IFN-γ-dependent anergy required a transfer of a high number of neonatal DO11.10/SCID T cells to develop. Thus, the frequency of neonatal T cell precursors rather than repertoire diversity exerts control on the development of organ-specific neonatal immunity. The Journal of Immunology, 2001, 167: 2585–2594.

Neonatal Immunity Develops in a Transgenic TCR Transfer Model and Reveals a Requirement for Elevated Cell Input to Achieve Organ-Specific Responses¹

Lequn Li, Kevin L. Legge,² Booki Min,³ J. Jeremiah Bell, Randal Gregg, Jacque Caprio, and Habib Zaghouani⁴

Department of Microbiology, University of Tennessee, Knoxville, TN 37996

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² Current address: Beirne B. Carter Center for Immunology Research, University of Virginia, 400 Lane Road, MR-4 Building, P.O. Box 801386, Charlottesville, VA 25908-1386.

³ Current address: Laboratory of Immunology, National Institute of Arthritis and Infectious Diseases, National Institutes of Health, Building 10, Room 11N314, Bethesda, MD 20892-1892.

⁴ Address correspondence and reprint requests to Dr. Habib Zaghouani, Department of Microbiology, University of Tennessee, M409 Walters Life Sciences Building, Knoxville, TN 37996. E-mail address: hzagh@utk.edu

⁵ Abbreviations used in this paper: PLP, proteolipid protein; EAE, experimental allergic encephalomyelitis; CD40L, CD40 ligand; HA, hemagglutinin.

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peptide given to mice on the day of birth in saline instead of Ig-PLP1 had no effect on the adult response to a challenge with PLP1 in CFA, and such animals were not protected against EAE (7, 14). Also, PLP1 in IFA given on the day of birth, although protective against EAE, generated a response to immunization with peptide in CFA characterized by a deviated T cell response in the spleen, and unresponsiveness in the lymph node (14). Consequently, delivery of peptide on Ig circumvents the use of adjuvant and confers to the peptide the ability to protect against autoimmunity by a unique mechanism involving lymph node deviation and IFN-γ-dependent splenic anergy (13, 22). In this report, we wished to address the question of whether such organ-specific regulation of neonatal immunity is related to the diversity of the T cell repertoire or whether it occurs when the neonatal immune system comprises a homogeneous population of TCR-transgenic T cells. To this aim, an Ag-specific TCR-transgenic neonatal T cell transfer system was developed which allowed for T cell homing and homeostasis within the host’s spleen. In this system, 1-day-old BALB/c newborns were given neonatal DO11.10-transgenic T cells (23) carrying a TCR specific for aa 323–339 of OVA, and homing analysis indicated that efficient transfer of the donor cells to the host’s spleen did occur. In addition, the frequency of DO11.10 cells reached 3% among host’s splenic CD4 lymphocytes by day 6 after transfer. Furthermore, when the newborn mice that received the neonatal DO11.10 T cells were given Ig-OVA, an Ig carrying the OVA peptide, and challenged with the OVA peptide at the age of 7 wk, they developed lymph node deviation and IFN-γ-dependent splenic anergy similar to the Ig-PLP1/SJL system (13, 14). However, to achieve such organ-specific neonatal immunity and induce the IFN-γ-dependent anergy, a high T cell transfer input was required. Moreover, we found that OVA peptide presentation on Igs interferes with the up-regulation of CD40 ligand (CD40L) on T cells, and such interference may play a role in the development of organ-specific neonatal immunity.

Materials and Methods

Mice

BALB/c mice (H-2d) were purchased from Harlan Sprague Dawley (Indiana, IN). DO11.10-transgenic mice expressing a TCR specific for chicken OVA323–339 in the context of the MHC class II molecule, I-A<sup>d</sup>, were previously described (23). DO11.10 mice were bred onto the SCID mouse background by day 6 after transfer.

Purification of CD44−KJ1-26<sup>+</sup> T cells

Because the spleen of DO11.10/SCID mice is smaller than that of non-DO11.10/SCID mice, we usually used 60 newborns and 9 adult mice to purify a sufficient number of neonatal and adult T cells. CD4<sup>+</sup>KJ1-26<sup>+</sup> T cells were purified by negative selection using the mouse CD4 Subset Mini Column Kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. The purity of T cells was 93% as assessed by double staining with KJ1-26 and anti-CD4 Abs using flow cytometry.

Transfer of neonatal CD44−KJ1-26<sup>+</sup> T cells into 1-day-old BALB/c mice

Neonatal DO11.10/SCID spleen cells were depleted of RBC by incubation in hypotonic lysis buffer. Subsequently, the percent of CD4<sup>+</sup>KJ1-26<sup>+</sup> T cells was determined by flow cytometric analysis as described (26), and the spleen cells were resuspended in 50 μl saline and injected s.c. on the back of neonatal BALB/c hosts (10 mice per group). The mice were sacrificed on day 10 after injection, and the spleens were removed for analysis of proliferative and cytokine responses.

