Gap Junction-Mediated Intercellular Communication between Dendritic Cells (DCs) Is Required for Effective Activation of DCs

Hiroyuki Matsue, Jian Yao, Keiko Matsue, Akiko Nagasaka, Hideaki Sugiyama, Rui Aoki, Masanori Kitamura and Shinji Shimada

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Gap junctions, formed by members of the connexin (Cx) family, are intercellular channels allowing direct exchange of signaling molecules. Gap junction-mediated intercellular communication (GJIC) is a widespread mechanism for homeostasis in organs. GJIC in the immune system is not yet fully understood. Although dendritic cells (DC) reportedly form cell-to-cell contact between DCs in nonlymphoid and lymphoid organs, GJIC between DCs remains unknown. In this study we examined whether DCs form GJIC. XS52 and bone marrow-derived DCs (BMDCs) were tested for GJIC by counting intercellular transfer of Lucifer Yellow microinjected into a cell. Either DC became effectively dye-coupled when activated with LPS plus IFN-γ and TNF-α. These results indicate that cell-to-cell contact and GJIC are required for effective DC activation. In addition, heptanol significantly inhibited the LPS- plus IFN-γ-induced up-regulation of the other costimulatory (i.e., CD80 and CD86) and MHC class II molecules expressed by BMDCs, and it significantly reduced their allostimulatory capacity. Among Cx members, Cx43 was up-regulated in dye-coupled BMDCs, and Cx mimetic peptide, a blocker of Cx-mediated GJIC, significantly inhibited the dye-coupling and activation, suggesting the involvement of Cx43. Thus, our study provides the first evidence for GJIC between DCs, which is required for effective DC activation. The Journal of Immunology, 2006, 176: 181–190.

Dendritic cells (DC) are widely distributed in the peripheral and lymphoid organs and are central in initiating immune responses and maintaining tolerance (1, 2). DCs are characterized by their highly potent capacities to 1) take up Ags and process them into proteolytic peptides; 2) load these peptides onto MHC class I and class II molecules; 3) migrate to T cell areas in draining lymph nodes (LNs); 4) deliver full T cell stimulation signals, including costimulatory signals; and 5) secrete T cell stimulatory and other immunoregulatory factors. Thus, DCs are well equipped with all the phenotypic and functional properties that are required for effective Ag presentation to the immune system (3). Each process has been studied extensively in both in vitro and in vivo experimental systems, and much has been learned about molecular mechanisms of Ag processing by DCs (4, 5); DC-dependent, Ag-specific T cell activation (6); and Ag-specific, T cell-dependent DC activation (7, 8). In particular, recent accumulating evidence indicates that the immunological synapse is an essential interface between DCs and T cells for Ag-specific bidirectional interactions (6, 9). However, little knowledge has been gained of DC-DC interactions during each process, presumably because no intensive attention has been focused on the interactions. Considering the fact that cell-to-cell contact occurs unavoidably in in vitro studies (i.e., conventional liquid cultures of DCs alone or DCs together with T cells), one can imagine that signal transductions associated with direct cell-to-cell contact between DCs may be involved in their biological activities. In addition, a recent elegant study using two-photon tissue-imaging techniques discovered the dynamics of DC-DC interactions in LNs of living mice. Indeed, this study revealed that resident steady-state DCs are enmeshed and occasionally clustered in LNs, and that immigrant mature DCs are integrated into such networks of DCs, where they encounter and search for Ag-specific T cells (10). Thus, physical DC-DC interactions are not artificial and inescapable consequences of carrying out studies in vitro, but, rather, they seem to have physiological relevance to the in vivo immune system.

Among several modes of cell-to-cell contact, gap junctions, which are formed by members of the connexin (Cx) family, are unique intercellular channels connecting adjacent cells to enable the passage of small molecules (<1000 Da) (11). These include various signaling molecules (e.g., cAMP, reactive oxygen species, and Ca2+). This process, termed gap junction-mediated intercellular communication (GJIC), is a widespread mechanism for the maintenance of homeostasis in tissues and organs. In the immune system, the existence and physiological role of GJIC between several types of immune cells have been reported (12, 13). Indeed,
many immune cells (e.g., T cells, B cells, macrophages, mast cells, and follicular DCs) have been found to form homotypic interactions with themselves or heterotypic interactions with other cell types via gap junctions (12, 13). With respect to functional aspects of GJIC, it is considered to be involved in hematopoiesis (14), T cell development in the thymus (15), inflammation (16), transendothelial migration of macrophages (16) and neutrophils (17), and Ab formation in the germinal center (18, 19). Very recently, Neijssen et al. (20) have revealed a novel mechanism of cross-presentation via GJIC. They found that activated human monocytes take up antigenic peptides and cross-present these peptides to Ag-specific CTLs by obtaining the peptide from nonlymphoid cells through gap junctions.

