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An Hour after Immunization Peritoneal B-1 Cells Are Activated to Migrate to Lymphoid Organs Where within 1 Day They Produce IgM Antibodies That Initiate Elicitation of Contact Sensitivity¹

Atsuko Itakura,^{2*} Marian Szczepanik,^{2†} Regis A. Campos,* Vipin Paliwal,* Monika Majewska,[†] Hiroshi Matsuda,[‡] Kiyoshi Takatsu,[§] and Philip W. Askenase^{3*}

Elicitation of contact sensitivity (CS), a classic example of T cell-mediated immunity, requires Ag-specific IgM Abs to trigger an initiation process. This early process leads to local recruitment of CS-effector T cells after secondary Ag challenge. These Abs are produced by the B-1 subset of B cells within 1 day after primary skin immunization. In this study we report the surprising observation that B-1 cells in the peritoneal cavity are activated as early as 1 h after naive mice are painted with a contact-sensitizing Ag on the skin of the trunk and feet to begin the initiation of CS. B-1 cells in the spleen and draining lymph nodes produce the initiating Abs by 1 day after immunization, when we found increased numbers of Ag-specific IgM Ab-producing cells in these tissues by ELISPOT assay. Importantly, we show that contact-activated peritoneal B-1 cells migrate to these lymphoid tissues and then differentiate into Ag-specific IgM Ab-producing cells, resulting in specific CS-initiating IgM Abs in the serum by 1 day. Furthermore, pertussis toxin, which is known to inhibit signaling via G protein-coupled chemokines, inhibited the migration of contact-activated peritoneal B-1 cells to the lymphoid tissues, probably due to BLR-1 (Burkitt lymphoma receptor-1). These findings indicate that within 1 h after contact skin immunization, B-1 cells in the peritoneal cavity are activated to migrate to the lymphoid tissues by chemokine-dependent mechanisms to produce serum Ag-specific IgM Abs within 1 day after immunization, leading to local recruitment of CS-effector T cells. *The Journal of Immunology*, 2005, 175: 7170–7178.

Cutaneous contact sensitivity (CS)⁴ is mediated by CD4⁺ or CD8⁺ CS-effector T cells, and is a subtype of delayed-type hypersensitivity (1). We recently discovered that the local recruitment of effector T cells at secondary challenge is due to a novel cascade involving Ag-specific IgM Abs that we call the initiating phase of CS, which is a process that culminates in Ag-specific endothelial cell activation (2, 3) to elicit responses (4).

The IgM Abs specific for priming Ag form complexes with challenge Ag leading to local activation of complement (4). This generates the C5a fragment that stimulates C5a receptors on local mast cells and platelets to release vasoactive mediators, such as serotonin and TNF- α (5–8). These mediators activate the endo-

thelium (2), which facilitates local T cell recruitment. These CS-initiating events occur within 2 h after secondary Ag challenge. Macroscopically they are detected as an edematous ear swelling response due to increased vascular permeability that peaks at 2 h after challenge (2, 9). This early 2-h initiating phase is responsible for elicitation of the classical late 24-h ear swelling phase mediated by the recruited CS-effector T cells.

Surprisingly, we found the Ag-specific CS-initiating IgM Abs are present in the circulation as early as 1 day after immunization (4). Furthermore, we showed that pan B cell-deficient JH^{-/-} mice (4), predominantly B cell-deficient μ MT mice (10), or B-1 cell-deficient *xid* mice with partial B-2 cell deficiency (11), had defective CS responses. Studies in these mice suggested that the CS-initiating IgM Abs are produced by the B-1 subset of B cells (4). Although the activated B-1 cells that are able to reconstitute defective CS responses in the B cell- and B-1 cell-deficient mice are found in spleens and draining lymph nodes by only 1 day after immunization (4), it is unclear whether these lymphoid tissues are the primary sites for activation of the CS-initiating B-1 cells.

In this report we demonstrate a new role of peritoneal B-1 cells in the elicitation of CS responses. We show that peritoneal B-1 cells are activated as early as 1 h after skin immunization. These 1-h immune peritoneal B-1 cells reconstitute CS in immunized *xid* mice that otherwise show impaired responses due to their B-1 cell deficiency (4). The i.p. transferred B-1 cells probably reconstitute CS by producing IgM Abs in the recipients within 24 h that locally initiate T cell recruitment after secondary Ag challenge in the skin to elicit the CS responses. However, lymphoid tissue cells do not acquire this reconstituting activity until 24 h after immunization, when we found increased Ag-specific IgM-producing cells in both spleen and draining lymph nodes. Accordingly, we show that i.p.

*Section of Allergy and Clinical Immunology, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06520; [†]Department of Human Developmental Biology, Jagiellonian University College of Medicine, Krakow, Poland; [‡]Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan; Department of Immunology and Microbiology, [§]Division of Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan.

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² A.I. and M.S. contributed equally to this work.

³ Address correspondence and reprint requests to Dr. Philip W. Askenase, Section of Allergy and Clinical Immunology, Department of Internal Medicine, Yale University School of Medicine, 300 Cedar street, New Haven, CT 06520-8013. E-mail address: philip.askenase@yale.edu

⁴ Abbreviations used in this paper: CS, contact sensitivity; BLC, B lymphocyte chemoattractant; BLR-1, Burkitt lymphoma receptor-1; iNKT cell, invariant NKT cell; OX, oxazolone; TNP-Cl, trinitrophenyl chloride.

transfer of 1-h CS-activated peritoneal cells subsequently leads to increased numbers of Ag-specific IgM-producing cells in the spleen. This apparent migration of 1-h immune B-1 cells from the peritoneal cavity to lymphoid tissues was inhibited by *in vitro* pretreatment with pertussis toxin, which is known to inhibit G protein-coupled chemokine receptors (12). Therefore, we conclude that B-1 cells are primarily activated in the peritoneal cavity within 1 h of cutaneous contact sensitization. These immune-activated B-1 cells then migrate to the spleen and draining lymph nodes by chemokine-dependent mechanisms, and within 1 day produce Ag-specific IgM Abs that are required for initiating the elicitation of CS. Collectively, these findings suggest a novel function of early-activated peritoneal B-1 cells in the effector phase of T cell-mediated immunity.

Materials and Methods

Mice

Specific pathogen-free male CBA/J and CBA/CaHN-btk-*xid*/J (*xid*) mice (5–8 wk old) and female C57BL/6, BALB/c, and μ MT mice (5–8 wk old) were obtained from the National Cancer Institute, National Institutes of Health, and The Jackson Laboratory and were rested for at least 1 wk. Female and male Burkitt lymphoma receptor-1^{-/-} (BLR-1^{-/-}) mice on a C57BL/6 background were obtained from Dr. F. Reinhold, Munich, Germany (13), and B cell-deficient JH^{-/-} mice were obtained from M. Shlomchik (Yale University Medical School, New Haven, CT). Experiments were performed according to guidelines of the Yale animal care and use committee.

Reagents

Picryl chloride (trinitrophenyl chloride (TNP-Cl); Nacalai Tesque), recrystallized twice with ethanol, and oxazolone (OX; Sigma-Aldrich) were stored in the dark. TNP-BSA was purchased from Bioscience Technologies. OX-BSA was made as described previously (14).