Lymph node and spleen T cell proliferation

Lymph node and spleen cells were incubated in 96-well flat-bottom plates at 4 and 10 x 10<sup>6</sup> cells/100 μl/well, respectively, with 100 μl stimulator. Ten days later, the mice were sacrificed, and their spleens and lymph nodes (axillary, lateral axillary, inguinal, and popliteal) were removed for analysis of proliferative and cytokine responses.

Antibodies

The anti-B7.1 (1G10, rat Ig-Ga), anti-B7.2 (2D10, rat Ig-Gb2), 3D1 (rat IgGb2), and rat anti-mouse α L chain mAbS were obtained from American Type Culture Collection (ATCC, Manassas, VA). Rabbit anti-mouse α2b Abs were purchased from Zymed Laboratories (San Francisco, CA). FITC-labeled anti-mouse IgG2a, FITC-labeled anti-mouse B220 (RA3-6B2), FITC-labeled anti-mouse CD11b (M1/70), PE-labeled anti-CD4 (RM4–5), PerCP-labeled anti-CD4 (RM4–5), biotin-labeled anti-CD40L (MR1), biotin-labeled anti-CD4 (GK1.5), and biotin-labeled hamster IgG were purchased from BD Pharmingen (San Diego, CA).
culture was continued for an additional 14.5 h. The cells were then harvested on a Triflux 1450 Microbeta Wallac Harvester, and incorporated \(^{3}H\) thymidine was counted using the Microbeta 270.004 software (EG&G Wallac, Gaithersburg, MD). The stimulators, OVA and HA\(^{110-120}\) peptides, were used at 10 \(\mu\)M. A control medium with no stimulator was included for each mouse and used as background.

**Measurement of cytokines by ELISA**

Spleen cells were incubated in 96-well round-bottom plates at 10 \(\times\) 10\(^5\) cells/100 \(\mu\)l well with 100 \(\mu\)l stimulator for 24 h. Cytokine production was then measured by ELISA according to PharMingen’s instructions using 100 \(\mu\)l Culture plates with rat anti-mouse II-2, IES6-1A12; rat anti-mouse IL-4, BV6D-24G2; and rat anti-mouse IFN-\(\gamma\), XMGI.2. The OD\(_{405}\) was measured on a Spectra Max 190 (Molecular Devices, Sunnyvale, CA) using SOFTmax PRO3.1.1. Graded amounts of recombinant mouse IL-2, IL-4, and IFN-\(\gamma\) were included in all experiments to construct standard curves. The concentrations of cytokines in supernatants was estimated by extrapolation from the linear portion of the standard curve.

**Measurement of cytokines by ELISPOT assay**

ELISPOT was used to measure cytokines produced by lymph node T cells during Ag stimulation as described (4, 13). HA-multiscreen plates (Millipore, Bedford, MA) were coated with 100 \(\mu\)l/well 1 M NaHCO\(_3\) buffer containing 2 \(\mu\)g/ml capture Ab. After an overnight incubation at 4\(^\circ\)C, the plates were washed three times with sterile PBS, and free sites were saturated with DMEM containing 10% FCS for 2 h at 37\(^\circ\)C. Subsequently, the blocking medium was removed, and 5 \(\times\) 10\(^5\) lymph node cells/100 \(\mu\)l well were added along with 100 \(\mu\)l Ag and incubated for 24 h at 37\(^\circ\)C in a 5% CO\(_2\) humidified chamber. The plates were then washed three times with PBS, followed by three washes with PBS-0.05% Tween. To each well, 100 \(\mu\)l biotinylated anti-IL-4, anti-IL-2 or anti-IFN-\(\gamma\) mAb were added, and the plates were incubated at 4\(^\circ\)C overnight. After three washes with PBS-0.05% Tween, 100 \(\mu\)l avidin-peroxidase (2.5 \(\mu\)g/ml) were added. The plates were then incubated for 1 h at 37\(^\circ\)C. Subsequently, spots were visualized by adding 200 \(\mu\)l substrate (3-aminio-9-ethylcarbazole; Sigma, St. Louis, MO) in 50 \(\mu\)M acetate buffer, pH 5.0, and counted under a dissection microscope. The anti-cytokine Abs pairs used here were those described for the ELISA technique.

**Flow cytometry analyses**

**Staining for CD4 and KJ1-26.** Splenocytes were incubated with 10 \(\mu\)g/ml anti-FeR (2.4G2) for 20 min at 4\(^\circ\)C. The cells were then labeled with anti-CD4-PE and purified KJ1-26 mAb (mouse IgG2a) at 30 min at 4\(^\circ\)C and washed with FACS buffer. An additional incubation with goat anti-mouse IgG2a-FITC Abs was conducted to detect bound KJ1-26. Events (10\(^5\) \(\times\) 10\(^5\)) were collected on a FACSscan flow cytometer (BD Biosciences, Mountain View, CA) and analyzed using CellQuest software 3.3 (BD Biosciences).

