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Group I mGlu receptor stimulation inhibits activation-induced cell death of human T lymphocytes

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1 The effects of L-glutamate on activation-induced cell death (AICD) of human activated (1 $\mu g\,ml^{-1}$ phytohemagglutinin plus 2 $U\,ml^{-1}$ interleukin-2; 8 days) T lymphocytes were studied by measuring anti-CD3 monoclonal antibody (10 $\mu g\,ml^{-1}$; 18 h)-induced cell apoptosis (Annexin V and propidium iodide staining).

2 L-Glutamate (1×10^{-8}–1×10^{-4} M) significantly ($P\leqslant0.01$) inhibited AICD in a concentration-dependent manner ($EC_{50}=6.3\times10^{-8}$ M; maximum inhibition $54.8\pm6.3\%$ at 1×10^{-6} M).

3 The L-glutamate inhibitory effect was pharmacologically characterized as mediated by group I mGlu receptors, since mGlu receptor agonists reproduced this effect. The EC_{50} values were: 3.2×10^{-7} M for (1*S*,3*R*)-ACPD; 4.5×10^{-8} M for quisqualate; 1.0×10^{-6} M for (*S*)-3,5-DHPG; 2.0×10^{-5} M for CHPG.

4 Group I mGlu receptor antagonists inhibited the effects of quisqualate 1.0×10^{-6} M. The IC_{50} values calculated were: 8.7×10^{-5}, 4.3×10^{-6} and 6.3×10^{-7} M for AIDA, LY 367385 and MPEP, respectively.

5 L-Glutamate (1×10^{-6} M; 18 h) significantly ($P\leqslant0.05$) inhibited FasL expression ($40.8\pm11.3\%$) (cytofluorimetric analysis), whereas it did not affect Fas signalling.

6 Expression of both $mGlu_1$ and $mGlu_5$ receptor mRNA by T lymphocytes and T-cell lines, as demonstrated by reverse transcriptase–PCR analysis, suggests that L-glutamate-mediated inhibition of AICD was exerted on T cells.

7 These data depict a novel role for L-glutamate in the regulation of the immune response through group I mGlu receptor-mediated mechanisms.
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Abbreviations: (1*S*,3*R*)-ACPD, (1*S*,3*R*)-1-aminocyclopentan-1,3-dicarboxylic acid; AICD, activation-induced cell death; AIDA, (*RS*)-1-aminoindan-1,5-dicarboxylic acid; CHPG, (*RS*)-2-chloro-5-hydroxyphenylglycine; DAG, diacylglicerol; (*S*)-3,5-DHPG, (*S*)-3,5-dihydroxyphenylglycine; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; GPCR, G-protein-coupled receptors; iGlu, ionotropic glutamate; IL, interleukin; INF, interferon; IP_3, inositol 1,4,5-triphosphate; L-AP4, L-α-amino-4-phosphonobutyrate; L-CCG-I, (2*S*,3*S*,4*S*)-α-(carboxycyclopropyl)glycine; LY 367385, (*S*)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid; mAb, monoclonal antibodies; MFI-R, median fluorescence intensity ratio; mGlu, metabotropic glutamate; MAPK, mitogen-activated protein kinases; MPEP, 2-methyl-6-(2-phenyl-1-ethynyl)-pyridine; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PI3K, phosphoinositide 3-kinases; TCR, T cell receptor; TNF, tumour-necrosis factor; TRAIL, tumour-necrosis factor-related apoptosis-inducing ligand

Introduction

L-Glutamate signalling is not restricted to the CNS since glutamate receptors have been identified in several non-neuronal cells including immune cells (Skerry & Genever, 2001; Hinoi *et al*., 2004; Boldyrev *et al*., 2005): human peripheral blood T cells posses high affinity L-glutamate binding sites on their surface (Kostanyan *et al*., 1997), and both rodent and human cells of the T lineage (thymocytes and lymphocytes) express glutamate receptors (Storto *et al*., 2000; Rezzani *et al*., 2003; Ganor *et al*., 2003; Pacheco *et al*., 2004; Boldyrev *et al*., 2005; Miglio *et al*., 2005b).

Two major types of glutamate receptors have been so far characterized: ionotropic (iGlu) and metabotropic (mGlu) receptors (Kew & Kemp, 2005). iGlu receptors are ligand-gated ion channels (Dingledine *et al*., 1999; McFeeters & Oswald, 2004), that, on the basis of their sequence homology and agonist preference, are classified into NMDA, AMPA and

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kainate receptor types. mGlu receptors are members of the family C of G-protein-coupled receptors (GPCR), including Ca^{2+} and Mg^{2+} receptors, $GABA_B$ receptors, a number of receptors found in the vomeronasal organ and receptors for sweet molecules (Pin *et al*., 2004). The eight mGlu receptor subtypes so far characterized are linked to several effector systems, and are accordingly divided into three groups (Conn & Pin, 1997). Group I ($mGlu_1$ and $mGlu_5$ receptor subtypes) stimulates formation of inositol 1,4,5-triphosphate (IP_3) and diacylglicerol (DAG), group II ($mGlu_2$ and $mGlu_3$ receptor subtypes) and group III ($mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$ receptor subtypes) induce reduction of intracellular cAMP levels.

