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# Invariant NKT Cells Rapidly Activated via Immunization with Diverse Contact Antigens Collaborate In Vitro with B-1 Cells to Initiate Contact Sensitivity<sup>1</sup>

Regis A. Campos,\* Marian Szczepanik,<sup>†</sup> Mariette Lisbonne,<sup>§</sup> Atsuko Itakura,<sup>‡</sup> Maria Leite-de-Moraes,<sup>§</sup> and Philip W. Askenase<sup>2‡</sup>

**In cutaneous contact sensitivity there is an early elicited innate cascade of complement, mast cells, and platelets activated via IgM Abs. This response is required to initiate the elicitation of acquired classical contact sensitivity by leading to local recruitment of effector T cells. We recently performed in vivo experiments showing that collaboration is required between innate-like invariant V $\alpha$ 14<sup>+</sup> NKT cells (iNKT) and the innate-like B-1 B cell subset to induce this initiation process. Contact sensitization triggers iNKT cells to produce IL-4 to coactivate the B-1 cells along with specific Ag for production of the initiating IgM Abs. We now describe in vitro collaboration of iNKT and B-1 cells. Normal peritoneal B-1 cells, incubated in vitro with soluble Ag, and with 1-h in vivo immune iNKT cells producing IL-4, are activated to mediate the contact sensitivity-initiation cascade. The three components of this process can be activated by different Ag. Thus, 1-h iNKT cell activation, B-1 cell stimulation, and generation of immune effector T cells can be induced by sensitization with three different Ag to respectively generate IL-4 and Ag-specific IgM Abs, to recruit the Ag-specific effector T cells. These findings have relevance to allergic and autoimmune diseases in which infections can trigger exacerbation of T cell responses to allergens or to autoantigens. *The Journal of Immunology*, 2006, 177: 3686–3694.**

**W**e recently described a cascade process consisting of innate immune components needed to recruit effector T cells for the elicitation of acquired immune contact and delayed-type hypersensitivity (1–3). In immunized mice, skin challenge with a low dose of the specific Ag elicits an immediate hypersensitivity-like process that is required for local recruitment of sensitized effector T cells to elicit the classical delayed responses. We have termed this process initiation of T cell recruitment (3). Initiation leads to activation of endothelium to enable recruitment of sensitized effector T cells into the local site within just 2 h (1–7). Recruited T cells with TCR- $\alpha\beta$  specific for Ag/MHC complexes on APC are activated to produce proinflammatory cytokines such as IFN- $\gamma$  (1, 6, 8). Locally generated IFN- $\gamma$  leads to recruitment of circulating leukocytes by inducing tissue cells to produce CXCR3 chemokines ligands, such as CXCL10 (IFN- $\gamma$ -inducible protein-10) (9). The chemokines locally recruit

the leukocytes to mediate local inflammation with classical 24- to 48-h tissue-swelling responses (9).

The initiation process begins within 30 min of secondary Ag challenge (6, 10) and is due to IgM Abs induced early after immunization (1). The challenge Ag forms local complexes with the IgM Abs (1, 2) that activate complement by the classical pathway to locally generate C5a (2, 6, 8, 11). C5a activates local mast cells and platelets (12–15) via their C5a receptors (6) to release vasoactive serotonin (16, 17) and TNF- $\alpha$  (6, 15). These mediators increase vascular permeability and induce expression of adhesion molecules, such as ICAM-1 and VCAM-1 on the luminal surface of local endothelium (18), to aid in recruitment of T cells into the tissues during a 2-h window that follows local Ag challenge (7).

In contact sensitivity (CS)<sup>3</sup> the B-1 B cells are activated within just 1 h (19) and migrate to the spleen via chemokines, to produce Ag-specific initiating IgM Abs that become present in the serum by just 1 day (1, 2, 19). Thus, CS or delayed-type hypersensitivity immunization in B cell- or B-1 cell-deficient mice, although inducing the effector T cells, fails because of absent IgM Ab to mediate initiation of T cell recruitment (1, 2). Adoptive cell transfer of 1-day immune FACS-sorted B-1 cells (1, 2) or 1-day immune serum IgM Abs from wild-type mice (2, 19), or specific IgM mAbs (20), reconstitute CS in B cell-deficient mice.

We recently began to characterize how the initiating B-1 cells are activated rapidly within just 1 h postimmunization. In CS induced by the reactive hapten Ag trinitrophenyl chloride, picryl chloride (TNP-Cl), or oxazolone (OX), we found that hepatic V $\alpha$ 14<sup>+</sup>J $\alpha$ 18<sup>+</sup> invariant NKT (iNKT) cells are activated within minutes following skin immunization (21). The iNKT cells release IL-4 needed to activate the B-1 cells via IL-4R and STAT-6 signaling (22). The B-1 cells are coactivated by TNP self protein Ag, most likely rapidly disseminated systemically from the skin site of

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<sup>3</sup> Abbreviations used in this paper: CS, contact sensitivity;  $\alpha$ -GalCer,  $\alpha$ -galactosylceramide; iNKT, invariant NKT; LMNC, liver mononuclear cell; OX, oxazolone; TNP, trinitrophenol; TNP-Cl, TNP chloride, picryl chloride.

immunization (23), to cross-link B-1 cell-specific surface IgM receptors.

In the current study, we developed an *in vitro* system to further evaluate collaboration between these innate immune cells in CS. We stimulate the initiating activity of normal peritoneal B-1 cells via a mixture of soluble specific Ag and *in vivo* immune activated iNKT cells. Activated iNKT cells are harvested from contact-sensitized mice just 1 h postimmunization as a potential source of IL-4. The *in vivo* activation of the iNKT cells to collaborate *in vitro* with B-1 B cells depends on cutaneous immunization, but not necessarily with the specific Ag. Immunization with noncross-reacting hapten also activated iNKT cells within 1 h. The heterologous Ag stimulation of collaboration between iNKT cells and B-1 cells led to recruitment of effector T cells sensitized to yet a third Ag. In contrast, nonspecific inflammation of the skin by contact painting with nonimmunogenic croton oil or phorbol ester failed to do so.

These studies show that CS-initiating B-1 B cells can be activated *in vitro* by specific soluble hapten-protein Ag and IL-4 produced by iNKT cells from hosts that are skin sensitized with either homologous or heterologous hapten Ag only 1 h previously. The findings suggest that immunization releases an endogenous glycolipid-like effect that is not due to nonspecific inflammation. This effect, together with Ag, results in rapid iNKT cell production of IL-4 needed to activate initiating B-1 cell function for elicitation of CS. The ability to *in vitro* activate the initiating activity of B-1 cells via collaborating immune iNKT cells may lead to further characterization of the function of these innate-like B-1 and iNKT cell subsets, and of their interactions that are required for recruitment of T cells in acquired cellular immune responses *in vivo*.

## Materials and Methods

### Mice

Specific pathogen-free male CBA/J, CBA/N-*xid*, and BALB/c mice of both sexes were from The Jackson Laboratory or the National Cancer Institute, National Institutes of Health. Breeders of BALB/c  $J\alpha 18^{-/-}$  iNKT cell-deficient mice were from M. Taniguchi (Chiba University, Chiba, Japan);  $V\alpha 14$  transgenic H-2<sup>d</sup> mice were from A. Bendelac (University of Chicago, Chicago, IL); and JH<sup>-/-</sup> pan B cell-deficient mice (H-2<sup>d</sup>) were from M. Shlomchik (Yale School of Medicine, New Haven, CT). Mice at four per group of 6–12 wk were rested at least 1 wk under specific pathogen-free conditions before use. Experiments were according to guidelines of the Animal Care and Use Committee at Yale University School of Medicine.

