Glutamate Released by Dendritic Cells as a Novel Modulator of T Cell Activation[1]

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Adaptive immune responses begin after productive immunosynaptic contacts formation established in secondary lymphoid organs by dendritic cells (DC) presenting the Ag to T lymphocytes. Despite its resemblance to the neurosynapse, the participation of soluble small nonpeptidic mediators in the intercellular cross-talk taking place during T cell–DC interactions remains poorly studied. In this study, we show that human DC undergoing maturation and in contact with T cells release significant amounts of glutamate, which is the main excitatory neurotransmitter in mammalians. The release of glutamate is nonvesicular and mediated by the DC-expressed X_c^- cystine/glutamate antiporter. DC-derived glutamate stimulating the constitutively expressed metabotropic glutamate receptor 5 impairs T cell activation. However, after productive Ag presentation, metabotropic glutamate receptor 1 is expressed in T cells to mediate enhanced T cell proliferation and secretion of Th1 and proinflammatory cytokines. These data suggest that, during T cell–DC interaction, glutamate is a novel and highly effective regulator in the initiation of T cell-mediated immune responses. *The Journal of Immunology,* **2006, 177: 6695–6704.**

As glutamate is the primary excitatory neurotransmitter, it is pivotal to the functioning of the CNS. Neuronal synaptic activity generates the transient release of this amino acid in the synaptic cleft, thereby contributing to neurotransmission (1). Glutamate may interact with either of two main multiple receptor types: namely, ionotropic glutamate receptors (iGluR)[4] (4), which form ion channels and mediate fast excitatory glutamate responses, and metabotropic GluR (mGluR), which are heptaspanning membrane receptors and belong to the superfamily of G protein-coupled receptors (1, 2).

Not only neurons, but also activated microglia, featuring a phenotype similar to brain macrophages, can release glutamate, which promotes neurotoxicity in neurons expressing ionotropic *N*-methyl-*D*-aspartate receptors (3, 4). Glutamate derived from brain macrophages is released via the cystine/glutamate antiporter (X_c^- system), a heterodimer composed of CD98 (H chain), commonly found in various transporters, and the xCT (L chain), which confers substrate specificity (5, 6). The X_c^- system mediates cystine uptake, which is intracellularly reduced to cysteine and subsequently used for glutathione biosynthesis (7), whereas exported glutamate is able to activate iGluR as well as mGluR (1).

In recent years, growing evidence has shown GluR expression in T lymphocytes (8–15), which suggests that glutamate plays an important role in the regulation of T cell-mediated immunity. In fact, a significant number of clinical studies have consistently shown strong correlation between immunodeficiency, such as occurs in AIDS (16–18) or in malignancies (19, 20), and the deregulation of plasma glutamate levels. However, both the cells producing glutamate and the physiological mechanisms underlying glutamate actions in the immune response remain far from being elucidated.

Adaptive immune responses are initiated when the Ag is presented to specific T cells by an APC (21, 22). The T cell–APC contact site is a highly organized supramolecular complex of cell surface receptors and associated signaling proteins known as the immunological synapse, which may modulate cell-fate decisions with respect to T cell maturation, activation, and differentiation (23, 24). The T cell–APC interaction may be viewed as the correlate of the neurosynapse in the nervous system. However, in contrast with what occurs in the nervous system where a high structural variety in neurotransmitters is found, the lymph node mediators reportedly released during the T cell–APC contact formation are classically polypeptidic molecules.

It has been reported recently that, like in neurological synapses of serotonergic neurons, dendritic cells (DC) release the small nonpeptidic mediator serotonin during T cell–DC contact formation (25). Subsequently, the DC-released serotonin acts on serotonin receptors expressed on the T cell surface promoting the decrease of intracellular cAMP levels, thereby enhancing T cell activation

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[4] Abbreviations used in this paper: iGluR, ionotropic glutamate receptor; mGluR, metabotropic GluR; AAA, α-aminoadipic acid; ADA, adenosine deaminase; CPCCOEt, 7-(hydroxyimino)cyclopropan-chromen-1a-carboxylate ethylester; DC, dendritic cell, iDC, immature DC; mDC, mature DC; MPEP, 6-methyl-2-(phenylethynyl)-pyridine; pAb, polyclonal antibody; SATg, extract of soluble Ags from *Toxoplasma gondii*; SEA, staphylococcal enterotoxin A; Var, attenuated varicella vaccine; β_2m, β_2-microglobulin.

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(25). Thus, this is the only available report in which a small nonpeptidic molecule classically viewed as neurotransmitter is also shown as immunomediator.

DC are the most potent APC specialized in the initiation of immune responses by directing the activation and differentiation of naive T lymphocytes (21, 22). Immature DC (iDC) reside in most tissues to uptake Ag; they are engaged when exposed to danger signals produced by microorganisms, inflammatory cytokines, nucleotides, and cell damage (26). Upon exposure to such factors, DC lose their phagocytic capacity, migrate to draining lymph nodes, and undergo a maturation process, acquiring high levels of membrane MHC, and costimulatory molecules such as CD80 and CD86. In the lymph nodes, mature DC (mDC) present the captured and processed Ag to specific T cells, thereby directing the development of immune responses (21, 22). Depending on the context, DC can stimulate the polarized outgrowth of distinct T cell subsets, including Th1 and Th2. Th1 or Th2 polarization orchestrates the immune effector mechanism most appropriate to combat the invading pathogen. Th1 cells promote cellular immunity protecting against intracellular infection and cancer, whereas Th2 cells promote humoral immunity, being a mechanism highly effective against extracellular pathogens, and play a role in tolerance mechanisms and allergic diseases (27).

Although many of the proteins involved in the T cell–DC interaction are well known, the possible role of soluble, small nonpeptidic mediators in the triggering of T cell activation remains weakly investigated. Because the interaction between T cells and DC has similarities to the neurological synapse, it is tempting to speculate the existence of immunomediators structurally similar to neurotransmitters. This study not only demonstrates the release of glutamate by DC during the T cell–DC contact formation, but also elucidates the role of the amino acid and of mGluR in regulating T cell proliferation and cytokine production.

Materials and Methods

Abs and reagents

FITC-conjugated mAbs against HLA-DR, CCR5, CD4, CD14, CD19, CD45RA, and IgG-γ1 isotype-matched control; PE-conjugated mAbs against HLA-DR, CXCR4, CD1a, CD11c, CD14, CD19, CD40, CD45, CD45RO, CD56, and IgG-γ1 isotype-matched control; and PerCP-conjugated mAbs against CD3 and IgG-γ1 isotype-matched control were purchased from BD Biosciences. PE-conjugated mAbs against CD80, CD83, and CD86 were from Coulter, and PE-conjugated mAb against CD209 was from eBioscience. The following polyclonal Abs (pAbs) were used: nonconjugated rabbit pAb against purified calf adenosine deaminase (ADA) (28); nonconjugated rabbit pAb against extracellular region of human mGlu1/5R (F1-Ab) (29); nonconjugated irrelevant rabbit IgG (Sigma-Aldrich); and secondary Ab PE-conjugated goat anti-rabbit (Sigma-Aldrich). α-Aminoadipic acid (AAA), 6-methyl-2-(phenylethynyl)-pyridine (MPEP), LPS, TNF-α, staphylococcal enterotoxin A (SEA), glutamate dehydrogenase, and glutamate oxidase were purchased from Sigma-Aldrich; alanine transaminase and NADP from Roche; sodium pyruvate from Life Technologies; and 7-(hydroxyimino)cyclopropan-chromen-1a-carboxylate ethylester (CPCCOEt) from Tocris. An extract of soluble Ags from *Toxoplasma gondii* (SATg) obtained from tachyzoites of the RH strain maintained in Swiss mice was provided by Argène Biosoft. An attenuated varicella virus vaccine preparation of OKA strain (Varilrix, here called Var) was from GlaxoSmithKline.