Active sensitization and elicitation of CS

All mice were immunized by contact painting 150 μ l of 5% TNP-Cl or 3% OX in ethanol/acetone (4/1) on a 1 \times 1-cm area of the shaved chest, abdomen, and feet unless otherwise indicated. On day 1 or 4, mice were challenged by topical application of 10 μ l of 0.4% TNP-Cl or OX in acetone/olive oil (1/1) on the ears, and ear thickness was measured by an observer unaware of the groups at 2 and/or 24 h after challenge. The net increase in ear thickness was calculated by subtracting ear thickness measured before challenge and was expressed as the mean millimeters \times 10⁻² \pm SE of four mice per group unless otherwise stated.

Isolation of B-1a, B-1b, and B-2 cells

Mice were contact skin immunized, and peritoneal cells were harvested at various time points by washing the peritoneal cavity with 5 ml of PBS containing heparin (5 U/ml). To obtain purified B-1a, B-1b, and B-2 cells, cells were stained with 0.1 μ g/10⁶ cells of FITC-conjugated anti-B220, PE-conjugated anti-Mac-1, and biotin-conjugated anti-CD5 (BD Pharmingen) at 4°C for 1 h, followed by staining with streptavidin-allophycocyanin at 4°C for 30 min. B-1a cells (B220⁺Mac-1⁺CD5⁺), B-1b cells (B220⁺Mac-1⁺CD5⁻), and B-2 cells (B220⁺Mac-1⁻CD5⁻) were sorted by FACS Vantage (BD Biosciences). The ratio of each cell population was B-1a:B-1b:B-2 = 1:1.6:3. Purity was usually >95–98%.

Adoptive cell transfer of CS

Peritoneal cells, sorted peritoneal cells, or mixed axillary and inguinal lymph node and spleen cells were prepared from the same number of donors as recipients, pooled, and transferred *i.p.* or *i.v.* as indicated in the figure legends. Some experiments used *xid*, JH^{-/-}, or μ MT recipients that previously were immunized by painting 150 μ l of 5 or 0.2% TNP-Cl or 3% OX to generate CS-effector T cells and then on day 3 received cell transfers. The recipients were challenged 1 day later, and ear thickness increases were measured 2 and/or 24 h after challenge and compared with thickness before challenge.

ELISPOT assay for specific IgM Abs

Single-cell suspensions were prepared from spleens and lymph nodes at various times after skin immunization and seeded into MultiScreen-IP

plates with Immobilon-P membranes (Millipore; 2 \times 10⁶ cells/well) previously coated with 50 μ l of TNP-BSA or OX-BSA (100 μ g/ml) in 35 mM NaHCO₃, 15 mM Na₂CO₃, and 0.02% NaN₃ (pH 9.5). Plates were incubated at 37°C overnight, washed with PBS three times, then three times with PBS containing 0.05% Tween 20, and incubated with 2 μ g/ml biotin-conjugated anti-mouse IgM mAb (BD Pharmingen) for 1 h, followed by incubation with streptavidin-peroxidase (Vector Laboratories; 1/200) for 1 h at 25°C. Spots were developed using 3-amino-9-ethylcarbazole substrate, and reaction was stopped by washing with H₂O. After drying in the dark, membranes were removed, and spots were counted under a phase-contrast microscope by a single observer unaware of the groups.

Determination of possible migration of peritoneal B-1 cells

Peritoneal cells were harvested from CBA/J mice 1 h after skin immunization and were transferred *i.p.* or *i.v.* into nonimmune *xid* mice or CBA/J mice. In some experiments, peritoneal cells were treated with 1 μ g/ml pertussis toxin (Sigma-Aldrich) for 3 h at 37°C and washed three times with PBS before transfer. Spleens were harvested on day 2, and the numbers of anti-TNP IgM-producing cells were measured by ELISPOT assay. To determine the ability of pertussis toxin-treated peritoneal cells to mediate 2 h ear-swelling responses, CBA/J recipients were ear challenged 1 day after cell transfer. We also determined the ability of peritoneal cells harvested 1 h after skin immunization and treated with pertussis toxin *in vitro* to produce Abs after *in vitro* stimulation with LPS (Sigma-Aldrich; 20 μ g/ml). After 48 h, cells were harvested and washed three times with medium, and the numbers of anti-TNP IgM-producing cells were measured by ELISPOT.

Statistics

Statistics were determined using the paired two-tailed Student's *t* test; *p* < 0.05 was taken as the level of significance.

Results

Peritoneal cells are activated within 1 h after immunization to mediate 2 h ear-swelling responses

We hypothesized that after skin immunization by painting with a contact-sensitizing Ag, B-1 cells eventually found in lymph nodes and spleen were activated in the peritoneal cavity and then migrated to these lymphoid tissues and acquired CS-initiating activity within 1 day. To test this hypothesis we harvested peritoneal cells at various times up to 24 h after contact immunization with TNP-Cl and then determined whether unimmunized mice that received adoptive transfer of these early immune peritoneal cell populations develop 2 h ear-swelling responses to ear challenge with TNP-Cl. In parallel experiments we determined numbers of anti-TNP IgM-producing cells in the spleen by ELISPOT assay.

Indeed, peritoneal cells harvested at only 1 h after immunization, on transfer into unimmunized mice, elicited 2 h responses in recipients when challenged 24 h after adoptive cell transfer (Fig. 1A, group B vs A). Transferred responses were comparable to reactions in 1-day actively sensitized mice (group E). Furthermore, 6 h immune peritoneal cells had the same activity (group C). In contrast, 24 h immune peritoneal cells transferred much less 2 h responsiveness (group D). As expected, peritoneal cells from non-immune mice did not transfer 2 h responses (data not shown and Fig. 2, groups B and F). Quantitative transfer of 1-h immune peritoneal cells showed a dose-dependent increase in 2 h responses (Fig. 1B, groups B–E vs A), and even cells from less than one donor successfully transferred 2 h responsiveness into one recipient (group E). The transfers with early activated peritoneal cells were not due to the nearby abdominal immunization, because we also detected the same activity in 1-h immune peritoneal cells derived from mice that were painted with TNP-Cl on their back (Fig. 1C, group E vs C vs A).

We tested whether the ability of 1-h immune peritoneal cells to transfer 2 h responses is Ag specific. Again, 1-h TNP-Cl immune peritoneal cells transferred 2 h ear-swelling responses in TNP-Cl-challenged recipients (Fig. 2, group C vs A). In contrast, these 1-h OX immune peritoneal cells were inactive in TNP-Cl-challenged