### Reagents

TNP-Cl (Nacalai Tesque) was recrystallized twice and stored protected from light. OX, croton oil, and PMA were from Sigma-Aldrich. TNP-BSA was obtained from Bioscience Technologies; anti-IL-4 (11B11) was obtained from the National Cancer Institute; and the isotype control was obtained from Sigma-Aldrich. FITC and dibutyl phthalate were obtained from Sigma-Aldrich. Murine rIL-4 was obtained from BD Pharmingen.

### Immunization and elicitation of CS

Mice were contact sensitized with 150  $\mu$ l of 5% TNP-Cl for active sensitization of normal mice or 0.2% for B cell-deficient recipients, or 3% OX in absolute ethanol and acetone (4:1), or 0.5% FITC in dibutyl phthalate. Responses were elicited on day 4 by painting ears with 10  $\mu$ l of 0.4% TNP-Cl or OX in acetone and olive oil (1:1), or 0.4% mixed TNP-Cl and OX. Ear thickness was measured with a micrometer (Mitutoyo) before challenge and then at 2 and 24 h by an observer unaware of experimental groups. Increases in ear thickness were expressed as the mean  $\pm$  SE  $\times 10^{-2}$  mm.

For sham sensitization, mice were shaved and painted with vehicle alone. For control sensitization, croton oil was diluted to 4% in ethanol:acetone (4:1) and 150  $\mu$ l was applied to the shaved skin and paws, as above. PMA was dissolved in DMSO and kept frozen at  $-20^{\circ}\text{C}$  until used. Thawed PMA was diluted in acetone ethanol (4:1) to 50  $\mu$ g per 150  $\mu$ l and similarly applied on skin.

As a positive control in some experiments, mice were injected *i.v.* with 2  $\mu$ g  $\alpha$ -Gal-Cer and then sacrificed at different times.

### Liver cell preparation to obtain enriched liver mononuclear cells (LMNC)

After sacrifice, liver was perfused with PBS via the portal vein until opaque, then strained (70  $\mu$ m; BD Biosciences), resuspended to 40% isotonic Percoll (Amersham Biosciences), and overlaid onto 60% isotonic Percoll. After centrifugation for 20 min at  $900 \times g$  at  $25^{\circ}\text{C}$ , the LMNC were isolated at the interface and 40% Percoll, combined, and washed with RPMI 1640 (Invitrogen Life Technologies) + 5% FBS (Gemini Bio-Products). Viability was  $>90\%$ .

### *In vitro* activation of naive B-1 cells mixed with LMNC from mice immunized *in vivo*

Peritoneal cells were obtained by lavage and used as a source of B-1 cells (15–20%). Simultaneously harvested syngeneic LMNC (35% iNKT cells) (2) were from mice contact sensitized 1 h previously with TNP-Cl or OX or FITC. These cell populations were incubated together with TNP-BSA (50  $\mu$ g/ml) for 60 min at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . Then after washing with sterile pyrogen-free  $3 \times$  PBS, the cell mixtures were transferred *i.p.* to syngeneic nonimmune recipients, or into B cell-deficient (JH<sup>-/-</sup>) or B-1 cell-deficient (CBA/N-*xid*) recipients, contact sensitized 3 days previously with 0.2% TNP-Cl. Recipients were challenged 24 h later on the ears with 0.4% TNP-Cl, and 2- and 24-h ear-swelling responses were determined.

In some experiments, LMNC from 1-h immune  $J\alpha 18^{-/-}$  or IL-4<sup>-/-</sup> or IFN- $\gamma^{-/-}$  BALB/c were used. We also used LMNC from nonimmune or 1-h TNP-Cl-immune  $V\alpha 14$  transgenic BALB/c mice. Elevated iNKT 85% of T cells (21) were further enriched by negative selection by magnetic bead depletion of non-T cells using a Pan T Cell Isolation Kit (MACS; Miltenyi Biotec) (21) to reach 98% purity.

In all experiments, peritoneal cells or FACS-sorted B-1 cells (of one donor per eventual recipient), and either total LMNC, or negatively selected enriched liver iNKT cells (also at one donor per eventual recipient) were incubated together *in vitro* with TNP-BSA, washed, and transferred. On average,  $1\text{--}2 \times 10^6$  peritoneal or 85% of LMNC T cells were obtained from each donor.

### Sorting of immune lymphoid B-1 cells

Spleen and lymph node cells from 1-day TNP-Cl-sensitized donors were stained at  $10^6\text{--}10^7/\text{ml}$  with anti-CD5 CyChrome, and anti-CD19 FITC, or anti-B220 PE, or anti-Mac-1 PE at 0.025  $\mu$ g per  $10^6$  cells, for 30 min on ice, and then washed with RPMI 1640. The stained cells were sorted with the FACS Vantage SE (BD Biosciences) to obtain  $\pm 98\%$  enriched CD19<sup>+</sup>CD5<sup>+</sup>Mac-1<sup>+</sup>B-1a cells, and CD19<sup>+</sup>CD5<sup>-</sup>Mac-1<sup>+</sup>B-1b cells at a ratio of B-1a cells to B-1b cells of 1:1.6. Then,  $6.5 \times 10^4$  of each subset was injected *i.v.* per mouse.

### Flow cytometry and binding of specific tetramers to iNKT cell TCR

PE- or allophycocyanin-labeled tetrameric mouse CD1d- $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) complexes that bind  $V\alpha 14i$  NKT cell TCR- $\alpha\beta$ , and unloaded control rCD1d  $\beta_2$ -microglobulin complexes without  $\alpha$ -GalCer as control were prepared (24). LMNC were resuspended in PBS staining buffer containing 2% BSA and 0.02%  $\text{NaN}_3$ , and then incubated for 15 min at  $4^{\circ}\text{C}$  with blocking 2.4G2 anti-Fc $\gamma$  mAb (BD Pharmingen) and blocking neutravidin (Molecular Probes). After washing, LMNC were stained for 30 min with PE- or allophycocyanin-labeled CD1d- $\alpha$ -GalCer tetramers, washed, incubated with FITC anti-TCR- $\beta$  and PerCP-Cy5.5 anti-CD4 mAb (BD Pharmingen) at  $25^{\circ}\text{C}$  for 20 min, and washed twice again. For intracellular staining, cells were then fixed with paraformaldehyde, permeabilized with saponin, and incubated with anti-IL-4 PE (clone 11B11) or anti-IFN- $\gamma$  PE (clone XMGI.2) or isotype controls (BD Pharmingen). Dead cells were excluded on the basis of forward and side scatter. Double tetramer and TCR- $\beta$ -positive cells were identified using a FACSCalibur (BD Biosciences). A minimum of  $5 \times 10^4$  events was acquired, and the results were analyzed using Mac CellQuest (BD Biosciences).

### Statistics

Statistics were performed using the paired two-tailed Student *t* test, and  $p < 0.05$  was taken as the level of significance.



## Results

### *Injected TNP-BSA induces 2-h ear-swelling responses to contact ear skin challenge with TNP-CI*

Cutaneous immunization for CS with the reactive hapten TNP-CI results in ability to elicit 2-h ear swelling to TNP-CI challenge only 1 day later (1, 4, 5). Because the 2-h CS-initiating response is strictly hapten specific, we hypothesized that immunization with the hapten conjugated to a heterologous carrier protein and injected in aqueous solution could induce similar responses. Indeed, intradermal immunization of naive CBA/J mice, with 100  $\mu\text{g}$  of TNP-conjugated BSA in PBS, induced significant 2-h ear-swelling responsiveness to TNP-CI challenge 1 day later (data not shown). Responses were similar to those of TNP-CI contact skin-immunized mice, compared with similarly ear-challenged nonimmune mice. We concluded that injection of mice with soluble TNP-protein Ag by various routes sufficed to immunize for rapid generation of the 2-h early component of CS at 1 day.

