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CD22 Expression Mediates the Regulatory Functions of Peritoneal B-1a Cells during the Remission Phase of Contact Hypersensitivity Reactions

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Although contact hypersensitivity (CHS) has been considered a prototype of T cell-mediated immune reactions, recently a significant contribution of regulatory B cell subsets in the suppression of CHS has been demonstrated. CD22, one of the sialic acid-binding immunoglobulin-like lectins, is a B cell-specific molecule that negatively regulates BCR signaling. To clarify the roles of B cells in CHS, CHS in CD22−/− mice was investigated. CD22−/− mice showed delayed recovery from CHS reactions compared with that of wild-type mice. Transfer of wild-type peritoneal B-1a cells reversed the prolonged CHS reaction seen in CD22−/− mice, and this was blocked by the simultaneous injection with IL-10 receptor Ab. Although CD22−/− peritoneal B-1a cells were capable of producing IL-10 at wild-type levels, i.p. injection of differentially labeled wild-type/CD22−/− B cells demonstrated that a smaller number of CD22−/− B cells resided in lymphoid organs 5 d after CHS elicitation, suggesting a defect in survival or retention in activated CD22−/− peritoneal B-1 cells. Thus, our study reveals a regulatory role for peritoneal B-1a cells in CHS. Two distinct regulatory B cell subsets cooperatively inhibit CHS responses. Although splenic CD1dhiCD5+ B cells have a crucial role in suppressing the acute exacerbating phase of CHS, peritoneal B-1a cells are likely to suppress the late remission phase as "regulatory B cells." CD22 deficiency results in disturbed CHS remission by impaired retention or survival of peritoneal B-1a cells that migrate into lymphoid organs. The Journal of Immunology, 2010, 184: 4637–4645.

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Abbreviations used in this paper: CHS, contact hypersensitivity; DNFB, 2,4-dinitrofluorobenzene; EAE, experimental autoimmune encephalomyelitis; LN, lymph node; MZ, marginal zone; QRT-PCR, quantitative RT-PCR; Ri, ratio of calcine- to PKH-26-labeled cells before i.p. injection; Ro, ratio of calcine- to PKH-26-labeled cells after elicitation.

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CHS is a cutaneous immune reaction that develops in two distinct phases: sensitization and elicitation (14, 15). In mice, primary skin painting with reactive hapten induces the CHS sensitization phase, in which effector T cells are sensitized by APCs. Subsequently, the elicitation phase is induced by re-exposure to the same hapten. Small numbers of sensitized Ag-specific T cells migrate from the circulation into the extravascular space at the skin challenge site and then interact again with Ag/peptide–MHCs on APCs. Activated T cells release proinflammatory cytokines, which then activate local tissue cells, leading to the characteristic late effector responses at 24–48 h (16–18). In the elicitation phase, the main effector cells have been demonstrated to be IFN-γ–producing CD8+ Tc1 cells (19–21). Thus, CHS is a prototypic T cell-mediated response.

The existence of “regulatory” B cells was originally suggested in delayed hypersensitivity reactions (22). Recently, we reported that CD19-deficient (CD19−/−) mice mount augmented CHS responses and that marginal zone (MZ) B cells, which are lacking in CD19−/− mice, have a regulatory role in CHS (12). Subsequently, splenic IL-10–producing CD1dhiCD5+ B cells were proven to normalize this augmented CHS reaction in CD19−/− mice or in wild-type mice depleted of CD20+ B cells (13). In contrast, in a collagen-induced arthritis model, transitional 2-MZ precursor cells were suggested to serve as regulatory B cells (9). Although it remains unclear whether these two populations are the same, IL-10 production appears to be a hallmark of these regulatory B cells. In addition to splenic regulatory B cells, studies have demonstrated that peritoneal B-1 cells are also an abundant source of IL-10 (23). Nonetheless, whether peritoneal B-1 cells play a regulatory role in inflammatory diseases remains to be determined.

B cell development, activation, and survival are elaborately regulated by the BCR and functionally interrelated cell-surface
receptors (24). CD22 is a B cell-specific transmembrane molecule that is a member of "sialic acid-binding immunoglobulin-like lectin" family of adhesion molecules (25). CD22 has ITIMs in its cytoplasmic domain and becomes phosphorylated in response to BCR ligation and other stimuli (26, 27). CD22 serves as an inhibitory coreceptor and modulates the BCR signal in response to cues from the local microenvironment (28). B cells from CD22-deficient (CD22−/−) mice exhibit exaggerated Ca2+ mobilization in response to BCR stimulation in vitro (29–32) and display hyperimmune responses. CD22−/− B cells predominantly undergo apoptosis (29, 34), which can be rescued by CD40 coligation. Thus, CD22 has significant and complex functions that regulate B cell activation and survival.

In this study, we examined the role of CD22 expression in CHS. CD22 deficiency resulted in prolonged CHS reactions, suggesting an inhibitory role for CD22 expression, especially during the late phase in elicitation of CHS. This study also demonstrates that peritoneal B-1a cells serve as regulatory B cells via IL-10 production.

Materials and Methods

Mice

C57BL/6 and BALB/c wild-type mice were purchased from Clea Japan (Shizuoka, Japan). CD22−/− (C57BL/6 × 129) mice were generated as described (32) and backcrossed onto a C57BL/6 strain 12 times. IL-10−/− (IL-10−/−) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice used were 8 to 12 wk of age and were housed in a specific pathogen-free barrier facility. All of the studies and procedures were approved by the Animal Committees of the International Medical Center of Japan and the Kanazawa University Graduate School of Medical Science.