**Staining for CD40L.** Staining for CD40L was conducted as previously described (28). Briefly purified CD4\(^+\) KJ1-26\(^+\) T cells from neonatal DO11.10/SCID mice were cultured with neonatal or adult BALB/c APCs and stimulated with OVA peptide or soluble Ig-OVA. Biotinylated anti-CD40L Ab or hamster Ig-G (1 mg/ml) was immediately added due to rapid down-regulation of CD40L (29). After an 8-h incubation, the cells were harvested and labeled with anti-CD4-PerCP, KJ1-26-FITC, and avidin-PE for CD40L.

**Staining for B7.1 and B7.2 costimulatory molecules.** Splenocytes from neonatal or adult BALB/c mice were cultured with OVA peptide (10 \(\mu\)M) or Ig-OVA (1 \(\mu\)M) for 24 h, and the cells were then harvested and double-stained with either anti-B7.1-PE or anti-B7.2-PE and anti-B220-FITC, anti-CD11b-FITC, or 33D1-FITC.

**Results**

**Presentation of OVA peptide on Igs**

In prior studies, we demonstrated that neonatal exposure to Ig-PLP1 primes the immune system to develop an unusual organ-specific secondary response upon challenge with PLP1 peptide later in life. Indeed, the lymph node T cells were deviated to Th2, and the splenic T lymphocytes exhibited a defective growth and differentiation (7, 13, 22). One key question in this study is whether such organ-specific regulation is related to the diversity of the T cell repertoire or whether it occurs when the target neonatal T cells represent a homogeneous population expressing one type of TCR. To address this issue, we used TCR-transgenic T cells to develop a neonatal transfer system suitable for these investigations. The DO11.10 transgenic mouse carrying the OVA-specific TCR (23) and the KJ1-26 anti-clonotypic Ab specific for such a TCR (30) are well characterized and provide useful tools to establish such a neonatal T cell transfer system. However, to test for the organ-specific T cell regulation, an Ig chimera expressing the OVA peptide for which the DO11.10 T cells are specific was needed. Thus, the nucleotide sequence coding for OVA peptide was inserted within the 91A\(_V_H\) gene (24), and sequencing analysis indicated that the D segment of complementarity determining region 3 region was deleted, and a nucleotide sequence coding for the full OVA\(_{323-339}\) peptide was inserted instead (Fig. 1, top). Also, the sequences surrounding the OVA insert were identical with those flanking the D segment within the parental 91A\(_V_H\) confirming that the OVA sequence was inserted in the correct reading frame. The chimeric 91A3\(_{V_H}\).OVA gene was then ligated to a BALB/c H2b constant region to form a complete H chain gene and cotransfected along with the parental 91A\(_V_L\) chain (25) into the non-Ig-producing myeloma B cell line SP2/0. Supernatant from drug-selected transfectants incubated on anti-mouse \(\gamma_2\)-coated microtiter plates allowed binding of \(^{121}\)I-labeled anti-mouse \(\kappa\) chain as did supernatant from transfectants expressing the parental IgG2b\(\times\)Ab, Ig-W (Fig. 1, bottom). These results indicate that the mutant H chain harboring the OVA peptide was able to pair with the parental L chain to form a complete Ig-OVA chimeric Ig. To assess for processing and presentation of the OVA peptide from Ig-OVA, a T cell activation assay was performed using splenic cells from DO11.10 mice that contain both the TCR-transgenic OVA-specific T cells and the APCs. As can be seen in Fig. 2, Ig-OVA-like free OVA peptide stimulated the DO11.10 T cells to proliferate, whereas the negative controls HA\(_{110-120}\) and Ig-HA, an Ig carrying HA\(_{110-120}\) peptide (31, 32), which are also restricted to H-2\(^d\) did not induce any proliferation. These results indicate that endo- cytotic processing of Ig-OVA released the OVA peptide that bound to MHC molecules and the complexes (MHC class II-OVA peptide) trigger activation of the DO11.10 T cells as with other peptides expressed on Igs (20, 31–34).

**Neonatal DO11.10 T cells develop uncommitted primary response on stimulation with Ag in vitro**

The secondary response of neonatally primed mice most likely derives from the primary response that arises upon exposure of neonatal T cells to the Ag. To ascertain that the neonatal DO11.10 T cells can develop a primary response rather than undergo cell death upon Ag presentation by neonatal APCs, we performed in vitro activation assays using Ig-OVA as Ag and neonatal splenocytes as APCs. Adult DO11.10 T cells were included to serve as reference. Also, adult splenocytes were used as APCs to discern any presentation discrepancy by the neonatal vs adult APCs. As can be seen in Fig. 3, the adult T cells upon stimulation with Ig-OVA presented on adult APCs proliferated (Fig. 3a), and produced IL-2 (Fig. 3b) and IFN-\(\gamma\) (Fig. 3c) but not IL-4 (Fig. 3d). The neonatal T cells, however, had reduced proliferation (Fig. 3a), produced lower amounts of the growth factor IL-2 (Fig. 3b), and were unable to secrete detectable levels of IFN-\(\gamma\) (Fig. 3c) or IL-4 (Fig. 3d). These results indicate that neonatal T cells develop a primary response upon exposure to Ag, but such a response displays no differentiation into the production of effector cytokines when compared with the primary response of adult DO11.10 T cells. Moreover, when neonatal APCs were used for presentation of Ig-OVA, both populations developed proliferative and IL-2 responses (Fig. 3, e and f), but neither neonatal nor adult T cells
produced IFN-γ or IL-4 (Fig. 3g). These results indicate that the neonatal APCs are unable to support differentiation of T cells into IFN-γ or IL-4 production in vitro.

