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Gap Junctions at the Dendritic Cell-T Cell Interface Are Key Elements for Antigen-Dependent T Cell Activation¹

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The acquired immune response begins with Ag presentation by dendritic cells (DCs) to naive T cells in a heterocellular cell-cell contact-dependent process. Although both DCs and T cells are known to express connexin43, a gap junction protein subunit, the role of connexin43 on the initiation of T cell responses remains to be elucidated. In the present work, we report the formation of gap junctions between DCs and T cells and their role on T cell activation during Ag presentation by DCs. In cocultures of DCs and T cells, Lucifer yellow microinjected into DCs is transferred to adjacent transgenic CD4⁺ T cells, only if the specific antigenic peptide was present at least during the first 24 h of cocultures. This dye transfer was sensitive to gap junction blockers, such as oleamide, and small peptides containing the extracellular loop sequences of connexin. Furthermore, in this system, gap junction blockers drastically reduced T cell activation as reflected by lower proliferation, CD69 expression, and IL-2 secretion. This lower T cell activation produced by gap junction blockers was not due to a lower expression of CD80, CD86, CD40, and MHC-II on DCs. Furthermore, gap junction blocker did not affect polyclonal activation of T cell induced with anti-CD3 plus anti-CD28 Abs in the absence of DCs. These results strongly suggest that functional gap junctions assemble at the interface between DCs and T cells during Ag presentation and that they play an essential role in T cell activation. *The Journal of Immunology*, 2009, 183: 277–284.

The adaptive immune response is initiated when dendritic cells (DCs),⁵ the most efficient APCs, present cognate Ag to T cells. DCs take up Ags and pathogens, generate MHC-peptide complexes at the cell surface, and migrate from the sites of Ag acquisition to secondary lymphoid organs. Once there, DCs can stimulate T lymphocytes through a mechanism that involves the assembly of a specialized supramolecular structure known as immunological synapse (1, 2). In contrast, naive T cells

travel to T cell areas of secondary lymphoid organs in search of DCs loaded with cognate peptide-MHC complexes (3). Once activated, T cells secrete IL-2 and proliferate vigorously generating effector T cells, which show an increase in CD69 and a reduction in CD62L surface expression, molecular markers of activated and naive T cells, respectively (4).

Immunological synapse assembly at the nanometer scale gap between a T cell and MHC-peptide-loaded APC provides the molecular signals leading to T cell activation. At this specialized area of physical contact between T cells and APCs, adhesion molecules and TCRs are actively segregated into distinct supramolecular complexes (5). Signaling through both TCR and adhesion molecules at the synapse contribute to increasing intracellular concentration of calcium required for T cell activation (6).

In several experimental cellular systems, it has been observed that appropriate intercellular adhesion is required for gap junction formation and stabilization (7). The heterocellular interaction mediated by cell adhesion molecules has also been suggested to favor the formation of intercellular junctions, such as gap junctions between DCs and T cells (8).

Gap junctions are constituted by a variable number of intercellular channels and are sites of cytoplasmic communication between contacting cells. Gap junction channels span the plasma membrane of two adjacent cells, and each cell contributes one-half of the channel, called hemichannel or connexon. Each hemichannel corresponds to an oligomer of six protein subunits termed connexins (Cx). According to their Cx composition, gap junction channels show different gating and permeability properties that define which ions and small molecules permeate this low resistance pathway (9).

The homocellular gap junctional communication between members of the immune system, such as mononuclear cells, has been shown to favor several cellular functions including secretion of metalloproteinases (10) and transfer of antigenic peptides (11). In addition, gap junction-mediated intercellular communication

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⁵ Abbreviations used in this paper: DC, dendritic cell; Cx, connexin; Ly, Lucifer yellow; AGA, 18 α -glycyrrhetic acid; BM-DC, bone marrow-derived dendritic cell; MHC II, MHC class II.

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between DCs has been shown to cause a more effective T cell activation by DCs (12). Although the formation of gap junctions between DCs have been documented with morphological and functional evidences (12, 13), the presence of this membrane specialization between APCs (Langerhans cells) and T cells has been only described at the light microscope level using an immunocytological method (14) and suggested as gap-junction-like structures at the ultrastructural level (14). Cx43 and functional gap junctions have also been detected at cell-cell contacts between a cell line of immortalized DCs and immortalized T cells (13). However, the existence of functional gap junctions between primary DCs and T cells and the role these structures may play in T cells physiology remains unknown.

In the present work, we report the expression of functional gap junctions between primary DCs and T cells and show that these channels are required for T cell activation. Our results show that in cocultures of DCs with T cells, heterocellular dye transfer is fully dependent on the presence of Ag and sensitive to gap junction blockers. Furthermore, gap junction inhibition was directly associated to a drastic reduction in T cell expression of activation markers and proliferative response, as revealed by a significant reduction in IL-2 secretion. This notion was further supported by experiments that show that gap junction blockers did not alter maturation, nor the expression of costimulatory molecules in DCs in the cocultures. In addition, gap junction blockade did not inhibit T cell activation in response to Ag-independent stimulation by CD3/CD28 engagement.

Materials and Methods

Mice and reagents

The C57BL/6, B10.BR, and V α 11/V β 3 C57BL/10-TgN TCR AND mice strains were from The Jackson Laboratory. AND \times B10.BR mice were bred and maintained at the Millennium Institute for Fundamental and Applied Biology. OT-I and OT-II mice were obtained from Jackson Laboratories and maintained at the animal facilities of the Pontificia Universidad Católica de Chile. All animal experiments were conducted according to institutional guidelines.

Lucifer yellow-CH (Ly), 18 α -glycyrrhetic acid (AGA), and oleamide were from Sigma-Aldrich. CFSE was from Molecular Probes. Peptides PCC₈₇₋₁₀₃ KKAERADLIAYLKQATAK, OT-II OVA₃₂₃₋₃₃₉, ISQAVHAAHAEINEAGR, and OT-I OVA₂₅₇₋₂₆₄ SIINFEKL were from New England Peptides. The gap junction inhibitor 1848 CNTQQPGCEN VCY (MW 1458.62) and control peptide 1849 SRPTEKTIF (MW 1078.23) were synthesized by standard solid-phase methods in an automated peptide synthesizer at the Peptide Synthesis Facility of the Albert Einstein College of Medicine (11).