Sensitization and elicitation of CHS

Mice were sensitized with 25 µl 0.5% 2,4-dinitro-1-fluorobenzene (DNFB; Sigma-Aldrich, St. Louis, MO), in acetone and olive oil (4:1), on shaved abdominal skin for two consecutive days. Five days later, CHS was elicited by applying 20 µl 0.25% DNFB on the right ear. As a negative control, the left ear was treated with acetone/olive oil alone. In some experiments, mice were treated with anti–IL-10 receptor mAb (250 µg, 1B1.3a; BD Pharmingen, San Diego, CA) or control IgG 2 d after DNFB challenge. Ear thickness was measured in a blinded manner with a micrometer (Mitutoyo, Kawasaki, Japan) before and after the challenge until day 10. The degree of CHS was expressed as a swelling of the hapten-challenged ear compared with that of the vehicle-treated ear in units of millimeter × 10−2 (mean ± SEM). As for sensitization with FITC, 400 µl 0.5% FITC (Sigma-Aldrich) in acetone and dibutyl phthalate (1:1) was applied on shaved abdominal skin of each mouse, followed by loading 20 µl 0.5% FITC on the right ear and acetone/dibutyl phthalate alone on the left ear (control) 5 d later. Ear thickness was evaluated as for DNFB-elicted mice.

Histology

Ear samples were taken before and 24 and 120 h after DNFB challenge and were fixed in 4% formalin for routine histology with H&E staining.

Immunofluorescence analysis

To examine splenic CD1d+CD5+ B cells and nodal regulatory T cells in CD22−/− and wild-type mice, spleens and inguinal lymph nodes (LN) were harvested from these mice before and 5 d after the sensitization with DNFB. Spleens and LN from nontreated mice were used as controls. Splenic cells were stained for three-color immunofluorescence analysis using FITC-conjugated anti-CD1d (1B1; BD Pharmingen), PE-conjugated anti-CD5 (53-7.3; BD Pharmingen), and PE-Cy5–conjugated anti-B220 Abs (RA3-6B2; BD Pharmingen) for 20 min at 4°C. Nodal cells were stained with FITC-conjugated anti-CD4 (GK1.5; BD Pharmingen), FITC-conjugated anti-CD8 (53-1.5; BD Pharmingen), and PE-Cy5–conjugated anti-Thy-1.2 (30-H12; BD Pharmingen) and PE-conjugated anti-Foxp3 Abs (MF-14; Biologend, San Diego, CA) using a BD Cytofix/Cytoperm kit (BD Pharmingen). Peritoneal B cells were stained using PE-conjugated anti-CXCR4 (2B11; ebiosis; San Diego, CA), PE-conjugated anti-CCR7 (2G8; BD Pharmingen), PE-conjugated anti-CCR6 (140706; R&D Systems, Minneapolis, MN), PE-conjugated anti-CCR7 (4B12; ebiosis), and PE-conjugated anti-CCR9 (ebioCW-1.2; ebiosis) after stimulation with LPS (10 µg/ml; Sigma-Aldrich) for 8 h. Labeled cells were analyzed on an Epics XL flow cytometer (Beckman Coulter, Miami, FL) with fluorescence intensity shown on a four-decade log scale. Positive and negative populations of cells were determined using unreactive isotype-matched Abs (Southern Biotechnology Associates, Birmingham, AL) as controls for background staining.

To examine peritoneal B-1a/B-1b cell populations, 5 ml PBS was injected into the abdominal cavity of sensitized mice 5 d after sensitization and then reccollected. Single-cell suspensions were stained using biotin-conjugated anti-CD11b. After being washed, cells were stained with streptavidine–PE-Cy5 (BD Pharmingen), FITC-conjugated anti-CD5 Ab, and PE-conjugated anti-B220 Ab, followed by flow cytometric analysis. To evaluate IL-10–producing B cells, cells were resuspended with LPS (10 µg/ml; Sigma-Aldrich), PMA (50 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich), and monensin (2 µM; ebiosis) for 5 h (13). For IL-10 detection, Fc receptors were blocked with mouse Fc receptor-specific mAb (2.4G; BD Pharmingen) before cell-surface staining, and then cells were fixed, permeabilized, and stained with PE-conjugated IL-10 mAb (JES5-16E3; BD Pharmingen).

Measurement of IL-10 concentrations

Secreted IL-10 was quantified using a mouse IL-10 ELISA kit (Invitrogen, Carlsbad, CA). Splenic B cells were purified with B220 mAb-coupled microbeads (Miltenyi Biotec, Auburn, CA) from wild-type and CD22−/− mice. CD1d+CD5+ B cells were collected using a FACSAria cell-sorting system (Becton Dickinson). Isolated CD1d+CD5+ B cells as well as CD5− B cells (3 × 106) were cultured in 200 µl RPMI 1640 culture medium containing 5% BSA and 10 mM HEPES in 96-well flat-bottom tissue culture plates at 37°C with 5% CO2 in the presence of 10 µg/ml LPS (Escherichia coli serotype 0111:B4, Sigma-Aldrich). After incubation for 72 h, IL-10 concentrations of cultured supernatant were quantified. All assays were carried out in triplicate.

Adoptive cell transfer

In adoptive cell transfers from sensitized donor mice to naive recipient mice, recipient mice were treated with DNFB as described above. Inguinal LNs, spleen, or peritoneal lavage were harvested 5 d later. T cells (2 × 106 cells) and B cells (2 × 106 cells) were isolated using anti–Thy-1.2– or anti-B220–coupled microbeads (Miltenyi Biotec) respectively, for positive selection via autoMACS (Miltenyi Biotec). These cells were adoptively transferred i.v.