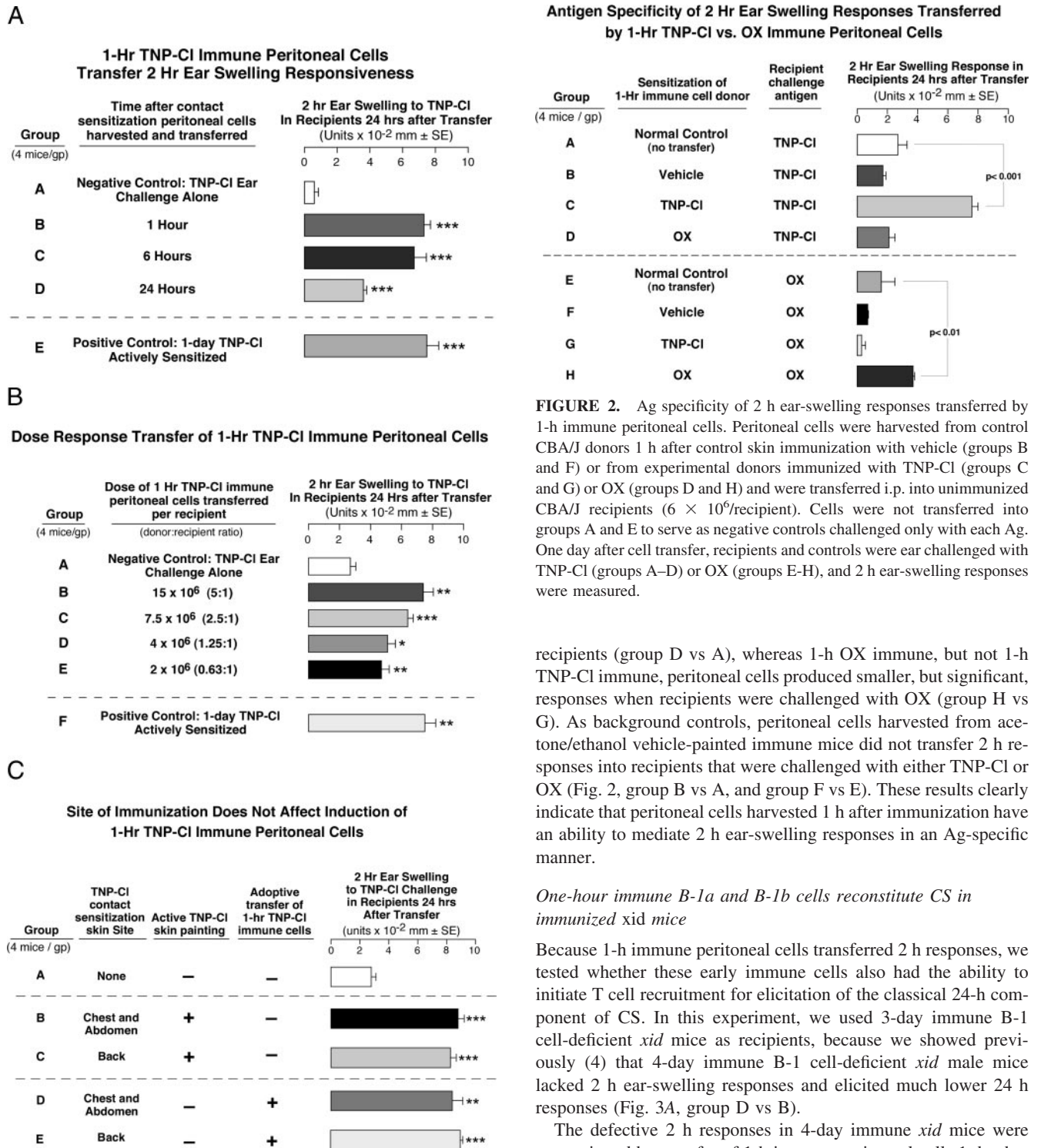


FIGURE 2. Ag specificity of 2 h ear-swelling responses transferred by 1-h immune peritoneal cells. Peritoneal cells were harvested from control CBA/J donors 1 h after control skin immunization with vehicle (groups B and F) or from experimental donors immunized with TNP-CI (groups C and G) or OX (groups D and H) and were transferred i.p. into unimmunized CBA/J recipients (6×10^6 /recipient). Cells were not transferred into groups A and E to serve as negative controls challenged only with each Ag. One day after cell transfer, recipients and controls were ear challenged with TNP-CI (groups A–D) or OX (groups E–H), and 2 h ear-swelling responses were measured.

recipients (group D vs A), whereas 1-h OX immune, but not 1-h TNP-CI immune, peritoneal cells produced smaller, but significant, responses when recipients were challenged with OX (group H vs G). As background controls, peritoneal cells harvested from acetone/ethanol vehicle-painted immune mice did not transfer 2 h responses into recipients that were challenged with either TNP-CI or OX (Fig. 2, group B vs A, and group F vs E). These results clearly indicate that peritoneal cells harvested 1 h after immunization have an ability to mediate 2 h ear-swelling responses in an Ag-specific manner.

*One-hour immune B-1a and B-1b cells reconstitute CS in immunized *xid* mice*

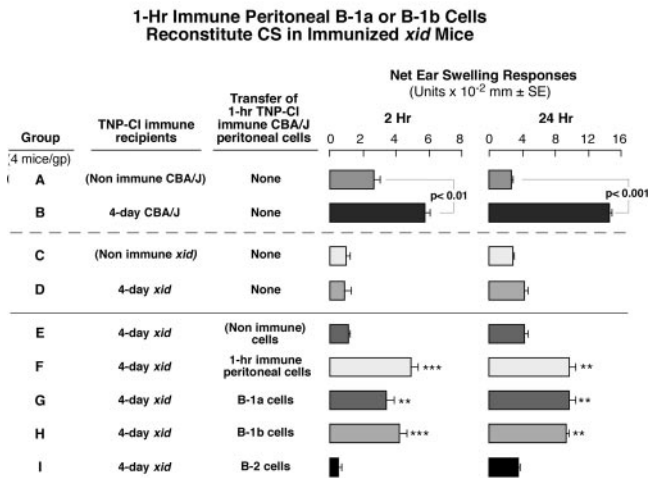
Because 1-h immune peritoneal cells transferred 2 h responses, we tested whether these early immune cells also had the ability to initiate T cell recruitment for elicitation of the classical 24-h component of CS. In this experiment, we used 3-day immune B-1 cell-deficient *xid* mice as recipients, because we showed previously (4) that 4-day immune B-1 cell-deficient *xid* male mice lacked 2 h ear-swelling responses and elicited much lower 24 h responses (Fig. 3A, group D vs B).

The defective 2 h responses in 4-day immune *xid* mice were reconstituted by transfer of 1-h immune peritoneal cells 1 day before challenge (day 3; Fig. 3A, left, group F vs D). Importantly, the restored 2 h responses then allowed elicitation of a significantly

FIGURE 1. Two hour ear-swelling responses mediated by 1-h TNP-CI immune peritoneal cells. A, Unimmunized CBA/J recipients were i.p. transferred with 1.5×10^7 peritoneal cells (donor:recipient ratio, 5:1) harvested at the indicated time points from donor's skin painted with the contact-sensitizing Ag TNP-CI (groups B–D), and the recipients were ear challenged with TNP-CI the next day. Mice in group E were actively immunized with TNP-CI 1 day before challenge as positive controls, and mice in group A were negative controls challenged only with TNP-CI. Two hour ear-swelling responses were determined. ***, $p < 0.001$ vs group A; $p < 0.01$ group C vs D. B, Unimmunized CBA/J recipients were transferred i.p. with the indicated decreasing numbers of 1-h TNP-CI immune peritoneal cells (groups B–E) and were ear challenged 1 day later. Mice in group F were actively immunized with TNP-CI on day 1 before ear challenge to serve as positive controls, and mice in group A were negative

controls challenged only with TNP-CI. *, $p < 0.02$; **, $p < 0.01$; ***, $p < 0.001$ (vs group A). C, CBA/J mice were unimmunized (group A) or immunized by contact painting with TNP-CI on the chest and abdomen (group B) or only on the back (group C), and were ear challenged the next day. Mice in groups D and E were transferred i.p. with 6×10^6 peritoneal cells (donor:recipient ratio, 2:1) harvested from 1-h immune CBA/J donors painted with TNP-CI on the chest and abdomen (group D) or on the back (group E) and were ear challenged the next day. Two hour ear-swelling responses were determined. **, $p < 0.01$; ***, $p < 0.001$ (vs group A).