Only a few circumstantial findings have been reported about gap junctions and/or Cx expression in various sets of DC types. In fact, follicular DCs, although not T cell-priming DCs, have been reported to up-regulate Cx43 expression and formation of gap junctions between each other or with B cells in the lymphoid germinal center upon antigenic challenge (18, 21). Thus, it has been proposed that these direct cell-to-cell interactions may play a role in synchronizing germinal center events during B cell maturation in response to antigenic challenge (18, 21). Among T cell-priming DCs, Cx43 expression by a specialized subset of DCs in the human epidermis (Langerhans cells) and DCs in the human appendix have been recently reported (20). In addition, gap junction-like structures between human Langerhans cells and T cells have been observed ultrastructurally in vitro and in vivo (22–24). However, GJIC between DCs has not yet been demonstrated. In this study we focus on homocellular GJIC of DCs and report that DCs form functional gap junctions between themselves only in response to defined stimuli that are required for effective DC activation.

**Materials and Methods**

**Animals**

C57BL/6 and BALB/c mice (6- to 8-wk-old females) were purchased from SLC Japan and maintained in the Animal Research Center facilities at University of Yamanashi. All animal experiments were approved by the institutional review board.

**Reagents and Abs**

We purchased LPS (Esherichia coli 0111:B4), Lucifer Yellow, heptanol, and rabbit anti-Cx43 Ab from Sigma-Aldrich and mouse rGMS-CSF from PeproTech. Mouse TNF-α, IFN-γ, anti-mouse TNF-α, and its control IgG were purchased from R&D Systems. The other mAbs were purchased from BD Pharmingen. Phosphorothioate-stabilized CpG oligodeoxynucleotide (ODN; TCCATGACGTTCCTGATGCT) was synthesized by Qiagen according to the sequences described by Hemmi et al. (25).

**Cell preparations**

XS52 is a long-term DC line established from the epidermis of a newborn BALB/c mouse (26); it was provided by Dr. A. Tashiro (University of Texas Southwestern Medical Center, Dallas, TX). XS52 DCs were maintained and expanded in the standard growth medium, i.e., complete RPMI 1640 supplemented with GM-CSF (2 ng/ml) and NS47 fibroblast culture supernatant (SUP; 5%) as previously described (27), and their phenotypic and functional features have been previously described (7, 26, 28, 29). Bone marrow-derived DCs (BMDCs) were generated from C57BL/6 mice in complete RPMI 1640 supplemented with 10 ng/ml GM-CSF as described previously (30). The resulting population containing >95% CD11c+ cells (DC preparations) was used on days 8–10 without additional purification. CD4+ T cell subsets were isolated from BALB/c splenocytes using the Mouse T Cell CD4 Subset Column Kit (R&D Systems) according to the manufacturer’s protocol, and the purity of the enriched CD4+ cells was >90%.

**Detection of GJIC**

GJIC was assessed by transfer of a membrane-impermeant fluorescent dye after a single cell microinjection with an automated microinjection system (Eppendorf) as described previously (31–33). Briefly, XS52 DCs and BMDCs (1 × 10⁶ cells/dish) in 35-mm culture dishes were stimulated for 24 h in the presence or the absence of LPS (100 ng/ml) and/or IFN-γ (1 or 30 ng/ml). In some experiments we added heptanol (0.5 mM), an inhibitor of GJIC, or vehicle, or heptanol (0.4% ethanol); a neutralizing anti-TNF-α Ab (1 μg/ml) or control Ab; or TNF-α (1 ng/ml) and/or IFN-γ (1 ng/ml). In other experiments BMDCs were stimulated for 24 h with 1 μM CpG ODN in the presence of either anti-TNF-α Ab (1 μg/ml) or control Ab. After washing cells with culture medium and adding new medium, adherent cells were microinjected with a mixture of Lucifer Yellow (10% in 3.5 mM lithium chloride) using an automated microinjection system at pressures of 500 hectopascals applied for 0.5 s. Immediately after microinjection, cells were observed under a fluorescence microscope to determine whether dye transfer had occurred. The incidence of dye-coupling was calculated by dividing the number of injected cells showing dye transfer to more than one neighboring cell by the total number of cells injected (a minimum of 10 per condition). Each dish was counted 100. For statistical evaluation, three or four dishes cultured under each condition were examined. Likewise, using a similar experimental design, BMDCs were tested for dye-coupling.