Generation of monocyte-derived DC and isolation of lymphocytes

Human PBMC from healthy individuals were obtained immediately after extraction from heparinized blood using the standard Ficoll gradient method. When indicated, cells from healthy donors having experienced varicella infection in their infancy, and cells from healthy individuals with SATg chronic infection (as defined by positive IgG serology), were studied. To obtain monocytes, PBMC (3×10^6 cells per ml) were incubated in serum-free XVIVO-15 medium (BioWhittaker) supplemented with 1% autologous serum, 50 μg/ml gentamicin (B. Braun Melsungen), and 2.5 μg/ml fungizone (DC medium; Bristol-Myers Squibb) for 2 h at 37°C in a humid atmosphere of 5% CO_2. Adherent cells were washed four times with prewarmed serum-free XVIVO-10 medium (BioWhittaker) and then cultured in DC medium at 37°C in a humid atmosphere of 5% CO_2. Cells were differentiated for 7 days to iDC, adding 1000 U/ml IL-4 (Prospec-Tany Technogene) and 1000 U/ml GM-CSF (Prospec-Tany Technogene) at days 0, 3, and 5. DC immunophenotyping was confirmed by flow cytometry using commercially available labeled mAbs against surface markers. Obtained iDC were $CD3^-$, $CD4^-$, $CD8^-$, $CD14^-$, $CD19^-$, $CD56^-$, HLA-DR^+, $CD80^+$, $CD83^+$, $CD86^+$, $CD1a^+$, $CD11c^+$, $CD40^+$, $CD45RO^+$, $CD45RA^-$, $CD209^+$, $CXCR4^+$, and $CCR5^-$. As a source of a T cell-enriched population for cocultures with autologous or allogeneic DC, nonadherent PBMC were collected after the 2-h period of plastic adherence and washed three times with XVIVO-10 medium. DC were cocultured with autologous or allogeneic T cells (see *Cocultures* section), or alone for maturation experiments, in 96-well plates (2×10^4 cells/well) in XVIVO-10 medium or, when indicated, in glutamate-free XVIVO-10 medium (see below) in the presence or absence of effectors in a final volume of 200 μl/well. DC were incubated for the indicated times at 37°C in a humid atmosphere of 5% CO_2. To obtain glutamate-free XVIVO-10 medium, normal XVIVO-10 medium was heated for 1 h at 70°C, cooled, and incubated 15 h at room temperature with 5 mM pyruvate and 1 μg/ml alanine transaminase. To inactivate alanine transaminase, medium was heated for 1 h at 70°C, cooled, and finally passed through 0.22-μm filter. To confirm glutamate elimination, its concentration was measured as indicated below.

Cocultures

DC were harvested at day 7, and fresh autologous or allogeneic (as indicated) T cells were prepared. DC were resuspended in DC medium (4×10^5 cells/ml) and, when indicated, pulsed at 37°C in a humid atmosphere of 5% CO_2 for 4 h with 100 pg/ml SEA. Alternatively, DC isolated from donors having experienced varicella infection in their infancy or from donors with SATg chronic infection were respectively pulsed with either 40 PFU/ml Var or 100 μg/ml SATg. Pulsed DC were washed four times and resuspended at 4×10^5 cells/ml with XVIVO-10 medium or, when indicated in figure legends, with glutamate-free XVIVO-10 medium. When T cell proliferation was measured, pulsed DC were gamma irradiated before coculture. Cocultures were performed in 96-well plates containing T cells (2×10^5 cells/well), effectors when indicated, and DC (2×10^4 cells/well) in XVIVO-10 medium or in glutamate-free XVIVO-10 medium at a final volume of 200 μl/well. Cocultures were incubated for the indicated times at 37°C in a humid atmosphere of 5% CO_2.

Determination of glutamate levels

At different times, supernatants were harvested and glutamate levels were determined in 96-well plates using a L-glutamic acid colorimetric method (Roche) according to the manufacturer's protocol using 40 μl of supernatants. Values of remainder glutamate levels in the used medium were determined and subtracted from values obtained for each supernatant.

Immunostaining

When direct-labeled Abs were used, cells were washed with PBS, resuspended at 2×10^6 cells/ml (50 μl/tube), and incubated with FITC-, PE-, and/or PerCP-conjugated Abs for 30 min at 4°C. Cells were washed with PBS, fixed with 1% formaldehyde in PBS, and analyzed by flow cytometry. FITC-, PE-, and PerCP-conjugated isotype-matched Abs were used for negative controls. When unlabeled primary pAbs were used, cells were washed with PBS, fixed in PBS containing 2% paraformaldehyde for 15 min at room temperature, and washed twice with PBS containing 20 mM glycine to quench the aldehyde groups. Cells were incubated with blocking buffer (10% heat-inactivated horse serum in PBS) for 30 min, then incubated with binding buffer (PBS containing 1% BSA, 20 mM glycine, and 0.1% NaN_3) for 15 min and labeled with F1-Ab (3 μg/ml), anti-ADA (20 μg/ml), or irrelevant rabbit IgG (20 μg/ml, as negative control of pAbs) for 1 h at room temperature. Cells were washed three times with binding buffer and stained with the secondary Ab PE-conjugated goat anti-rabbit IgG (1/20) during 1 h at room temperature. Finally, cells were washed three times with binding buffer, and flow cytometry analysis was performed with an EPICS profile flow cytometer (Coulter). Cell populations were selected by forward and side light-scatter parameters.

Relative mRNA levels measurements

Total RNA was extracted from cells using the TRIzol reagent (Sigma-Aldrich) according to the manufacturer's protocol. Aliquots of 1 μg of total RNA, 0.5 μg of oligo(dT) (Sigma-Aldrich) as primers, and 2 μl of dNTPs

(mix, 5 mM each; Sigma-Aldrich) were dissolved with sterile RNase-free water (total volume of 12 μl), heated at 65°C for 5 min, and cooled at 4°C. Then, 4 μl of first-strand buffer 5× (250 mM Tris-HCl, 375 mM KCl, and 15 mM MgCl (pH 8.3); Invitrogen Life Technologies), 2 μl of 100 mM DTT (Invitrogen Life Technologies), and 1 μl of RNase inhibitor (RNasa OUT; Invitrogen Life Technologies) were added and incubated at 42°C for 2 min. The reaction was started by the addition of 1 μl (200 U) of SuperScript II reverse transcriptase (Invitrogen Life Technologies), incubated at 42°C for 50 min, and finished at 70°C for 15 min. The cDNAs were amplified using a commercially available kit LightCycler FastStart DNA Master Plus SYBR Green I (Roche). Each reaction was conducted with 2 μl of cDNA, 4 μl of 5× MasterMix (FastStart DNA Master Plus SYBR Green I), 0.5 μM of each primer, and sterile RNase-free water at final volume of 20 μl. Real-time PCR amplification was conducted using the LightCycler instrument. Specific primers were selected according to GenBank database resource, and conditions of cycles, times, and temperatures for amplification were as follows: mGlu1R, amplimer 453 bp (forward) 5′-ACCCGGTC CTCCTGCCCAACA-3′, (reverse) 5′-CGTCCATTCCGCTCTCCCCA TAA-3′, 30 cycles (5 s at 95°C, 5 s annealing at 69°C, 19 s at 72°C, and acquiring 4 s at 79°C); mGlu5R, amplimer 661 bp (forward) 5′-TCCTG GCCACCCTGTTTGTTACTG-3′, (reverse) 5′-GTGGCACTGAGGCT GACCGAGAAA-3′, 30 cycles (5 s at 95°C, 5 s annealing at 69°C, 27 s at 72°C, and acquiring 4 s at 78°C); xCT, amplimer 363 bp (forward) 5′-CCTGGCATTTGGACGCTACATT-3′, (reverse) 5′-TTTTCAGGGTTT TCTACTTCTTCA-3′, 45 cycles (5 s at 95°C, 5 s annealing at 68°C, 15 s at 72°C, and acquiring 4 s at 79°C); and β_2-microglobulin (β_2m), amplimer 107 bp (forward) 5′-ACACAACTGTGTTCACTAGC-3′, (reverse) 5′-CAACTTCATCCACGTTCACC-3′, 30 cycles (5 s at 95°C, 10 s annealing at 58°C, 4 s at 72°C, and acquiring 4 s at 85 °C). Relative levels of receptor mRNAs were calculated and normalized by β_2m mRNA levels as control using (30) the following formula: Relative mRNA expression = $2^{-(\text{Ct of receptor} - \text{Ct of } \beta_2\text{m})} \times 10^3$. All cDNA samples were amplified in triplicates.