A



B

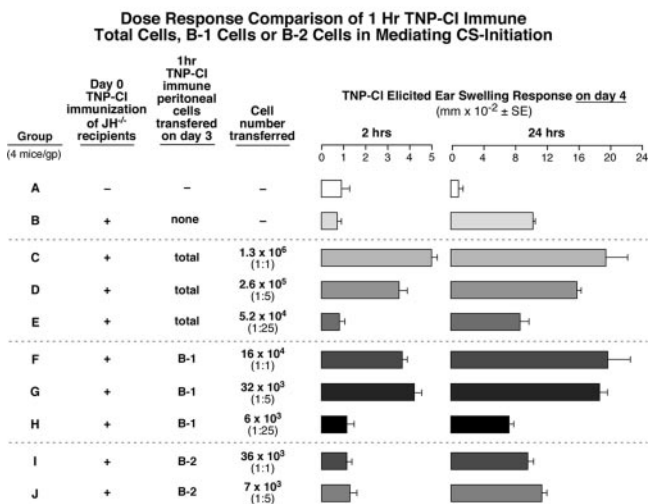


FIGURE 3. Reconstitution of 2 and 24 h CS responses in immunized B-1 cell-deficient *xid* mice and B cell-deficient *JH^{-/-}* mice by transfer of CS-initiating, 1-h immune purified peritoneal B-1a vs B-1b vs B-2 cells. *A*, Peritoneal cells were harvested from nonimmune CBA/J mice (group E) or 1-h TNP-CI immune CBA/J mice (group F), and some 1-h TNP-CI immune peritoneal cells were sorted into B-1a cells (B220⁺Mac-1⁺CD5⁺; group G), B-1b cells (B220⁺Mac-1⁺CD5⁻; group H), or B-2 cells (B220⁺Mac-1⁻CD5⁻; group I). The 1-h immune cell populations were i.p. transferred into *xid* mice that were immunized with TNP-CI 3 days later (7.5×10^6 peritoneal cells, 4×10^4 B-1a cells, 6.3×10^4 B-1b cells, or 1.2×10^5 B-2 cells). One day later, the immunized *xid* recipients were ear challenged with TNP-CI, and 2 and 24 h ear-swelling responses were measured. **, $p < 0.01$; ***, $p < 0.001$. *B*, Peritoneal cells or sorted B-1a cells (B220⁺CD5⁺) or B-2 cells (B220⁺CD5⁻) were harvested from 1-h TNP-CI immune BALB/c mice. The cell populations were transferred i.p. into 3-day TNP-CI immune, pan B cell-deficient *JH^{-/-}* mice at the cell number and the donor:recipient ratios shown. One day later, recipients were ear challenged with TNP-CI, and their 2 and 24 h ear-swelling responses were measured.

stronger 24-h component of CS in the immune *xid* recipients that almost reached the level seen in actively 4-day immune CBA/J mice (Fig. 3A, right, group F vs B). In contrast, peritoneal cells harvested from unimmunized CBA/J mice could not reconstitute either 2 or 24 h responses (group E vs D). Thus, 1-h immune peritoneal cells not only mediate 2 h ear-swelling responses, but

also can initiate full CS responses due to the early 2-h component leading to T cell recruitment to consequently elicit the late 24-h component.

To test whether B-1 cells are responsible for the CS-initiating activity of 1-h immune peritoneal cells, we attempted to reconstitute CS in 4-day immune *xid* mice with 1-h immune sorted peritoneal B-1a, B-1b, or B-2 cells. Both B-1a cells (B220⁺Mac-1⁺CD5⁺) and B-1b cells (B220⁺Mac-1⁺CD5⁻) harvested from 1-h immune CBA/J mice reconstituted both 2- and 24-h components of CS in 4-day immune *xid* mice (Fig. 3A, groups G and H vs E), whereas B-2 cells (B220⁺Mac-1⁻) failed (group I vs E). We confirmed that B-1a cells, but not B-2 cells, are responsible for CS-initiating activity of 1-h immune peritoneal cells by transferring various numbers of 1-h immune sorted B-1a or B-2 cells into 4-day immune pan B cell-deficient *JH^{-/-}* mice (Fig. 3B). Surprisingly, as few as 3.2×10^4 B-1a cells were able to reconstitute defective CS in TNP-CI-immune pan B cell-deficient *JH^{-/-}* mice (group G vs B). Thus, we concluded that peritoneal B-1 cells, including both B-1a and B-1b cells, are activated as early as 1 h after contact immunization to eventually initiate the elicitation of CS responses.

Lymphoid cells do not acquire the activity to mediate 2 h ear-swelling responses until 24 h after immunization

We tested for possible migration of transferred immune B-1 cells from the peritoneal cavity to the lymphoid tissues. Transfer of 1-h immune peritoneal cells again led to 2 h responses in unimmunized ear-challenged recipients (Fig. 4A, group A). However, 24-h immune cells also harvested from the peritoneal cavity had lost this ability (group C). In contrast and as shown previously, 24-h immune lymphoid cells clearly transferred 2 h responsiveness to non-immune CBA/J mice (group D). However, lymphoid cells were not yet active at 1 h after immunization (group B) when peritoneal cells have active CS-initiating function (group A). One hour peritoneal cells or 1-day lymphoid cells transferred 2 h activity either i.p. or i.v. (Fig. 4B). This coincident loss of activity in the peritoneal cells and gain of activity in lymphoid cells 24 h after immunization suggest that immune activated peritoneal B-1 cells leave the peritoneal cavity and migrate to the lymphoid tissues by 1 day after immunization.

Ag-specific IgM-producing cells are present in lymphoid tissues as early as 1 day after immunization

We determined whether 1-day immune lymphoid cells mediate 2 h ear-swelling responses via production of specific IgM Abs. We used ELISPOT assay to measure Ag-specific IgM-producing cells in lymphoid tissues early after contact immunization.

Nonimmune CBA/J mice had background numbers of natural anti-TNP IgM-producing cells in the spleen (Fig. 5A, left, group A). We found a significant increase as early as 1 day after immunization (group B), and by 2 days their number had nearly doubled (group C). Background levels of anti-TNP IgM-producing cells were undetectable in unimmunized CBA/J lymph nodes, were first detected by 1 day after immunization, and were progressively greater at 1 and 4 days (Fig. 5A, right, groups C and D). In contrast, *xid* B-1 cell-deficient mice had <20% of anti-TNP IgM-producing cells vs CBA/J mice in the spleen before immunization and showed no response to TNP-CI immunization (Fig. 5A, left, groups E–H). Surprisingly, we found significantly greater numbers of anti-TNP IgM-producing cells in 2-day immune *xid* lymph nodes, which then increased dramatically by day 4 (Fig. 5A, right), probably due to B-2 cells.