To ascertain that the lack of differentiation in the primary response of neonatal T cells is related to developmental factors rather than the nature of Ag, we performed similar experiments with free OVA peptide and assessed for proliferation and cytokine production. As can be seen in Fig. 4, whereas the adult T cells proliferated (Fig. 4a) and produced IL-2 (Fig. 4b), IFN-γ (Fig. 4c), and IL-4 (Fig. 4d) upon stimulation with OVA peptide presented on adult APCs, the neonatal T cells had reduced proliferation (Fig. 4a), produced lower amounts of the growth factor IL-2 (Fig. 4b), and were unable to secrete detectable levels of IFN-γ or IL-4 (Fig. 4c and d). Again as with Ig-OVA, the neonatal T cells developed a primary response upon exposure to the peptide, but such a response displayed a lack of differentiation into the production of IFN-γ or IL-4 when compared with the primary response of adult DO11.10 T cells. Furthermore, when neonatal APCs were used for presentation of OVA peptide, both populations developed proliferative and IL-2 responses (Fig. 4, e and f), but neither neonatal nor adult T cells produced IFN-γ or IL-4 (Fig. 4, g and h). These results indicate that the neonatal APCs are unable to support differentiation of T cells into IFN-γ or IL-4 production in vitro. Overall, neonatal DO11.10 T cells stimulated with Ig-OVA or free OVA peptide presented by neonatal APCs develop a primary response in which T cell polarization could not occur.

Effective homing of neonatal DO11.10 T cells to the spleen of newborn BALB/c recipients

The neonatal DO11.10 T cells have shown the ability to proliferate and produce the growth factor IL-2 upon stimulation with Ag in vitro. Thus, the DO11.10 system would provide a suitable source of T cells to establish a neonatal T cell transfer system if appropriate homing could occur upon transfer into BALB/c neonates. To investigate this matter, varying numbers of 1-day-old DO11.10/SCID splenic cells containing defined numbers of DO11.10 T cells (CD4+/KJ1-26+) were transferred into BALB/c neonates via the facial vein, and the cells were assessed for homing to the recipients’ spleen. As can be seen in Fig. 5, 4 days after transfer, the number of CD4+/KJ1-26+ T cells rose from undetectable in normal 4-day-old BALB/c mice (Fig. 5a) to 16, 22, and 25 per 1000 CD4+ splenocytes in the mice recipient of DO11.10/SCID splenocytes containing 2, 10, and 30×10^3 DO11.10 cells, respectively (Fig. 5, b–d). When the homing was assessed 6 days after transfer, the frequency increased from 22 to 26 and 25 to 30 cells per 1000 CD4+ splenocytes in the mice recipient of 10 and 30×10^3 DO11.10 cells, respectively (Fig. 5, e and f). These results indicate that neonatal DO11.10 T cells are able to home to and populate the spleen of neonatal BALB/c recipients to an extent similar to that of an adult transfer system (26).
containing 100 donates, and a few hours later the hosts were given a saline solution.

DO11.10/SCID mice were transferred into 1-day-old BALB/c neonates. To this aim, neonatal T cells from 1-day-old mice were collected, and after 72 h, proliferation was measured by $[^3]$H]thymidine incorporation as described in Materials and Methods. HA$_{110-120}$ and Ig-HA (31, 32), Ags presented by H-2$,^b$, like OVA peptide and Ig-OVA, were used as negative control. Stimulation index represents the ratio of cpm obtained by incubation of the splenocytes without Ag to cpm obtained in the presence of Ag. Each point represents the mean of triplicates.