**Phenotypic analyses**

XS52 DCs (1 × 10⁶ cells/ml/well; 24-well plates) were stimulated for 24 h with or without LPS (100 ng/ml) and/or IFN-γ (1 ng/ml) in the presence or the absence of the indicated concentrations of heptanol. Vehicle (0.4% ethanol) was added to the experimental panels without heptanol. In some experiments, anti-TNF-α Ab (1 or 5 μg/ml) or control Ab was added. The samples were then examined for the expression of CD40 using FACScanLibur (BD Immunocytometry Systems). The viabilities of DCs were assessed by measuring propidium iodide (PI) uptake by DCs. We established a no-cell-to-cell contact condition by seeding cells in T75 flasks at a low cell density (5 × 10⁴ cells/ml) and then stimulating them for 24 h with or without LPS (100 ng/ml) plus IFN-γ (1 ng/ml) in the presence or the absence of 0.5 mM heptanol. Cells were examined for surface expression of CD40 as described above. Likewise, using a similar experimental design, BMDCs were examined for the expression of CD40 in CD11c− populations. To evaluate the effects of GJIC on CD80, CD86, and I-Ab expression by BMDCs, cells (1 × 10⁶ cells/ml/well) were simultaneously stimulated for 5 h with or without LPS (100 ng/ml) plus IFN-γ (1 ng/ml) in the presence of vehicle alone (0.4% ethanol) or with heptanol (2.5 mM). Cells were then examined for the surface expression of CD80, CD86, and I-Ab as described above. The viabilities of DCs, assessed by measuring PI uptake, were not significantly different between each condition.

**Allo-MLR assay**

We wanted to evaluate the impact of GJIC between DCs on Ag-presenting capacity. We therefore avoided the putative effects of GJIC between DCs and T cells or between T cells by using fixed DCs (after stimulation) as stimulator cells. To do so, BMDCs derived from C57BL/6 mice (1 × 10⁶ cells/ml/well; 24-well plates) were stimulated for 5 h with or without LPS (100 ng/ml) plus IFN-γ (1 ng/ml) in the presence of vehicle alone (0.4% ethanol) or with heptanol (2.5 mM). Cells were added to 50 µl/well of Dulbecco’s PBS (Ca²⁺ and Mg²⁺ free) were washed twice with HBSS and then fixed with 2% paraformaldehyde in HBSS on ice for 20 min. After extensive washing the cells, the fixed DCs were used as stimulator cells. In a one-way allo-MLR assay, CD4+ T cells freshly purified from BALB/c splenocytes using the Mouse T Cell CD4 Subset Column Kit (R&D Systems; 1 × 10⁵ cells/well) were cocultured with the fixed DCs (1 × 10⁶ cells/well) described above for 5 days. Proliferative responses were measured by [3H]thymidine uptake (1 μCi/well) in the last 8 h.

**Experiments using GAP27, a Cx mimetic peptide**

GAP27 (SRPTKETIFH) (19), a synthetic peptide corresponding to a sequence in the second extracellular loop of Cx43, and a control peptide termed Scramble (SRTRFPTEK) was designed by a randomly shuffled sequence of GAP27, were synthesized by Qligen. The purity of both peptides assessed by HPLC was >98%. None of the reagents contained detectable amounts of endotoxin as tested by Endosafe-PTS (Charles River Japan). Peptides were dissolved in a minimum volume of DMSO and added to cell cultures as described below. No effect of the solvent added (0.5%) was observed. XS52 DCs and BMDCs were stimulated with LPS (100 ng/ml) plus IFN-γ (1 ng/ml) for 23 h. After washing the cells with growth medium, GAP27 or Scramble peptide (50 µg/ml) was added 1 h before 0.02% EDTA and PI dye transfer assay. To examine the effects of the peptides on CD40 expression, the peptides were added simultaneously when cells were stimulated for 24 h with LPS or LPS plus IFN-γ.
Quantitative real-time PCR