### *In vitro activation of naive peritoneal cells with soluble Ag and IL-4 induces the ability to transfer 2-h ear-swelling responsiveness*

We tested whether in vitro incubation of naive peritoneal cells enriched in B-1 cells (25) with aqueous soluble hapten-protein conjugate could similarly activate the B-1 cells. Previous in vivo results suggested that coactivation of the B-1 cells with specific Ag together with IL-4 was required to induce the 2-h activity (21, 22). Thus, we added murine rIL-4 to soluble TNP-BSA Ag. Naive peritoneal cells incubated at 37°C for 1 h with TNP-BSA with IL-4 (20 mg/ml) were washed and transferred i.p. to naive recipients. Mice were challenged with TNP-CI 1 day after transfer to allow for in vivo production and systemic circulation of IgM Abs in recipients (1, 20). Data presented in Fig. 1A show that peritoneal cells exposed to TNP-Ag + IL-4 in vitro transfer the early component of CS (group B), compared with similarly TNP-CI ear-challenged control mice that received no transfers (group A).

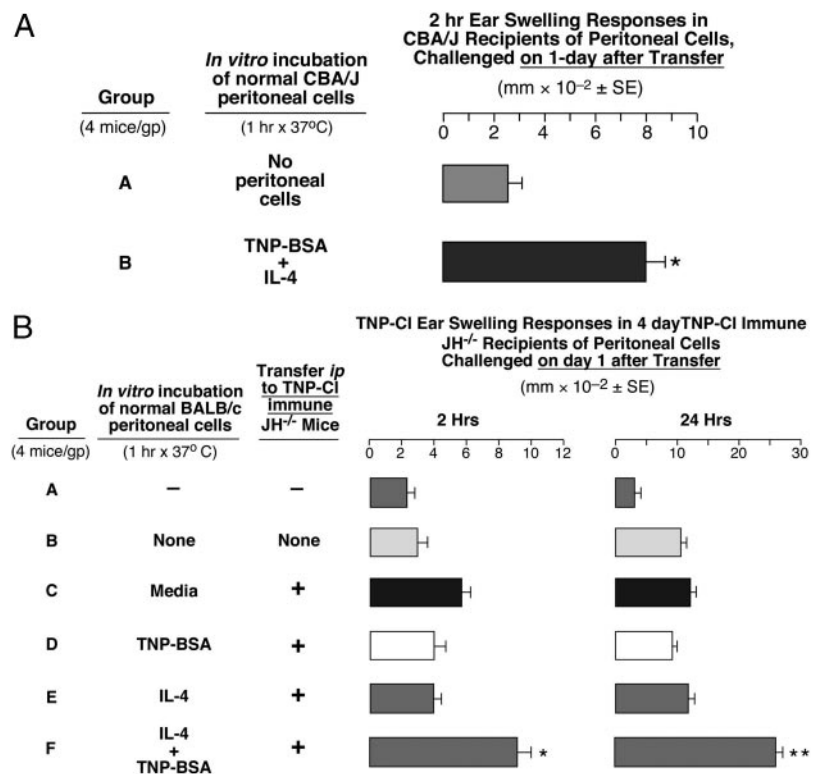
### *Normal BALB/c peritoneal cells activated in vitro with TNP-BSA and IL-4 transfer CS initiation that recruits T cells*

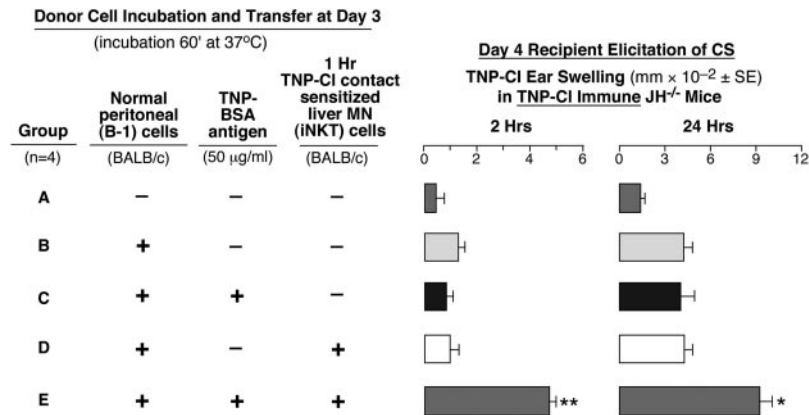
The results above suggested that incubation of presumed normal B-1 cells among the peritoneal cells with the soluble hapten-protein TNP-BSA, together with IL-4, was sufficient to activate the B-1 cells to mediate the early 2-h component of CS responses. We tested whether this activated peritoneal B-1 cells for their CS-initiating function of recruiting CS effector T cells to mediate the late 24-h component of CS. Thus, we incubated normal BALB/c H-2<sup>d</sup> peritoneal cells with TNP-BSA, together with IL-4, for 1 h at 37°C. After washing, we transferred these cells i.p. to JH<sup>-/-</sup> H-2<sup>d</sup> pan B cell-deficient mice contact immunized 3 days previously with TNP-CI. These mice have defective CS because of the requirement for immunized B-1 cells to produce IgM Abs to initiate recruitment of CS effector T cells (1). Accordingly, Fig. 1B shows that TNP-CI-sensitized JH<sup>-/-</sup> mice fail to elicit the early 2-h and late 24-h CS response (group B), compared with challenged nonimmune controls (group A). In contrast, in vitro incubation of normal BALB/c peritoneal cells with TNP-BSA plus IL-4 activated the cells to enable them to transfer 2-h CS (group F, left). Because these JH<sup>-/-</sup> recipients were previously TNP-CI immunized, they had generated effector T cells (1). Thus, the early 2-h CS component mediated by the in vitro-activated peritoneal cells reconstituted the ability to elicit the classical 24-h late component CS responses (group F, right). As controls, transferred peritoneal cells similarly incubated just with medium (group C), or just with TNP-BSA (group D), or with IL-4 without Ag (group E) did not allow 24-h CS to develop in the immunized B cell-deficient mice. We concluded that incubation in vitro with a combination of the TNP-BSA plus IL-4 induced 2-h initiating activity in presumed peritoneal B-1 cells, leading to in vivo recruitment of CS effector T cells to reconstitute 24-h CS.

### *iNKT from 1-h contact-sensitized donors provide IL-4 to activate CS-initiating peritoneal B-1 cells in vitro*

In contact-sensitized mice, activation of the B-1 cells for CS-initiating activity is provided by liver iNKT cells stimulated within

**FIGURE 1.** In vitro activation of naive peritoneal cells with TNP-BSA and IL-4 induces ability to transfer 2-h ear-swelling responses to TNP-CI challenge, which initiate the recruitment of effector T cell in CS. **A**, Peritoneal cells were harvested from naive wild-type CBA/J mice and then incubated in vitro for 1-h at 37°C with a mixture of TNP-BSA (50  $\mu\text{g}/\text{ml}$ ) and IL-4 (20 ng/ml). After harvest and washing, the cells were transferred i.p. to naive CBA/J mice that were challenged 1-day later on the ears with 0.4% TNP-CI (group B). Ear-swelling responses at 2 h were compared with those of negative controls receiving TNP-BSA and IL-4 without peritoneal cells (group A) after subtraction of background responses in mice just ear challenged with TNP-CI, but not transferred (group not shown). \*,  $p < 0.01$ , group B vs A. **B**, Peritoneal cells from naive BALB/c H-2<sup>d</sup> donors were incubated in vitro with TNP-BSA plus IL-4 (group F), vs TNP-BSA alone (group D), vs IL-4 alone (group E) for 60 min at 37°C. Then cells were harvested, washed, and transferred i.p. to JH<sup>-/-</sup> H-2<sup>d</sup> mice that 3 days previously had been contact sensitized with TNP-CI. Then 1 day following transfers, recipients were challenged and 2- and 24-h swelling responses were measured and compared with those of similar TNP-CI-immunized JH<sup>-/-</sup> mice that received no transfers (group B), or received peritoneal cells incubated in medium alone (group C), or negative control nonimmune JH<sup>-/-</sup> mice just ear challenged (group A). \*,  $p < 0.01$ , group F vs groups A–E. \*\*,  $p < 0.001$ , group F vs A–E.