Cytokine determination

IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ levels were determined using a BD Cytometric Bead Array Human Th1/Th2 cytokine kit (BD Bioscience) according to the manufacturer's protocol using 50 μl of conveniently diluted coculture supernatants.

Proliferation assays

In the last 18 h of coculture, cells were pulsed with 1 μCi thymidine/well ([^{3}H]methyl, 2 Ci/mmol; Moravek Bioquemicals) and fixed in 3.7% formaldehyde for 30 min, harvested onto filters, and extensively washed using a cell harvester (LKB 1295-001; Wallac). Tritium incorporation was determined by a liquid scintillation counter (1205 Betaplate; Wallac). When indicated proliferation was determined by the CFSE method. In these experiments, cells were stained with 1 μl of 5 mM CFSE/10 × 10^6 cells as indicated by the manufacturer's protocol using the CellTrace CFSE cell proliferation kit (Molecular Probes). CFSE dilution analysis was performed using an EPICS profile flow cytometer (Coulter). Cell populations were selected by forward and side light-scatter parameters. The number of division cycles was determined as described previously (31).

Results

DC undergoing maturation release glutamate

To examine whether DC can release glutamate, freshly obtained DC from healthy donors were cultured in a glutamate-free medium in the presence or absence of SEA, a superantigenic protein which upon binding to MHC class II molecules promotes their cross-link on the APC surface (32). DC were cultured for different time intervals, and the glutamate concentration was determined in the culture medium supernatant. Although DC released glutamate weakly in the absence of SEA (Fig. 1*A*), the concentration of the glutamate accumulated in the bulk medium was significantly different on day 6 vs on day 1 ($p < 0.005$). In the presence of SEA, the release of glutamate was markedly higher (Fig. 1*A*). To determine whether SEA stimulates the release of glutamate in a dose-dependent fashion, glutamate levels were evaluated in supernatants of DC stimulated with increasing SEA concentrations ranging from 100 fg/ml to 100 ng/ml. The results show that the amount of glutamate released by DC was dependent on the dose of SEA used to stimulate DC, with an EC_{50} value ~100 pg/ml (Fig. 1*B*). In further assays, the concentration of SEA used was 1 ng/ml, which gives ~75% of the maximal glutamate release by DC (Fig. 1*B*). Interestingly, 1 ng/ml SEA promoted an increase ranging from 1.3- to 4-fold in the glutamate concentration released by DC (Fig. 1, *A* and *C*). Not only was the SEA-induced increase in glutamate levels observed in every donor tested (averaging from 15 to 30 μM on day 4), but the difference in the average value with respect to untreated cells for the five donors was also statistically significant ($p < 0.0001$; Fig. 1*C*). Because glutamate derived from macrophages in the brain (5) is released through the cystine/glutamate antiporter (X_c^- system), experiments were performed using AAA, an inhibitor of this transporter, in DC subjected to the action of different danger signals capable of inducing maturation in these cells. The results show that the release of glutamate from DC was inhibited by AAA (Fig. 1*D*), which suggests the involvement of the cystine/glutamate antiporter X_c^- system. In addition, these results show that stimuli that induce DC maturation, such as LPS or TNF α (TNF-α), which greatly increased expression of HLA-DR, CD80, CD83 and CD86 (data not shown), enhanced the capacity of DC to release glutamate (Fig. 1*D*).

DC release glutamate during the T cell–DC interaction

The capacity of DC to release glutamate in cocultures with T cells under conditions approximating those found in lymph nodes during Ag presentation was analyzed. When DC were cocultured with autologous T cells, a significant time-dependent increase in glutamate concentration in the coculture medium supernatant was observed either using nonpulsed DC or SEA-pulsed DC (Fig. 2*A*). Glutamate levels were however markedly higher when T cells were cocultured with SEA-pulsed DC, thus indicating that high levels of glutamate are released during Ag-specific T cell–DC contacts. Not only was the time-dependent release of glutamate during T cell–DC interaction observed in every donor tested (averaging from 10 μM on day 1 to 50 μM on day 4), but the difference in the average value with respect to untreated cells for the five donors was statistically significant ($p < 0.0001$; Fig. 2*B*). To confirm that glutamate is released through the cystine/glutamate antiporter X_c^- system, SEA-pulsed DC were cocultured with autologous T cells in the presence of increasing concentrations of the cystine uptake inhibitor AAA, and glutamate levels were measured in the coculture medium supernatants. As shown in Fig. 2*C*, the release of glutamate during the T cell–DC interaction was inhibited by the cystine uptake inhibitor in a dose-dependent manner with an IC_{50} of 1000 ± 100 μM. There was a background level of glutamate when T cells were cultured in glutamate-free medium in the absence of DC. The contribution of T cells to the extracellular concentration of glutamate observed in cocultures was low, and it was not affected by treatment with the cystine uptake inhibitor AAA (Fig. 2*C*).

To corroborate that the X_c^- system operates in DC but not in T cells, we analyzed the mRNA expression of xCT (L chain of the cystine/glutamate antiporter) using a quantitative RT-PCR method. Consistent with previous reports describing low cystine membrane transport activity in T cells (33, 34), very low xCT mRNA expression levels in T cells were observed (Fig. 3). In contrast, DC exhibited significant xCT mRNA expression (Fig. 3).