In adoptive cell transfers from naive donors to sensitized recipient mice, donor mice were sensitized by DNFB. T cells and B cells were isolated as above. To collect B-1a and B-1b cells, peritoneal cells were stained with FITC-conjugated anti-CD5, PE-conjugated anti-B220, and PE-Cy5–conjugated anti-CD11b Abs and then isolated using a FACSaria cell-sorting system. These cells were also transferred i.v. to recipient mice. In adoptive cell transfers from sensitized donors to sensitized recipient mice, donor mice and recipient mice were sensitized as above, and 5 d later cells were isolated and transferred as described above.

Ear thickness of the recipients, at least five mice per group, was measured with a micrometer before the transfers. Twenty-four hours later, the recipients were challenged on the right ear with 20 µl 0.25% DNFB as above and on the left ear with acetone/olive oil alone. The subsequent increases in ear thickness were determined at 24, 120, and 240 h after challenge. The thickness of the control ears was subtracted from experimental responses to yield net ear swelling.

Quantitative RT-PCR

Peritoneal CD5−B220+CD5+ B220− cells taken before sensitization and 2 d after DNFB challenge as mentioned-above were homogenized in Isogen S (Wako, Tokyo, Japan), and total RNA was isolated following the manufacturer’s instructions. RNA concentration was determined using nanodrop (NanoDrop Technologies, Wilmington, DE) by the A260 value of the samples. Total RNA was reverse-transcribed to cDNA using the Reverse Transcription System with random hexamers (Promega, Madison, WI). Quantitative RT-PCR (QRT-PCR) was performed using the Applied Biosystems 7500 Sequence Detection System (Applied Biosystems) according to the manufacturer’s instructions. TaqMan probes and primers for IL-10, TGF-β, and GAPDH were purchased from Applied Biosystems.
B cell migration assays

After wild-type recipient mice were sensitized with DNFB, peritoneal cells were obtained from wild-type and CD22−/− donor mice, and peritoneal CD5+ B cells were isolated as above. B cells derived from wild-type mice were labeled with calcein-AM (Invitrogen), and equivalent amounts of B cells from CD22−/− mice were labeled with PKH-26 (Sigma-Aldrich). The ratios of calcein-AM to PKH-26 before i.p. injection were examined by flow cytometry. Subsequently, both labeled cell populations were injected i.p. into wild-type recipient mice. One day after injection, recipient mice were elicited as above. Spleen and cervical LNs were obtained from recipient mice at day 1 and day 5 postelicitation, and the ratios of calcein- to PKH-26–labeled cells in these were examined by flow cytometry. Subsequently, both labeled cell populations were injected i.p. into wild-type recipient mice. One day after injection, recipient mice were elicited as above. Spleen and cervical LNs were obtained from recipient mice at day 1 and day 5 postelicitation, and the ratios of calcein- to PKH-26–labeled cells in these were examined by flow cytometry.

Statistical analysis

All of the data are shown as mean ± SEM. Student t test was used for determining the level of significance of differences between two groups, and Steel-Dwass multiple comparison test was used among three or more groups. A p value < 0.05 was considered statistically significant.

Results

Recovery from CHS in delayed in CD22−/− mice

To assess whether CD22 expression plays a role in CHS, wild-type and CD22−/− mice were challenged with DNFB after sensitization, and ear swelling was measured before and after challenge. In both mice, ear thickness was increased equivalently on day 1 postelicitation (Fig. 1A). In wild-type mice, ear swelling responses reached a peak on day 3 and returned to normal by approximately day 7. In contrast, ear swelling was prolonged in CD22−/− mice, resulting in a significant increase of ear swelling from day 5 to day 10 compared with that in wild-type mice. Thus, CD22−/− mice exhibited prolonged CHS reactions to DNFB. This delayed recovery was not limited to CHS against DNFB, because CD22−/− mice exhibited similarly prolonged responses to FITC application (Fig. 1B). Thus, CD22−/− mice experience a more persistent CHS reaction.

The degree of CHS reaction was also assessed histopathologically (Fig. 1C). Cellular infiltrates and edema were observed in the DNFB-painted ears of both CD22−/− and wild-type mice 2 d after challenge. In both groups of mice, the majority of the infiltrating cells were polymorphonuclear cells and lymphoid cells. By 10 d after challenge, cellular infiltration and edema had almost disappeared in the ears of wild-type mice, whereas the ears of CD22−/− mice still showed marked cellular infiltration and edema. Therefore, CD22−/− mice exhibited a delayed recovery in CHS, both clinically and pathologically, compared with that of wild-type mice.

Splenic CD1d6CD5+ B cells are normally present in CD22−/− mice

Because CD22 expression is restricted to B cells, a delayed recovery of CHS in CD22−/− mice suggests that regulatory mechanisms by B cells are impaired in CD22−/− mice. IL-10–producing CD1d6CD5+ B cells in the spleen, termed B10 cells, have been demonstrated to play a suppressive role in CHS (13). IL-10–producing CD1d6CD5+ B cells have a CD21hiCD23lo phenotype, which resembles that of MZ B cells. Because CD22−/− mice lack MZ B cells (29–32), we next examined splenic CD1d6CD5+ B cells in CD22−/− mice to assess the possibility that the delayed recovery of CHS in CD22−/− mice resulted from the loss of CD1d6CD5+ B cells. We examined the percentages of these cells in CD22−/− and wild-type mice before and after sensitization (Fig. 2A). The percentages of splenic CD1d6CD5+ B cells in total splenic cells from CD22−/− and wild-type mice were 1.9 ± 0.3 and 2.0 ± 0.3% before sensitization and 3.0 ± 0.3 and 3.1 ± 0.4% after sensitization. Thus, although the phenotype of splenic CD1d6CD5+ B cells resembles that of MZ B cells, the CD1d6CD5+ B population is distinct from MZ B cells and is present in untreated CD22−/− mice.