**FIGURE 2.** Presentation of Ig-OVA chimea to DO11.10 T cells. Splenocytes (4 x 10$^5$ cells/well) from DO11.10 mice were incubated with graded amounts of peptides or affinity chromatography-purified Ig chimera; 72 h later, proliferation was measured by $[^3]$H]thymidine incorporation as described in Materials and Methods. HA$_{110-120}$ and Ig-HA (31, 32), Ags presented by H-2$,^b$, like OVA peptide and Ig-OVA, were used as negative control. The results were then compared with those of the Ig-W recipient mice and among the cell transfer groups. As can be seen in Fig. 6, the mice given Ig-OVA on the day of birth, like those recipients of Ig-W, developed equivalent proliferative responses in the lymph node upon immunization with OVA peptide at adult age. The number of transferred neonatal DO11.10 T cells had only a slight influence on these lymph node proliferative responses. However, in the lymph node, both endogenous and transferred DO11.10 T cells slightly increased the proliferation. These responses are specific because in vitro stimulation with HA$_{110-120}$ Peptide did not induce any significant proliferation (not shown). At the cytokine level, while the lymph node cells of Ig-W recipient mice produced IL-2 and IFN-γ, those from the Ig-OVA recipient group produced IL-4 instead (Fig. 6). Stimulation of these lymph node cells with HA$_{110-120}$ peptide did not induce any IL-2, IL-4, or IFN-γ (not shown). Moreover, although the Th1 response in Ig-W recipient mice was significant only when the neonatal transfer was conducted with 30 x 10$^3$ DO11.10 T cells, the deviated responses in Ig-OVA recipient mice were significant with all the transfer numbers. Because control BALB/c mice that had not received neonatal T cell transfer but were tolerized with Ig-OVA on the day of birth developed lower but similar lymph node proliferative and IL-4 response (not shown), we believe that the IL-4 seen in the murine recipients of the T cell transfer is produced by both endogenous BALB/c and transferred DO11.10 T cells. In the spleen, although the murine recipients of Ig-W developed proliferative responses with all transfer regimens, those exposed to Ig-OVA did not (Fig. 7, top row). Moreover, the Ig-W group produced both type 1 (IL-2, and IFN-γ) and type 2 (IL-4) cytokines. In contrast, the Ig-OVA group although displaying proliferative unresponsiveness had significant IL-2 production when the transfer was conducted with 30 x 10$^3$ neonatal DO11.10 cells, but IFN-γ was lacking and IL-4 was greatly reduced. Overall, the results indicate that a high neonatal T cell input is required to obtain nonproliferative splenic T cells that produce IL-2 but not IFN-γ. In the spleen, IL-2 production most likely emanates from DO11.10 T cells, because mice that did not receive any cell transfer, but were exposed to Ig-OVA, showed no measurable IL-2 or IFN-γ in their splenic responses. However, in the lymph node, both endogenous and DO11.10 T cells may be involved in IL-4 production as expected to Ig-OVA in mice receiving no transfer induced IL-4 production. The finding that DO11.10 T cells are detectable in both lymphoid organs of these mice (Fig. 8) provides support for their contribution to the responses in both the spleen and lymph node. In addition, because their frequency in the Ig-OVA-treated mice (Fig. 8, e and d) is slightly increased relative to the control animals given Ig-W instead (Fig. 8, a and b), this may reflect a response of Ag experienced vs naive cells and further attest to their differential function (see Figs. 6 and 7).
The production of IL-2 by the splenic T cells despite proliferative and IFN-γ unresponsiveness suggests that the cells may be anergic as was observed in the polyclonal SJL/Ig-PLP1 system (13, 22). To address this issue, the splenic cells were stimulated anergic as was observed in the polyclonal SJL/Ig-PLP1 system of OVA or HA110 peptide stimulation of the splenic cells with OVA peptide in the presence of stimulation with Ag, they regained full responsiveness. Indeed, stimulation of the splenic cells with OVA peptide compared with adult T cells. CD4+ KJ1-26+ T cells (5 x 10⁴ cells/well) were purified from the spleen of 50 neonatal or 10 adult DO11.10/SCID mice and incubated with an optimal concentration (10 μM) of OVA or HA110-120 peptide and irradiated (3000 rad) adult (a-d) or neonatal (e-h) BALB/c splenocytes as APCs (5 x 10⁵ cells/well). After 48 h incubation, 1 μCi [³H]thymidine/well was added and proliferation (a and e) was measured 14.5 h later. Duplicate cultures were prepared and incubated for 24 h, and the supernatant was used to measure IL-2 (b and f), IFN-γ (c and g), and IL-4 (d and h) by ELISA. Each bar represents the mean ± SD of triplicate wells. In vitro stimulation with the negative control HA110-120 peptide was included for each mouse, and there was no significant proliferation or cytokine production.

FIGURE 4. Neonatal T cells develop distinct primary response to OVA peptide compared with adult T cells. CD4+ KJ1-26+ T cells (5 x 10⁴ cells/well) were purified from the spleen of 50 neonatal or 10 adult DO11.10/SCID mice and incubated with an optimal concentration (10 μM) of OVA or HA110-120 peptide and irradiated (3000 rad) adult (a-d) or neonatal (e-h) BALB/c splenocytes as APCs (5 x 10⁵ cells/well). After 48 h incubation, 1 μCi [³H]thymidine/well was added and proliferation (a and e) was measured 14.5 h later. Duplicate cultures were prepared and incubated for 24 h, and the supernatant was used to measure IL-2 (b and f), IFN-γ (c and g), and IL-4 (d and h) by ELISA. Each bar represents the mean ± SD of triplicate wells. In vitro stimulation with the negative control HA110-120 peptide was included for each mouse, and there was no significant proliferation or cytokine production.

The production of IL-2 by the splenic T cells despite proliferative and IFN-γ unresponsiveness suggests that the cells may be anergic as was observed in the polyclonal SJL/Ig-PLP1 system (13, 22). To address this issue, the splenic cells were stimulated with OVA peptide in the presence of IL-12, and proliferation and IFN-γ production were measured. The results presented in Fig. 9 show that when the splenic T cells were supplied with IL-12 during stimulation with Ag, they regained full responsiveness. Indeed, stimulation of the splenic cells with OVA peptide in the presence of IL-12 restored both proliferation and IFN-γ production to levels significantly higher than stimulation with OVA peptide alone. In addition, the restoration of the response is Ag specific as stimulation of the cells with IL-12 in the presence of the irrelevant peptide HA110-120 did not lead to significant responses.