The IgM Ab response in the spleen induced by contact immunization is Ag specific (Fig. 5B). When anti-TNP IgM-producing

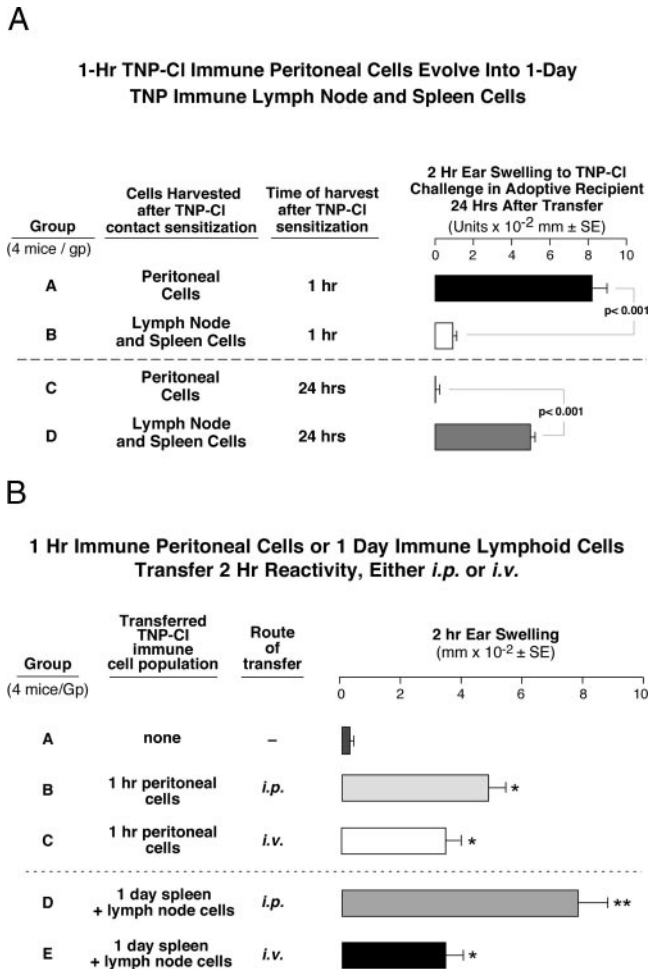


FIGURE 4. Lymphoid cells transfer 2 h ear-swelling responses 24 h after immunization, but are not yet active 1 h after immunization. *A*, Peritoneal cells (3×10^6) and mixed spleen and lymph node cells (7×10^7) were harvested from CBA/J donors at 1 or 24 h after TNP-CI immunization and were respectively transferred *i.p.* or *i.v.* into unimmunized CBA/J recipients. One day later, the recipients were ear challenged with TNP-CI, and 2 h ear-swelling responses were measured. Data are expressed as the net ear-swelling responses by subtracting control ear swelling in mice that were challenged with TNP-CI without receiving cell transfer. *B*, One-hour TNP immune CBA/J peritoneal cells or 1-day immune mixed spleen and lymph node cells were transferred to normal CBA/J recipients at a ratio of two donors to one recipient by the *ip* or *iv* route. Twenty-four hours later, ear swelling induced by local ear challenge with TNP-CI was determined and compared with that in identically challenged controls that did not receive transfers.

cells were measured in spleens harvested from mice immunized with TNP-CI vs the irrelevant Ag, OX, anti-TNP IgM-producing cells again were increased in mice immunized with TNP-CI (Fig. 5B, group B vs A), whereas OX immunization did not change their number (group C vs A). Taken together, Ag-specific IgM Abs likely produced by B-1 cells probably account for CS-initiating activity of 24-h immune lymphoid cells.

Ag-specific IgM Abs released into the circulation within 1 day after immunization are responsible for initiation of CS

We next attempted to reconstitute the defective 2- or 24-h component of CS in immunized *xid* mice by transfer of serum harvested from CBA/J mice at various times after immunization. Again, positive control 1-day immune lymphoid cells successfully reconstituted 24 h ear-swelling responses in 4-day TNP-CI immune

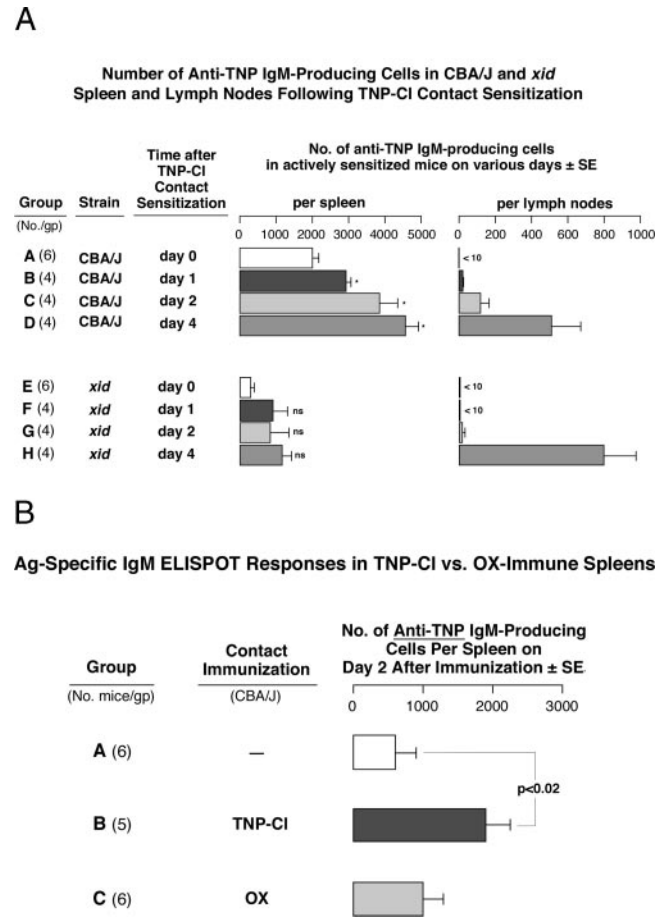


FIGURE 5. Early induction of Ag-specific IgM Ab responses by contact immunization in CBA/J mice vs B-1 cell-deficient *xid* mice. *A*, Spleens and draining lymph nodes were harvested from CBA/J mice or *xid* mice at the indicated times after contact skin immunization with TNP-CI, and the number of anti-TNP IgM-producing cells was measured by ELISPOT assay. *, $p < 0.01$ (vs group A). *B*, The numbers of anti-TNP IgM-producing cells in spleen from unimmunized CBA/J mice and from 2-day TNP-CI or OX immune CBA/J mice were measured by ELISPOT assay.

xid mice (Fig. 6A, group A vs B). Kinetic testing of serum transfers showed that both 6- and 12-h immune serum failed to reconstitute 24 h responses in immune *xid* mice (groups C and D). In contrast, the serum became as active as responses transferred by the 1-day immune lymphoid cells 24 h after immunization (group E vs B).

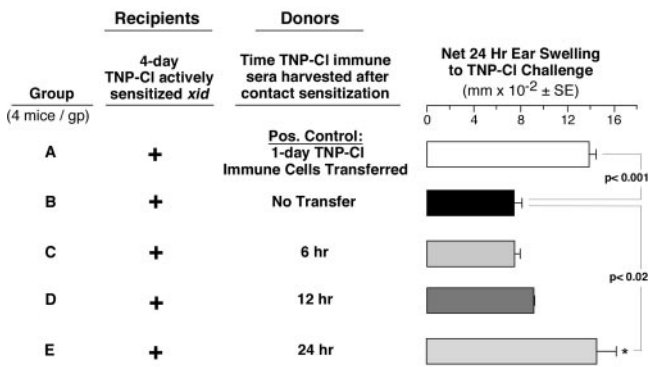
We demonstrated previously that CS-initiating activity in 1-day immune serum was Ag specific (4). To directly demonstrate that Ag-specific IgM Abs in 1-day immune serum were responsible for initiation of CS, we determined whether 1-day immune serum lost CS-initiating activity by passing through an anti-Ig μ -chain column or an anti- κ L chain column. Importantly, the fraction of 1-day immune serum passing through either an anti- μ -chain column, or an anti- κ L chain column no longer had CS-initiating activity (Fig. 6B, groups E and F vs D). Therefore, we concluded that B-1 cells mediated CS-initiating activity through rapid production of serum Ag-specific IgM Abs by 1 day after immunization.