Through these receptors, L-glutamate modulates several lymphocyte functions: (i) it potentiates T-cell responses (intracellular Ca^{2+} ($[Ca^{2+}]_i$) rises) to anti-CD3 monoclonal antibodies (mAb) or phytohemagglutinin (PHA) by acting on NMDA and non-NMDA iGlu receptors (Lombardi *et al*., 2001); (ii) it induces reactive oxygen species formation and modulates T-cell activation by acting on NMDA receptors (Boldyrev *et al*., 2004; Miglio *et al*., 2005b); (iii) it increases lymphocyte adhesion to extracellular matrix proteins and cell motility by acting on AMPA iGlu receptors (Ganor *et al*., 2003); (iv) it modulates the properties of $K_v1.3$ channels by acting on mGlu receptors and non-NMDA iGlu receptors (Poulopoulou *et al*., 2005); (vi) it induces intracellular Ca^{2+} signals and early gene (*c-jun* and *c-fos*) expression by acting on group I mGlu receptors (Miglio *et al*., 2005a).

Upon T-cell receptor (TCR) triggering, resting T cells are activated to proliferate and to produce cytokines. Restimulation of activated T cells through the TCR induces an apoptosis program termed activation-induced cell death (AICD). AICD is a crucial mechanism for maintenance of peripheral tolerance and limiting ongoing immune responses (Lenardo *et al*., 1999; Green *et al*., 2003; Marrack & Kappler, 2004). Current data suggest that AICD consists of an inductive phase, triggered by TCR stimulation, followed by an effector phase, which activates cell death (Budd, 2001; Hildeman *et al*., 2002). Inhibition of AICD by actinomycin D or cycloheximide (Shi *et al*., 1990) suggests that the inductive phase induces transcription of a new set of genes required to activate the cell-death program. The interface between the two phases appears to be linked to molecules belonging to the tumour-necrosis factor (TNF) and TNF receptor superfamilies, whose expression and function are closely regulated by TCR stimulation (Kroemer *et al*., 1995). Several members of the TNF receptor superfamily act as 'death receptors' and include the TNFR-I, the DR4 and DR5 receptors for tumour-necrosis factor-related apoptosis-inducing ligand (TRAIL), and Fas (CD95/APO-1). Binding of these receptors by their ligands activates a caspase cascade and induces apoptosis (Sharma *et al*., 2000). AICD is inhibited by microenvironmental signals acting on T cell costimulatory molecules, such as CD28, H4/ICOS, several adhesion molecules, and cytokine receptors (Aoudjit & Vuori, 2000; Palmer *et al*., 2001; Carreno & Collins, 2002; Kerstan & Hunig, 2004).

The aim of this study was to investigate the effect of L-glutamate on T-cell AICD. Results show that micromolar concentrations of L-glutamate significantly inhibit AICD; this effect is mediated by group I mGlu receptor activation and is partially achieved by down regulation of FasL expression.

Methods

Cell cultures

Peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation onto Ficoll (Lymphoprep, Nycomed, Oslo, Norway) of heparinized venous blood obtained from healthy volunteers (Boyum, 1968). T cells were purified by panning to remove $CD11b^+$, $CD45RO^+$ and $HLA\text{-}DR^+$ cells with the appropriate mAbs, followed by use of the $CD4^+$/$CD8^+$ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch-Gladbach, Germany). This approach provided >99% cells displaying the phenotype $CD3^+CD4^+CD8^+CD45RA^+CD14^-CD16^-$, as assessed by flow cytometry. Cells were activated with PHA ($1\,\mu g\,ml^{-1}$) and cultured in RPMI 1640 supplemented with fetal calf serum (FCS; 10% $v\,v^{-1}$), human recombinant IL-2 ($2\,U\,ml^{-1}$), L-glutamine (2 mM), penicillin ($100\,U\,ml^{-1}$) and streptomycin ($100\,\mu g\,ml^{-1}$).