Exposure to Ag induces a higher expression of B7 molecules on adult vs neonatal monocytes/macrophages

We have shown above that both APCs and T cells contribute to the make up of the primary neonatal response. Also, we have found that neonatal exposure to Ig-OVA followed by an active immunization with OVA peptide at adult age gave rise to a response that includes IL-4 secreting lymph node T cells and IL-2-producing anergic splenic T cells whose proliferative and IFN-γ responsiveness could be restored with exogenous IL-12. In this section we attempted to define factors whose expression may be subject to developmental control and thereby play a critical role in the induction of neonatal immunity. Because B7 molecules on APCs provide important costimulatory functions for T cell activation and differentiation (35–37), we monitored the expression of both B7.1 and B7.2 on neonatal splenic APCs upon incubation with Ig-OVA or free OVA peptide and compared their expression pattern with adult APCs. As can be seen in Fig. 10, on stimulation with Ig-OVA, both adult and neonatal 33D1⁺ (mostly dendritic cells) and B220⁺ (mostly B cells) expressed B7.1 and B7.2 to the same
FIGURE 6. Newborn BALB/c mice receiving neonatal DO11.10 T cells and exposed to Ig-OVA develop deviated lymph node T cell responses on challenge with OVA peptide at the age of 7 wk. Groups of 1-day-old BALB/c mice (4–6/group) were given i.v. in the facial vein 2,000 (2K), 10,000 (10K), or 30,000 (30K) neonatal DO11.10/SCID T cells and injected with 100 μg Ig-W (■) or Ig-OVA (□) within 24 h. When the mice reached the age of 7 wk, they were challenged with 125 μg OVA peptide in CFA; 10 days later, the lymph node cells (5 × 10^5 cells/well) were stimulated with 10 μM OVA peptide. Proliferation (top row) was measured by [3H]thymidine incorporation after 3 days incubation. Production of IL-2 (second row), IFN-γ (third row), and IL-4 (bottom row) were measured after 24 h by ELISPOT as described in Materials and Methods. Ig-OVA was used at 100 μg/mouse on the day of birth because this amount was found to be optimal for induction of organ-specific regulation of neonatal immunity in the SJL/Ig-PLP1 system (13, 14). Because both memory and naive T cells will be available in Ig-OVA-treated mice, the use of 125 instead of the usual 300 μg OVA peptide for immunization at adult age would be more effective in recalling Ag-experienced T cells rather than priming naive endogenous BALB/c cells. The in vitro stimulation used 10 μM peptide, which was defined optimal in this setting. ELISPOT was used to measure cytokines instead of ELISA because treatment with Igs induced highly proliferative T cells in the lymph node that reabsorb cytokine and can alter the significance of results (13). The indicated values represent the mean ± SD of four to six individually tested mice. In vitro stimulation with the negative control HA110–120 peptide was included for each mouse, and there was no significant proliferation (mean cpm was 775 ± 200 for the six tested mice) or cytokine production (all cytokines tested were below detectable level). The mice used for these experiment are those described in Fig. 6.

FIGURE 7. Exposure of newborn BALB/c mice recipient of neonatal DO11.10 T cells to Ig-OVA allowed for IL-2 but suppressed IFN-γ secretion and proliferative responsiveness upon challenge with OVA peptide at the age of 7 wk. Groups of 1-day-old BALB/c mice (4–6/group) were given i.v. in the facial vein 2,000 (2K), 10,000 (10K), or 30,000 (30K) neonatal DO11.10/SCID T cells and injected with 100 μg Ig-W (■) or Ig-OVA (□) within 24 h. After 7 wk, the mice were challenged with 125 μg OVA peptide in CFA. Ten days later, the animals were sacrificed, and the splenic cells (1 × 10^6 cells/well) were stimulated with 10 μM OVA peptide. Proliferation (top row) was measured by [3H]thymidine incorporation after 3 days incubation. Production of IL-2 (second row), IFN-γ (third row), and IL-4 (bottom row) were measured after 24 h of stimulation by ELISA as described in Materials and Methods. The indicated values represent the mean ± SD of four to six individually tested mice. In vitro stimulation with the negative control HA110–120 peptide was included for each mouse, and there was no significant proliferation (mean cpm was 775 ± 200 for the six tested mice) or cytokine production (all cytokines tested were below detectable level). The mice used for these experiment are those described in Fig. 6.

CD40L on activated T cells has been shown to ligate CD40 on APCs and trigger IL-12 production, which promotes T cell differentiation into the Th1 phenotype (38, 39). Also, prior studies indicated that the splenic anergic T cells when recalled with Ag at adult age display an inability to up-regulate CD40L and trigger IL-12 production by APCs (22). Consequently, these cells were unable to differentiate and produce IFN-γ (22). Because the neonatal TCR-transgenic T cell transfer system gives rise to splenic anergic T cells upon exposure to Ig-OVA and immunization with OVA peptide, it provides a practical system to investigate whether it may be that monocytes/macrophages function as the major APCs in neonates and may contribute to the biased neonatal responses to Ag through the differential expression of B7 molecules relative to adult counterparts.

