Connexin43 Is Dispensable for Phagocytosis

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Macrophages that lack connexin43 (Cx43), a gap junction protein, have been reported to exhibit dramatic deficiencies in phagocytosis. In this study, we revisit these findings using well-characterized macrophage populations. Cx43 knockout (Cx43<sup>−/−</sup>) mice die soon after birth, making the harvest of macrophages from adult Cx43<sup>−/−</sup> mice problematic. To overcome this obstacle, we used several strategies: mice heterozygous for the deletion of Cx43 were crossed to produce Cx43<sup>+/+</sup> (wild type [WT]) and Cx43<sup>+/−</sup> fetuses. Cells isolated from 12- to 14-d fetal livers were used to reconstitute irradiated recipient animals. After reconstitution, thioglycollate-elicited macrophages were collected by peritoneal lavage and bone marrow was harvested. Bone marrow cells and, alternatively, fetal liver cells were cultured in media containing M-CSF for 7–10 d, resulting in populations of cells that were >95% macrophages based on flow cytometry. Phagocytic uptake was detected using flow cytometric and microscopic techniques. Quantification of phagocytic uptake of IgG-opsonized sheep erythrocytes, zymosan particles, and Listeria monocytogenes failed to show any significant difference between WT and Cx43<sup>−/−</sup> macrophages. Furthermore, the use of particles labeled with pH-sensitive dyes showed equivalent acidification of phagosomes in both WT and Cx43<sup>−/−</sup> macrophages. Our findings suggest that modulation of Cx43 levels in cultured macrophages does not have a significant impact on phagocytosis. The Journal of Immunology, 2013, 190: 000–000.

Connexin43 (Cx43) is a multimeric protein conduit that functions to connect the cytoplasms of two cells. Cx43 is expressed in multiple cells and organs of the immune system, and has been suggested to contribute to immune function (1–3). For almost a decade, our laboratory has been engaged in the study of Cx43, specifically its contribution to macrophage function. The most extensive of these studies involve the use of radiation chimeras to produce mice that lack Cx43 in cells of hematopoietic origin (4). Equivalent reconstitution from Cx43<sup>+/+</sup>, Cx43<sup>+/−</sup>, and Cx43<sup>−/−</sup> fetal liver cells is commonly observed in these animals, and populations of inflammatory macrophages are proportionately high for cells of donor origin (>98%). There are no obvious immune defects in these animals (4). Furthermore, macrophages derived from Cx43<sup>+/+</sup>, Cx43<sup>+/−</sup>, and Cx43<sup>−/−</sup> fetal liver cells have been analyzed for bacterial killing, revealing no obvious defect in bactericidal activity (A.M. Glass, T.D. Nguyen, and S.M. Taffet, unpublished observations). Throughout these studies, we have found no evidence that Cx43 is required for phagocytosis, or that macrophages generated from mice that lack Cx43 display any impairment of phagocytic uptake, a prerequisite for bactericidal activity, as compared with macrophages derived from wild type (WT) animals.

The lack of a role for Cx43 in macrophage phagocytosis is in sharp contrast with a study by Anand et al. published in 2008 (5).

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Abbreviations used in this article: Cx43, connexin43; MFI, mean fluorescence intensity; sRBC, sheep erythrocytic; WT, wild type.

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10% FBS and 5% penicillin/streptomycin) for 24 h to allow for selective adherence of contaminating cells (such as fibroblasts and fetal liver resident macrophages).

After genotyping, nonadherent fetal liver cells were collected, pooled according to genotype, and plated at a concentration of 1 × 10⁶ nucleated cells/plate in nontissue culture–treated 100-mm petri dishes (BD Falcon). Cells were grown in complete medium supplemented with supernatant from L929 cells, a source of M-CSF, for 7–10 d (6). Mature macrophages, which were adherent to the dishes, were harvested after incubation with cold PBS + 2 mM EDTA.