DC maturation and T cell–DC interaction contribute to the glutamate release

To determine whether glutamate release is a general feature of Ag presentation and due to the fact that SEA simply needs to bind to class MHC II molecules to act as superantigen, experiments with DC presenting the following Ags structurally and mechanistically distinct from SEA were performed. First, the extract of soluble Ag

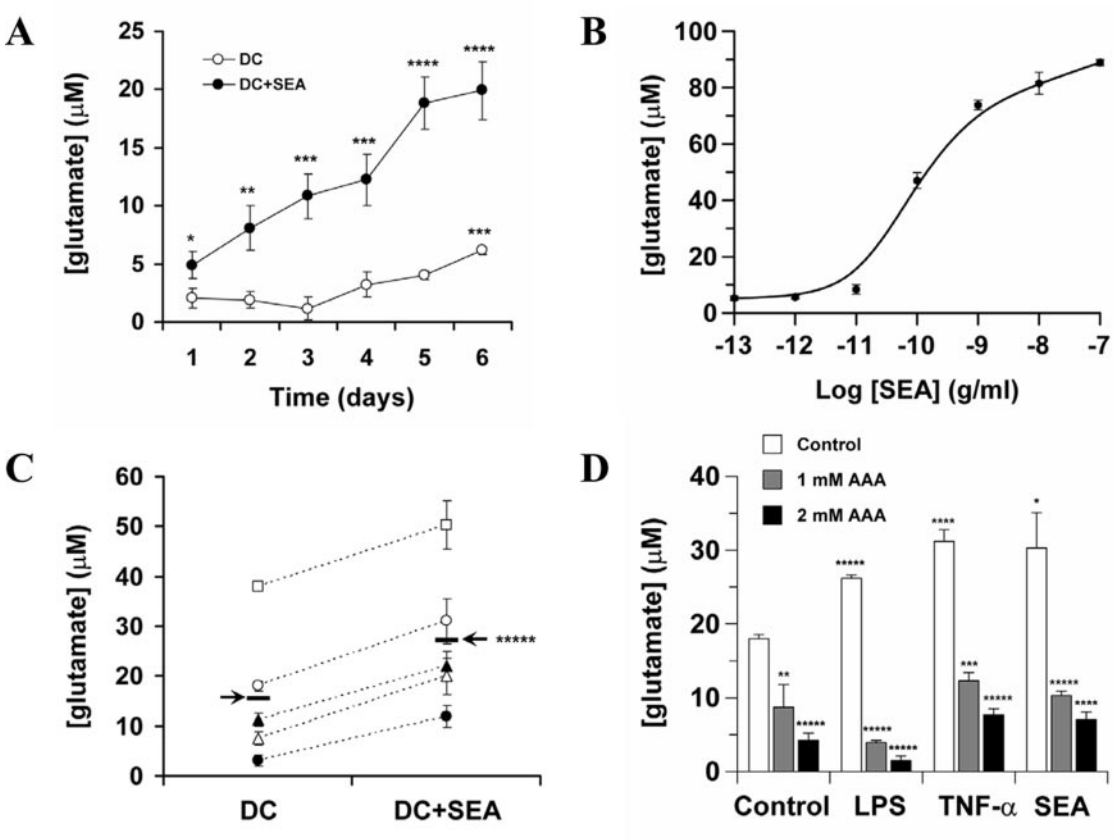

FIGURE 1. Glutamate release by DC. *A*, DC were cultured in glutamate-free medium for different time intervals in the absence or presence of 1 ng/ml SEA, and glutamate levels in the culture medium were determined. *B*, DC were cultured in glutamate-free medium for 6 days in the presence of increasing concentrations of SEA, and glutamate levels in the culture medium were determined. *C*, DC from five different donors were cultured in glutamate-free medium for 4 days in the absence or presence of 1 ng/ml SEA, and glutamate levels in the culture medium were determined. Each symbol represents the value of the glutamate level obtained in DC cultures from a healthy donor. Average values are indicated by ▬ (arrows). *D*, DC in the absence (control) or presence of 1 μg/ml LPS, 1000 U/ml TNF-α (TNF-α), or 1 ng/ml SEA were incubated without or with 1 or 2 mM AAA in glutamate-free medium for 4 days, and glutamate levels in the culture medium were determined. Data represent the mean ± SD of triplicates. Representative data from one of five (*A*) or three (*B* and *D*) independent experiments are shown. Statistical analysis was performed by unpaired (*A* and *D*) or paired (*C*) Student's *t* test (treated vs untreated at day 1 (*A*), untreated (*C*), and untreated control (*D*); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.0005$; *****, $p < 0.0001$).

from SATg, which requires its capture and processing to be presented to T cells, was used. Second, an attenuated live virus (Var preparation), which involves the infection of DC and, subsequently, its processing and presentation to T cells, was used. Finally, nonpulsed iDC were used as alloantigens to activate T cells from HLA-incompatible individuals. This latter Ag presentation does not require either capture or Ag processing. In cocultures of SATg-pulsed DC with autologous T cells from donors with chronic infection by SATg (documented by IgG serology), glutamate levels were significantly higher than those released in cocultures with nonpulsed DC (Fig. 4*A*). Similar results were obtained when attenuated varicella virus was used as the Ag in cocultures with T cells from healthy young individuals who experienced varicella infection in their infancy (Fig. 4*A*). In addition, when nonpulsed iDC were used to stimulate HLA-incompatible T cells, the levels of glutamate released were higher than the amounts determined in cocultures with autologous T cells (Fig. 4*A*). It should be noted that, in all these experiments, cocultures of nonpulsed iDC with autologous T cells led to a moderate increase of glutamate release, compared with DC alone. This result is similar to that described in Fig. 2*A*, i.e., there is a basal release of glutamate in cocultures of nonpulsed DC with autologous T cells, further indicating that the simply contact between autologous DC and T cells in the absence of Ag stimulation and exogenous maturational stimuli causes a release of glutamate by DC.

To know whether glutamate release promoted by exogenous maturation signals (for instance, by LPS) is additive to the glutamate release caused by signals derived from Ag-dependent contacts with autologous T cells, autologous and allogeneic cocultures were performed in the presence or absence of LPS (Fig. 4*B*). As expected, LPS treatment of DC increased the basal glutamate release, and cocultures of iDC with autologous T cells also gave a basal increase in the glutamate levels. In contrast, when iDC were used to stimulate allogeneic T cells, there was a high increase in the glutamate release, which did not increase further by LPS action (Fig. 4*B*).

Group I mGluR are expressed in T cells, but not in DC

Although mGluR expression on the T cell surface has been described previously (8, 11–15), the mGluR expression in DC still remains unexplored. Therefore, to determine whether DC-released glutamate acts on group I mGluR via an autocrine mechanism, group I mGluR expression on the DC surface was assayed. Using a pAb directed against an extracellular region common to both members of group I mGluR (29), the expression of mGlu1R and/or mGlu5R, was analyzed in iDC or TNF-α-matured DC. Activated T cells were used as a positive control for mGlu1/5R expression (Ref. 12 and Fig. 5*A*), and immunolabeling of ADA was used as positive control for DC surface expression (Ref. 35 and Fig. 5*A*). The results demonstrated that neither iDC nor mDC express group

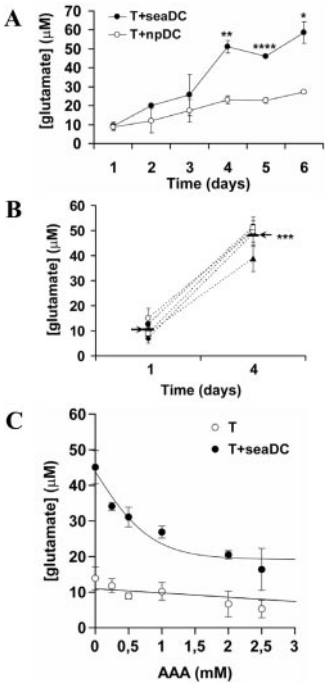

FIGURE 2. Glutamate released by DC cocultured with T cells. *A*, T cells were cocultured with either autologous nonpulsed DC (T+npDC) or autologous SEA-pulsed DC (T+seaDC) in glutamate-free medium. Cocultures were incubated for different time intervals, and glutamate levels in the culture medium were determined. *B*, Cocultures of T cells with autologous SEA-pulsed DC from five different donors were incubated for 1 or 4 days in glutamate-free medium, and the glutamate levels in the culture medium were determined. Each symbol represents the value of the glutamate level obtained from a healthy donor. Average values are indicated by black bars (arrows). *C*, T cells alone (T) or cocultured with autologous SEA-pulsed DC (T+seaDC) were incubated with increasing concentrations of AAA for 4 days in glutamate-free medium, and glutamate levels in the culture medium were determined. Data represent the mean ± SD of triplicates. Representative data from one of five (*A*) or three (*C*) independent experiments are shown. Statistical analysis was performed by unpaired (*A*) or paired (*B*) Student's *t* test (treated vs untreated (*A*) or values in day 4 vs values in day 1 (*B*); *, $p < 0.001$; **, $p < 0.0005$; ***, $p < 0.0001$; ****, $p < 0.00005$).