Exposure to Ig-OVA hinders up-regulation of CD40L expression on neonatal T cells

In contrast, neonatal CD11bCD18^+ cells (mostly monocytes/macrophages) had a much lower expression of both B7.1 and B7.2 molecules than adult CD11bCD18^+ cells. Similar results were obtained when the incubation was conducted with free OVA peptide (not shown). Evaluation of the frequency of the three types of APCs in the neonatal and adult spleen indicated that CD11bCD18-positive cells represent the majority (43%) of neonatal splenic cells (Table I). In the adult spleen, however, 50% of the cells expressed the B220 marker, 9% were CD11bCD18 positive, and 3% stained with 33D1 Ab. Based on these observations,
FIGURE 8. Detection of DO11.10 T cells in both lymph node and spleen on challenge with OVA peptide of adult mice receiving T cell transfer and Ig-OVA at the neonatal stage. Groups of 1-day-old BALB/c mice (four to six per group) were given i.v. in the facial vein 30 \times 10^6 neonatal DO11.10/SCID T cells and injected with 100 \mu g Ig-W (a and b) or Ig-OVA (c and d) within 24 h. After 7 wk, the mice were challenged with 125 \mu g OVA peptide in CFA; 10 days later, the lymph node and spleen were analyzed by staining for presence of CD44^+KJ1-26^+ double-positive DO11.10 T cells ex vivo. The percentages of double-positive cells among total CD4 cells are indicated in the upper right corner of each quadrant. Tol., tolerized; FL1-H and FL2-H, fluorescence intensity.

FIGURE 9. Restoration of proliferation and IFN-\gamma production by the anergic spleen T cells by stimulation with Ag in the presence of IL-12. A group of 1-day-old BALB/c mice were adoptively transferred with 30,000 neonatal DO11.10/SCID cells and injected with 100 \mu g Ig-OVA within 24 h. At the age of 7 wk, the mice were challenged with 125 \mu g OVA peptide in CFA; 10 days later, the animals were sacrificed and the spleen cell (1 \times 10^6 cells/well) were stimulated with 10 \mu M OVA peptide in the presence of 1 or 10 U/ml IL-12. Proliferation (a) was measured by [\textsuperscript{3}H]thymidine incorporation after 3 days of incubation. Production of IFN-\gamma (b) was measured by ELISA after 24 h incubation. The indicated values represent the mean \pm SD of five individually tested mice. In vitro stimulation with OVA peptide without exogenous IL-12 and stimulation with the negative control HA\textsubscript{10-120} peptide in the presence of IL-12 was included for control purposes. Results are those obtained with 10 U/ml IL-12.

FIGURE 10. Differential expression of B7 molecules on adult and neonatal CD11bCD18-positive cells. Neonatal and adult splenocytes (1 \times 10^6 cells/mouse) were incubated with 1 \mu M (150 \mu g/ml) Ig-OVA for 24 h. Subsequently, the cells were double stained with Abs to either B7.1 (top row) or B7.2 (bottom row) and Mac-1 (left column), 33D1 (middle column), or B220 (right column) as described in Materials and Methods. Results are histograms gated on Mac-1^−, 33D1^−, and B220^+ cells, respectively. FL1-H and FL2-H, fluorescence intensity.

The results illustrated in Fig. 11 indicate that incubation of neonatal T cells with adult or neonatal APCs without Ag does not induce up-regulation of CD40L (Fig. 11, a and d). When OVA peptide was added to the culture, 69% of neonatal CD44^+KJ1-26^+ T cells, expressed CD40L when the APCs were of adult origin (Fig. 11b). However, the number of cells with significant levels of CD40L expression declined to 45% when the APCs were from neonatal source (Fig. 11e). In contrast, when Ig-OVA was used for stimulation, the expression of CD40L was at background levels whether the APCs were from neonatal or adult mice (Fig. 11, c and f). Overall, presentation of OVA peptide on neonatal APCs leads to a decline in the expression of CD40 ligand as was observed in other systems (40) while exposure to Ig-OVA imprints a defective up-regulation of CD40L expression on the T cells.

Discussion

The findings reported in this manuscript show that organ-specific regulation of secondary responses in neonatally tolerized mice can develop when the responder cells derive from a homogeneous population of T cells expressing a transgenic TCR but a high input of neonatal T cells is required. Indeed, when the neonatal transfer was conducted with a low to moderate number of DO11.10 T cells (2 to 10 \times 10^3 cells/mouse), the lymph nodes developed IL-4 producing proliferative T cells but the spleen had no significant responses (Fig. 6 and 7). This pattern is similar to the endogenous responses of BALB/c mice that were not given any T cell transfer before tolerization with Ig-OVA on the day of birth (not shown). However, when the neonatal transfer used 30 \times 10^3 DO11.10 cells (which leads to a 3% frequency of CD44^+KJ1-26^+ T cells among the total CD4^+ T cells in the spleen of the BALB/c host, see Fig. 5), the lymph node response was stronger and the spleen showed significant levels of responses (Fig. 11, c and d).