**Generation of radiation chimeric mice**

Radiation chimeric mice were generated as previously described (4). Cx43+/− mice on a CD45.1 background were mated to produce CD45.1+ Cx43+/+ and Cx43−/− fetuses from which fetal liver cell suspensions were prepared as described above. A total of 1 × 10⁶ fetal liver cells were transferred to an irradiated (950 cGy) CD45.2 + BALB/c host by retro-orbital injection. After allowing 10 wk for reconstitution, we analyzed peripheral blood for the donor marker (CD45.1). Greater than 97% of peripheral blood leukocytes were donor derived (Cx43+/+ 99.1 ± 1.0 versus Cx43−/− 97.5 ± 0.5).

**Preparation of phagocytic targets**

sRBCs (Innovative Labs) were washed in PBS and labeled with CFSE (Cell Trace; Invitrogen) according to manufacturer’s specifications. Immediately before use in a phagocytosis assay, labeled erythrocytes were incubated for 30 min with fractionated rabbit anti-sheep RBC antisera (Sigma) diluted 1:10, followed by washing with PBS before addition to macrophages. Zymosan A from Saccharomyces cerevisiae (Sigma) was fluorescently labeled using the DyLight 649 labeling kit (Thermo Scientific) or using pHrodo red succinimidyl ester kit (Invitrogen) according to manufacturer’s instructions. Phagocytic target concentration and fluorescence were determined using a hemocytometer and fluorescence microscope.

**L. monocytogenes**

*Listeria* expressing a nonsecreted form of GFP and a secreted recombinant protein containing the amino acid sequence SINFEKL (Lm-PASFLAG) was generated previously (7).

**Phagocytosis assays**

Fetal liver-derived and bone marrow–derived macrophages were plated in nontissue culture–treated 24-well plates (CytoOne) at a density of 5 × 10⁵ cells/well in complete medium. Cells were allowed to adhere for at least 12 h, and DMEM was replenished 30 min before assessment of phagocytosis.

**Phagocytosis of sRBCs**

IgG-opsonized sRBCs were added to wells at a target/macrophage ratio of 100:1 (to ensure an abundance of available targets) or 10:1. During incubation, cultures were maintained at 37 ºC and 5% CO₂. At 20-, 40-, and 60-min time points, external sRBCs were lysed by a 1-min incubation with distilled water. cultures were washed with PBS to remove remaining sRBC fragments, and macrophages in PBS + 2 mM EDTA were placed on ice for several minutes to allow release from the surface of the dish. Fetal liver-derived macrophage suspensions were filtered using 70-μm nylon mesh, transferred to round-bottom tubes (BD Falcon), and kept on ice until flow cytometric analysis. The bone marrow–derived macrophages from radiation chimeric animals were not filtered before analysis.

**Phagocytosis of sRBCs by fluorescence microscopy**

Fetal liver-derived macrophages or thioglycolate-elicited macrophages, from radiation chimeric animals, were allowed to adhere to 18-mm diameter glass coverslips (Fisherbrand) placed in the wells of a 12-well plate at a density of 5 × 10⁵ cells/well. The following day, IgG-opsonized, fluorescently labeled sRBCs were added to each well. After 20 min of incubation, wells were washed three times with PBS and placed on ice.

For macrophages derived from fetal livers, fixation was achieved using 1% paraformaldehyde. Nonspecific secondary Ab binding to macrophage Fcy receptors was blocked by incubation with 2.4G2 hybridoma supernatant (8). Incompletely internalized sRBCs were labeled using Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen). After several washes, cells were permeabilized using 0.5% Triton X-100 in PBS and mounted to slides using Prolong Gold with DAPI (Invitrogen). Images were taken at ×200 magnification using a Nikon Eclipse E800 fluorescence microscope equipped with a Spot RF Slider camera. Images were randomized and analyzed in a blinded fashion for the number of internalized sRBCs per macrophage.

For thioglycolate macrophages, noninternalized sRBCs were lysed using distilled water, followed by several washes in PBS. Cells were fixed in 1% paraformaldehyde and placed in a randomized configuration in the wells of a new 12-well plate. An inverted fluorescence microscope (Olympus IX51) was used to count the number of sRBCs per cell.