Antibodies

The following mAbs from BD Biosciences were used: FITC-conjugated anti-I-A/I-E (2G9); FITC-conjugated anti-V α 11 (RR8-1); FITC-conjugated anti-CD69 (H1.2F3); PE-conjugated anti-CD11c (HL3); FITC-conjugated anti-CD80/B7.1 (16-10A1); FITC-conjugated anti-CD86/B7.2 (GL1); PE-conjugated anti-CD62L (MEL-14); PE-conjugated rIgGak (R35-95) isotype control; PE-conjugated anti-CD40 (3/23); PerCP-conjugated anti-CD4 (RM4-5); purified anti-I-Ek (14-4-4S). F(ab')₂ from previously characterized rabbit anti-Cx43 polyclonal Ab were used (14). F(ab')₂ were obtained using the Pierce preparation kit, as described (15). FITC or TRITC-conjugated anti-rabbit was obtained from DakoCytomation. Anti-mouse CD3 (2C11) and anti-CD28 (PV-1) were donated by Dr. Randolph Noelle, Dartmouth Medical School (Lebanon, Nit).

Cell isolation

Spleen DCs of AND \times B10.BR mice were obtained as previously reported (16). Bone marrow-derived DCs (BM-DC) of C57BL/6 mice were prepared as previously described (17).

Naive CD4 T cells were obtained from the thymus of AND \times B10.BR 4- to 6-wk-old mice depleted of APCs by negative selection using I-Ek mAb plus MACS conjugated with anti-mouse IgG Abs (Miltenyi Biotec). Transgenic thymocytes contained >75% of mature CD4⁺ T cells, of which >90% expressed the transgenic V α 11 chain. H-2K^b/OVA₂₅₇₋₂₆₄-specific

CD8⁺ T cells and I-A^b/OVA₃₂₃₋₃₃₉-specific CD4⁺ T cells were obtained from lymph nodes suspensions from OT-I and OT-II transgenic mice respectively, as previously described (18, 19).

Dye coupling

Spleen DCs (2.5 \times 10⁶ cells) were cocultured with AND \times B10.BR CD4⁺ T cells (5 \times 10⁶ cells). Ly (5% weight to volume Ly dissolved in 150 mM LiCl) was microinjected into DCs through glass microelectrodes by brief overcompensation of the negative capacitance circuit in the amplifier until the impaled cell was brightly fluorescent. Gap junctional communication was tested by observing whether dye transfer occurred to T cells during the first minutes after dye injection, as described previously (13). 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 in each experiment multiplied by 100. In all experiments, dye coupling was tested in a minimum of 10 microinjected DCs. Dye coupling was observed in an inverted microscope equipped with xenon arc lamp illumination and a Nikon B filter (excitation wavelength 450–490 nm; emission wavelength above 520 nm). Similar dye coupling experiments were made with bone marrow derived DCs (2.5 \times 10⁶ cells) and OT-II CD4⁺ T cells or OT-I CD8⁺ T cells (5 \times 10⁶ cells).

Detection of Cx43 in DCs and T cells

Spleen DCs (1 \times 10⁶ cells) were cocultured with AND \times B10.BR CD4⁺ T cells (1 \times 10⁶ cells) for 18–20 h. Then, the cells were stained for CD4 and CD11c to label T cells and DCs, respectively. Cells were then fixed with Cytotfix/Cytoperm and permeabilized with PermWash 1 \times (BD Pharmingen). Subsequently, DCs and T cells were stained with a F(ab')₂ rabbit polyclonal anti-Cx43 Ab followed by FITC-conjugated anti-rabbit Ab and analyzed by flow cytometry on a FACScan (BD Biosciences) using the CellQuest program.

Cx43 was also detected by immunofluorescence using the F(ab')₂ of the anti-Cx43 Ab prepared as described above. For all immunostaining experiments, cells seeded on coverslips were fixed at –20°C with 70% ethanol for 30 min, and washed three times with ice cold PBS and then with PBS containing 10% normal goat serum (Zymed) for 30 min. CD11c⁺ DCs were double stained for MHC class II (MHC II) and Cx43 by first incubating cells overnight at 4°C with a fluorescent-labeled mouse anti-MHC II mAb (IgG1, 1/500) diluted in PBS-10% normal goat serum followed by three rinses in PBS and an overnight incubation at 4°C with rabbit anti-Cx43 F(ab')₂ Ab (1/1500). After washing three times with PBS, cells were incubated at room temperature for 3 h with a goat anti-rabbit IgG (Fab) conjugated to TRIC. After several washes, the coverslips were mounted in Fluoromount and examined by epifluorescence in a confocal laser-scanning microscope (Olympus FV1000) with a \times 63 objective. Confocal images were taken with two lasers (argon 488 nm and helium/neon 543 nm).