Next, IL-10 secretion from splenic CD1d6CD5+ B cells was investigated. IL-10 secretions from CD1d6CD5+ B cells were significantly higher than those from non-CD1d6CD5+ B cells in both wild-type and CD22−/− mice (p < 0.01 for each; Fig. 2B). However, the difference between CD1d6CD5+ B cells from wild-type mice and those from CD22−/− mice was not significant, although IL-10 secretion from CD22−/− B cells was slightly increased (Fig. 2B). Therefore, CD1d6CD5+ B cells from CD22−/− mice have a capability of secreting IL-10 similarly to that for those from wild-type mice. Furthermore, the numbers of IL-10–producing CD1d6CD5+ B cells in CD22−/− mice were significantly higher in CD22−/− mice than those from wild-type mice (p < 0.05; Fig. 2C).

The percentages of CD4+Foxp3+ regulatory T cells in inguinal LNs were also examined. They represented 2.8 ± 0.4% of live cells present before sensitization and 3.2 ± 0.4% of live cells after sensitization in CD22−/− mice. There was no significant difference from the percentages present in wild-type mice (2.7 ± 0.5% before sensitization and 3.5 ± 0.5% after sensitization; data not shown).
compared with that in wild-type recipients. Likewise, when inguinal LN cells from sensitized CD22^-/- mice were adoptively transferred to naive CD22^-/- or naive wild-type mice, there was no significant difference in ear swelling between recipient CD22^-/- and naive wild-type mice on day 2, whereas recipient CD22^-/- mice showed a more significantly increased response than recipient wild-type mice on day 7 (p < 0.05) and day 10 (p < 0.001). When the donor cells were from CD22^-/- mice, the difference in the magnitude of the responses was not observed compared with that of wild-type donor cells. Therefore, although CHS is fully transferable to naive mice with sensitized LN cells from the donor regardless of CD22 deficiency in the donor mice, CD22 deficiency in the recipient mice prolongs the duration of the responses once CHS is induced. Next, B or T cells were purified from inguinal LNs from sensitized mice and were transferred to wild-type or CD22^-/- mice. T cells were able to induce initial CHS reactions at a magnitude similar to those observed in whole-cell transfers (Fig. 3A, 3B). Between wild-type and CD22^-/- recipients transferred with wild-type T cells (Fig. 3B, middle panel), there is more significant difference on day 7 (p < 0.001) than whole-cell transfers. CHS did not occur when B cells alone were transferred to naive mice (Fig. 3C). Collectively, these results suggest that CD22 expression in recipient mice is required for the optimal late-phase suppression of the CHS reactions.

Peritoneal B-1a cells of wild-type mice suppress prolonged CHS reactions in recipient CD22^-/- mice

To further investigate the delayed recovery from the CHS reaction in CD22^-/- mice, B or T cells from the spleen, inguinal LN, and peritoneal cavity were isolated from unsensitized wild-type mice and transferred to CD22^-/- mice that were sensitized 5 d before (Fig. 4A). Serum transfer was also performed to examine the participation of Abs. Recipient CD22^-/- mice were elicited for CHS 1 d after transfer. In all cases, CHS occurred equivalently to that in mice injected only with PBS. Ear swelling in CD22^-/- mice was not altered by the transfer of nodal or splenic B cells, all fractions of T cells, or sera from wild-type mice. Notably, however, the CHS reaction in CD22^-/- mice was resolved in a manner comparable to that in wild-type mice by peritoneal B cell transfer from unsensitized wild-type mice (Fig. 4A). Furthermore, this result was also observed when B and T cells were isolated negatively and positively and when a sensitized wild-type donor was used (data not shown).

The transfer of splenic, nodal, and peritoneal B and T cells as well as sera from unsensitized CD22^-/- to wild-type mice that were sensitized 5 d before was also examined (Supplemental Fig. I). Recipient wild-type mice were elicited for CHS 1 d after transfer. In all cases, CHS occurred and resolved equivalently to mice that were injected only with PBS. This result was observed equally when sensitized wild-type donor cells were used. Consequently, these data suggest that peritoneal B cells from wild-type mice, whether sensitized or not, were capable of amending the prolonged CHS reactions in CD22^-/- mice. Also, peritoneal B cells from CD22^-/- mice did not have the ability to exacerbate CHS reactions in wild-type mice.

To investigate further the inhibitory functions of peritoneal B cells, peritoneal B cells of unsensitized wild-type mice were separated into B220^+CD11b^-CD11b^- cells (B-1a cells), B220^+CD5^- CD11b^- cells (B-1b cells), and B220^-CD5^-CD11b^- cells (B-2 cells). Each fraction was injected into CD22^-/- mice 5 d after sensitization (Fig. 4B). Although peritoneal B-1b or B-2 cells from wild-type mice did not affect ear swelling in CD22^-/- mice, the CHS reaction ceased when peritoneal B-1a cells from wild-type mice were transferred to CD22^-/- mice. This result was also observed using wild-type donors that were sensitized 5 d before.