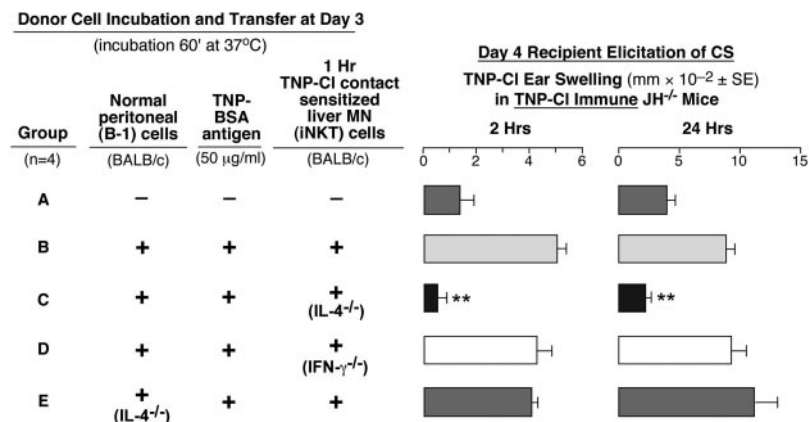
**FIGURE 2.** LMNC activated 1 h previously by cutaneous CS-activated LMNC induce CS-initiating function of peritoneal (B-1 cells) in vitro. Normal BALB/c H-2<sup>d</sup> peritoneal cells were incubated in vitro with TNP-BSA and with LMNC from BALB/c donors contact sensitized 1 h previously with TNP-Cl (group E). Cells were transferred i.p. to JH<sup>-/-</sup> H-2<sup>d</sup> recipients that were immunized 3 days previously with 0.2% TNP-Cl. The day after transfers, recipients were challenged on the ears with TNP-Cl, and 2- and 24-h swelling was measured. These responses were compared with those transferred by peritoneal cells incubated with LMNC without TNP-BSA alone (group D), or with TNP-BSA alone (group C), or just with medium (group B). All recipient groups were compared with TNP-Cl-immunized JH<sup>-/-</sup> mice that received no transfers and were ear challenged similarly (group A). \*\*, *p* < 0.001, group E vs groups A–D. \*, *p* < 0.02, group E vs groups A–D.

1 h postimmunization (21, 22). We tested whether LMNC rich in iNKT cells harvested from mice contact sensitized just 1 h previously could substitute for added IL-4 to coactivate B-1 cells in vitro to initiate CS. Thus, we incubated normal BALB/c peritoneal cells, together with Ag, plus added LMNC from 1-h TNP-Cl contact-sensitized donors. After washing and i.p. transfer, this cell mixture led to CS-initiating activity that mediated the early 2-h component of CS, and as a consequence reconstituted late 24-h CS in TNP-Cl-immunized JH<sup>-/-</sup> recipients (Fig. 2, group E vs groups B–D).

To further test whether added LMNC from 1-h TNP-Cl contact-sensitized donor mice could substitute in vitro for IL-4 required to coactivate CS-initiating B-1 cells with TNP-BSA, we used LMNC from IL-4<sup>-/-</sup> mice contact sensitized 1 h previously. As before, wild-type BALB/c-derived 1-h TNP-Cl contact-sensitized LMNC added with TNP-BSA to normal peritoneal cells stimulated CS-initiating activity (Fig. 3, group B), compared with immunized and challenged JH<sup>-/-</sup> mice that did not receive transfers (group A).

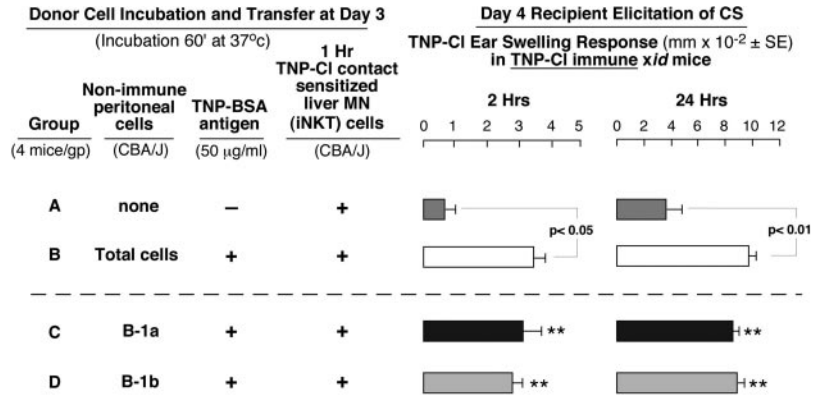
Importantly, 1-h immune LMNC from IL-4<sup>-/-</sup> mice were inactive (group C), while immune LMNC from IFN-γ<sup>-/-</sup> donors reconstituted CS responses (group D) similar to wild-type 1-h immune LMNC donors (group B). Furthermore, when peritoneal cells from IL-4<sup>-/-</sup> mice were incubated with wild-type 1-h immune LMNC, there also were positive results, ruling out peritoneal cells as a source of IL-4 (Fig. 3, group E).

To determine whether IL-4 derived from iNKT cells in the LMNC mixture was responsible for inducing the CS-initiating activity in vitro, we compared peritoneal cells coincubated with Ag and 1-h immune LMNC from Jα18<sup>-/-</sup> BALB/c background donors deficient in iNKT cells. Again, only the incubation and transfer of the three components (nonimmune peritoneal cells, TNP-BSA Ag, and 1-h TNP-Cl-immune LMNC) reconstituted CS in TNP-Cl-immunized JH<sup>-/-</sup> recipients (data not shown). However, when 1-h TNP-Cl-immunized LMNC from Jα18<sup>-/-</sup> donors were similarly used with Ag-stimulated peritoneal cells, no CS-initiating activity was generated (data not shown). Also, in vitro anti-IL-4



**FIGURE 3.** LMNC from IL-4<sup>-/-</sup>-immunized donors fail to activate CS-initiating peritoneal B-1 cells in vitro. Peritoneal cells from BALB/c H-2<sup>d</sup> mice were incubated in vitro with TNP-BSA, together with either 1-h TNP-Cl-immune LMNC from wild-type mice (group B), or from IL-4<sup>-/-</sup> mice (group C), or IFN-γ<sup>-/-</sup> mice (group D). In another group, peritoneal cells from IL-4<sup>-/-</sup> mice were incubated with 1-h immune wild-type LMNC and TNP-BSA (group E). Each cell mixture was transferred i.p. to separate groups of 3-day TNP-Cl-immune JH<sup>-/-</sup> H-2<sup>d</sup> recipients. Then, 1 day later, recipients were ear tested with TNP-Cl, and 2- and 24-h swelling responses were measured, and compared with immunized and challenged JH<sup>-/-</sup> mice not transferred (group A). Statistics: at 2 h, *p* < 0.01, group C vs group B; *p* < 0.02, group C vs groups D and E. At 24-h, *p* < 0.001, group C vs group B; *p* < 0.01, group C vs groups D and E.