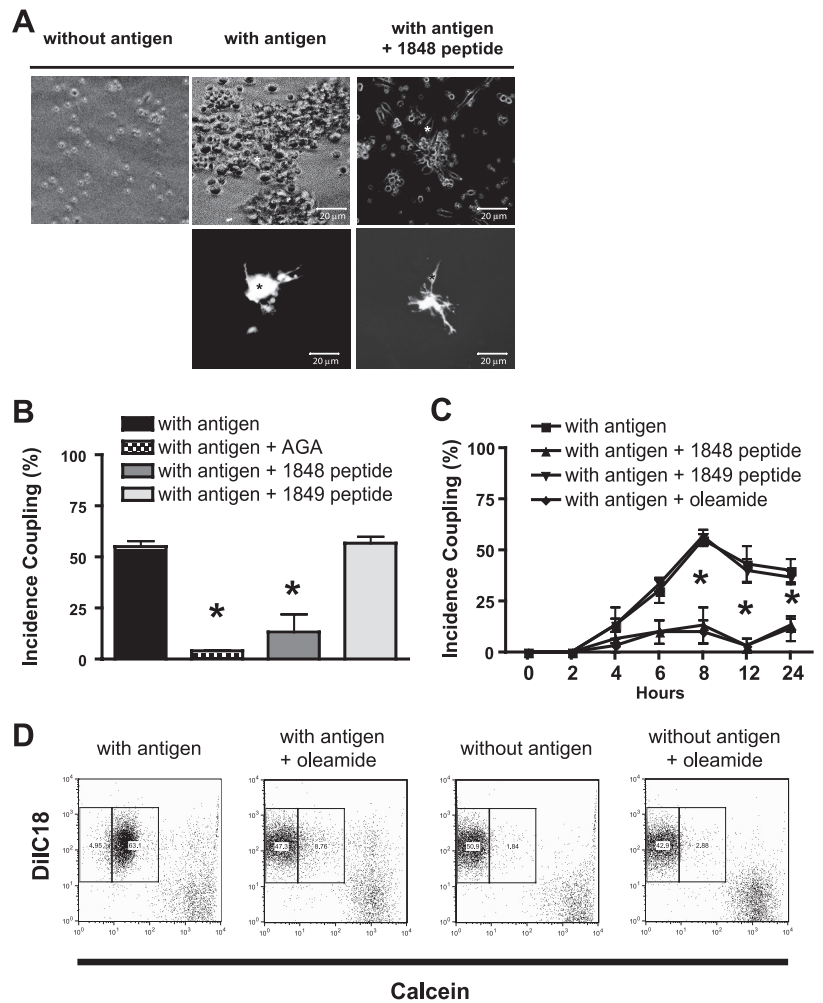
Expression of T cell activation markers and cluster formation

Spleen DCs (4 \times 10⁵ cells) were cocultured with AND \times B10.BR CD4⁺ T cells (2 \times 10⁶ cells) plus peptide in the absence or presence of a gap junction blocker. CD4⁺ T cells were double-stained either for CD4 and CD69 after 20 h or for CD4 and CD62L after 76 h of coculture and analyzed by flow cytometry. In some experiments, polyclonal T cell activation was done in the absence or presence of gap junction blockers in plates coated with 1 μ g/ml anti-CD3 and 1 μ g/ml anti-CD28. The effect of oleamide on cluster formation was established in cocultures of spleen DCs with AND \times B10.BR CD4⁺ T cells plus peptide in the absence or presence of a gap junction blocker. After 36 h of coculture, individual wells were photographed and the surface area and number of clusters determined.

Cytokine production

For cytokine secretion, spleen DCs were cocultured at different ratio (DC:T cells 1:1, 1:2, 1:4, 1:8) with 1 \times 10⁵ AND \times B10.BR CD4⁺ T cells on 96-well culture dishes in the absence or presence of a gap junction blockers. After 20 h of coculture, supernatants were harvested and tested for mouse IL-2 using an ELISA kit (BD Pharmingen) according to the manufacturer's instructions. The detection limits for IL-2 was 1.6 pg/ml. Similar experiments were made in cocultures of BM-DC with either OT-II CD4⁺ T cells or OT-I CD8⁺ T cells at 1:1 ratio (10⁵ cells each per well). In some experiments, cytokine production was determined after polyclonal T cells activation in the absence or presence of gap junction blockers in plates coated with 1 μ g/ml anti-CD3 and 1 μ g/ml anti-CD28.

FIGURE 1. Transfer of Lucifer yellow between DCs and T cell occurs through gap junctions. Naive CD4 T cells were cocultured with DCs without Ag or with Ag plus 100 μ M BGA, 200 μ M 1848, or 1849 peptide. *A*, CD4 T cell (5×10^6 cells) were cocultured with spleen DCs (2.5×10^6 cells) for 8 h without Ag (*left*), with Ag (*middle*), or with Ag plus 1848 peptide (*right*). DCs were microinjected with Lucifer yellow and dye transfer to T cells analyzed. *Upper panel*, Phase-contrast view of the fluorescent field showed in the lower panel. The asterisk denotes the microinjected DC and arrows indicate Lucifer yellow transfer to T cells. *B*, Graphs showing the incidence of dye coupling between DCs and T cells. The effect of the gap junction inhibitors AGA and peptide 1848 as well as of the control peptide 1849 are also shown. Each bar corresponds to the average \pm SD ($n = 3$). *C*, Time course of incidence of dye coupling between DCs and T cells. The effect of the gap junction inhibitors oleamide and peptide 1848, as well as of the control peptide 1849 are also shown. Each bar corresponds to the average \pm SD ($n = 3$). *D*, Calcein transfer from calcein-labeled DCs (1×10^6 cells) to DiIC18-labeled CD4 T cells (5×10^6 cells) in the presence or absence of antigenic peptide with or without oleamide, and determined by flow cytometry after 8 h of coculture ($n = 3$).*, $p < 0.05$ with respect to coculture with Ag.



Proliferation assay

Purified spleen DCs were cocultured with CFSE-labeled AND \times B10.BR CD4⁺ T cells in flat-bottom 24-well plates as described (20). Unless stated, 2×10^5 DCs/well were used to stimulate 1×10^6 CD4 T cells. Cells were cocultured in complete medium for 3 or 5 days and thereafter their level of CFSE were analyzed by flow cytometry.

Statistical analysis

Data are presented as mean \pm SD and were analyzed using one-way ANOVA or two-way ANOVA with Bonferroni correction where appropriate.

Results

Heterocellular gap junctional communication in cocultures of isogenic DCs and T cells

Gap junctional communication has been detected in independent homocellular cultures of either activated DCs or T cells (11–13, 21). Also, formation of heterocellular gap junctions between allogeneic DCs and T cells in cocultures has been suggested using morphological techniques (8, 22). To investigate whether gap junctional communication might also occur between isogenic T cells and DCs, we cocultured DCs purified from spleen with naive TCR-transgenic CD4⁺ T cells (TCR AND) in the presence of the specific peptide PCC_{87–103}, which is recognized in the context of I-E^k. After 8 h of coculture, DCs were microinjected with Ly and dye transfer to T cells was observed during the next few minutes (Fig. 1, *A*, *middle* and *B*), with an incidence of dye coupling of $55 \pm 3\%$. Dye transfer was drastically reduced when cells were

cocultured in the presence of peptide 1848, which is known to inhibit Cx43 gap junctions (Fig. 1, *A*, *right* panel and *B*) (incidence of coupling $10 \pm 2\%$). Blockade of intercellular dye transfer was also observed 10 min after the application of 100 μ M of the gap junction blocker AGA ($5 \pm 1\%$) (Fig. 1*B*). In contrast, cocultures treated with control peptide 1849, a connexin mimetic peptide which lacks two isoleucine residues required for membrane insertion (24), showed an incidence of dye coupling of $60 \pm 1\%$, comparable to that found in cells cocultured without inhibitors (Fig. 1*B*). Because there were no cell clusters in cocultures incubated without Ag (Fig. 1*A*, *left*), intercellular dye transfer was not observed in the absence of cognate Ag on the DC surface. Additionally, dye coupling was observed starting at 4 h up to 24 h of coculture, with the maximum coupling (dye transfer) at 8 h (Fig. 1*C*).