FIGURE 2. Splenic CD1d^hi^CD5^+^ cells in wild-type and CD22^-/- mice before and after sensitization. A, Spleens were harvested before and 5 d after the sensitization with DNFB. Splenic B cells were stained for two-color immunofluorescence analysis using FITC-conjugated anti-CD1d and PE-conjugated anti-CD5. Labeled cells were analyzed on a flow cytometer with fluorescence intensity shown on a four-decade log scale. Positive and negative populations of cells were determined using unreactive isotype-matched Abs as controls for background staining. Results shown are representative of those from six 2-mo-old mice. Numbers represent the percentages of CD1d^hi^CD5^+^ cells. B, IL-10 secretion from splenic B cells. B220^+^CD1d^hi^CD5^+^ B cells and B cells other than the CD1d^hi^CD5^+^ population (3 x 10^5 for each) were obtained from wild-type and CD22^-/- mice and cultured with LPS for 72 h. Supernatant IL-10 concentrations were measured by ELISA. Each group contains three mice. *p < 0.01. C, IL-10-producing population in B220^+^CD1d^hi^CD5^+^ B cells from CD22^-/- and wild-type mice. Representative data of histograms demonstrate cytoplasmic IL-10 expression before and after LPS, PMA, and ionomycin stimulation. Percentages indicate IL-10^+^ cell frequencies. Dashed lines represent negative controls stained with isotype-matched control Ab.

CD22 expression in recipient mice influenced the duration of CHS reactions

To examine the cause of delayed recovery from CHS in CD22^-/- mice, bulk inguinal LN cells from wild-type mice were adoptively transferred i.v. to unsensitized CD22^-/- mice or unsensitized wild-type mice 5 d after sensitization. Then 1 d after cell transfer, 20 µl 0.25% DNFB was loaded onto the ears of recipient CD22^-/- or wild-type mice. As shown in Fig. 3A, when they were injected with whole cells, ear swelling on day 2 was comparable between recipient CD22^-/- and wild-type mice. By contrast, ear swelling remained significantly augmented in CD22^-/- mice transferred with whole cells on day 7 (p < 0.05) and day 10 (p < 0.001)
Thus, although the possibility that Ab engagement during B220+ CD5+CD11b+ cell isolation may influence the results cannot be completely excluded, these data suggest that peritoneal B220+ CD5+CD11b+ B-1a cells have the ability to suppress prolonged CHS reactions in CD22−/− mice.

It has been already demonstrated that CD22−/− mice have increased numbers of peritoneal B-1 cells (29–32); however, detailed characterization in a C57BL/6 background has not been reported. As is shown in Fig. 5A, the percentages of B220+ CD11b+ B cells (whole B-1 cells) in total peritoneal lymphocytes were 10.0 ± 3.8% (1.6 ± 0.6 × 10⁶ cells) in wild-type mice and 28.8 ± 5.1% (4.9 ± 0.9 × 10⁶ cells) in CD22−/− mice. Thus, peritoneal B-1 cells were significantly increased in CD22−/− mice (p < 0.05). B220+CD11b+CD5− B-1a cells were increased in CD22−/− mice (2.9 ± 0.5 × 10⁶; 17.2 ± 3.8% of peritoneal B cells) compared with those in wild-type mice (0.7 ± 0.2 × 10⁶; 4.5 ± 1.6%; p < 0.05). Peritoneal B-1b cells were also increased in CD22−/− mice, because the percentages of B220+CD11b+CD5− B-1b cells were 5.6 ± 1.9% (0.9 ± 0.4 × 10⁶) in wild-type mice and 11.5 ± 2.3% (1.9 ± 0.4 × 10⁶) in CD22−/− mice (p < 0.05). Thus, peritoneal B-1a cells and B-1b cells are both increased in CD22−/− mice compared with those in wild-type mice, although this does not explain the mechanisms by which CD22−/− mice exhibited augmented CHS response.

CD22 deficiency does not affect IL-10 production from peritoneal B cells after elicitation

Regulatory B cells have been demonstrated to suppress T cell-mediated inflammatory reactions through IL-10 secretion. Although CD22−/− mice had more B-1 cells, the cytokine-producing abilities of these cells were unknown. Therefore, we examined IL-10 production by peritoneal B cells before sensitization and after elicitation. Peritoneal B cells were harvested from wild-type and CD22−/− mice before sensitization and 2 d after elicitation and analyzed by flow cytometry and QRT-PCR. In both wild-type and CD22−/− mice, <0.5% of peritoneal B-1a cells were positive for intracellular IL-10 before sensitization and 2 d after elicitation (Fig. 5B). Fig. 5C shows IL-10 mRNA expression in peritoneal B cells. After elicitation, IL-10...
mRNA expression was significantly increased in both types of mice compared with that seen before sensitization \( (p, 0.01) \). Between wild-type and CD22\(^{-/-}\) mice, expression levels of IL-10 were equivalent before sensitization and after elicitation. Also, there was no significant difference in TGF-\( \beta \) mRNA expression between wild-type and CD22\(^{-/-}\) peritoneal B cells before and after elicitation (data not shown). When IL-10 secretion was evaluated by ELISA, there was also no significant difference between wild-type and CD22\(^{-/-}\) peritoneal B-1a cells (Fig. 5D).

Collectively, IL-10 secretion from peritoneal B cells of CD22\(^{-/-}\) mice was increased after elicitation, similarly to that of wild-type mice.

**IL-10 contributes to CHS remission**

Although IL-10 secretion from splenic regulatory B cells has been demonstrated to play an essential role in their suppressive functions, this may not be the case with peritoneal B cells. Therefore, IL-10 contributions to the suppression of CHS by peritoneal B-1a cells were assessed with a blocking mAb against the IL-10 receptor. Coinjection of isotypic control mAb and wild-type peritoneal B-1a cells into sensitized CD22\(^{-/-}\) recipient mice 2 d after elicitation resulted in suppression of the late phase CHS response to wild-type levels, as was observed previously (Fig. 6A). In contrast, IL-10 receptor mAb injection during transfer of wild-type peritoneal B-1a cells into sensitized CD22\(^{-/-}\) mice significantly delayed CHS recovery \((p < 0.01\) at day 10), which was similar to the course observed in CD22\(^{-/-}\) mice. IL-10 receptor mAb injection alone further augmented CHS response when compared with IL-10 receptor mAb injection with wild-type peritoneal B-1a cell transfer on days 2 and 7, although the difference was not significant (Fig. 6A).