**FIGURE 4.** FACS-sorted B-1a and B-1b cells are activated in vitro by 1-h immune liver iNKT cells to initiate CS. Total peritoneal cells (group B), or FACS-sorted B-1a cells (group C, CD19<sup>+</sup>CD5<sup>+</sup>Mac-1<sup>+</sup>), or B-1 B cells (group D, CD19<sup>+</sup>, CD5<sup>-</sup>, Mac-1<sup>+</sup>), from nonimmune CBA (H-2<sup>k</sup>) mice were incubated in vitro with TNP-BSA together with LMNC from syngeneic mice that were contact sensitized with TNP-CI 1 h previously. Cells were transferred i.p. ( $6.5 \times 10^4$  per recipient) to 3-day TNP-CI-immune *xid* H-2<sup>k</sup> mice that were ear tested 1 day later with TNP-CI, and 2- and 24-h CS ear swelling was determined. Responses were compared with similarly challenged immune *xid* mice receiving LMNC alone (group A). \*\*,  $p < 0.02$ , groups C and D vs group A.



mAb treatment of 1-h TNP-CI-immune LMNC from wild-type donors inhibited the generation of CS-initiating activity (data not shown), compared with isotype-treated controls. We concluded that IL-4 produced by V $\alpha$ 14<sup>+</sup>J $\alpha$ 18<sup>+</sup> iNKT cells in the 1-h immunized LMNC most likely was responsible for activating the CS-initiating activity of peritoneal B-1 cells in vitro.

*Sorted peritoneal B-1a and B-1b cells are activated in vitro by 1-h immunized liver iNKT cells to initiate CS*

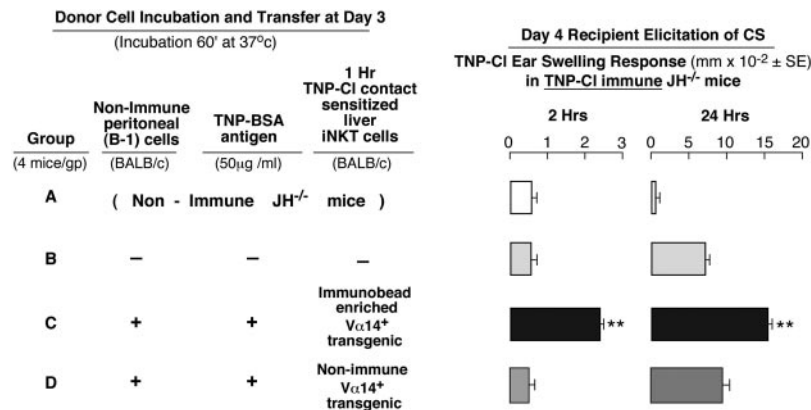
The preceding experiments used normal mixed peritoneal cells in vitro. We determined whether B-1 cells among these mixed peritoneal cells were responsible, by positively selecting B-1 cells in peritoneal cells via FACS sorting, before in vitro treatment. Furthermore, we tested whether the two putative subsets of B-1 cells, i.e., B-1a cells (CD5<sup>+</sup>B220<sup>+</sup>Mac1<sup>+</sup>) and B-1b cells (CD5<sup>-</sup>B220<sup>+</sup>Mac1<sup>+</sup>), were involved. Again, mixed peritoneal cells were activated for CS-initiating activity by the mixture of Ag and 1-h immune LMNC (Fig. 4, group B). When 1-h TNP-CI contact-sensitized LMNC were transferred alone, no CS-initiating activity was obtained (group A). In contrast, sorted B-1a cells (group C) and sorted B-1b cells (group D) were both suitable targets among the peritoneal cells for activation of CS-initiating activity by TNP-BSA Ag plus 1-h TNP-CI-immunized iNKT cells.

*Negative selected 1-h activated V $\alpha$ 14<sup>+</sup> TCR transgenic iNKT cells stimulate peritoneal B-1 cells in vitro to initiate CS*

To confirm that iNKT cells in LMNC produced IL-4 required to activate B-1 cells in vitro, we used enriched V $\alpha$ 14 J $\alpha$ 18 iNKT cells. These were from the livers of V $\alpha$ 14 TCR transgenic mice on a BALB/c background that normally has 65% iNKT cells among TCR- $\beta$ <sup>+</sup> LMNC, compared with 35% in wild type, and following TNP-CI sensitization rapidly rises to 85% of LMNC (21). Thus, the iNKT cells in LMNC of 1-h immunized V $\alpha$ 14 TCR transgenic mice were enriched to 98% by negative selection. Enriched iNKT cells from V $\alpha$ 14 transgenic mice also mediated in vitro stimulation of CS-initiating activity in B-1 cells (Fig. 5, group C), while non-immune LMNC from the V $\alpha$ 14 TCR transgenic mice were inactive (group D). We concluded that the V $\alpha$ 14 iNKT cells among 1-h TNP-CI-immune LMNC were responsible for stimulating CS-initiating activity of Ag-stimulated peritoneal B-1 cells in vitro.

*Heterologous Ag contact sensitization activates liver iNKT cells to stimulate CS-initiating B-1 cells in vitro*

The above experiments exclusively used 1-h TNP-CI-immune LMNC. It was possible that activation of the iNKT cells to collaborate with B-1 cells in vitro might be restricted to this immunizing hapten. Therefore, we tested whether sensitization with OX,



**FIGURE 5.** Enriched 1-h TNP-CI-activated V $\alpha$ 14 TCR transgenic iNKT cells stimulate peritoneal B-1 cells in vitro to initiate CS. Peritoneal cells from nonimmune BALB/c H-2<sup>d</sup> mice were incubated in vitro with TNP-BSA together with 1-h TNP-CI-immune LMNC from V $\alpha$ 14 TCR transgenic H-2<sup>d</sup> mice (iNKT cells 65% of T cells) (group D). In group C, 1-h TNP-CI-immune transgenic iNKT cells (iNKT cells 85% of T cells) were enriched further to 98% iNKT cells. In group D, normal peritoneal cells and TNP-BSA were incubated in vitro with LMNC from nonimmune V $\alpha$ 14 TCR transgenic mice as a control. Following incubation and washing, the mixed cells of each group were transferred into 3-day TNP-CI-immune H-2<sup>d</sup> JH<sup>-/-</sup> mice. Recipients were ear tested with TNP-CI 24 h later, and 2- and 24-h components of CS were determined. Responses in recipients were compared with 4-day TNP-CI-immune JH<sup>-/-</sup> mice that received no transfers (group B), and with nonimmune JH<sup>-/-</sup> mice TNP-CI challenged only (group A). \*\*,  $p < 0.001$ , group C vs groups A and B.