To compare the phagocytic capacity of WT and Cx43−/− macrophages, we prepared histograms showing the number of particles per cell on the horizontal axis. A relative cell number is reported on the vertical axis to normalize for unequal numbers of cells from each group. Relative cell number = (number of macrophages in each bin/total number of cells counted) × 100. At least 100 cells were counted for each experiment. For both sets of data, averages were analyzed using Student’s t test. No significant differences (p < 0.05) were observed in any parameter.
Phagocytosis of labeled Zymosan

Either DyLight 649 (DL649)-labeled or pHrodo-labeled zymosan particles were added to cultured macrophages at a target/macrophage ratio of 100:1. For each Cx43 genotype studied, two identical 24-well plates were used: one plate that was incubated at 37˚C and 5% CO2 to measure phagocytic uptake, and a second plate incubated on ice at 4˚C for the same period. The 4˚C plate was used to establish a baseline measurement of particle adherence to macrophages for comparison with actual phagocytic uptake. After 60 min, the wells of both the 37˚C and 4˚C plates were washed three times to remove unbound/unengulfed zymosan particles. Macrophages were incubated on ice with PBS + 2 mM EDTA, lifted by pipetting, filtered, and kept on ice until flow cytometric analysis.

Phagocytosis of L. monocytogenes and Ag presentation assay

Listeria uptake and Ag presentation assays were performed as previously described (9). In brief, overnight Listeria cultures were used to inoculate

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** WT and Cx43−/− macrophages are equally capable of phagocytosing opsonized sRBCs. (A) WT (black line) and Cx43−/− (gray line) macrophages were incubated for 20 min with IgG-opsonized, CFSE-labeled sRBCs and analyzed by flow cytometry (shaded black peak represents macrophages incubated with unlabeled sRBCs). (B) Percentage of cells positive for CFSE fluorescence after 20 min of incubation with opsonized sRBCs (average and SEM of eight experiments). (C) Histogram of phagocytosis by WT (black bars) and Cx43−/− (gray bars) fetal liver-derived macrophages after 20 min of incubation with sRBCs. Histograms were normalized for cell counts by dividing the number of cells in each bin by the total number of cells counted. At least 100 cells were counted for each genotype.

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Bone marrow–derived macrophages from radiation chimeric mice reconstituted with fetal liver cells from WT and Cx43−/− mice are equally capable of phagocytosing of sRBCs. (A) Fluorescence histograms comparing sRBC uptake of WT (black dashed lines) and Cx43−/− (red lines) bone marrow–derived macrophages after 20-, 40- and 60-min coincubation. Macrophages were treated with particles at 10:1 (left panels) and 100:1 (right panels) particle/macrophage ratios. Gray shaded peaks represent nonfluorescent sRBCs. (B) Kinetic plot of phagocytosis by bone marrow–derived macrophages from radiation chimeric animals reconstituted with WT (black dashed lines) and Cx43−/− (red lines) fetal liver cells. Macrophages were incubated with 100:1 (closed symbols) and 10:1 (open symbols) sRBC/macrophage ratios for 20-, 40- and 60-min time points before analysis by flow cytometry (average and SEM for three experiments).
BHI broth and grown to log phase. WT or Cx43−/− fetal liver-derived macrophages were suspended at a concentration of 2 × 10^6 cells/ml in IMDM supplemented with 10% FBS, but without the addition of antibiotic. Macrophages were infected with midlog phase Listeria at multiplicity of infection = 20 for 1 h at 37˚C, followed by washing and resuspension in IMDM containing 5 μg/ml gentamicin (Cellgro) to kill any remaining extracellular bacteria. Infected cells were incubated at 37˚C, and aliquots were harvested for analysis at 30-min intervals.

To identify Listeria-derived SINVFEKl, we incubated macrophages in 2.4G2 supernatant, followed by staining with Alexa Flour 647 (AF647; Invitrogen)–conjugated 25-D1.16 mAb (10). Flow cytometry was used to identify infected cells using GFP fluorescence. In addition, the mean fluorescence intensity (MFI) of AF647 was used to quantify surface Kβ-SINVFEKL expression in GFP+ cells.