That IL-10 secretion from peritoneal B cells is critical for the suppression of the late phase CHS response was confirmed using IL-10\(^{-/-}\) mice. When peritoneal CD5\(^+\) B cells from wild-type or IL-10\(^{-/-}\) mice were injected into sensitized CD22\(^{-/-}\) recipient mice, IL-10\(^{-/-}\) B-1a cells failed to resolve the ear swelling on days 7 and 10 (Fig. 6B). Thus, the ability of regulatory peritoneal B-1a cells to bring about prolonged CHS in CD22\(^{-/-}\) mice depends upon IL-10.

**FIGURE 5.** IL-10 expression in peritoneal B-1a cells from wild-type and CD22\(^{-/-}\) mice. A, Peritoneal B-1 cell profile of CD22\(^{-/-}\) mice. Single-cell suspensions of the peritoneal lavage from wild-type and CD22\(^{-/-}\) mice were stained using biotin-conjugated anti-CD11b. After being washed, the cells were stained with streptavidin-PE-Cy5, FITC-conjugated anti-CD5, and PE-conjugated anti-B220 Abs, followed by flow cytometric analysis. These results are representative of those obtained with six 2-mo-old mice. B, Peritoneal CD5\(^+\)B220\(^+\) cells from wild-type and CD22\(^{-/-}\) mice were stained for intracellular IL-10 before sensitization and 2 d after DNFB challenge. C, Peritoneal CD5\(^+\)B220\(^+\) cells before sensitization and 2 d after DNFB challenge were collected from wild-type and CD22\(^{-/-}\) mice (five per group), and RNA was extracted. The mRNA levels of IL-10 were analyzed by QRT-PCR and normalized with internal control GAPDH. Data are shown as mean \( \pm \) SEM from five mice. D, IL-10 secretion was determined by ELISA. Naive peritoneal CD5\(^+\)B220\(^+\) cells from wild-type and CD22\(^{-/-}\) mice were cultured in media alone or containing LPS for 24 h. \( p < 0.05; \) **\( p < 0.01.\)

**FIGURE 6.** The suppression of CHS by peritoneal B-1a cells depends upon IL-10. A, Peritoneal CD5\(^+\) B cells (B-1a cells) from unsensitized wild-type mice and/or control or IL-10 receptor-specific mAb were transferred to CD22\(^{-/-}\) mice 3 d after DNFB challenge. The bottom bars indicate ear swelling of nontransferred wild-type mice. B, Peritoneal B-1a cells from unsensitized wild-type or IL-10 receptor-specific mAb were transferred to CD22\(^{-/-}\) mice 3 d after DNFB challenge. The bottom bars indicate ear swelling of nontransferred wild-type mice. \( \ast \ast p < 0.01.\)
Fewer CD22<sup>−/−</sup> peritoneal B-1a cells reside in lymphoid organs after elicitation

It has been suggested that CD22 binding to endogenous ligands is required for the migration of mature recirculating B cells to the bone marrow. Because CD22<sup>−/−</sup> mice had considerably reduced numbers of recirculating B cells in the bone marrow, we assessed the migration of CD22<sup>−/−</sup> B-1a cells to lymphoid organs using a two-color in vivo migration assay (35). Equivalent numbers of purified peritoneal CD5<sup>+</sup> B cells from CD22<sup>−/−</sup> mice labeled with the intravital fluorochrome PKH-26 and purified peritoneal CD5<sup>+</sup> B cells from wild-type mice labeled with the intravital fluorochrome calcein were subjected to i.p. adoptive transfer into wild-type recipient mice. One day after cell transfer, the right ears of recipient mice were elicited by DNFB. B cell distribution was assessed by comparing the ratio of calcein- to PKH-26–labeled cells collected from cervical LNs and spleens at day 1 and day 5 after elicitation (Ro) with the ratio of calcein- to PKH-26–labeled cells before i.p. injection (Ri). Cells with equivalent migratory properties distribute evenly and generate Ro/Ri ratios approaching 1 (Fig. 7). On day 1, wild-type and CD22<sup>−/−</sup> B cells were distributed in spleen and cervical LNs at similar frequencies. However, Ro/Ri ratios were significantly increased to 1.58 ± 0.48 in spleen (p < 0.05) and 1.76 ± 0.54 in LNs (p < 0.01) on day 5. These data reflect that fewer CD22<sup>−/−</sup> peritoneal B cells relative to wild-type peritoneal B cells existed in lymphoid organs 5 d after elicitation. This was also similarly observed when these cells were injected into CD22<sup>−/−</sup> mice (1.52 ± 0.55 in spleen, p < 0.05; 1.83 ± 0.77 in LNs, p < 0.05 on day 5). Therefore, fewer CD22<sup>−/−</sup> peritoneal B cells remained in lymphoid organs compared with wild-type peritoneal B cells after elicitation. To examine the migratory functions of CD22<sup>−/−</sup> peritoneal B cells, cell-surface densities of B cell-expressing chemokine receptors were examined in peritoneal CD5<sup>+</sup> B cells from CD22<sup>−/−</sup> and wild-type mice. Constitutive expression levels of CXCR4, CXCR5, CCR6, CCR7, and CCR9 were almost identical between CD22<sup>−/−</sup> and wild-type B-1a cells (data not shown). After LPS stimulation, CXCR4 expression was slightly higher in CD22<sup>−/−</sup> B-1a cells (Supplemental Fig. 2). By contrast, CCR6 expression was slightly lower in CD22<sup>−/−</sup> B-1a cells. Nonetheless, these modest differences in expression levels of these chemokine receptors may not be likely to cause the difference in the migration.