Human monocytes were isolated from heparinized venous blood of healthy volunteers as previously described (Brunelleschi *et al*., 2001) and their purity was assessed with the pan-leukocyte anti-CD45 (HLE-1) and the anti-CD14 (Leu-M3) monoclonal antibodies (Ziegler-Heitbrock, 2000). Monocyte-derived macrophages (MDM) were prepared by culturing monocytes for 7–8 days in RPMI 1640 medium containing FCS (10% $v\,v^{-1}$), L-glutamine (2 mM), HEPES (10 mM), penicillin ($100\,U\,ml^{-1}$) and streptomycin ($100\,\mu g\,ml^{-1}$). MDM were defined as macrophage-like cells by evaluating the decreased expression of CD14 and absence of CD1a according to Gantner *et al*. (1997).

Jurkat (clone E6-1, human leukaemic T cells), FRO (human leukaemic T cells), SUP-T1 (VB) (human leukaemic T cells), H9 (human cutaneous T cell lymphoma), HuT-78 (human cutaneous T-cell lymphoma), and THP-1 (human leukaemic monocytic cells) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). Jurkat, FRO, SUP-T1, H9, and THP-1 cells were maintained in RPMI 1640 medium containing FCS (10% $v\,v^{-1}$), L-glutamine (2 mM), $NaHCO_3$ ($1.5\,g\,l^{-1}$), glucose ($4.5\,g\,l^{-1}$), HEPES (10 mM), sodium piruvate (1.0 mM), penicillin ($100\,U\,ml^{-1}$) and streptomycin ($100\,\mu g\,ml^{-1}$). HuT-78 cells were maintained in RPMI 1640 medium containing FCS (20% $v\,v^{-1}$), L-glutamine (4 mM), $NaHCO_3$ ($1.5\,g\,l^{-1}$), penicillin ($100\,U\,ml^{-1}$) and streptomycin ($100\,\mu g\,ml^{-1}$).

L(tk^-), mouse fibroblast cells, stable transfected with recombinant human $mGlu_{1b}$ receptor or human $mGlu_{5a}$ receptor (Lin *et al*., 1997) were a generous gift from Professor F. Moroni (University of Florence, Italy). They were cultured in Dulbecco's Modified Eagle Medium supplemented with dialyzed FCS (10% $v\,v^{-1}$), L-glutamine (2 mM), penicillin ($100\,U\,ml^{-1}$), streptomycin ($100\,\mu g\,ml^{-1}$) and G418 ($500\,\mu g\,ml^{-1}$). To induce the expression of recombinant receptors, the cells were treated with dexamethasone (1×10^{-6} M) for 18 h.

Apoptosis assays

In the AICD assay, activated T cells (day 8 of culture) were washed twice in phosphate-buffered saline and then cultured (5×10^4 cell $well^{-1}$) in 96-well flat-bottomed plates coated with anti-CD3 mAb ($10\,\mu g\,ml^{-1}$) in L-glutamate free medium (Neurobasal medium), supplemented with B27 supplement, penicillin ($100\,U\,ml^{-1}$) and streptomycin ($100\,\mu g\,ml^{-1}$) in the

absence or presence of different drug concentrations. After a 18 h incubation, live cells were counted with the Trypan blue exclusion test or by flow cytometric detection of apoptotic cells after staining with propidium iodide and Annexin V. Assays were performed in triplicate and analysed blind by an observer. Annexin V staining was performed using the Annexin-V-Fluos kit (Boehringer Mannhein, Gmbh, Germany). Briefly, cells from each well were stained with Annexin V (Annexin-V-Fluos kit, Boehringer Mannhein, Gmbh, Germany) 20 μg ml^{-1} and propidium iodide (1 μg ml^{-1}) in 140 mM NaCl, 5 mM $CaCl_2$ and 10 mM HEPES pH 7.4 and analysed by flow cytometry, that was set to acquire cells for 10 s. Live cells were those not displaying shrunken/hypergranular morphology and unstained by propidium iodide or Annexin V (Ramenghi *et al*., 2000). Results are expressed as percentage of relative cell loss calculated as follows: 100−(total live cell count in the assay well/total live cell count in the respective control well) × 100. Cells in the control wells were treated with experimental medium alone. Spontaneous cell loss in the control wells was always $<10\%$ of the seeded cells.

Evaluation of Fas-induced apoptosis was performed using the same protocol on activated T cells cultured for 18 h with the anti-Fas mAb (CH11 IgM; 1 μg ml^{-1}).

FasL expression

FasL expression was evaluated by cytofluorimetric analysis using a fluorescein isothiocyanate (FITC)-conjugated anti-FasL mAb (Ancell, Bayport, MN, U.S.A.). Non-specific background fluorescence was evaluated with the appropriate isotype-matched control mAb (Becton Dickinson, San Jose, CA, U.S.A.). Levels of membrane-bound FasL were increased by blocking FasL cleavage before treatement with 3×10^{-5} M of GM6001, a broad spectrum matrix metalloproteinase inhibitor (Meng *et al*., 2004). FasL expression was expressed as median fluorescence intensity ratio (MFI-R) of total T lymphocytes according to the following formula: MFI-R = MFI of sample histogram (arbitrary units)/MFI of control histogram (arbitrary units).