Furthermore, to verify that gap junction inhibitors were not affecting cluster formation, cocultures of DCs and T cells plus antigenic peptide in the presence or absence of varying amounts of oleamide or 1848 peptide, were analyzed at different time periods by confocal microscopy and the number of DC-T cell conjugates measured. Importantly, we observed no significant differences in the number or size of DC-T cell conjugates formed at different time points (Fig. 2*C* and data not shown). To corroborate that the dye was actually transferred to the T cells through the newly formed gap junction, we cocultured calcein-loaded DCs with DiIC18-labeled CD4 T cells in the presence or absence of antigenic peptide with or without oleamide, and determined by flow

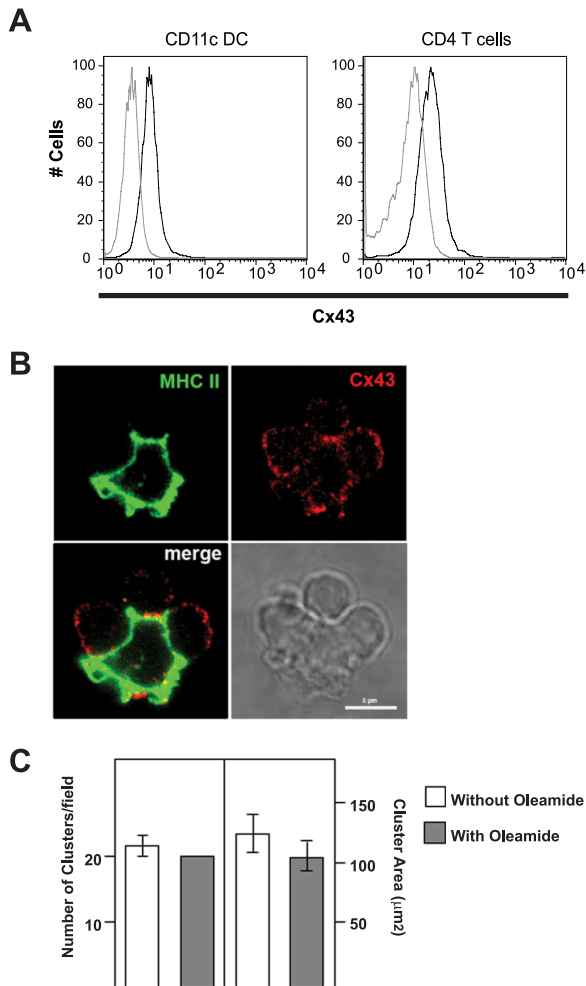


FIGURE 2. Both DCs and T cells express Cx43 immunoreactivity during Ag presentation. Naive CD4 T cells (5×10^6 cells) were cocultured with DCs (2.5×10^6 cells) for 18–20 h. Cells in coculture were stained for CD4 and CD11c to identify T cells or DCs, respectively. Then, cells were fixed, permeabilized, and stained for Cx43 and either analyzed by flow cytometry (A) or immunofluorescence (B). A, Flow cytometry analysis for Cx43 reactivity (black line) and control isotype (gray line) on DCs (left) and CD4 T cells (right). These results were representative of three independent experiments. B, Confocal immunofluorescence of Cx43 (red, top right), MHC II (green, top left), colocalization of Cx43 and MHC II (bottom left) and phase contrast view of the field (bottom right), $n = 3$. In the confocal immunofluorescence at least 50 different T:DC conjugates were analyzed. Scale bar, 5 μm . C, Cocultures of DCs and T cells plus antigenic peptide, in the presence or absence of oleamide, were photographed at 8 h to count the number of clusters and measure their size. Each bar represents the average \pm SD ($n = 3$).

cytometry the transfer of calcein from DCs into the T cells. As shown in Fig. 1D, DiIC18-labeled T cells acquire calcein from DCs, in a process that is inhibited by the presence of oleamide. These results strongly suggest that gap junctions assembled at the DC-T cell interface mediate dye transfer between these two cells and that this occurs only in the presence of cognate Ag.

DCs and T cells express Cx43

To further demonstrate the presence of gap junction subunits in DCs and T cells, we conducted flow cytometry experiments with an Ab specific for the Cx43 protein. Previous studies using flow cytometry, immunocytochemistry and immunofluorescence have detected the presence of Cx43 in isolated mouse DCs (8, 12, 13),

as well as in T cells derived from human peripheral blood (23). In addition, the Cx43 mRNA has been detected in DCs using a semi-quantitative RT-PCR (13). These observations prompted us to analyze whether both DCs and T cells expressed Cx43 under the coculture conditions used in our experimental setting. DCs and T cells were harvested from 8 to 20 h of coculture and tested for Cx43 reactivity. As shown in Fig. 2, both cell types were Cx43 positive as tested by confocal microscopy (Fig. 2B) and flow cytometry (Fig. 2A). In contrast, cocultures of DC-T cell incubated in the absence of the antigenic peptide showed very low Cx43 reactivity (data not shown), suggesting that the presence of Cx43 is associated to the presence of Ag and T cell activation. Furthermore, confocal microscopy data indicate that at 20 h of cocultures of DCs with T cells incubated with the antigenic peptide, Cx43 was detected in both DCs and T cells. In addition, it was observed that in 44% of the cells Cx43 colocalized with MHC II in discrete areas (Fig. 2B, data not shown), which corroborates with dye coupling that we observed at this time (Fig. 1C). These observations suggest that during Ag presentation, Cx43 colocalizes with other molecules present at the immunological synapse.