Table I. Relative frequency of APCs among neonatal and adult splenic cells

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<tr>
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<th>CD11bCD18^+</th>
<th>33D1^+</th>
<th>B220^+</th>
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<tbody>
<tr>
<td>Adult</td>
<td>9</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>Neonatal</td>
<td>43</td>
<td>1</td>
<td>10</td>
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*a* Splenic cells from adult (8-wk-old) and newborn (1-day-old) BALB/c mice were incubated with 1 \mu g/ml FITC-labeled Mac-1, 33D1, or B220 Ab for 30 min on ice, and expression of the specific markers was analyzed by flow cytometry. The percent of cells expressing a specific marker is among total splenic cells.
significant IL-2 secretion without any proliferation or IFN-γ production. Moreover, when these IL-2 producing cells were stimulated with OVA peptide in the presence of IL-12 they regained the ability to proliferate and produce IFN-γ (Fig. 9). The implication of DO11.10 cells in the secondary response is supported by the increase in their frequency relative to cells who have not seen the Ag at the neonatal period (Fig. 8). Overall, these results generated with a homogeneous T cell population are similar with those obtained in the SJL/J Ig-PLP1 system where the responder T cells likely derive from a diverse T cell repertoire. Therefore, it may be that cell frequency rather than diversity plays a critical role in the generation of organ-specific neonatal responses and particularly the splenic IL-12/IFN-γ-dependent anergy. The SJL/J mouse which lacks negative selection against PLP1 peptide during fetal and neonatal stages comprises an unusually elevated frequency of PLP1-specific T cells (1/2-0,000 CD4⁺ T cells) in its naive repertoire (41). This mouse strain also develops IFN-γ-dependent anergy on neonatal tolerance with Ig-PLP1 (13, 22). Thus the frequency of specific precursors most likely promotes organ-specific regulation of neonatal immunity. Moreover, while in free peptide driven neonatal tolerance repertoire diversity has been shown to rescue splenic responsiveness by promoting a biased Vβ gene usage and deviation to Th2 (42). In our Ig-peptide model of neonatal immunity the frequency of neonatal T cell precursors seems to bypass diversity and preserve a type of nonpolarized anergic T cells that could be driven to full responsiveness by exogenous IL-12 or IFN-γ (Refs. 13 and 22 and this study).

Given the fact that the organ-specific responses were readily inducible in SJL/J mice, where the frequency of PLP1-specific T cells is remarkably high (41), and reproducible with the homogeneous DO11.10/SCID system when a high number of T cells were transferred there must be a mechanism by which cell frequency promotes different type of responses in the lymph node vs the spleen. On a speculative basis, high frequency may sustain lymphocyte trafficking and supply of Ag helps broaden circulation within diverse organs and tissues (43). Consequently, various types of APCs subject to diverse environmental control may be involved in neonatal Ag presentation leading to specific regulation of the responses. In fact we have demonstrated that neonatal APCs play a critical role in the development of T cell responses in vitro (Fig. 3 and 4). In addition, the frequency of specific types of APCs in the spleen (Table I) and most likely within other organs is variable and expression of costimulatory molecules on these APCs is subject to differential regulation (Fig. 10). Thus, it is logical to envision a relationship between frequency, trafficking, and exposure to various types of APCs in the control of neonatal immunity. In fact, it has previously been shown that different types of APCs promote different outcomes in neonatal immunity (5). Finally, the organ-specific regulation of neonatal immunity occurs with peptide presented on Ig but not with free peptide (13, 14). The experiment presented in Fig. 11 indicated that free OVA peptide presented on neonatal APCs displayed a quantitative regulation of CD40L expression as has been observed in another antigenic system (40). However, when Ig-OVA was used for stimulation, CD40L expression on the neonatal T cells could not occur (Fig. 11). We have previously shown that the anergic splenic T cells of the secondary response in adult SJL mice also display an intrinsic defect for up-regulation of CD40L when stimulated with free PLP1 peptide (22). CD40L is required for cross-linking CD40 on APCs to trigger the production of IL-12 (38, 39) and IL-12 is a key cytokine for Th1 differentiation. Therefore, we postulate that neonatal exposure to Ig-OVA triggers activation of neonatal T cells without up-regulation of CD40L. Consequently, depending on the local environment differentiation may proceed with little or no IL-12 giving rise to cells fully differentiated to Th2 and others committed to Th1 but nondifferentiated. Alternatively, activation in the absence of CD40L may generate IL-2-producing nonpolarized T cells that are still susceptible to undergo differentiation to either Th1 or Th2 depending on the environment.

Overall, we believe that both the frequency of T cell precursors at the neonatal stage and the form of Ag contribute to the generation of organ-specific regulation of neonatal immunity.

**References**


ROLE OF CELL FREQUENCY IN NEOnatal IMMUNITY