**Flow cytometry**

Flow cytometry was performed using an LSRII or LSRFortessa cytometer (Becton Dickinson). Phenotypic analysis of cultured fetal liver-derived macrophages was achieved by preincubation with 2.4G2 supernatant followed by staining anti-CD11b–APC/Cy7 and anti–F4/80-PE Abs (BioLegend). Analysis of thioglycollate-elicited and bone marrow–derived macrophages was performed using anti-CD45.1-APC/Cy7, anti–CD11b–APC, and anti–F4/80–PE Abs (BioLegend). For macrophage phenotyping and analysis of phagocytic activity, at least 1 × 10^6 live cell events were recorded. Analysis was performed using FlowJo software (Tree Star). Data were analyzed using Student r test, and no significant differences (p < 0.05) were found.

**Results**

**Derivation of Cx43-deficient macrophages from cultured fetal liver and bone marrow cells**

Cx43-deficient (Cx43−/−) mice die soon after birth, making the generation of macrophages from bone marrow impractical (11). Therefore, we used several approaches to obtain WT and Cx43−/− macrophages: 1) derivation from hematopoietic cells harvested from fetal livers, 2) the harvest of thioglycollate-elicited peritoneal macrophages from radiation chimeric mice consisting of irradiated WT animals reconstituted with WT or Cx43−/− fetal liver cells, and 3) production of bone marrow–derived macrophages from radiation chimeric animals.

After 7–10 d in culture in the presence of M-CSF–containing media, fetal liver–derived cells from WT and Cx43−/− fetuses were found to be predominantly macrophages, as demonstrated by their expression of the macrophage markers CD11b and F4/80 (Fig. 1A). Similarly, macrophages prepared in an identical manner from bone marrow cells collected from radiation chimeric mice were overwhelmingly both donor derived, as identified by the marker CD45.1, and expressed both CD11b + and F4/80 + (Fig. 1B). Importantly, no discrepancy in the proportion of positively stained cells was observed between WT or Cx43−/− macrophages.

**Cx43-deficient macrophages phagocyte sRBCs**

We next sought to characterize the phagocytic capacity of WT and Cx43−/− macrophages. First, the Fcγ receptor–mediated phagocytic capacity of macrophages was assayed using IgG-opsonized sRBCs as a target. WT and Cx43−/− fetal liver–derived macrophages were equally capable of sRBC uptake (Fig. 2A). Importantly, there was no difference in fluorescence intensity between these two populations (MFI = 54017 ± 2122 WT versus 54899 ± 2018 Cx43−/−), as is evidenced by the overlapping fluorescence histograms. The vast majority (94.6% WT versus 95.6% Cx43−/−) of cultured macrophages from both genotypes were positive for uptake of sRBCs after 20 min of incubation (Fig. 2B), increasing to nearly 100% of cells after 60 min (data not shown). As evidence that this uptake was FcR dependent, nonopsonized sRBCs were taken up at a rate ∼20-fold less (<5% of cells at 20 min) than that of opsonized targets.

The phagocytic capacity of WT and Cx43−/− fetal liver-derived macrophages after 20 min of incubation with sRBCs was also quantified microscopically. The resulting histograms demonstrate the phagocytic capabilities of WT and Cx43−/− macrophages were comparable (WT versus Cx43−/−: 5.5 ± 0.3 versus 6.1 ± 0.2 particles/cell; Fig. 2C). A similar assay was also performed on thioglycollate-elicited macrophages from radiation chimeric animals and again, no major difference in phagocytic ability was observed (WT versus Cx43−/−: 4.3 ± 0.4 versus 3.6 ± 0.3, on average; Supplemental Fig. 1).