The relative expression of Cx43 mRNA under each condition was determined by real-time PCR using the ABI PRISM 5500 Sequence Detection Systems (Applied Biosystems) with SYBR Green I dye (Qiagen) according to the manufacturers’ instructions. XS52 DCs (1 x 10⁶ cells/ml) and BMDC (1 x 10⁶ cells/ml) were cultured for 8 h in the presence or the absence of LPS (100 ng/ml) and/or IFN-γ (1 ng/ml). Total RNA was isolated using TRIzol (Invitrogen Life Technologies), and cDNA was synthesized using a SuperScript system (Invitrogen Life Technologies). Primers corresponding to each mouse Cx43 and G3PDH were designed using Primer Express software (Applied Biosystems) as follows: 5’-CAATTCCGCGCCCAATCC-3’ and 5’-TTTGCTGCCTGTAATGGC-3’ for Cx43, and 5’-CGGTTCTTCCGCCACCTT-3’ and TGTCACTTTG GCAAGGTTTCT for G3PDH. Cycle threshold numbers (Ct) were derived from the exponential phase of PCR amplification. Fold differences in the expression of gene x in the cell populations y and z were derived by 2^(-ΔΔCt), where k = (Ct - CxG3PDH) y - (Ct - CxG3PDH) z.

Western blot analysis

Western blotting was performed as described previously (33). Briefly, equal amounts of cell lysates were separated by 10% SDS-polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membranes. After blocking with 3% BSA in PBS, the membranes were incubated with anti-Cx43 Ab. After washing with PBS-0.1% Tween 20, filters were probed with HRP-conjugated sheep anti-rabbit IgG or rabbit anti-mouse IgG. Immunoreactivity was detected by the ECL system (Amersham Biosciences). To assess the amount of Cx43 protein loaded, filters were treated with 2% SDS and 100 mM 2-ME in 62.5 mM Tris-HCl (pH 6.8) for 30 min at 60°C and reprobed for β-actin. Films were scanned, and the ODs of the bands were measured with Image (Scion).

Statistical analyses

Comparisons between two groups were performed using Student’s t test. Statistical evaluations of dye-coupling experiments are described above. Each experiment was repeated at least twice to assess reproducibility.

Results

GJIC between XS52 DCs in response to defined stimuli

Although freshly isolated human monocytes/macrophages have recently been reported to develop functional gap junctions after treatment with LPS plus IFN-γ or TNF-α plus IFN-γ (16), the possible existence of GJIC between DCs remained undetermined. Therefore, we examined whether DCs would form functional gap junctions. To test this, we first performed dye-coupling experiments using a Langerhans cell-like DC line, XS52, established from mouse epidermis. XS52 DCs were cultured with their growth medium at a relatively high density (1 x 10⁶ cells/35-mm culture dish) and then tested for intercellular dye transfer. A, Representative dye-uncoupled and dye-coupled XS52 DCs. No dye transfer occurred without LPS and IFN-γ, whereas dye transfer to adjacent cells occurred in response to LPS and IFN-γ. Arrows in microscopic phase-contrast images indicate cells that were microinjected with Lucifer Yellow. B, The data shown are the mean ± SD (n = 3 dishes) incidence of dye-coupling (percentage) from a minimum of 10 microinjected cells/dish and are representative of three independent experiments. *, p < 0.05; **, p < 0.01 (brackets indicate the groups compared).

Impact of a gap junction inhibitor on LPS- plus IFN-γ-induced activation of XS52 DCs

To explore the functional aspects of GJIC between DCs, we examined the effect of a GJIC inhibitor on CD40 expression (as a representative of activation markers) by DCs. Consistent with a previous report (36), XS52 DCs cultured in their growth medium...
only marginally express CD40, representing an immature phenotype of DCs (Fig. 3, A and B). LPS significantly enhanced CD40 expression, and LPS plus IFN-γ further augmented CD40 expression synergistically (Fig. 3A). Heptanol significantly inhibited CD40 expression induced by LPS plus IFN-γ (up to 80%) in a dose-dependent manner (Fig. 3, B and C), suggesting that the disruption of GJIC between DCs inhibited the synergistic effects of LPS and IFN-γ on their CD40 expression. Because anti-TNF-α Ab inhibited dye-coupling between DCs induced by LPS plus IFN-γ (Fig. 2B), we hypothesized that anti-TNF-α Ab can also inhibit LPS- plus IFN-γ-induced up-regulation of CD40 expression by disrupting GJIC between DCs. Indeed, CD40 expression augmented by LPS plus IFN-γ was significantly inhibited by anti-TNF-α Ab (Fig. 3D). Importantly, anti-TNF-α Ab had no significant effect on CD40 expression induced by LPS alone, indicating that DC-derived TNF-α is required for synergistic augmentation of CD40 expression induced by LPS and IFN-γ. Taken together, these results suggest that synergistic up-regulation of CD40 expression by XS52 DCs is mediated by GJIC induced by IFN-γ plus TNF-α that is secreted by the cells in response to LPS.