Gap junction inhibitors block T cell activation in cocultures with Ag-pulsed DCs

The colocalization of Cx43 with MHC II and the fact that the presence of cognate Ag was needed for Cx43 expression led us to investigate whether Cx43 and gap junction formation is also involved in T cell activation by DCs. Thus, spleen DCs were cocultured with naive CD4⁺ T cells in the presence of antigenic peptide. In parallel, cocultures were set in the presence of a gap junction blocker (100 μM oleamide). After 20 and 76 h of coculture, expression levels of CD69 and CD62L were determined by flow cytometry. As shown in Fig. 3A, CD4⁺ T cells cocultured with DCs in the absence of Ag maintained a high expression of the naive T cell marker CD62L ($67 \pm 4\%$). In contrast, the percentage of T cells expressing CD62L decreased when cells were cocultured in the presence of the antigenic peptide ($28 \pm 5\%$), an indication of T cell activation (Fig. 3A). Interestingly, when the cocultures containing Ag were treated with 100 μM oleamide, the percentage of T cells expressing the naive T cell marker, CD62L, was restored to the level observed in the absence of Ag (Fig. 3A) ($56 \pm 5\%$).

Conversely, a high percentage of CD4⁺ T cells express the early activation marker CD69 when the cocultures were performed in the presence of Ag compared with coculture without Ag (Fig. 3B). Under the presence of Ag, the gap junction inhibitor, oleamide, in the cocultures drastically reduced CD69 expression in T cells to basal levels (Fig. 3B). Furthermore, we evaluated whether inhibition of gap junction formation in the cocultures could also impair IL-2 secretion by T cells. To directly investigate this possibility, we cocultured spleen DCs with T cells with or without Ag, in the presence of chemical or peptidic gap junction inhibitors (100 μM oleamide or 200 μM of the 1848 peptide). As a control peptide, we also incubated Ag-pulsed cells with peptide 1849. After 20 h, the supernatants were harvested and analyzed for the presence of IL-2. As expected, our results show that T cells cocultured with DCs in the absence of Ag did not secrete IL-2 (Fig. 3C). In contrast, when T cells were cocultured in presence of Ag they secreted significant levels of IL-2. However, when cocultures were set in the presence of antigenic peptide plus the gap junction inhibitor peptide 1848 or oleamide, we observed a drastic reduction of IL-2 secretion by T cells (Fig. 3C). In contrast, no reduction on IL-2 release was observed when the control peptide 1849 was used (Fig. 3C). Altogether these results suggest that gap junctions participate in T cell activation and that inhibition of gap junction formation can impair T cell activation during Ag presentation by DCs.

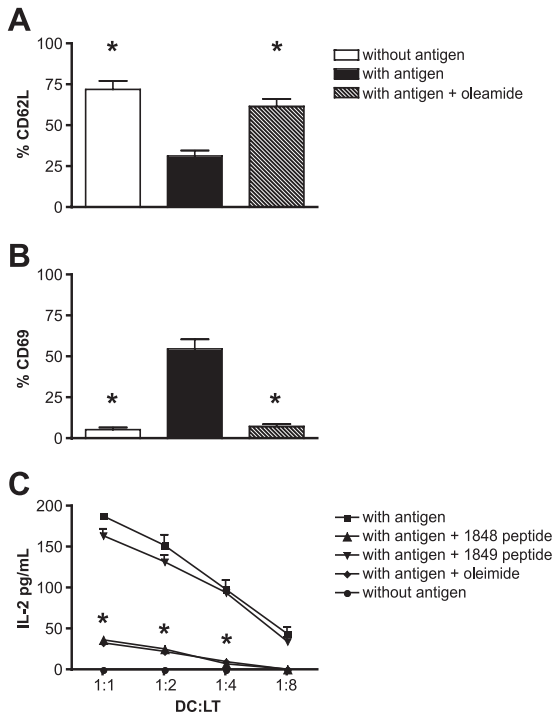


FIGURE 3. Inhibition of gap junction formation during DC-T cell coculture prevents T cell activation. Naive CD4 T cells were cocultured with DCs without Ag or with Ag plus 100 μ M oleamide, 200 μ M of the 1848 peptide, or 200 μ M of the 1849 control peptide. *A*, Flow cytometry analysis of CD62L expression on CD4 T cell (2×10^6 cells) after coculture for 76 h with spleen DCs (4×10^5 cells). *B*, Analysis by flow cytometry of CD69 on CD4 T cell after 20 h of coculture with spleen DCs. *C*, Supernatants obtained after 20 h of coculture of CD4 T cells (1×10^5 cells) with different amounts of DCs were tested for mouse IL-2 using an ELISA kit. Each bar corresponds to the average \pm SD ($n = 3$). *, $p < 0.05$ with respect to coculture with Ag.

To verify that gap junction inhibitors did not impair T cell activation by acting directly on the T cells, we determined the effect of gap junction blockade on polyclonally activated CD4⁺ T cells. We observed that under these conditions oleamide or peptide 1848 had no effect on T cells IL-2 secretion (Fig. 4A) or CD69 expression (Fig. 4B).

Likewise, to rule out that gap junction blockers could alter the maturation of DCs and thus influence their capacity to activate T cells, spleen DCs were cocultured with naive CD4⁺ T cells in the presence of antigenic peptide, with or without a gap junction blocker (100 μ M oleamide), and the expression levels of MHC II, CD80, CD86, and CD40 were determined by flow cytometry on DCs. After 20 h of coculture, no significant impairment on DC maturation or on the expression of costimulatory molecules by DCs as a result of gap junction blockade (Fig. 5). Collectively, these results suggest that gap junction blockers inhibit T cell activation by directly interfering with gap junction assembly between DCs and T cells.