Peritoneal CS-initiating B-1 cells migrate to spleen to produce Ag-specific IgM Abs

To directly examine the migration of Ag-specific peritoneal B-1 cells, we transferred 1-h immune CBA/J peritoneal cells *i.p.* into unimmunized *xid* mice, and 2 days later measured anti-TNP IgM-producing cells in the recipient spleens by ELISPOT assay. We

A

Kinetics of CS-Initiating Activity Transferred by Immune Sera Obtained During the First Day After TNP-CI Contact Sensitization



B

1-day TNP-CI Immune Serum IgM Antibodies Reconstitute CS in TNP-CI Immunized B-1 Cell-Deficient *xid* Mice

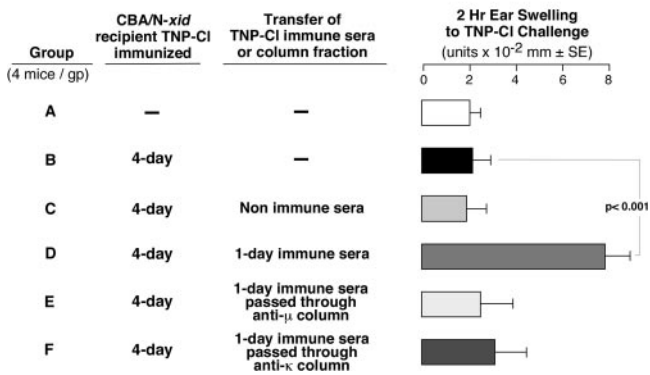


FIGURE 6. Ag-specific IgM Abs are responsible for CS-initiating activity of 1-day immune serum. *A*, Serum was harvested from TNP-CI immunized CBA/J mice at the indicated time points. Then each serum was passively transferred i.v. into separate groups of mice at a dose of 0.75 ml/recipient. The recipients were B-1 cell-deficient CBA/N *xid* mice that had been contact sensitized with TNP-CI 4 days previously. Mice in group A were transferred with 5 × 10⁵ 1 day TNP-CI immune lymph node and spleen cells to serve as a positive control. One day later, recipients were challenged with TNP-CI, and 24 h ear-swelling responses were measured. *B*, Sera were harvested from normal CBA/J mice or 1-day TNP-CI immune mice and transferred i.v. into 3-day immune *xid* mice (0.75 ml/recipient). Mice in groups E and F received 1-day TNP-CI immune serum that was passed through anti-μ H chain or anti-κ L chain columns. One day later, recipients were ear challenged with TNP-CI, and 2 h ear-swelling responses were measured.

used *xid* mice as recipients, because unimmunized B-1 cell-deficient *xid* mice have many fewer background anti-TNP IgM-producing cells in the spleen compared with unimmunized CBA/J mice (Fig. 5*A*, left, group E vs A). Thus, the use of *xid* recipients would aid detection of increases derived from transferred cells. When nonimmune *xid* mice received 1-h immune peritoneal cells, their spleens contained significantly higher numbers of anti-TNP IgM-producing cells 48 h later than *xid* mice that received non-immune CBA/J peritoneal cells (Fig. 7, group B vs A).

We also examined IgM-producing activity in the peritoneal cavity of recipients of 1-h immune or nonimmune cells and found no detectable anti-TNP IgM-producing cells in recipient *xid* mice regardless of donor immunization (Fig. 7, groups C and D). In

Number of Anti-TNP IgM-Producing Cells in Non-Immune Recipient *xid* Mice 2 Days after the Transfer of 1-Hr TNP-CI Immune CBA/J Peritoneal Cells

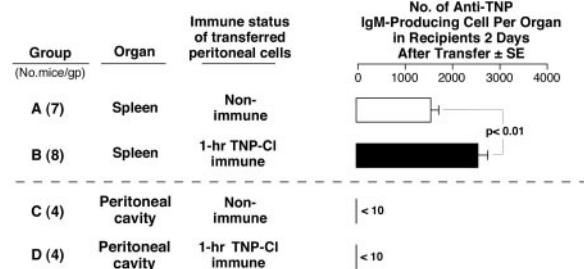


FIGURE 7. Peritoneal B-1 cells migrate to the spleen to produce Ag-specific IgM Abs. Nonimmune (groups A and C) or 1-h TNP-CI immune peritoneal cells (groups B and D) were transferred i.p. into unimmunized *xid* mice (9 × 10⁶ cells/recipient), and the numbers of anti-TNP IgM-producing cells per spleen and peritoneal cavity were measured by ELISPOT assay 2 days later.

addition, we harvested peritoneal cells 1 h after immunization and immediately measured anti-TNP IgM-producing cells by ELISPOT assay. We found almost no anti-TNP IgM-producing cells in both nonimmune and 1-h immune peritoneal cells (non-immune, 1.6 ± 1.0; 1-h immune, 0.0 ± 0.0). Therefore, we concluded that peritoneal B-1 cells activated within 1 h after immunization are unable to produce Abs in the peritoneal cavity. Instead, they migrate to the spleen, where they gain the ability to produce Ag-specific IgM Abs to initiate CS.

Pertussis toxin inhibits migration of peritoneal B-1 cells and suppresses their CS-initiating ability

To test whether migration of TNP-specific peritoneal B-1 cells to the spleen in CS involves chemokine receptor signaling, we used a bacterial toxin that can inhibit signaling from G protein-coupled chemokine receptors (12). Again, transfer of 1-h immune peritoneal cells increased the number of anti-TNP IgM-producing cells in recipient spleens (Fig. 8*A*, group B vs A). When these harvested cells were treated in vitro with pertussis toxin, peritoneal B-1 cells specific for TNP appeared to no longer migrate to the spleen because the ELISPOT assay was negative (group C vs B).

More than 95% of cells were alive after the treatment with pertussis toxin as determined by trypan blue dye exclusion test (data not shown). To exclude the possibility that peritoneal cells did migrate to the spleen, but had lost ability to produce Abs, we determined Ab production from 1-h immune peritoneal cells stimulated with LPS in vitro and found no difference in the number of anti-TNP IgM-producing cells between the untreated group and the pertussis toxin-treated groups (data not shown).

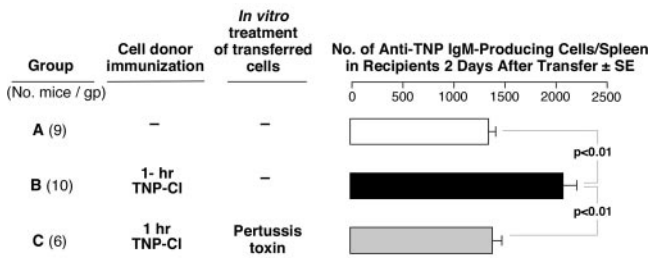
Importantly, when 1-h immune peritoneal cells were treated with pertussis toxin before i.p. transfer into naive recipients, they no longer mediated 2 h ear-swelling responses (Fig. 8*B*, group E vs C). This also was the case when pertussis toxin-treated peritoneal cells were i.v. transferred (group D vs B). Therefore, we concluded that peritoneal B-1 cells are activated by 1 h after immunization to migrate to the spleen to produce anti-TNP IgM CS-initiating Abs by 1 day. This mechanism involves pertussis toxin-sensitive chemokine receptor(s), which are required to mediate 2-h ear-swelling responses.