I mGluR on the cell surface (Fig. 5*A*). Similar results were obtained with LPS-matured DC (data not shown). To test whether group I mGluR are expressed in those T cells in contact with DC, T cells were cocultured with superantigen-pulsed DC for different time intervals and the mRNAs for mGlu1R and mGlu5R were quantified by real-time RT-PCR. The results revealed that, while mGlu1R mRNA levels were undetectable at time zero of coculture, the receptor was expressed beginning on day 1 of coculture, attaining high levels on day 3 (Fig. 5*B*). In contrast, the mRNA for mGlu5R was constitutively expressed in T cells (Fig. 5*B*), and its expression did not decrease after coculture.

Glutamate released by DC during T cell–DC contact formation modulates cytokine production

In the lymph nodes, cytokine production constitutes the culmination of the productive immunosynaptic contacts formation between DC and T cells (36). As SEA binds to MHC class II, the T cell response triggered in our experimental model is $CD4^+$ mediated (37). Two major $CD4^+$ T cell subsets are now known, Th1 and Th2, each exhibiting a particular cytokine pattern. Th1 or Th2 polarization orchestrates the immune effector mechanism that is most effective against the invading pathogen (27). Based on the important role that cytokines play in the T cell response,

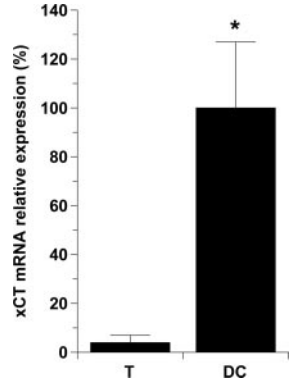

FIGURE 3. xCT mRNA expression in DC and T cells. Total RNA was isolated from T cells (T) and DC, and quantitative RT-PCR analysis of xCT mRNA levels was conducted as indicated in *Materials and Methods*. To obtain a better comparison of relative mRNA expression, values are represented as percentage of xCT mRNA expressed by DC. Data represent the mean ± SD of triplicates. Representative data from one of three independent experiments are shown. Statistical analysis was performed by unpaired Student's *t* test (*, $p < 0.05$).

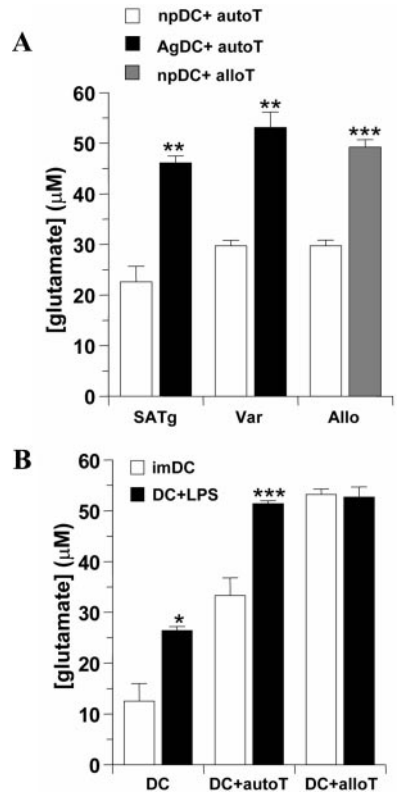

FIGURE 4. Release of glutamate triggered by presentation of different Ags or by allogeneic recognition. *A*, Nonpulsed DC (npDC) or DC pulsed (AgDC) with either 100 μg/ml extract of soluble Ags from SATg or 40 PFU/ml Var were cocultured with autologous (autoT) or allogeneic (alloT) T cells in glutamate-free medium. *B*, Nonpulsed DC were cultured alone (DC) or cocultured with either autologous (DC+autoT) or allogeneic (DC+alloT) T cells in glutamate-free medium in the absence (DCim) or presence of LPS (DC+LPS). *A* and *B*, Cultures were incubated for 4 days, and glutamate levels in the culture medium were determined. Data represent the mean ± SD of triplicates. Representative data from one of three independent experiments are shown. Statistical analysis was performed by unpaired Student's *t* test (black/gray bars vs respective white bars; *, $p < 0.005$; **, $p < 0.0005$; ***, $p < 0.00005$).

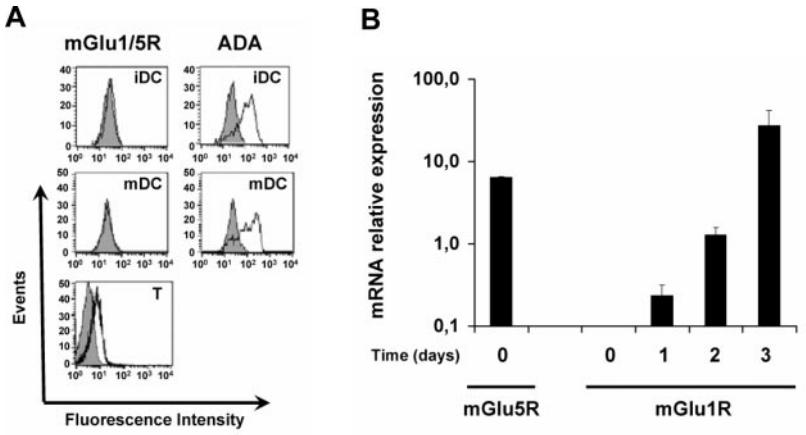

FIGURE 5. mGlu1R and mGlu5R expression in DC and T cells. *A*, iDC or DC matured with 1000 U/ml TNF-α for 48 h in glutamate-free medium (mDC) were immunostained using 3 μg/ml primary anti-mGlu1/5R Ab F1-Ab (mGlu1/5R) or using 20 μg/ml anti-adenosine deaminase (ADA) Ab as a positive control for DC surface expression, followed by incubation with a PE-conjugated goat anti-rabbit secondary Ab (dilution 1/20). As a positive control for mGlu1/5R expression, activated T cells (T) were immunolabeled with F1-Ab followed by PE-conjugated goat anti-rabbit secondary Ab. Analysis was performed by flow cytometry (white histograms). Negative controls (gray histograms) were obtained by using an irrelevant rabbit IgG (20 μg/ml) as primary Ab. *B*, T cells were cultured with SEA-pulsed autologous DC in glutamate-free medium for different time intervals, and total RNA was isolated. Quantitative RT-PCR analysis of mGlu1R and mGlu5R mRNA levels were performed as indicated in *Materials and Methods*. Data represent the mean $\pm$ SD of triplicates. Representative data from one of three independent experiments are shown.

experiments aimed to determine the role of group I mGluR in Th1, Th2, and proinflammatory cytokine secretion during the T cell–DC interaction were conducted. To determine the global effects of glutamate in cytokine production, cocultures of SEA-pulsed DC and T cells were treated with alanine transaminase plus pyruvate, thereby depleting the medium of glutamate. Cytokine secretion