**Impact of cell-to-cell contact on activation of XS52 DCs**

To explore the functional significance of GJIC between DCs, we stimulated individual XS52 DCs with LPS (100 ng/ml) plus IFN-γ (1 ng/ml) under conditions that prevented cell-to-cell contact. To achieve this condition, we cultured XS52 DCs at a very low cell density, in which most cells adhered to the culture plate with few, if any, cell-to-cell contacts (Fig. 4A, photograph). Interestingly, under this condition, LPS plus IFN-γ only modestly induced CD40 expression by XS52 DCs, whereas the same stimuli robustly induced CD40 expression when there was significant cell-to-cell contact (Fig. 4B). Again, heptanol inhibited this LPS- plus IFN-γ-induced CD40 up-regulation (Fig. 4B), but had no effect on the modest CD40 expression without cell-to-cell contact (Fig. 4A). These results suggest that cell-to-cell contact and GJIC are required to activate DCs synergistically in response to LPS plus IFN-γ. Alternatively, soluble factors (i.e., TNF-α) secreted by XS52 DCs in response to LPS may be lower than the levels required to synergistically stimulate DC together with IFN-γ, because secreted TNF-α may become diluted under low cell density conditions. To exclude this possibility, XS52 DCs at a low or high cell density, as indicated in Fig. 4, A and B, were cultured for 24 h with high cell density SUPs, which were collected 24 h after these cells at a high cell density had been stimulated in the presence of LPS (100 ng/ml) plus IFN-γ (1 ng/ml). Under the condition without cell-to-cell contact, XS52 DCs that were cultured with the SUP only modestly up-regulated their CD40 expression. This up-regulation was at a comparable level to that of the cells stimulated by LPS plus IFN-γ (Fig. 4C, upper panel). In sharp contrast, under cell-to-cell contact conditions, the SUPs markedly augmented CD40 expression by XS52 DCs. This augmentation was comparable to the response of cells with cell-to-cell contact to LPS plus IFN-γ (Fig. 4C, lower panel). Therefore, it is likely that direct cell-to-cell contact is required for the synergistic activation of DCs, although we cannot exclude the possibility of contributions from unstable soluble factor(s) generated by stimulated DCs.

**GJIC between BMDCs**

To broaden our observations obtained using a DC line, in the next sets of experiments, we determined whether BMDCs also form functional gap junctions. As shown in Fig. 5A (left panel) and B, BMDCs failed to form significant dye-coupling without stimuli or in response to a single stimulation with LPS, IFN-γ, or TNF-α. In sharp contrast, when BMDCs were stimulated with LPS plus IFN-γ or TNF-α plus IFN-γ, significant dye-coupling (30–35%) was observed (Fig. 5, A (right panel) and B). Anti-TNF-α Ab significantly inhibited LPS- plus IFN-γ-induced dye-coupling between BMDCs, whereas control IgG had no effect (Fig. 5C). Similarly, anti-TNF-α Ab inhibited CpG ODN- plus IFN-γ-induced dye-coupling (Fig. 5D). These results indicated that BMDCs also form GJIC in response to the other combination of stimuli (i.e., CpG ODN plus IFN-γ) in addition to the same stimuli that induced GJIC between XS52 DCs, and that TNF-α, which is secreted by
BMDCs in response to both combinations of stimuli, is required for GJIC. Indeed, TNF-α was detected in the SUPs from BMDCs stimulated with LPS plus IFN-γ (6.5 ± 0.45 or 4.3 ± 0.32 ng/ml, respectively). We next asked whether BMDCs, like XS52 DCs, are effectively activated by LPS plus IFN-γ only when there is cell-to-cell contact. Individual cell stimulation modestly up-regulated CD40 expression, and heptanol did not affect this up-regulation (Fig. 5E, upper panel). In contrast, cell-to-cell contact was required to effectively up-regulate CD40 expression by BMDCs in response to LPS plus IFN-γ, and heptanol significantly inhibited the up-regulation (Fig. 5E, lower panel). In summary, these observations indicate that BMDCs, like XS52 DCs, form GJIC, and that cell-to-cell contact and GJIC are required for effective DC activation.