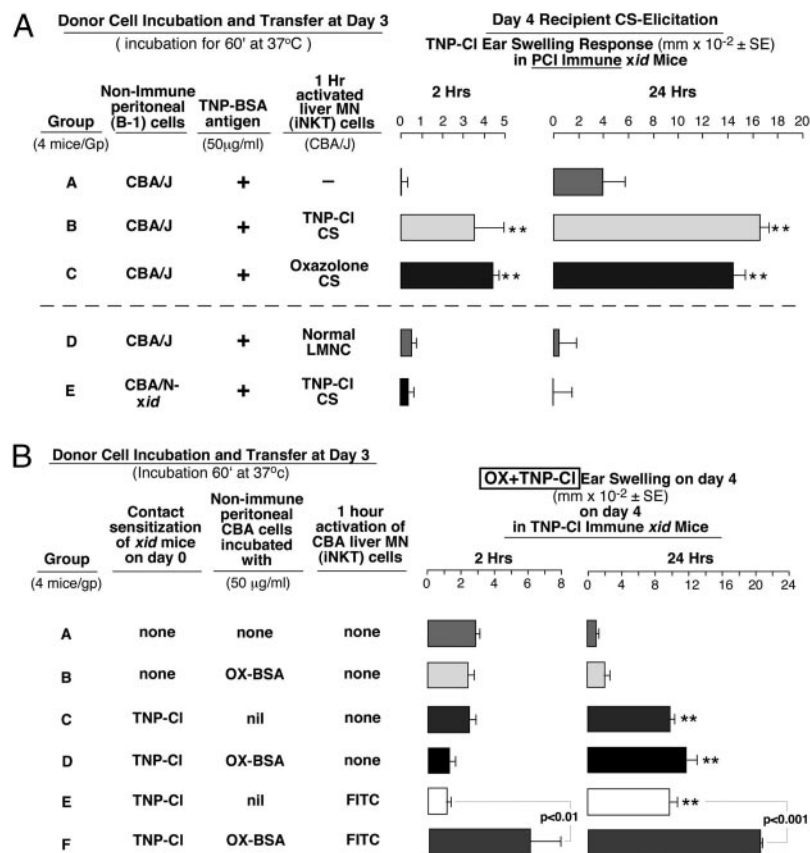


another well-known and noncross-reactive cutaneous sensitizer, could provide similar results. CS induced by OX, similar to CS induced by TNP-CI, has an early 2-h initiation phase required for the elicitation of the 24-h effector phase, and both phases are defective in  $J\alpha 18^{-/-}$  iNKT cell-deficient mice (data not shown).

Thus, we used wild-type CBA/J (H-2<sup>k</sup>) mice immunized with OX as a different contact sensitizer for donors of 1-h immune LMNC. We tested for reconstitution of defective CS in CBA/N-*xid* recipient mice (H-2<sup>k</sup>) previously immunized with the heterologous Ag TNP-CI. These mice are predominately deficient in B-1 cells, and thus fail to elicit CS (1). As before, the 1-h TNP-CI-immunized LMNC stimulated CS-initiating activity (Fig. 6A, group B), compared with control peritoneal cells just exposed to TNP-BSA Ag (group A). In addition, contact sensitization 1 h previously with the noncross-reactive contact sensitizer OX also activated LMNC to collaborate with TNP-BSA Ag-stimulated B-1 cells (group C), compared with controls of normal LMNC from nonimmunized donors (group D). To further establish that the B-1 cells in the peritoneal cell mixture were the target of iNKT cell activation in vitro, we used peritoneal cells of B-1 cell-deficient *xid* mice incu-

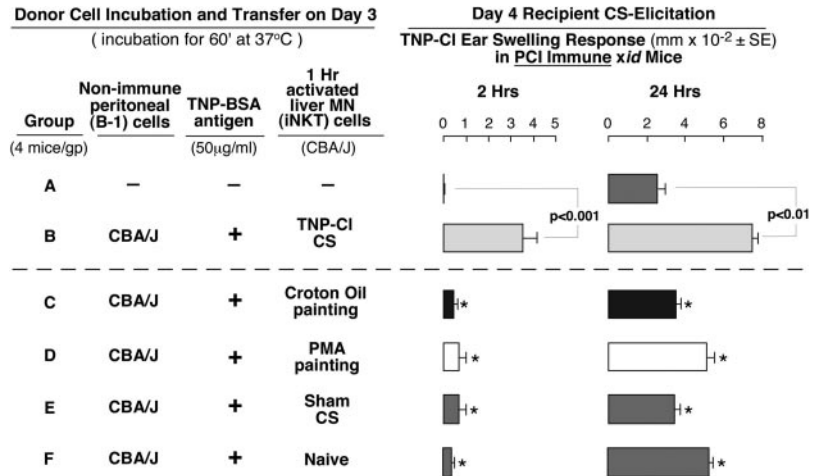
bated with TNP-BSA Ag and LMNC from 1-h TNP-CI-immune mice. The *xid* B-1 cell-deficient peritoneal cells were inactive following incubation with the 1-h TNP-CI-immune LMNC (group E).

These experiments show that sensitization with a different contact-sensitizing hapten also rapidly stimulates the iNKT cells in the 1-h immune LMNC to coactivate the B-1 cells incubated with the heterologous Ag TNP-CI. It thus appeared that contact immunization per se led to rapid activation of the iNKT cells, because sensitization with two Ag gave similar results. We tested this point further in an experiment involving three different contact-sensitizing Ag: TNP-CI, OX, and FITC. We found that immunization with FITC as the third contact-sensitizing Ag stimulated 1-h LMNC to collaborate with OX-BSA-incubated peritoneal cells to initiate CS to TNP-CI in *xid* mice previously immunized with TNP-CI (Fig. 6B, group F). Responses in recipients exposed to three Ag and challenged with a mixture of OX and TNP-CI were significantly greater than those in previously immunized recipients just transferred with 1-h FITC-sensitized LMNC (group E), or just OX-BSA-incubated peritoneal cells (group D), which were equivalent to just TNP-CI-immunized *xid* mice (group C). Therefore, activation



**FIGURE 6.** Contact skin immunization with noncross-reactive sensitizer Ag activates liver iNKT cells to stimulate CS-initiating B-1 cells in vitro, but nonspecific skin inflammation does not. **A**, Peritoneal cells from nonimmune CBA/J H-2<sup>k</sup> mice were incubated in vitro with TNP-BSA and with LMNC from CBA/J donors that were immunized on the skin 1 h previously with either TNP-CI (group B) or OX, a noncross-reactive hapten (group C), or were from nonimmune mice (group D). In another group, peritoneal cells from CBA/N-*xid* H-2<sup>k</sup> B-1 cell-deficient mice were incubated with TNP-BSA plus 1-h TNP-CI-immune LMNC from CBA/J mice (group E). The cell mixtures of each group were transferred i.p. to 3-day TNP-CI-immune *xid* H-2<sup>k</sup> recipients that were ear tested with TNP-CI the next day to determine 2- and 24-h CS ear swelling. These responses were compared with negative controls consisting of *xid* recipients of CBA/J peritoneal cells incubated with TNP-BSA, but without LMNC, and then similarly transferred and ear tested (group A).  $p < 0.001$  vs group A. **B**, Three separate Ag can stimulate the CS-initiating B-1 cells, the required iNKT LMNC, and the CS effector T cells. Peritoneal cells from CBA/J mice were incubated in vitro with OX-BSA together with LMNC from mice contact sensitized 1 h previously with FITC. After incubation and washing, the cell mixture was transferred into *xid* recipients sensitized 3 days previously with TNP-CI (Fig. 8B; group F). The next day, ears were challenged with a final mixture of 0.4% OX and 0.4% TNP-CI, and 2- and 24-h swelling responses were compared with nonimmune control groups of *xid* mice just challenged (group A), or mice receiving peritoneal cells just incubated with OX-BSA-incubated peritoneal cells (group D), or TNP-CI-immune *xid* mice receiving peritoneal cells incubated with medium alone (group C), or OX-BSA alone (group D), or LMNC just stimulated in vivo by FITC contact sensitization (group E). \*\*,  $p < 0.01$ , groups C–E vs groups A and B.

**FIGURE 7.** Evaluation of contact sensitization with TNP-Cl or OX, vs croton oil or PMA. Peritoneal cells from nonimmune CBA/J H-2<sup>k</sup> mice were incubated in vitro with TNP-BSA and LMNC harvested either from donors skin immunized 1 h before by contact sensitization with TNP-Cl (group B), or from mice similarly skin painted with nonimmunogenic croton oil (group C), or with proinflammatory PMA (group D), or sham sensitized (shaving and painting just with the acetone-ethanol vehicle) (group E), or from nontreated donors (group F). Then, cell mixtures were transferred i.p. to 3-day TNP-Cl-immune CBA/N-*xid* H-2<sup>k</sup> recipients that were ear tested with TNP-Cl 1 day later, and 2- and 24-h CS ear swelling was determined. These responses were compared with those of TNP-Cl-immune *xid* H-2<sup>k</sup> mice that received no transfers and were ear challenged similarly (group A). \*,  $p < 0.01$  vs group B.