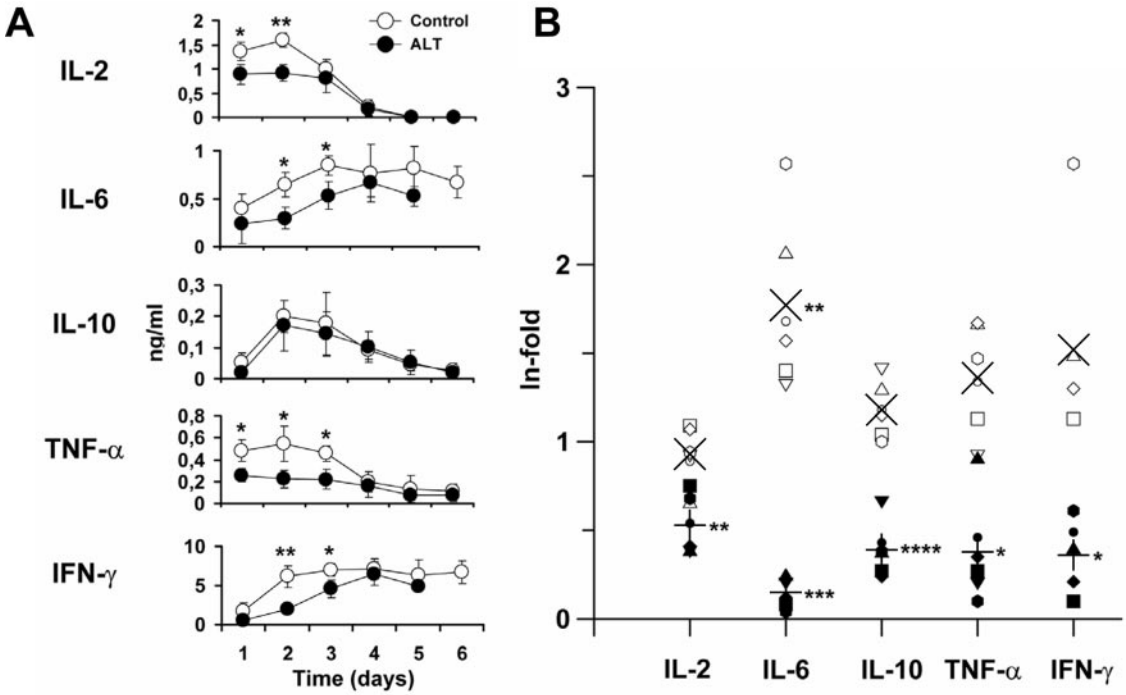

FIGURE 6. Effects of glutamate depletion and of group I mGluR antagonists on cytokine secretion during T cell–DC interaction. SEA-pulsed DC were cocultured with autologous T cells for different time intervals and cytokine levels in the culture medium were determined as indicated in *Materials and Methods*. *A*, Time course of cytokine production in the absence (control) or presence of 1 μg/ml alanine transaminase plus 5 mM pyruvate (ALT). Representative data from one of three independent experiments are shown. Data represent the mean $\pm$ SD of triplicates. *B*, Cytokine production by autologous cocultures obtained from six different healthy donors incubated for 2 days in the absence or presence of either 1 μM MPEP (open symbols) or 100 μM CPCCOEt (solid symbols). Each symbol represents the value of the level of cytokine produced by cells from a healthy donor. Irrespective of the donor, experiments conducted in the absence of T cells gave values of cytokine production lower than 20 pg/ml. In the absence of DC, cytokine levels produced by T cells were lower than 20 pg/ml, except for IL-6, which ranged between 60 and 130 pg/ml. Variations on cytokine production promoted by mGluR antagonists (MPEP or CPCCOEt) are expressed as the ratio of the values obtained in the presence vs in the absence of the compound. Average values obtained after the treatment with MPEP (x) or with CPCCOEt(+) are indicated. Statistical analysis was performed by unpaired (*A*) or paired (*B*) Student's *t* test (treated vs untreated; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.0005$).

was then determined in the coculture medium supernatant at different time intervals. The absence of glutamate led to impaired Th1 (IL-2 and IFN-γ) and proinflammatory (IL-6 and TNF-α) cytokine production (Fig. 6*A*). More importantly, when mGlu5R was antagonized by the specific antagonist MPEP, a significant increase (~2-fold) in the IL-6 secretion was observed, whereas when mGlu1R was antagonized by the specific antagonist CPCCOEt, impaired IL-2, IL-6, IL-10, TNF-α, and IFN-γ production was observed (Fig. 6*B*). IL-4 production was also tested, but it proved undetectable either in the absence or presence of GluR agonist, antagonists, or alanine transaminase. As a positive control, IL-4 was measured in supernatants of autologous T cell–DC cocultures from four HIV$^+$ patients in which the Th1/Th2 balance is impaired (38). In these conditions, production of IL-4 was 170 ± 50 pg/ml. Moreover, in the presence of adenosine deaminase, which has a costimulatory effect on cytokine production (35), IL-4 levels were enhanced up to 1000 ± 300 pg/ml.

DC-released glutamate acting upon mGluR modulates T cell proliferation

Another important feature of Ag presentation by DC in the lymph nodes is T cell proliferation, which constitutes the last phase of the activation process (36). To determine the role that DC-released glutamate plays via group I mGluR in T cell proliferation, a series of experiments measuring T cell proliferation were performed in which DC and T cells were cocultured in the presence of group I mGluR antagonists, glutamate-depleting agents, or the cystine uptake blocker AAA. When mGlu5R was antagonized by the specific antagonist MPEP, enhanced proliferation was observed, whereas in presence of the mGlu1R-specific antagonist CPCCOEt, T cell proliferation was impaired (Fig. 7*A*). In contrast, when SEA-pulsed DC were cocultured with autologous T cells in the presence of glutamate-depleting agents such as glutamate oxidase, glutamate dehydrogenase plus NADP, or alanine transaminase plus pyruvate, the global effect resulting from the absence of glutamate over the entire period of coculture was antiproliferative (Fig. 7*B*). In addition, when superantigen-pulsed DC were cocultured with autologous T cells in glutamate-free medium in the presence of increasing concentrations of the cystine/glutamate antiporter inhibitor AAA, which inhibits glutamate release from DC (Figs. 1*D* and 2*C*), a dose-dependent inhibitory effect on T cell proliferation was observed (Fig. 7*C*). Proliferation in cocultures performed in glutamate-free medium was similar to that measured in complete medium (data not shown). This indicates that the lack in the culture medium of the nonessential amino acid glutamate is not the cause of the functional responses detected by using the mGluR antagonists or the inhibitor of the cystine/glutamate antiport.

As indicated above, mGlu1R expression is induced by T cell activation, and its stimulation promotes a costimulatory effect. To determine whether mGlu1R antagonism affects the number of cells in division, the number of division cycles or both in CD4$^+$ T cells stimulated by SEA-pulsed DC, proliferation was quantified by the CFSE method. As shown in Fig. 8, the specific mGlu1R antagonist CPCCOEt promoted a significant decrease in the number of cells in division without significant effect in the average number of division cycles (Fig. 8*A*). Similar results were obtained when glutamate release was inhibited by the blocker of cystine/glutamate antiporter in cocultures of SEA-pulsed DC and autologous T cells (Fig. 8*B*).

Discussion

The data presented in this work provide evidence that during the maturation process, DC release physiologically relevant glutamate amounts (Fig. 1), which are high enough to activate GluR (2).