Impact of GJIC between DCs on Ag presentation

Examining the expression of CD40 as a representative of costimulatory molecules, we found that its expression by DCs is regulated by GJIC between DCs under cell-to-cell contact conditions (Figs. 4C and 5E). We then asked whether the other important costimulatory molecules, which are essential for Ag presentation, are also regulated by GJIC. Under the cell-to-cell contact condition, heptanol significantly inhibited up-regulation of the other costimulatory molecules (i.e., CD80 and CD86) and MHC class II molecules (I-Ab) that was induced by LPS plus IFN-γ (Fig. 6A). These results suggest that GJIC between DCs may be involved in regulating the magnitudes of costimulatory signals under conditions in which GJIC between DCs are formed.

We then asked whether GJIC between DCs affects alloantigen presentation. To address this, we performed one-way MLR using C57BL/6-derived BMDCs as APC and BALB/c-derived CD4+ T cells as responder T cells. To avoid contributions of putative GJIC between DCs and T cells or between T cells and to solely evaluate the impact of GJIC between DCs, BMDCs were fixed with paraformaldehyde after LPS plus IFN-γ stimulation with or without heptanol. As shown in Fig. 6B, LPS- plus IFN-γ-stimulated BMDCs induced marked proliferation of CD4+ T cells isolated from an allogeneic strain compared with BMDCs receiving no
BMDCs constitutively expressed Cx43 mRNA (analyzed by RT-PCR), and its expression was up-regulated (8-fold) in response to LPS (determined by quantitative real-time RT-PCR). However, IFN-γ had no significant effect on the level of LPS-induced Cx43 mRNA expression (Fig. 7A). Reflecting the regulation of Cx43 mRNA, Cx43 proteins were detected in BMDCs without stimuli, and the significant up-regulation of its phosphorylated form was observed in cells stimulated with LPS. IFN-γ seemed to slightly dampen this up-regulation (Fig. 7B). These results suggested that the regulation of Cx43 expression in response to these stimuli may be linked to functional GJIC between BMDCs.

**Impact of a Cx mimetic peptide on GJIC between DCs**

To explore whether Cxs are involved in GJIC between DCs, we used a Cx mimetic peptide, GAP27 (SRPTEKTFII), whose amino acid sequence corresponds to part of the extracellular loop 2 of Cx43 (35). This peptide includes the sequence SRPTEK, which is present in most Cxs (35). Therefore, GAP27 has been widely used to inhibit GJIC as a relatively universal gap junction inhibitory peptide, which inhibits not only Cx43-mediated GJIC, but also the other Cx-mediated GJIC (35). As a control, we synthesized a peptide (termed Scramble) consisting of all the amino acids of GAP27, but with their order scrambled. As shown in Fig. 8A, GAP27, but not Scramble, significantly inhibited LPS- plus IFN-γ-induced dye-coupling in both DCs, indicating that Cxs are involved in GJIC between DCs. We next examined the functional effects of GAP27 on CD40 expression by DCs. Under conditions with cell-to-cell contact of XS52 DCs or BMDCs, GAP27 did not inhibit the moderate LPS-induced CD40 expression (Fig. 8B). In contrast, GAP27 significantly, but moderately, inhibited LPS plus IFN-γ-induced up-regulation of CD40 expression by XS52 DCs, and it completely inhibited the up-regulation by BMDCs to the level of CD40 up-regulation by LPS alone (Fig. 8B), whereas Scramble had no effect. These results more specifically confirmed that Cxs are involved in GJIC-mediated activation of DCs. Considering the CD43 expression by DCs, GJIC between BMDCs seems to be associated with Cx43 expression.