Functional gap junctions between DCs and T cells are fundamental for T cell proliferation

To address the question whether a persistent inhibition of T cells activation by gap junction blockade also resulted in a suppression of T cell proliferation, we examined the role of gap junction assembly between DCs and T cells in the proliferations of T cells. DCs were cocultured with CFSE-labeled CD4⁺ T cells for 3 or 5 days in the presence or absence of gap junction inhibitors and then

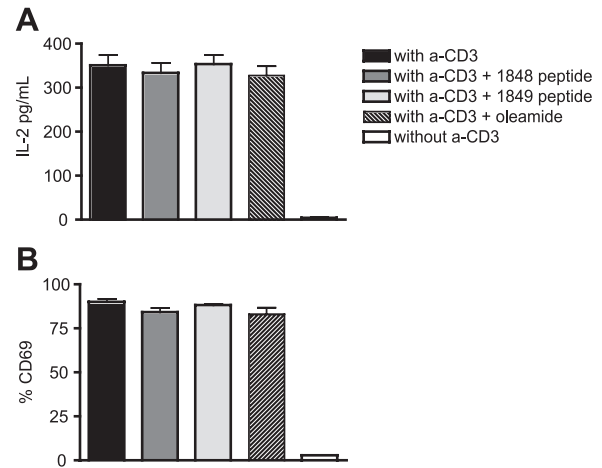


FIGURE 4. Gap junction blockers do not affect polyclonal activation of T cells. Naive CD4 T cells were cultured with anti-CD3 and anti-CD28 Abs in the presence of either 100 μ M oleamide, 200 μ M of the 1848 peptide, or 200 μ M of 1849 control peptide or without Abs. *A*, Supernatants were harvested after 20 h and tested for mouse IL-2 using an ELISA kit; *B*, CD4 T cell were analyzed for CD69 expression by flow cytometry. Each bar corresponds to the average values obtained from three independent experiments \pm SD.

the proliferation of the T cell was determined by assaying of CFSE dilution on the resulting effector T cells. Our results show that at day 3, CFSE-labeled T cells proliferated normally when cocultured with DCs in presence of Ag, whereas CFSE-labeled T cells cocultured with Ag plus the gap junction blocker oleamide showed no significant proliferation (Fig. 6A). At day 5, CFSE-labeled T cells cocultured with Ag plus oleamide presented a significantly delayed proliferation when compared with the controls (Fig. 6A).

To exclude that oleamide affects T cell proliferation directly, we performed proliferation assays using CFSE-labeled T cells activated with anti-CD3 plus anti-CD28 in presence or absence of

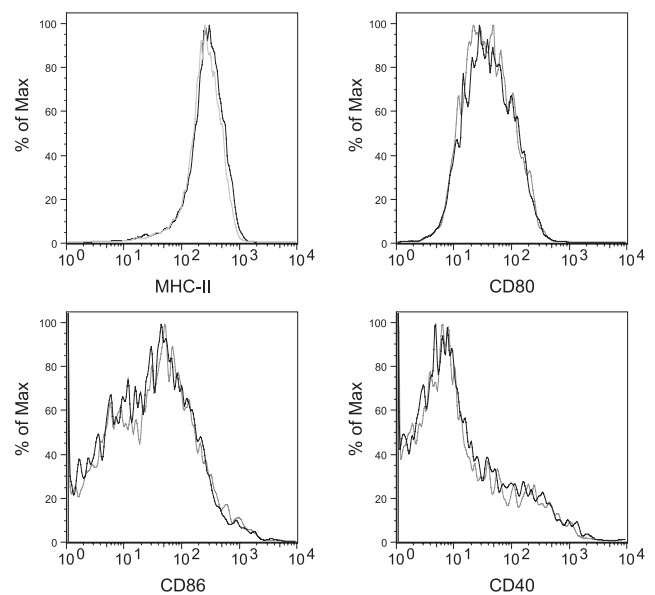
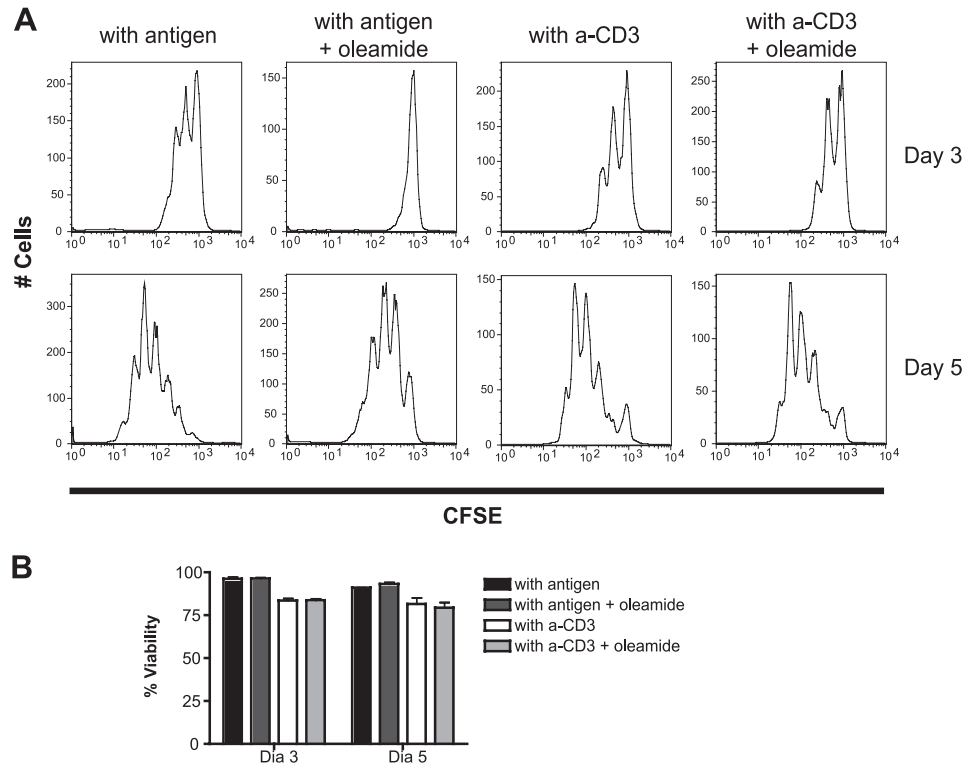


FIGURE 5. Gap junction blockers do not affect the expression of DCs maturation markers. Naive CD4 T cells were cocultured with DCs plus Ag in presence (black) or absence (gray) of 100 μ M oleamide. After 20 h, DCs were analyzing for the expression of MHC-II, CD80, CD86, and CD40. The data are representative of two independent experiments.