Peritoneal B-1a cells from wild-type mice, but not from CD22<sup>−/−</sup> mice, improve recovery from CHS reactions in CD19<sup>−/−</sup> mice

Recently, we reported that CHS reactions are augmented by a deficiency of CD19, a positive B cell response regulator, due to the absence of regulatory B cells in the spleen (12). When CHS reactions induced by DNFB in CD22<sup>−/−</sup> mice were compared with those in CD19<sup>−/−</sup> mice, CHS reactions in CD19<sup>−/−</sup> mice were augmented during both early (day 1 to day 3) and late (day 5 to day 10) phases, whereas in CD22<sup>−/−</sup> mice the acute phase of the CHS reactions was similar to that in wild-type mice and only recovery in the late phase (recovery phase) was delayed (Fig. 8A). Because we have demonstrated above that peritoneal B-1a cells have the potential to play a regulatory role in the recovery phase of CHS in CD22<sup>−/−</sup> mice, we examined further the role of peritoneal B-1a cells in augmented CHS reactions of CD19<sup>−/−</sup> mice by adoptive cell transfer. Un sensitized wild-type or CD22<sup>−/−</sup> peritoneal B-1a cells were purified and transferred to CD19<sup>−/−</sup> mice that were sensitized 5 d before. Recipient CD19<sup>−/−</sup> mice were elicited for CHS 1 d after transfer. As shown in Fig. 8B, in both cases, CHS in CD19<sup>−/−</sup> recipient mice was more prominent than that in control, unmanipulated wild-type mice and showed significant delays in recovery. CD19<sup>−/−</sup> recipient mice that received wild-type cells recovered from CHS equivalently to nontransferred wild-type mice, whereas ear swelling remained on day 10 in CD19<sup>−/−</sup> recipient mice that received CD22<sup>−/−</sup> cells. Collectively, these results suggest that wild-type peritoneal B-1a cells have an inhibitory role during the late phase of CHS reactions in CD19<sup>−/−</sup> mice, but not during the early phase, and that CD22<sup>−/−</sup> peritoneal B-1a cells were impaired in this inhibitory role.

Discussion

CD22 is a B cell-specific transmembrane molecule that negatively regulates B cell responses to a range of extracellular signals (32). The current study has demonstrated that CHS was more prolonged in CD22<sup>−/−</sup> mice (Fig. 1). When various fractions of splenic,
B-1 CELLS AS REGULATORY B CELLS IN CHS

FIGURE 8. Peritoneal B-1a cell transfer from wild-type and CD22<sup>−/−</sup> to CD19<sup>−/−</sup> mice in CHS. A, CHS responses of CD22<sup>−/−</sup>, CD22<sup>−/+</sup>, and wild-type mice. Mice were sensitized and elicited as above by DNFB. B. Peritoneal B-1a cells from unsensitized wild-type and CD22<sup>−/−</sup> mice were transferred to CD19<sup>−/−</sup> mice. The bottom bars indicate ear swelling of nontransferred wild-type mice. CHS was elicited by DNFB 1 d after transfer. Ear swelling was assessed as described in Fig. 3. ∗p < 0.05; ∗∗p < 0.01.

nodal, and peritoneal lymphocytes derived from wild-type donors were transferred to sensitized CD22<sup>−/−</sup> recipients, only transfer of peritoneal CD5<sup>−</sup>CD11b<sup>+</sup> B-1a cells extinguished prolonged ear swelling of CD22<sup>−/−</sup> mice (Fig. 4). Furthermore, peritoneal B-1a cells from IL-10<sup>−/−</sup> mice failed to suppress CHS response (Fig. 6B). Recent studies have extensively clarified that inhibitory subsets of lymphocytes play crucial roles during inflammatory and immune responses. In addition to regulatory T cells (36), we have recently demonstrated that splenic CD19<sup>+</sup>CD5<sup>+</sup> B cells play a suppressive role in CHS (13). The current study has demonstrated that peritoneal B-1 cells also serve as regulatory B cells in CHS via IL-10 production.