One-hour immune peritoneal cells from BLR-1^{-/-} mice do not have CS-initiating activity

Because B lymphocyte chemoattractant (BLC) preferentially attracts B-1 cells (15), we examined whether BLC mediates the migration of peritoneal B-1 cells to the spleen in CS through its

A

**Pertussis Toxin Inhibits Migration of
1-Hr TNP-CI Immune Peritoneal B-1 Cells to the Spleen**



B

**Inhibited Transfer of 2 Hr Ear Swelling Responses of
1 Hr Pertussis Toxin-treated Peritoneal Cells by i.p. or i.v. Route**

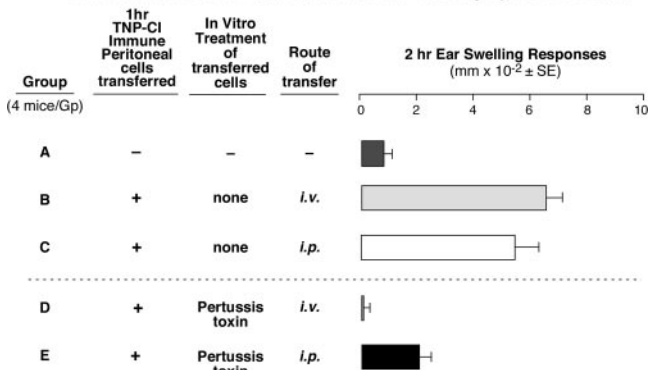


FIGURE 8. Previous in vitro treatment with pertussis toxin inhibits Ag-specific peritoneal B-1 cell migration to produce anti-TNP IgM Abs in the spleen and the ability of B-1 cells to mediate 2 h ear-swelling responses. A, Nonimmune (group A) or 1-h TNP-CI immune peritoneal cells (groups B and C) were harvested from CBA/J donors and were treated with either diluent alone (group B), or 1 μ g/ml pertussis toxin (group C) for 3 h at 37°C. After washing with PBS three times, the cell populations were transferred into unimmunized *xid* mice (9×10^6 cells/recipient), and the numbers of anti-TNP IgM-producing cells in recipient spleens were measured 2 days later by ELISPOT assay. B, Nonimmune peritoneal cells (group A) or 1-h TNP-CI immune CBA/J peritoneal cells (groups B–E) were transferred i.v. (groups B and D) or i.p. (Groups C and E). Recipients were naive CBA/J mice (3×10^6 /recipient). Cells were treated in vitro with either PBS (groups B and C) or 1 μ g/ml pertussis toxin (groups D and E). One day later, recipients were ear challenged with TNP-CI, and 2 h ear-swelling responses were measured.

specific receptor BLR-1. As shown previously (6), μ MT mouse ear challenged 1 day later had impaired 2 h ear-swelling responses due to lack of B cells (Fig. 9, group D vs B), whereas wild-type C57BL/6 mice showed intact CS responses (group B vs A). The impaired responses were reconstituted by transfer of peritoneal cells from 1-h immune, but not from nonimmune, wild-type C57BL/6 mice (groups F and E vs D). However, peritoneal cells from 1-h immune BLR-1^{-/-} mice did not have CS-initiating activity (group G), indicating that signaling through BLR-1 is required for peritoneal B-1 cells to initiate CS. We concluded that activation of BLR-1 by BLC was probably required for the migration of 1-h immune activated peritoneal B-1 cells to the spleen for subsequent production of specific IgM Abs that initiate elicitation of CS responses.

Discussion

B-1 cells are well known as a source of natural background IgM Abs and also IgM specific for autoantigens (11). In this study we studied the required involvement of B-1 cells in initiation of the elicitation of classical T cell-mediated allergic cutaneous CS responses. We showed that B-1 cells in the peritoneal cavity, which is the normal primary site of B-1 cells in mice, are activated within 1 h after skin immunization to lead to initiation of the elicitation of CS responses in the skin (Fig. 10).

B-1 cells in 1-h immune peritoneal cells mediate CS initiation

The very rapid activation of peritoneal B-1 cells within 1 h after contact immunization extends previous findings that B-1 cells can provide very rapid responses to immunization with Ag (16), but our results demonstrate their activation at the very earliest possible time after immunization in the skin. Such very rapid activation might be explained by previous priming with cross-reactive Ag from the environment and/or by normal flora or self Ag, resulting in heightened signaling properties in B-1 cells, as indicated by their constitutive activation of STAT-3, ERK, and NF-AT in non-immunized mice (11, 16–18).

Likely migration of immune peritoneal cells to lymphoid tissues to initiate CS

Our data show that peritoneal cells acquire CS-initiating activity with completely different kinetics from lymphoid tissue cells and suggest that this can be accounted for by rapid activation of B-1 cells in the peritoneal cavity within 1 h, and then their migration to

Reconstitution of CS in Immunized B Cell-Deficient μ MT Mice with 1-Hr Immune Peritoneal Cells from Wild Type vs. BLR1^{-/-} Mice

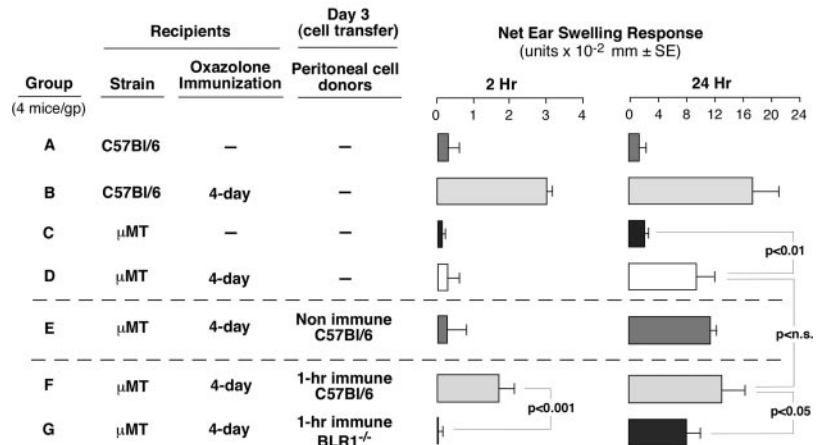
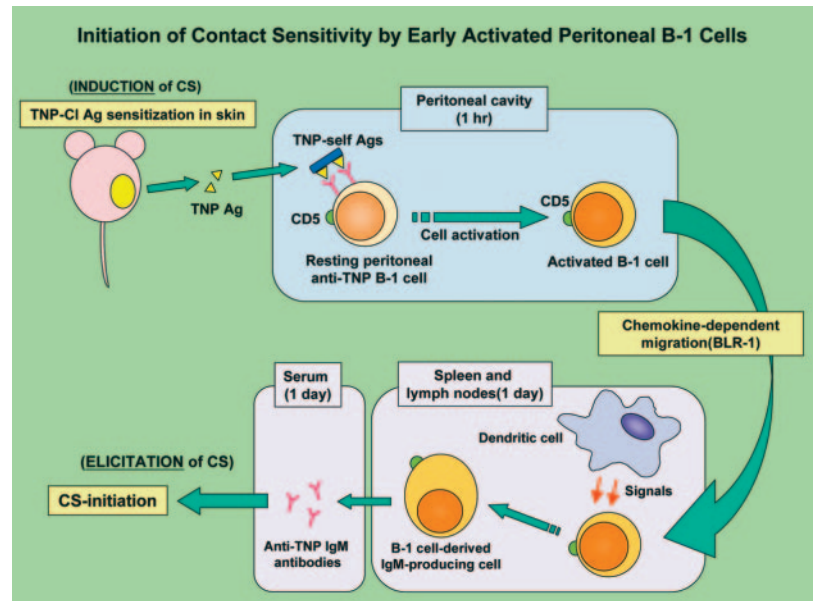


FIGURE 9. One-hour immune peritoneal cells from BLR-1^{-/-} mice do not reconstitute 2 h responses in B cell-deficient μ MT mice. Peritoneal cells were harvested from nonimmune C57BL/6 mice (group E), 1-h OX immune C57BL/6 mice (group F), or 1-h OX immune BLR-1^{-/-} mice (group G). Peritoneal cells then were transferred i.p. into 3-day OX immune μ MT mice. Each recipient received 10^6 peritoneal cells from C57BL/6 mice or 1.6×10^7 peritoneal cells from BLR-1^{-/-} mice to provide an equal number of B-1 cells. Mice were challenged with OX, and 2 h ear-swelling responses were measured 1 day later. Mice in groups A–D that did not receive cell transfer were challenged 4 days after immunization to serve as controls.