FIGURE 6. Inhibition of gap junction formation during DC-T cell coculture affects T cell proliferation. **A**, CFSE-labeled naive CD4 T cells (1×10^6 cells) were cocultured with DCs (2×10^5 cells) plus Ag or with anti-CD3 and anti-CD28 Abs in the presence or absence of 100 μ M oleamide. After 3 days (*upper panels*) or 5 days (*lower panels*) T cell proliferation was analyzed by flow cytometry. The data shown are representative of one of three independent experiments. **B**, Viability percentage of CD4 T cells at day 3 and 5, from the different groups described in **A**. The viability was evaluated through propidium iodide exclusion by flow cytometry ($n = 3$).



oleamide. In all cases, CFSE-labeled T cells presented the same proliferation rate (Fig. 6A).

Finally, the effects described above were not the consequence of oleamide or the gap junction inhibiting peptide 1848 on T cell viability, since in all experiments we observed no difference on the viability as determined by propidium iodide or trypan blue exclusion (Fig. 6B and data not shown).

The inhibition of gap junctions between DCs and T cells blocks both CD8⁺ and CD4⁺ T cell activation

To determine whether gap junctions are important for activating CD8⁺ T cells, as well as CD4⁺ T cells, BM-DCs from C57BL/6

mice were cocultured with naive CD8⁺ OT-I and CD4⁺ OT-II T cells in presence of activating concentrations of OT-I or OT-II antigenic peptides, respectively (25, 26). In parallel, cocultures were set in the presence of gap junction blockers (either 100 μ M Oleamide or 200 μ M of peptide 1848). After 20 h of coculture, the supernatants were harvested and analyzed for the presence of IL-2. Our result show that both OT-I and OT-II T cells cocultured with BM-DCs in the presence of either chemical or peptidic gap junction inhibitors drastically decrease IL-2 secretion (Fig. 7, A and B) compared with the controls. In contrast, this effect was not observed when the control peptide 1849 was included in the cocultures (Fig. 7). These data suggest that inhibition of gap junctions

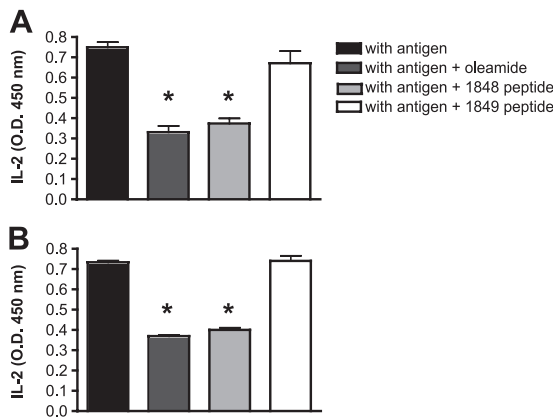


FIGURE 7. Gap junction blockers prevent activation of both CD8 and CD4 T cells by DCs. OT-I (A) and OT-II (B) T cells were cocultured with C57BL/6 BM-DCs at 1:1 ratio in the presence of H-2K^b-OVA or I-A^b-OVA peptides respectively plus either 100 μ M oleamide, 200 μ M 1848 peptide, or 200 μ M 1849 control peptide. After 20 h, supernatants were harvested and evaluated for mouse IL-2 using an ELISA kit. Each bar corresponds to the average \pm SD ($n = 2$). Statistical analyses (Student's *t* test) are relative to treatments with OT-I or OT-II peptide. *, $p > 0.05$.

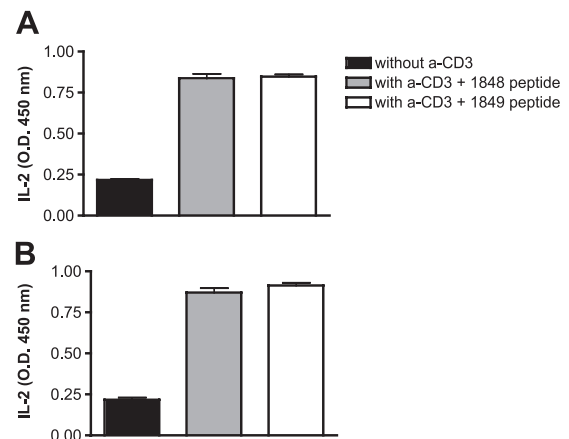


FIGURE 8. Gap junction blockers do not affect polyclonal activation of CD8 and CD4 T cells. Naive OT-I (A) or OT-II (B) T cells were cultured with anti-CD3 and anti-CD28 Abs in the presence of 200 μ M 1848 peptide or 200 μ M 1849 control peptide or without Abs. After 20 h, supernatants were harvested and tested for mouse IL-2 using an ELISA test. Each bar corresponds to the average \pm SD ($n = 2$).

affects equally the activation of CD8⁺ and CD4⁺ T cell during the Ag presentation by DCs.

Similarly to AND T cells, activation of OT-I or OT-II T cells by CD3/CD28 cross-linking was not impaired by gap junction blockade, because no significant reduction of CD3/CD28-induced IL-2 secretion by these cells was observed after incubating with the gap junction blocking peptide (Fig. 8).

In summary, the results presented in this study reveal a fundamental role for gap junction assembly at the interface between DCs and T cells for Ag-specific activation and proliferation of T cells.