B-1 cells are long-lived and self-renewing B cells that mainly reside in peritoneal and pleural cavities, although they are also found in the spleen in smaller numbers. B-1 cells are defined by a pattern of surface marker expression, B220<sup>+</sup>, IgM<sup>hi</sup>, IgD<sup>−</sup>, CD9<sup>+</sup>, CD43<sup>+</sup>, and CD23<sup>hi</sup>, as opposed to conventional circulating B-2 cells that are B220<sup>+</sup>, IgM<sup>lo</sup>/<sup>hi</sup>, IgD<sup>+</sup>, CD9<sup>−</sup>, CD43<sup>−</sup>, and CD23<sup>lo</sup> (37–39). B-1a cells express CD5<sup>+</sup>, whereas the other B-1 sister populations of B-1b cells share all surface markers with B-1a cells except CD5 expression. B-1 cells are the major source of natural Abs, which are polyreactive and weakly autoreactive. They recognize Ags from many common pathogens and thus are very important for the early response to bacterial and viral infections (40). Previous studies have demonstrated the possible contribution of B-1 cells to immune disorders. In CHS, a promoting, rather than inhibitory, role for peritoneal B-1 cells has been reported. Using Btk-defective xid mice on a CBA background, Tsuji et al. (41) have demonstrated that Ag-specific IgM Abs from peritoneal B-1 cells are required for the recruitment of effector T cells in the early elicitation phase. By contrast, Btk<sup>−/−</sup> mice on a C57BL/6 background have been reported to exhibit augmented CHS in response to DNFB (42). Similarly, we reported that CD19<sup>−/−</sup> mice, which have decreased numbers of B-1a cells but normal B-1b cell numbers, exhibited augmented CHS responses. The discrepancy of these results may be due to the differences in mouse backgrounds and/or haplotypes. Nonetheless, in the current study, the early elicitation phase of CHS in CD22<sup>−/−</sup> mice was similar to that in wild-type mice, suggesting that the initiation of CHS responses is not impaired in CD22<sup>−/−</sup> mice. Furthermore, serum transfer from CD22<sup>−/−</sup> mice, whether they were sensitized or not, did not exacerbate CHS in wild-type mice (Fig. 4). Collectively, it is unlikely that IgM Abs cause the prolonged CHS in CD22<sup>−/−</sup> mice. In fact, cell transfer experiments have elucidated that peritoneal B-1 cells have a regulatory function in CHS. Intriguingly, Lyn-deficient mice, which display hyperactive B-1 cells and IgM hyperglobulinemia, display augmented severity of EAE (43). Lyn is a Src kinase family member abundantly expressed in B cells and is responsible for CD22 phosphorylation. For augmented EAE in Lyn-deficient mice, IgM Abs from B-1 cells are suspected to be responsible. However, depletion of CD5<sup>+</sup> B-1 cells during the induction phase of the disease resulted in an increase in the incidence of EAE and in the clinical score, whereas depletion during the effector phase of EAE also decreased the severity and the incidence (44). Recently, Shimomura et al. (45) have also reported a regulatory role for B-1 cells in chronic colitis. Thus, during inflammatory responses, B-1 cells may have complex roles that vary depending on the phase of the response.

Paradoxically, although CD22<sup>−/−</sup> mice have more B-1a cells that can produce IL-10 at wild-type levels, peritoneal B-1a cells from wild-type mice, but not CD22<sup>−/−</sup> mice, can condense the late phase of CHS. Itakura et al. (46) have reported that peritoneal B-1 cells are activated to migrate to lymphoid organs immediately after sensitization. The migration assay of adoptively transferred peritoneal B-1 cells in the current study showed that a significantly smaller number of peritoneal B cells from CD22<sup>−/−</sup> mice, relative to those from wild-type mice, were observed in lymphoid organs in recipient mice 5 d after elicitation, whereas there was no significant difference on day 1. It has been demonstrated that CD22<sup>−/−</sup> B cells predominantly undergo apoptosis in response to BCR stimulation and thus show reduced proliferation in vitro (29, 34). Thus, our data may imply an impairment of survival of CD22<sup>−/−</sup> peritoneal B cells. Alternatively, CD22 deficiency may result in defective cell–cell interactions in activated B cells. CD22 constitutively binds with other B cell-surface glycoproteins, such as CD45 and IgM, in cis (27, 47, 48). Thus, CD22 on most B cell surfaces is “masked” and unable to bind exogenous ligands; it has been reported that CD22 becomes “unmasked” upon cellular activation (49). Therefore, it is possible to hypothesize that CD22 deficiency may impair the retention of peritoneal B cells by binding exogenous ligands in lymphoid organs. Also, CD22 ligands are expressed on sinusoidal endothelial cells of bone marrow, and interaction of CD22 has been implicated in the homing of recirculating B cells to the bone marrow (50). Taken together, either retention or survival of peritoneal B cells may be disrupted in CD22<sup>−/−</sup> mice.

Although both CD22<sup>−/−</sup> and CD19<sup>−/−</sup> mice showed augmented CHS responses, the reaction pattern appeared different. The most definitive difference is that the remission phase, and not the acute phase, is disturbed in CD22<sup>−/−</sup> mice, whereas both acute and remission phases are altered in CD19<sup>−/−</sup> mice (Figs. 1, 8A). Like CD22, CD19 is a B cell-specific cell-surface molecule. We have demonstrated that CD19<sup>−/−</sup> mice lack splenic IL-10–producing CD1<sup>hi</sup>CD5<sup>+</sup> B cells that suppress CHS (13). Although the cell-surface phenotype of CD1<sup>hi</sup>CD5<sup>+</sup> B cells resembles that of MZ B cells, these two populations appear distinct because CD22<sup>−/−</sup> mice lack MZ B cells but possess a normal number of CD1<sup>hi</sup>CD5<sup>+</sup> B cells. These results imply that CHS is suppressed in the remission phase by a different pathway from that in the acute phase and that peritoneal B-1a cells have an inhibitory role in the remission phase of CHS. This is also supported by the adoptive cell transfer of wild-type peritoneal B-1a cells into sensitized CD19<sup>−/−</sup> recipients, which extinguished prolonged ear swelling of CD19<sup>−/−</sup> mice in the late phase because CD19<sup>−/−</sup> mice have
markedly decreased numbers of peritoneal B-1a cells (51). Alterna-
tively, it might be possible that peritoneal B-1a cells that migrate to lymphoid tissue may develop into CD1dCD5+ regul-
atory B cells. Collectively, in CHS, the elicitation phase can be divided into an acute phase and a remission phase, and splenic CD1dCD5+ B cells and peritoneal B-1a cells have distinct inhibitory roles in CHS that are mediated via IL-10.

Disclosures
The authors have no financial conflicts of interest.

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