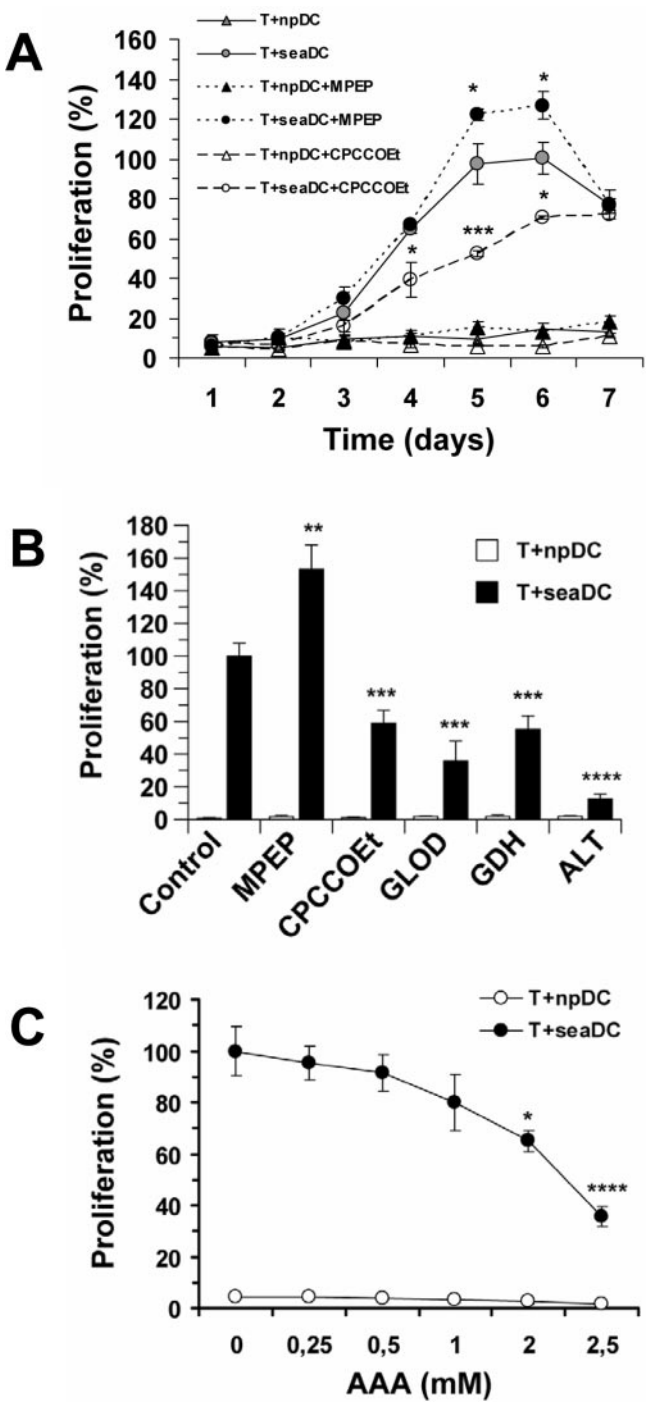

FIGURE 7. Effects of glutamate depletion, X_c^- system inhibition, and group I mGluR antagonists on T cell proliferation. *A*, Nonpulsed (npDC) or SEA-pulsed (seaDC) DC were cocultured with autologous T cells (T) in the absence or presence of either 1 μM MPEP or 100 μM CPCCOEt for different time intervals. *B*, Nonpulsed or SEA-pulsed DC were cocultured for 6 days with autologous T cells in the absence (control) or presence of 1 μM MPEP, 100 μM CPCCOEt (CPCCOEt), 20 mU/ml glutamate oxidase (GLOD), 20 mU/ml glutamate dehydrogenase plus 200 μM NADP (GDH), or 1 μg/ml alanine transaminase plus 5 mM pyruvate (ALT). *C*, Nonpulsed or SEA-pulsed DC were cocultured for 6 days with autologous T cells in glutamate-free medium in the presence of increasing concentrations of AAA. In *A–C*, proliferation was determined as [^{3}H]thymidine incorporation. Values are expressed as percentages with respect to the control at day 6. Data represent the mean ± SD of triplicates. Representative data from one of three independent experiments are shown. Statistical analysis was performed by unpaired Student's *t* test (treated vs control). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.0001$.

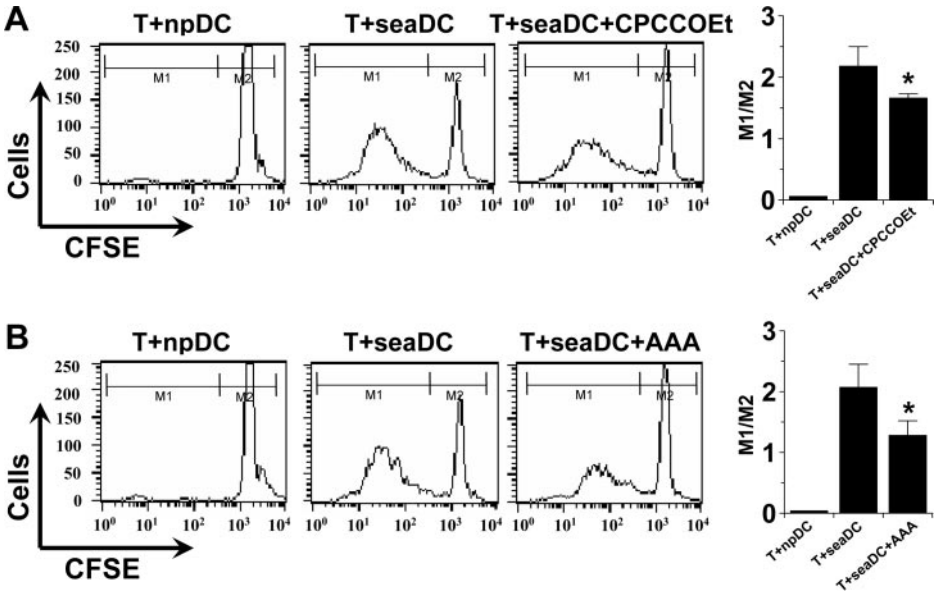

FIGURE 8. Antagonism of mGlu1R on T cells or inhibition of glutamate release from DC during T cell–DC interaction induce a decrease on the number of proliferating cells. T cells were loaded with CFSE as indicated in *Materials and Methods*. *A*, CFSE-loaded T cells were cocultured with autologous nonpulsed (npDC) or SEA-pulsed (seaDC) DC in complete culture medium in the absence or presence of 100 μM of the mGlu1R selective antagonist CPCCOEt (CPCCOEt) for 6 days. *B*, CFSE-loaded T cells were cocultured with autologous nonpulsed (npDC) or SEA-pulsed (seaDC) DC in glutamate-free medium in the absence or presence of 2 mM AAA for 6 days. After coculture incubation, cells were immunostained with PE-conjugated mouse anti-CD4 mAb as indicated in *Materials and Methods*. $CD4^+$ cells were gated and distribution of CFSE in proliferating $CD4^+$ T cells was analyzed by flow cytometry. *A* and *B*, Representative histograms from one of three independent experiments are shown. Each experiment was performed in triplicates. *A* and *B*, Proliferating (M1) and nonproliferating (M2) populations were gated, and the ratio of M1 to M2 was determined (*right panel*). Statistical analysis was performed by unpaired Student's *t* test (treated vs T+seaDC; $*$, $p < 0.05$).