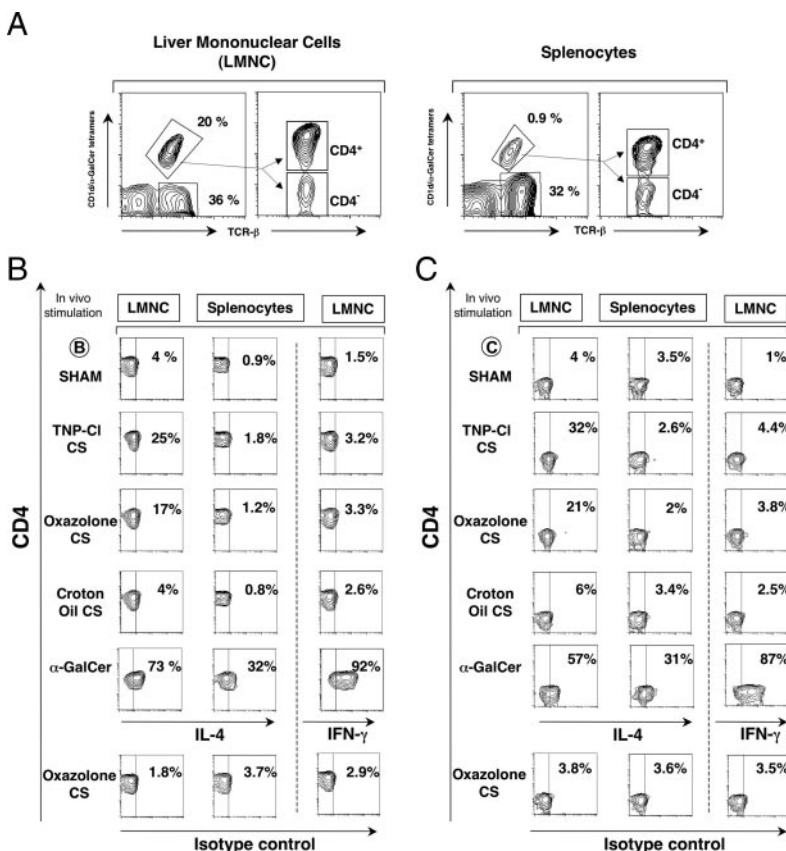
of the iNKT cells to stimulate the CS-initiating process did not depend on the particular contact Ag used to immunize the donors, because 1-h LMNC from TNP-Cl (Fig. 6A, group B), or OX-immune (Fig. 6A, group C), or FITC-immune (Fig. 6B, group F) mice coactivated B-1 cells along with soluble TNP-BSA Ag or OX-BSA Ag to reconstitute CS responses due to TNP-Cl.

#### Attempted *in vitro* activation of peritoneal B-1 cells for CS initiation by LMNC stimulated *in vivo* nonspecifically

The above results suggest that immunization per se was needed to activate the iNKT cells. However, it was possible that nonspecific inflammation in the skin at the time of contact immunization may have been a common factor responsible for nonspecific release of endogenous mediators that led to activation of the iNKT cells. This

is especially possible in contact sensitization, in which sensitizing chemicals are quite irritating and known to activate NF- $\kappa$ B-dependent local inflammation (26). Therefore, we tested whether non-specific skin inflammation induced by contact skin painting with purely proinflammatory, but nonimmunogenic haptens could activate the LMNC similarly. We showed that compared with skin painting with the known sensitizers TNP-Cl or OX, similar skin application of proinflammatory croton oil or PMA, a powerful ingredient in croton oil, does not induce CS (data not shown).

Fig. 7 shows that these inflammatory, but nonimmunogenic contact stimulants could not substitute for painting the skin with the Ag TNP-Cl. Again, TNP-Cl immunization stimulated LMNC to induce CS-initiating activity in B-1 cells (group B). In contrast, neither croton oil skin painting (group C) nor PMA painting (group



**FIGURE 8.** IL-4 is preferentially produced in liver iNKT cells within minutes after contact skin immunization, but not by nonspecific inflammation. CBA/J mice were TNP-Cl skin immunized or sham immunized, and 30 min later their LMNC and splenocytes were harvested and stained with APC-CD1d- $\alpha$ -GalCer tetramers, anti-TCR- $\beta$ , anti-CD4, and intracellularly for IL-4, or IFN- $\gamma$ , or with isotype control. They were analyzed by flow cytometry and gated on tetramer and TCR- $\beta$ -positive cells (iNKT cells), or tetramer-negative T cells (conventional  $\alpha\beta$ -T cells) (A, left plots in LMNC vs splenocytes). The percentage of iNKT cells and conventional T cells is shown in A. iNKT cells were further gated on CD4<sup>+</sup> or CD4<sup>-</sup> T cells (i.e., CD4<sup>+</sup> and CD4<sup>-</sup> iNKT cells) (A, right plots in LMNC and splenocytes). This resulted in 86% CD4<sup>+</sup> and 14% CD4<sup>-</sup> iNKT cells among LMNC and 82% CD4<sup>+</sup> and 18% CD4<sup>-</sup> iNKT cells among splenocytes. Both IL-4 and IFN- $\gamma$  production by gated CD4<sup>+</sup> iNKT (B) and CD4<sup>-</sup> iNKT (C) were evaluated in mice contact sensitized with TNP-Cl (second row), OX (third row), croton oil (fourth row), or sham-treated mice (first row) (B and C). As positive control for iNKT cell stimulation,  $\alpha$ -GalCer was injected i.v. at 2  $\mu$ g per mouse, and 30 min later the IL-4 and IFN- $\gamma$  production by both CD4<sup>+</sup> and CD4<sup>-</sup> populations of iNKT cells was evaluated. The results are from a representative of three experiments. The numbers in the upper right corner quadrants of the contour plots (B to C) represent the percentages of IL-4- or IFN- $\gamma$ -positive cells among the gated population. Isotype control staining for intracellular cytokines is shown in the bottom rows.



D) was active. In this experiment, we also compared LMNC from naive donors to LMNC from sham-immunized donors that were shaved and skin painted just with the acetone:ethanol vehicle used with TNP-Cl or OX contact sensitization, and neither activated the iNKT cells (groups E and F). We concluded that skin immunization, with different contact-sensitizing Ag, and not the accompanying skin inflammation or irritation, led to activation of iNKT cells in the liver to produce IL-4 that with specific Ag coactivated peritoneal B-1 cells to mediate CS initiation.

*Rapid preferential production of IL-4 in LMNC iNKT cells following immunization with Ag, but not by nonspecific inflammation*

iNKT cells in LMNC (Fig. 8A, left) and splenocytes (Fig. 8A, right) were separated into CD4<sup>+</sup> and CD4<sup>-</sup> subpopulations and evaluated for intracellular IL-4 and IFN- $\gamma$  expression rapidly after contact skin painting with various agents compared with sham-sensitized mice (Fig. 8, B and C, first row). Compared with isotype controls (Fig. 8, B and C, last row), there was an increase in the percentage of IL-4-expressing cells in CD4<sup>+</sup> (Fig. 8B) and CD4<sup>-</sup> (Fig. 8C) hepatic iNKT cells at only 30 min following contact sensitization with TNP-Cl or OX, but not by croton oil (Fig. 8, B and C, second and third row, first box). In contrast, iNKT splenocytes did not produce IL-4 following sensitization with TNP-Cl or OX (Fig. 8, B and C, middle column). Importantly, these contact sensitizations did not induce IFN- $\gamma$  production by either liver (Fig. 8, B and C, third column) nor splenic iNKT cells (data not shown). The injection of the positive control  $\alpha$ -GalCer induced much higher expression of IL-4 and coincident IFN- $\gamma$  in both populations (Fig. 8, B and C, next to last row). Furthermore, TNP-Cl and OX sensitization did not stimulate IL-4 expression in conventional T cells (data not shown). We concluded that contact immunization with Ag induced rapid and preferential expression of IL-4 in hepatic and not splenic iNKT cells that was not due to nonspecific skin inflammation.