**Discussion**

The present study provides in vitro evidence for the existence of functional gap junctions between DCs, which occur only in response to specific combinations of defined stimuli (i.e., LPS plus IFN-γ or TNF-α plus IFN-γ; Figs. 1, 2, and 5). When LPS plus IFN-γ were used to induce GJIC between DCs, DC-derived TNF-α was required for GJIC between DCs, indicating that LPS- plus IFN-γ-induced GJIC is mediated by TNF-α secreted by DCs in response to LPS (Figs. 2 and 5). In addition, other combinations of stimuli (i.e., CpG ODN plus IFN-γ) induced GJIC between DCs, and this was also mediated by DC-derived TNF-α (Fig. 5D). With respect to functional aspects, cell-to-cell contact and GJIC were required for the synergistic induction of CD40 expression by LPS plus IFN-γ or TNF-α plus IFN-γ. This suggests that GJIC plays a role in synergistic DC activation by the combinations of defined stimuli (Figs. 3–5). In addition, the expression of the other important costimulatory molecules (i.e., CD80 and CD86) and MHC class II molecules appeared to be similarly regulated by GJIC between DCs, affecting their costimulatory capacity (Fig. 6). We also found that Cx43, at least, was involved in GJIC between DCs (Figs. 7 and 8). To our knowledge, this is the first report documenting GJIC between DCs.

We detected marginal dye-coupling between DCs under control conditions or when the cells were given a single stimulus. These
observations were consistent with a previous study that documented no functional gap junctions formed between mouse peritoneal macrophages or human monocytes/macrophages in response to a single stimulus (e.g., LPS or IFN-γ) (16, 37). Conceivably, under steady-state conditions, DCs as well as macrophages in nonlymphoid and lymphoid tissues may not form homocellular GJIC. Considering the dynamic movements of DCs in enmeshed networks of cells observed in nonlymphoid tissue (e.g., Langerhans cells) (38) and lymphoid tissue (e.g., LNs) (10), one may consider that GJIC between steady-state DCs might be shut off. To form efficient homocellular GJIC between DCs, we found that two humoral factors (i.e., LPS plus IFN-γ or TNF-α plus IFN-γ) are the minimal required stimuli. The LPS- plus IFN-γ-induced dye-coupling between X552 DCs or between BMDCs was almost completely inhibited by a neutralizing anti-TNF-α Ab, and TNF-α plus IFN-γ mimicked the effect of LPS plus IFN-γ. Thus, LPS- plus IFN-γ-induced dye-coupling between DCs was mediated by the autocrine action of DC-derived TNF-α. LPS is the best-described microbiobially derived inducer of inflammation (39) and is also the most widely used activator of DCs. It has been reported that IFN-γ synergistically enhances LPS-triggered inflammatory events by TNF-α that is induced by LPS, thus enhancing inflammatory responses at sites of bacterial infection (40, 41).

In fact, TNF-α synergizes with IFN-γ in the induction of many genes, including MHC classes I and II, adhesion molecules, costimulatory molecules, and cytokines (42), although many processes initiated by LPS are independent of the intermediary production of TNF-α (43). In this regard, LPS- plus IFN-γ-induced GJIC between DCs completely depends on DC-derived TNF-α. Similarly, Cpg ODN- plus IFN-γ-induced GJIC is mediated by DC-derived TNF-α. Considering that LPS or Cpg ODN is a ligand for TLR4 or TLR9, respectively, it is conceivable that any stimuli (including ligands for TLRs) that induce TNF-α secretion by DCs may induce GJIC between DCs in cooperation with IFN-γ. With respect to the source of IFN-γ, activated NK cells and T cells are considered to be the principal source of IFN-γ. In addition to well-studied Ag-specific, bidirectional DC-T cell interactions, recent studies revealed bidirectional interactions between DCs and NK cells leading to various in vitro effects (i.e., activation, cytokine production, proliferation, survival of each cell type, and NK cell lysis of DCs) (44, 45). It has been shown that peripheral inflamed tissues (46) and Ag-stimulated draining LNs (47) are two locations of in vivo interactions for NK cells and DCs. In addition, a certain subtype of DCs has been reported to produce IFN-γ upon stimulation (48). Accordingly, DCs may form GJIC at peripheral inflammatory sites and in Ag-stimulated draining LNs where TNF-α