FIGURE 10. Hypothetical cascade of CS initiation by early-activated peritoneal B-1 cells. Cutaneous contact sensitization generates TNP-conjugated self protein and peptide Ag that stimulate TNP-specific B-1 cells in the peritoneal cavity within 1 h after skin sensitization. The rapidly activated B-1 cells migrate to the spleen and lymph nodes through chemokine-dependent mechanisms. The B-1 cells then produce TNP-specific IgM Abs within 1 day after skin sensitization in these lymphoid organs. The anti-TNP IgM Abs are present in the circulation by 1 day after immunization. After secondary Ag challenge of the skin, the IgM Abs mediate CS initiation to enable local T cell recruitment to lead to the effector T cell inflammatory response.



the spleen and lymph nodes along with production of the CS-initiating IgM Abs within 1 day (Fig. 10). Thus, in contrast to peritoneal cells, lymphoid cells in spleen and lymph nodes were not able to transfer 2 h responsiveness within 1 h after immunization, but became active by 1 day after immunization. In fact, ELISPOT assay showed no production of Abs by activated B-1 cells in the peritoneal cavity, but there were significantly increased numbers of anti-TNP IgM-producing cells in both spleen and lymph nodes by 1 day in immune CBA/J mice, and early ear-swelling activity was found in 1-day immune sera. These cells and serum IgM Abs are probably responsible for the specific IgM-mediated CS-initiating activity found at 1 day in these lymphoid tissues. The finding that *xid* mice did not mount early IgM responses to TNP-Cl skin immunization at 1 day also suggests that anti-TNP IgM-producing cells are derived from B-1 cells that are absent in *xid* mice.

Although naive CBA/J mice have significant numbers of anti-TNP IgM-producing cells in the spleen, neither naive lymphoid tissue cells nor normal serum have CS-initiating activity. Thus, natural anti-TNP IgM Abs in naive mice are not sufficient to initiate CS. Therefore, a small quantitative increase induced after immunization may be responsible for CS initiation. Another possibility is that anti-TNP IgM Abs produced after immunization are qualitatively different from those of naive mice. The induced anti-TNP IgM Abs might have higher affinity that can allow more efficient immune complex formation with Ag and local activation of complement.

Thus, from these data we concluded that peritoneal B-1 cells activated by primary skin immunization were unable to produce Abs in situ, but their migration to the lymphoid tissues is required to produce CS-initiating, hapten-specific IgM Abs. A similar finding was made in a recent study of B-1 cell-derived natural IgM Abs specific for the carbohydrate determinant Gal α 1,3Gal, which is important in hyperacute organ transplantation rejection (19). In this case, without intentional immunization, peritoneal B-1 cells also were shown to require migration to the spleen over an undetermined time to become specific IgM-producing cells (19). However, it is not clear that peritoneal B-1 cells have to migrate to lymphoid tissues for differentiation into Ab-producing cells. The B-1 cells may need signals from local lymphoid cells such as dendritic cells for further maturation or activation. In this regard, the dendritic cells could produce TNF family ligands, such as a proliferation-inducing ligand and B cell-activating factor, that re-

cently have been shown to enhance differentiation of B cells into plasma cells (20).

Role of chemokine receptors in migration of CS-initiating B-1 cells

To determine whether migration of peritoneal B-1 cells to lymphoid tissues in CS is dependent on chemokine receptors, we treated 1-h immune peritoneal B-1 cells with pertussis toxin, which is known to inhibit signaling through G protein-coupled chemokine receptors. We found that in vitro pretreatment with pertussis toxin inhibited the migration of 1-h activated peritoneal B-1 cells to the spleen, reducing local production of anti-TNP IgM Abs. Therefore, we concluded that skin contact sensitization rapidly activated peritoneal B-1 cells to migrate to lymphoid tissues via signals mediated through chemokine receptors. Our results suggest that BLR-1 is involved in the migration of B-1 cells in CS, because 1-h immune wild-type peritoneal cells mediated significantly greater CS initiation than those from BLR-1^{-/-} mice.

What signals are required for rapid activation of B-1 cells in the peritoneal cavity? It has been shown that Ag receptor stimulation is not sufficient to induce activation of B-1 cells, because CD5 that is expressed on B-1a cells, but not on B-2 cells, negatively regulates IgM receptor-mediated signaling in B-1 cells (21, 22). In addition, non-CD5-mediated, negative regulation of IgM receptor signaling has been reported (23).

We recently discovered that very rapid activation of V α 14J α 18 invariant NKT cells (iNKT cells) to produce IL-4 after skin immunization is required for dual activation of the CS-initiating B-1 cells along with the immunizing Ag (24). Surprisingly, iNKT cells produce IL-4 as early as 7 min after skin immunization (R. A. Campos, M. Szczepanik, A. Italcara, M. Lisbann, N. Dey, Maria C. L. de Moraes, and P. W. Askenase, unpublished observations). Therefore, rapid release of IL-4 from iNKT cells together with hapten Ag dispersed from the skin site of contact immunization (25) may lead to required dual activation of peritoneal B-1 cells within 1 h after skin sensitization. In addition to the multiple activities of IL-4 on B cells, such as inhibition of apoptosis and enhancement of proliferation, and particularly IgE Ab production, IL-4 also was shown to promote migration of B cells to the spleen (26). Therefore, early iNKT cell-derived IL-4 may be involved not only in the activation of CS-initiating B-1 cells, but also in their

consequent migration to lymphoid tissues to then produce the CS-initiating Ag-specific IgM Abs.

Summary

We have demonstrated that peritoneal B-1 cells are activated as early as 1 h after skin immunization to eventually lead to the initiation of T cell recruitment during elicitation of CS responses. Furthermore, we showed that the 1-h immune peritoneal B-1 cells migrate to lymphoid tissues probably via chemokines to produce the Ag-specific IgM Abs that are required for the initiation process that is needed to recruit T cells in the elicitation phase of CS (Fig. 10). These findings provide new insights into the CS-initiating cascade process that leads to *in vivo* T cell recruitment in CS. A similar initiating process has been identified in delayed-type hypersensitivity responses after immunization with soluble protein Ag (27). These findings lead us to propose that this novel role of peritoneal B-1 cells in T cell-mediated immunity in CS may also underlie the recruitment of effector T cells in other disease models. This may include collagen-induced arthritis (28–30) and experimental autoimmune encephalomyelitis (31–33) that are known to involve Abs, complement, and mast cells. Although B-1 cell-derived IgM Abs are preferentially stimulated early after immunization, evolution of the Ab-mediated initiation process later involves B-2 cell-derived Ab isotypes (4, 34). Thus, for T cell recruitment at later times after immunization, mast cells may be stimulated to recruit T cells indirectly via B-2 cell-derived complement-activating isotypes (i.e., IgG2a and IgG2b). In addition, other B-2 cell-derived Ab isotypes capable of directly activating mast cells via their FcRs (i.e., IgG1 and IgE) may mediate analogous initiating events (34) that guide T cell recruitment in more chronic cellular immune inflammation.

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Disclosures

The authors have no financial conflict of interest.

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