Bone marrow–derived macrophages were produced from radiation chimeric mice reconstituted with WT or Cx43−/− fetal liver cells. These cells were >99% donor derived. Both WT and Cx43−/− macrophages were exposed to fluorescently labeled opsonized sRBCs and were phagocytic. This phagocytosis was dependent on time and target concentration. Both populations exhibited identical mean fluorescence intensities at 20-, 40-, and 60-min time points when treated with a specific amount of sRBCs. This identity was observed at a particle/macrophage ratio of 100:1, as well as 10:1 (Fig. 3).

**Cx43-deficient macrophages can phagocytose zymosan and the resultant phagosomes acidify normally**

We next sought to measure the ability of Cx43−/− fetal liver-derived macrophages to phagocytose zymosan, another commonly used target for in vitro phagocytosis. In contrast with targets opsonized with IgG, uptake of zymosan by macrophages is mediated not by FcRs, but by several different receptors, including...
Analysis of Kb-SIINFEKL was limited to live GFP+ cells only. One representative experiment of three is shown.

To confirm that Cx43 is not required for phagocytosis of zymosan, we next treated WT and Cx43−/− macrophages with zymosan particles labeled with pHrodo. pHrodo is a pH-sensitive dye that increases in fluorescence intensity several fold in response to decreasing pH, as occurs during the acidification of a maturing phagosome. Again, the majority of cells were found to be positive for pHrodo-zymosan after 60 min of incubation at 37°C (98.6% of WT versus 98.9% of Cx43−/−; Fig. 5). Importantly, the dramatic increase (WT versus Cx43−/−: 71.1- versus 72.2-fold) in pHrodo fluorescence at 37°C as compared with cells incubated at 4°C indicates that the zymosan particles were successfully incorporated into maturing phagosomes. No significant difference in the percentage of pHrodo-zymosan–positive cells or in the fluorescence intensity of live cells was observed between WT and Cx43−/− fetal liver-derived macrophages. Together with the results using DL649-zymosan, this is strong evidence that Cx43 is dispensable for the phagocytosis of zymosan particles.

Cx43-deficient macrophages are able to phagocytose Listeria and present Listeria-derived Ags on MHC class I molecules

We next measured the ability of Cx43−/− macrophages to phagocytose a physiologically relevant target: pathogenic bacteria. We chose the Gram-positive facultative intracellular bacterium Listeria for these assays. This is an ideal target because immunity to Listeria is dependent on phagocytosis and subsequent killing by activated macrophages (14). For these studies, we used a recombinant Listeria strain (Lm-PASFLAG) that expresses a nonsecreted form of GFP as well as a secreted protein that includes the model MHC class I binding peptide SIINFEKL. The nonsecreted GFP was used to identify macrophages that have internalized Listeria, whereas the secreted SIINFEKL-containing recombinant protein was used to measure the ability of macrophages to process and present foreign Ags (7). Notably, internalization of Listeria in this model system

FIGURE 5. Fetal liver-derived macrophages from WT and Cx43−/− mice can phagocytose zymosan and are capable of phagosome acidification. (A) WT (black line) and Cx43−/− (gray line) macrophages were incubated with zymosan particles labeled with pHrodo for 60 min at 4°C (left panel) or at 37°C (right panel; shaded black peak represents macrophages not treated with zymosan). (B) Percentage of cells positive for labeled zymosan after incubation at 4°C (left panel) or at 37°C (right panel). (C) MFI of pHrodo signal in macrophages incubated at 4°C (left panel) or at 37°C (right panel; averages from three experiments, error bars depict SEM).