**FIGURE 5.** GJIC between BMDCs. A. Representative dye-uncoupled and dye-coupled BMDCs. No dye transfer occurred without LPS and IFN-γ, whereas dye transfer to adjacent cells occurred in response to LPS and IFN-γ. Arrows in microscopic phase-contrast images indicate cells that were microinjected with Lucifer Yellow. B. LPS plus IFN-γ or TNF-α plus IFN-γ induces GJIC by BMDCs. BMDCs were cultured for 24 h with or without the indicated stimuli at the same concentrations used in Fig. 2D. These cells were tested for dye transfer assay. The data shown are the mean ± SD of incidence of dye coupling (percentage). C and D, TNF-α is required for LPS- plus IFN-γ-induced or Cpg ODN- plus IFN-γ-induced GJIC by BMDCs. BMDCs were cultured for 24 h with LPS and IFN-γ (C) or with Cpg ODN and IFN-γ (D) in the presence of anti-TNF-α mAb or control IgG. These cells were then tested for dye transfer assay. The data shown are the mean ± SD incidence of dye coupling (percentage). The data for dye-coupling in this figure are representative of two independent experiments and are the mean ± SD (n = 4 dishes) incidence of dye-coupling (percentage) from microinjected cells (a minimum of 10) per dish. *, p < 0.05; **, p < 0.01 (brackets indicate the groups compared). E, LPS- plus IFN-γ-induced up-regulation of CD40 expression by BMDCs depends on cell-to-cell contact. BMDCs were plated in T75 flasks at a low cell density (5 × 10^4 cells/ml; upper panel) or in wells of 24-well plates at a high cell density (1 × 10^5 cells/ml; lower panel), then were stimulated for 24 h with or without LPS plus IFN-γ in the presence or the absence of 0.5 mM heptanol or vehicle. The cells were examined for surface expression of CD40. The data shown are the mean ± SD mean fluorescence intensity (MFI) of CD40 expression from triplicate cultures. ***, p < 0.01 (brackets indicate groups compared).
and IFN-γ are elaborated by several types of immune cells. These GJIC may, in turn, contribute to the synergistic and effective activation of DCs induced by particular combinations of stimuli.

Lindquist et al. (10) recently generated transgenic mice in which CD11c<sup>+</sup> DCs expressed enhanced yellow fluorescent protein under the control of a CD11c promoter. Using two-photon laser-scanning microscopy, they were able to visualize the dynamics of resident steady-state DCs and/or immigrant mature DCs in LNs of live transgenic mice. They found that most resident DCs formed dense networks of cells touching each other with their dendrites, and that DCs often formed tightly packed clusters, especially in the border zone between T cell zones and B cell follicles, where acquired immune responses are actively initiated. They also examined the migration of mature DCs from the periphery into the LNs by intradermal injection of DCs expressing another fluorescent protein together with LPS. Injected DCs were initially more motile than steady-state DCs, then they dispersed and integrated into the sessile resident DC networks and clusters, where the immigrant DCs became sessile. These elegant observations prompted them to propose that immigrant mature DCs in the sessile networks and clusters may be well positioned to form stable interactions with Ag-specific T cells or relay Ags to other resident DCs by releasing exosomes (49) or apoptotic bodies (50) to increase efficient interactions with Ag-specific T cells. Considering the physical contact between resident DCs and between resident and immigrant DCs, one can envision that cell-to-cell communication via gap junctions plays a role in transmitting activation signals from immigrant DCs to resident DCs. In addition, Ag-bearing mature DCs from the periphery may transfer antigenic peptides to resident DCs in draining LNs through gap junctions (20). Although we demonstrated the existence of functional GJIC between DCs in vitro, it will next be crucial, although challenging, to determine the in vivo relevance to understand the physiological roles played by GJIC between DCs.

It is equally important to note several key questions that remain to be clarified. First, one must determine the molecular identities and quantities of signaling molecules transmitted via gap junctions, which are involved in effective DC activation. Second, the molecular mechanisms by which a combination of defined stimuli (i.e., TNF-α plus IFN-γ) is required for GJIC must be elucidated. It is also important to determine the involvement of other members of the Cx family besides Cx43 in GJC between DCs. Third, it will be intriguing to determine whether DCs and other cell types can form heterocellular GJIC to carry out some of their tasks. DCs, in performing their tasks as listed in the introduction, physically interact with many cell types. These include neighboring cells in the periphery (e.g., keratinocytes in skin) where resident DCs reside, endothelial cells of lymphatic vessels, and lymphocytes in LNs. In particular, it is important to determine whether GJIC between DCs and T cells exists, and if it plays a role in bidirectional interactions during Ag presentation. If so, would these gap junctions be localized to immunological synapses between DCs and T cells? Finally, during this study we realized the importance of direct homocellular interactions, which become apparent even when one examines heterocellular biological interactions. For example, when Ag-specific DC-T cell interactions are studied, it is not clear how direct heterocellular interactions (e.g., GJIC) between DCs or between T cells contribute to the biological outcomes for each cell type. We
References