## Discussion

We describe for the first time an *in vitro* collaboration between iNKT cells and B-1 B cells. This collaboration is required to lead to initiation of the *in vivo* recruitment of effector T cells for elicitation of CS. Remarkably, skin sensitization of different mice with three different reactive haptens (TNP, OX, and FITC) used to induce CS was able to stimulate the *in vitro* collaborative function of hepatic iNKT cells within only 1 h. The liver iNKT cells were activated to produce sufficient IL-4 *in vitro* to stimulate naive peritoneal B-1 cells along with specific soluble hapten-protein Ag. Flow cytometry confirmed that contact sensitization with the hapten Ag OX led to rapid expression of IL-4 in hepatic iNKT cells, as shown previously with TNP-Cl sensitization (22). In contrast, contact painting with nonspecific inflammatory croton oil or PMA did not stimulate any similar IL-4 expression. Thus, rapid iNKT cell stimulation via sensitizing Ag led to IL-4 production. Along with Ag stimulation, this activated Ag-specific B-1 cells to migrate from the peritoneal cavity to the spleen, for production of specific CS-initiating IgM Abs within 1 day (19). Following subsequent skin testing, IgM Abs produced in the spleen and distributed systemically via the blood (19) led to recruitment of effector T cells to mediate cutaneous CS responses.

*In vitro activation of B-1 cells by Ag plus IL-4*

Both Ag stimulation of B-1 cells *in vitro* and the role of IL-4 in B-1 cell responses have received little prior attention. Most studies instead have found that B-1 cells are distinctly hyporeactive when stimulated *in vitro* (27, 28). Also, B-1 cell responses mostly have

been noted to involve IL-5, although IL-4 stimulation has been reported (29). Our prior studies show that activation of B-1 cells for initiating function in CS requires their expression of IL-4R $\alpha$  and STAT-6 signaling (22). These findings suggest that this pathway is responsible for the dual *in vitro* activation of the B-1 cells by iNKT cell-derived IL-4 and specific Ag, as shown in this study. We ruled out a possible role of IL-4 produced by the B-1 cells themselves, by using B-1 cells from IL-4<sup>-/-</sup> mice. Additionally, we ruled out participation of other cells in the peritoneal cell mixture, by showing that FACS-sorted B-1a or B-1b cells were the targets for *in vitro* activation by the early immune iNKT cells. We also ruled out a role for B-1 cells in the LMNC by showing that 1-h immune LMNC from B-1 cell-deficient *xid* mice were still active, and that 1-h hapten immune LMNC without Ag-stimulated B-1 cells were inactive.

*Diverse contact sensitizers activate iNKT cells to collaborate in vitro with B-1 cells*

How specific Ag immunization leads to very rapid activation of iNKT cells in the liver is a major unanswered question raised by these studies. We have shown that three different contact sensitizers can activate hepatic iNKT cells to stimulate the initiating function of peritoneal B-1 cells *in vitro*. We postulate that cutaneous release or availability of an endogenous glycolipid-like effect is involved (30). This endogenous effect is postulated to act in a manner analogous to  $\alpha$ -Gal-Cer, which is a model glycolipid Ag derived from a marine sponge that binds to CD1d on APC to strongly activate iNKT cells via their V $\alpha$ 14 J $\alpha$ 18 TCR.

There are at least three ways to postulate how this could occur. First, the iNKT cells may already be interacting with endogenous glycolipids presented in the liver by CD1d expressed on local APC. In this case, immunization in the skin may result in release of factors that can promote this interaction, leading to preferential production of IL-4.

A second related possibility is that Ag immunization releases factors, again possibly from dendritic cell-type cells, that lead to increased expression of CD1d (31), or NK ligands (32), or MHC class II (J. Gompertz, unpublished observations) on APC that are engaged in presenting the endogenous glycolipids to iNKT cells. This could lead to early rapid IL-4 production, because iNKT cells are known to already express IL-4 mRNA (33).

A third possibility that we favor is that immunization in the skin locally releases previously unavailable endogenous glycolipid-like substances, similar to those described recently (30). These rapidly drain to the liver to bind to CD1d on APC. This could occur directly without internal processing to stimulate very rapid production of IL-4 needed to activate the B-1 cells.

Ag immunization, but not nonspecific inflammation produced by skin painting with croton oil nor with PMA, generates the  $\alpha$ -Gal-Cer-like effects. These findings suggest that the postulated release of endogenous glycolipids may be part of, or associated with processes responsible for the uptake of specific Ag. This could be performed by specialized Ag-handling cells, which might be related to Langerhans cells in the skin, or to dendritic cells in the skin or tissues, or possibly other cells.

*Soluble Ags activate iNKT cells to collaborate with B-1 cells*

Our data suggest that contact with Ag in the skin may not be the only way to lead to early iNKT cell and B-1 cell collaboration, because injection of hapten-protein conjugates also rapidly led to the ability to elicit 2-h ear-swelling responses. Whatever the exact mechanism for rapid early activation of iNKT cells to produce IL-4 to activate B-1 cells, our data show that this stimulation enables initiation of T cell recruitment to elicit 24-h CS responses.

This most likely is due to rapid production by the activated B-1 cells of specific IgM Abs that following skin challenge activate complement locally to generate C5a to mediate vasoactivity that results in the local recruitment of CS effector T cells (1–6, 8, 10). Thus, rapid effects of Ag at immunization, which most likely are CD1d dependent, lead to activation of collaboration between iNKT cells and B-1 cells via IL-4, leading to effector T cell recruitment for classical CS effector responses.

### Biological and clinical relevance

In an allergic disease like asthma, or an autoimmune disease like arthritis, immunologically driven local inflammation is largely mediated by the recruitment of effector T cells into the tissues. Thus, Th2-type allergen peptide MHC-specific T cells are recruited into airway tissues in allergic asthma, and Th1-type autoantigen peptide MHC-specific T cells, resulting from a breakdown of tolerance, are recruited into the synovium in arthritis. In both instances, exacerbations of disease often follow infections with bacteria or viruses. The data of the current study show that immunization with an unrelated Ag can trigger an innate initiation cascade that can lead to recruitment of immune T cells. Thus, Ag of an infectious agent could trigger B cells to produce Abs that could initiate T cell-mediated inflammation to a heterologous Ag. These initiating Abs can recruit T cells into tissues containing Ag and APC-expressing allergen or autoantigen peptides to trigger local activation, respectively, of specific Th2- or Th1-type T cells to produce proinflammatory cytokines. Thus, some Th2 or Th1 effector responses could depend on Ag of the infectious agents stimulating an innate-like iNKT cell and B-1 cell collaboration. This would generate Abs that combine locally with their specific microbial Ag to mediate the initiation process needed to recruit effector T cells of different Ag specificities into the tissues to meet their Ag peptide/MHC complexes on local APC. Therefore, new therapeutic approaches with a focus on the iNKT cell and B-1 cell collaboration could possibly intervene at an early phase in these disease processes.

### Disclosures

The authors have no financial conflict of interest.

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