Piani and Fontana (5) reported that brain macrophages release glutamate in the range of 6 to 40 μM per 10^5 cells, which is comparable to the amount of glutamate released by DC (2–25 μM per 10^5 cells). Therefore, both types of APC are able to release similar levels of glutamate, which indeed are high enough to activate group I mGluR (2). The parallelism between DC maturation and glutamate release suggests that this amino acid could play a physiological role during the Ag presentation to T cells. In this regard, DC released physiologically relevant concentrations of glutamate during T cell–DC interactions (Fig. 2). In addition, it is likely that the glutamate released into the microenvironment where the T cell–DC contacts are established reaches local concentrations that would be higher than those measured in the culture medium supernatant.

Both, DC maturation (Fig. 1) and T cell–DC contact (Figs. 2 and 4) induced release of glutamate above basal levels, and they were additive in cocultures of DC and autologous T cells but not in cocultures of allogeneic cells (Fig. 4*B*). All these results suggest that the release of glutamate is triggered by not only maturation, but also signals derived from nonspecific interactions involved in the T cell–DC contact, which would be enhanced during specific-Ag presentation. The inhibition by a blocker of cystine uptake (Figs. 1*D* and 2*C*) and the fact that DC expressed relevant levels of mRNA for xCT (Fig. 3), the L chain of X_c^- system (5, 6), indicates that the cystine/glutamate antiporter is expressed and mediates the glutamate release by DC.

In lymph nodes, the microenvironment is tightly regulated, and therefore the local concentration of glutamate released by DC during the T cell–DC contact would be the proper for regulate the T cell activation during Ag presentation. In fact, the K_D values recorded for members of the group I mGluR family fluctuate from 10 to 60 μM (2), indicating that these receptors can be activated by DC-released glutamate. Our results show clearly that group I mGluR were not expressed on DC (Fig. 5*A*), therefore indicating that it is likely that the target of DC-released glutamate are receptors expressed on T cells. In addition, when DC were matured with TNF-α or LPS in the presence of AAA, a blocker of cystine/glutamate antiporter, expression of the maturation markers (HLA-DR, CD80, CD83, and CD86) did not significantly change (data not shown). Therefore, the possibility of an indirect effect of glutamate on T cell activation by affecting DC maturation through unidentified GluR can be ruled out. Whereas mGlu5R was constitutively expressed in resting T cells, mGlu1R was expressed only after 24–48 h of coculture with superantigen-pulsed DC (Fig. 5*B*). Previously, it has been reported that mGlu1R expression is induced in PHA-stimulated T cells in the absence of DC (12). Therefore, mGlu1R expression seems to be dependent on T cell activation. This finding suggests that, early on, during the formation of T cell–DC contacts, DC-released glutamate, activates the constitutively expressed mGlu5R in T cells, whereas at later stages, the amino acid activates inducible mGlu1R.

Cytokine production plays a key role in the T cell activation (39–41) and T cell phenotype polarization (27). On assessing the effect of group I mGluR stimulation by DC-released glutamate on cytokine secretion, our results indicate that, shortly after T cell–DC contact establishment, DC-released glutamate acts on the mGlu5R expressed in T cells, impairing IL-6 production (Fig. 6*B*). However, 48 h later, DC-released glutamate acts via mGlu1R, which is already expressed on the T cell surface, thereby counteracting the effect promoted via mGlu5R on IL-6 production and also enhancing the secretion of TNF-α, Th1 cytokines (IL-2 and IFN-γ), and IL-10. Importantly, the mGlu1R-mediated increase in TNF-α production (Fig. 6*B*) may constitute a positive feedback mechanism for the release of glutamate by DC, as shown in Fig. 1*D*. In addition, the mGlu1R-mediated rise in the IFN-γ and IL-2 secretion (Fig. 6*B*) would potentiate a Th1-polarized response,

thus promoting cellular immunity and protection against intracellular infection (27), whereas the mGlu1R-induced increase on the IL-6 production (Fig. 6*B*) could promote costimulation of T cell activation (39, 40). Interestingly, IL-10 levels were not affected by treatment with alanine transaminase plus pyruvate (Fig. 6*A*), but by the mGlu1R antagonist CPCCOEt (Fig. 6*B*). As IL-10 has been implicated in the inhibition of chemotactic migration of $CD4^+$ T cells (42–44), it is likely that the action of glutamate impairing IL-10 production described by others (10) occurs via another GluR type, probably iGlu3R, which participates in the regulation of adhesion and chemotactic migration (10). Thus, those iGluR expressed in resting T cells (10) stimulated by plasma glutamate (10–50 μM) (45, 46) could allow the chemotactic migration of T cells in peripheral tissues. These cells could be retained in the lymph nodes for some days by the action of DC-released glutamate, the latter acting on the inducible mGlu1R expressed following productive Ag presentation.

Our results indicate that, when glutamate acts via the constitutively expressed mGlu5R, it inhibits T cell proliferation, while when acting via the inducible mGlu1R, it enhances T cell proliferation (Fig. 7*A*). In contrast, when SEA-pulsed DC were cocultured with autologous T cells in the presence of glutamate-depleting agents such as glutamate oxidase, glutamate dehydrogenase plus NADP, or alanine transaminase plus pyruvate, the global effect resulting from the absence of glutamate over the entire period of coculture was antiproliferative (Fig. 7*B*). In addition, when T cell–DC cocultures were incubated with increasing concentrations of the cystine uptake blocker, the T cell proliferation was inhibited in a dose-dependent manner (Fig. 7*C*), thus demonstrating that the glutamate released by the X_c^- system expressed on DC during T cell–DC contacts formation is essential to allow a robust T cell proliferation. Consequently, the glutamate released by DC acts initially through the mGlu5R, impairing the T cell proliferation, but later mGlu1R stimulation overcomes the mGlu5R-triggered signaling and allows robust T cell proliferation. In contrast, the DC-released glutamate that acts via inducible mGlu1R, potentiates IL-2 secretion (Fig. 6*B*), thereby promoting enhanced T cell proliferation after productive immunosynaptic contact formation (Fig. 7*A*). Because T cell costimulation via IL-2 and via IL-6 reportedly follows two independent pathways (39, 40), the DC-released glutamate acting via mGlu1R promotes T cell costimulation through two parallel ways, one by counteracting the mGlu5R-mediated effect, thereby restoring and enhancing the IL-6 levels, and another by enhancing IL-2 production.

In summary, our data demonstrate that glutamate is a novel immunomediator in the intercellular cross-talk established between DC and T cells. Upon maturation, DC release glutamate through the cystine/glutamate antiporter X_c^- system. This DC-released glutamate acts early during T cell–DC interaction via mGlu5R, which is positively coupled to the adenylate cyclase (12), thereby impairing the IL-6 production and, consequently, T cell proliferation. Upon T cell activation, following productive Ag presentation, glutamate acts on the inducible mGlu1R, which are coupled to the ERK-pathway (12). In this way, glutamate causes the attenuation of the mGlu5R-triggered effects as well as the enhancement of Th1 and proinflammatory cytokine secretion, thus inducing costimulation. These findings suggest the existence of a physiologically relevant mechanism whereby DC, during Ag presentation in the lymph nodes, generate a suitable microenvironment, initially geared toward impeding T cell activation, but then upon engagement of T cells, toward providing strong costimulatory action. It is predicted that the deregulation of glutamate release by DC could promote erroneous cell-fate decisions with respect to T cell maturation, activation, and differentiation. Such findings would have important implications for pathophysiology of autoimmunity as well as pathophysiology of immunodeficiency diseases.

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Disclosures

The authors have no financial conflict of interest.

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