FIGURE 6. Cx43-deficient macrophages are capable of phagocytosis of L. monocytogenes and presentation of a foreign Ag. (A) Flow cytometry histogram of gated live cells demonstrating engulfment of GFP+ Listeria at 90 min postinfection; black line represents Listeria uptake by WT macrophages and gray line represents uptake by Cx43−/− cells. (B) Percentage of WT and Cx43−/− macrophages positive for GFP fluorescence at 90 min postinfection (graph representative of three independent experiments). (C) Kinetics of surface Kb-SIINFEKL production in WT (circle) and Cx43−/− (square). Analysis of Kb-SIINFEKL was limited to live GFP+ cells only. One representative experiment of three is shown.
requires phagocytosis, because mice lack the receptors required for nonphagocytic uptake of these bacteria (15). WT and Cx43−/− macrophages were infected with Listeria and analyzed by flow cytometry. No differences were observed in either the percentage of cells positive for Listeria (63.1% in WT versus 63.3% in Cx43−/−; Fig. 6B) or in the number of Listeria ingested, as displayed in the histogram of GFP fluorescence (Fig. 6A). There was also no difference in the kinetics of surface expression of K-β2MIF and Cx43−/− cells. This suggests that there are no major differences in the abilities of WT- and Cx43−/− macrophages to ingest Listeria and process Listeria-derived proteins for presentation on MHC class I molecules.

Discussion
In contrast with a previous report (5), we provide evidence in this article that modulation of Cx43 levels does not have a major impact on macrophage phagocytosis of opsonized rRBC, zymosan particles, or Listeria. The discrepancies between the results reported by Anand et al. (5) and this study may lie in the nature of the model systems studied.

Because animals unable to express Cx43 die soon after birth, the direct harvest of knockout macrophages or the culture of bone marrow–derived macrophages is essentially impossible (11). To circumvent this issue, Anand et al. (5) harvested mouse fetal livers as a source of macrophages. In their protocol, macrophages were selected by adherence to coverslips, based on the method of Morris et al. (16). These resident fetal cells were identified as mostly (>95%) macrophages based on expression of CD45. Although it was used as a macrophage marker in the study by Anand et al. (5), it is widely accepted that CD45, also known as leukocyte common Ag, is present on many cell types derived from hematopoietic cells.

That the adherent population of fetal liver cells contains such a high proportion of macrophages is at odds with Morris et al. (16), who found that the resident fetal liver population was composed of only 50% macrophages, based on the more specific marker F4/80. Our own analysis of this adherent, resident cell population from fetal liver found that only a limited number of the cells could be characterized as macrophages, making analysis of phagocytosis by flow cytometry impossible. Therefore, it is possible that the results from Anand et al. (5) can be explained by contamination of cultures by nonmacrophage constituents of fetal livers. It is also not clear whether there may be a difference in the maturation of resident macrophages within the fetal liver of a Cx43−/− mouse.

In the model system used in this study, pluripotent cells derived from fetal livers or bone marrow harvested from radiation chimeric mice were differentiated to the monocyte/macrophage lineage by culture in an M-CSF–containing medium. Thus, our system has the advantage that cells from WT and Cx43−/− fetuses were cultured under controlled and identical conditions. This resulted in a population of cultured cells from WT and Cx43−/− fetuses that were >95% macrophages, as assessed by the markers CD11b and F4/80 (Fig. 1). Although CD11b is found on a range of myeloid cell types, F4/80 is specific for murine macrophages (17). The technique used in this study for deriving macrophages in this manner is based on an established protocol for the production of macrophages from bone marrow suspensions that has been adapted to the use of fetal livers for studying Cx43 deletion caused by perinatal lethality (18, 19). Under these conditions, cells displayed typical phenotypic macrophage properties with no defect in phagocytosis.

Phagocytosis is essential for the survival of metazoan species. In this study, mice were generated with immune cells lacking Cx43 through the use of radiation chimeric animals (4). Recipients of Cx43−/− fetal liver cells appeared healthy overall. Peritoneal macrophages elicited by injection of thioglycollate broth into animals reconstituted by WT and Cx43−/− donors were >98% donor derived and had similar proportions of CD11b+ cells. Furthermore, peritoneal macrophages from WT and Cx43−/− donors were equally capable of phagocytosis (Supplemental Fig. 1). In previous experiments with Cx43−/− chimeric mice, animals have survived for prolonged periods, in some cases, >6 mo, without morbidity or mortality; a feat that would not be expected of animals that have been immunocompromised by reconstitution with poorly phagocytic macrophages. That result and the data presented in this article clearly demonstrate that there is no primary role for Cx43 in macrophage phagocytosis and phagosome maturation.

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Disclosures
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