Discussion

It has been well established that initiation and regulation of Ag-specific immune responses involves direct contact between T cells with Ag-presenting DCs (1). In the present study, we provide evidence supporting the notion that gap junctions are indeed formed between Ag-presenting DCs and T cells and these structures are required for efficient T cell activation by DCs. It was recently reported that TNF- α plus IL-1 β , as well as IFN- γ induce transient gap junctional communication between monocytes/macrophages or primary DCs and DC-derived cell lines (10, 12, 13). In contrast, in the experiments reported here, no additional cytokine stimuli were required, in addition to the specific Ag, to observe dye coupling between DCs and T cells. Because in ~50% of the cells dye-coupling between DCs and T cells starts after 6 h of coculture (Fig. 1C), this suggests that this period is needed for the establishment of the necessary factors and conditions to induce the functional expression of heterocellular gap junctions. In our experiments, transfer of the fluorescent indicator Ly occurs within seconds after microinjection into the DC, reinforcing the idea that functional gap junctions are actually formed between DCs and T cells and ruling out other mechanisms of dye transfer such as membrane fragments enclosing cytoplasm fragments. Furthermore, as shown in Fig. 1D the transfer of the indicator dye calcein from dendritic cells to T cells is blocked by the gap junction blocker oleamide. We discard that calcein seen in these experiments is a product of fluorescence remaining in the conjugated DC-T cells, because the number of DC-T clusters are similar in the controls and in the cocultures treated with oleamide (Fig. 2C). If the calcein-fluorescence in the T-DC cocultures, was coming from the conjugated DC, we should observe similar percentage of calcein-fluorescence in culture treated or not with oleamide. That is not the case (Fig. 1D).

The fact that the presence of cognate Ag is an absolute requirement for establishing functional gap junction channels between DCs and T cells indicates that Ag presentation by an APC is needed for the generation of the appropriate condition for gap junction assembly. It has been shown that TCR binding to the cognate peptide-MHC complex is required for the assembly of a stable and persistent T cell-APC synapse, which leads to T cell activation and proliferation (1). Our data indicate that T cells and DCs express very little gap junction subunit Cx43 in the absence of TCR/peptide-MHC complex. In contrast, Cx43 expression is greatly enhanced after 20 h of coinubation in the presence of cognate peptide-MHC complexes. This observation is consistent with recent reports (27) indicating that increasing doses of Ag enhance the duration of T cell-DC interaction from minutes to hours and that the interaction between APC and T cells generates a polarization of actin filaments and microtubules, leading to the transport of cell surface molecules including the TCR and costimulatory molecules (28). Similar events could lead to the transport of Cx subunits to the cell surface and the formation of functional gap junctions. Because we detected Cx43 in both DCs and T cells and this Cx is known to form homotypic gap junction channels (9), it

is likely that Cx43 alone or assembled with compatible Cxs constitutes the heterocellular gap junctions described in this study to assemble at the interface between DCs and T cells.

Numerous reports show that L-selectin (CD62L) is present in naive T cells and that it diminishes its expression after T cell activation (29–32). In contrast, surface expression of CD69, and IL-2 secretion are hallmark of T cell activation (29–33). Our data show that presentation of cognate Ag by DCs is followed by a decrease in the expression of CD62L and an increase in the expression of CD69 and IL-2 secretion by T cells, an indication of T cell activation. These responses require gap junction formation, because the addition of gap junction blockers have a strong inhibitory effect on the expression of these T cell activation markers. The observation that the specific gap junction inhibitor 1848 (but not the control 1849 peptide) also produced this inhibition of activation provides further support to this notion. In a normal immune response, T cell activation leads to a robust proliferation of these cells. By way of CFSE dilution experiments, in this study we show that T cell proliferation is also dependent on gap junction formation, because the inclusion of gap junction inhibitors abrogated or significantly retarded T cell proliferation.

For the initiation of an immune response, after Ag capture in the periphery, DCs must migrate to the nearest secondary lymphoid organ and present Ags as peptides loaded onto MHC molecules to Ag-specific T cells. Recently, it was reported that skin Ag-presenting Langerhans cells show high expression of MHC II and Cx43, and that DCs in the appendix also showed the formation of Cx43 gap junctions between MHC II⁺ cells and surrounding cells (11). Although several molecules, including the TCR, MHC-peptide complex and various adhesion and costimulatory molecules, have been described as constituents of the immunological synapse, to our knowledge gap junction subunits have not been previously described as part of this supramolecular structure. Using confocal laser microscopy, we were able to establish that Cx43 colocalizes with MHC II molecules on Ag-loaded DCs during the interaction with T cells. These data would suggest that gap junctions could also assemble at the immunological synapse and contribute to T cell activation, which is consistent with the observation that assembly of functional gap junctions is needed for efficient T cell activation.

Gap junctions connect the cytoplasmic content of contacting cells, allowing for the nonspecific exchange of small molecules, such as ions, second messengers, and small peptides, thus providing for a mechanism to functionally coordinate the interacting cells. Two recent reports have addressed the nature of the molecules transferred via gap junctions between cells of the immune system. Neijssen et al. (10) reported the transfer of small peptide (1,800 molecular mass) between Cx43 transfected cells as a mechanism for cross-priming of APCs and cytotoxic T cell recognition. More recently, Schmitt and coworkers (34) reported that Tregs mediate their suppressive activity through the transfer of cAMP, a potent inhibitor of T cell proliferation and IL-2 synthesis, from the regulatory T cells to the responder T cells. Although they did not unambiguously identify the Cxs involved, they showed a blockade of cAMP transfer and IL-2 mRNA expression in the presence of a gap junction inhibitory peptide mimicking the extracellular loop of Cx43. In the cell-cell interaction reported here, neither the cluster formation (Fig. 2C) nor TCR activation (Figs. 4, 6, and 8) was affected with the inhibition of gap junctions, suggesting that the identification of the small molecules exchanged between DCs and T cells may prove more elusive to identify. Besides the obvious transfer of ions such as Ca²⁺, numerous other candidate molecules need to be analyzed. The fact that the size and number of the

clusters formed in the presence or absence of oleamide are identical is a further confirmation that the effect of oleamide is due to its known inhibitory effect on gap junctions rather than to a secondary effect on the immunological synapse or cluster configuration.

In summary, we have identified functional gap junctions that appear to operate under conditions of physiological T cell activation, and which appear to be required for effective T cell activation and proliferation. We believe that these data provide new insights in our understanding of the involvement of gap junctions in T cell activation and in the mechanisms of initiation and regulation of the adaptive immune response. Finally, the assembly of gap junctions at the membrane interface between T cells and Ag-loaded DCs could be considered as a new checkpoint for the modulation of T cell activation.

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Disclosures

The authors have no financial conflict of interest.

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