RNA isolation and reverse transcriptase (RT)–PCR

Total RNA was isolated using the GenElute™ mammalian total RNA miniprep kit (Sigma-Aldrich, Milan, Italy) according to the manufacturer's instructions. Total RNA (5 μm) were reverse-transcribed using the ThermoScript™ RT–PCR kit (Invitrogen, Milan, Italy). Aliquots were amplified by emi-nested ($mGlu_5$ receptor) or nested ($mGlu_1$ receptor) PCR with 2.5 U of Taq polymerase (Invitrogen, Milan, Italy). The primers and protocols we used were reported in Table 1. Amplification products were separated by electrophoresis on 2% agarose gels and visualized with ethidium bromide.

Statistical analysis

Results are expressed as means ± s.e.m. of n experiments. Significance was assessed with Student's t-test for paired varieties with $P \leqslant 0.05$ as the cutoff. Data were fitted as sigmoidal concentration–response curves and analysed with a four-parameter logistic equation. The molar concentration of an agonist that produces 50% of the maximal possible effect of that agonist (EC_{50}) and the molar concentration of an

Table 1 PCR primers and protocols used in this study

Template	*Primers*	*Exon*	*Size (bp)*	*Denaturation*	*Annealing*	*Extension*	*Cycles*
HmGluR1	*External*						
NM_000838[a]	Forward 5′-TTCGAGATGAGAAGGATGGG-3′	1	707	94°C for 30 s	60°C for 30 s	72°C for 60 s	30
	Reverse 5′-CTCGTGTTAGTGTCCAGCC-3′	3					
	Internal						
	Forward 5′-ACAAGCATCGACCTGAGTGA-3′	1	234	96°C for 15 s	63°C for 30 s	72°C for 30 s	35
	Reverse 5′-GCGTTGCTGTAGATTTTGTCA-3′	2					
HmGluR5	*External*						
NM_000842[a]	Forward 5′-CGATCCTATTCGATGAGAATGGA-3′	4	1075	94°C for 30 s	TD[b] 65-60°C for 30 s	72°C for 90 s	30
	Reverse 5′-GTGGCACTGAGGCTGACCGAGAAAA-3′	7					
	Internal						
	Reverse 5′-GACCCCAGTTGGCATGCCTTGC-3′	6	310	94°C for 20 s	TD[b] 65-60°C for 20 s	72°C for 20 s	30
GAPDH	Forward 5′-GGTCGGAGTCAACAACGGATTTGG-3′	2	1000	96°C for 30 s	60°C for 30 s	72°C for 45 s	25
NM_002046[a]	Reverse 5′-ACCACCCTGTTGCTGTAGCCA-3′	9					

[a]Accession number NCBI sequence database (GenBank).
[b]TD: Touch down program (annealing temperature decreases of 1°C for the first 10 cycles.

antagonist that reduces the response to an agonist by 50% (IC_{50}) values were determined with a nonlinear regression model using the software Origin version 6.0 (Microcal Software, Northampton, MA, U.S.A.).

Drugs and chemicals

(1*S*,3*R*)-1-Aminocyclopentan-1,3-dicarboxylic acid ((1*S*,3*R*)-ACPD), AMPA, (*RS*)-1-aminoindan-1,5-dicarboxylic acid (AIDA), L-α-amino-4-phosphonobutyrate (L-AP4), (2*S*,3*S*,4*S*)-α-(carboxycyclopropyl)glycine (L-CCG-I), (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG), (*S*)-3,5-dihydroxyphenylglycine ((*S*)-3,5-DHPG), kainate, (*S*)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid (LY 367385), NMDA, 2-methyl-6-(2-phenyl-1-ethynyl)-pyridine (MPEP) and quisqualate were purchased from Tocris Cookson Ltd (Northpoint, U.K.). GM6001 was from Calbiochem (Milan, Italy). Dulbecco's Modified Eagle Medium, RPMI 1640, Neurobasal medium, FCS, dialyzed FCS and B27 supplement were obtained from Gibco (Life Technologies, Milan, Italy). Recombinant human IL-2 was from Biogen (Geneva, Switzerland). Anti-CD3 (OKT3), anti-CD11b (OKM1), anti-HLA-DR (L243), and anti-CD45RO (UCHL1) mAbs were purified by affinity chromatography on protein G-Sepharose 4 fast flow columns (Amersham Pharmacia Biotech, Uppsala, Sweden) from the hybridoma supernatants. Anti-CD14 (Leu-M3), anti-CD1a (HI149) and anti-CD45 (HLE-1) mAbs were from Becton Dickinson (San Jose, CA, U.S.A.). Anti-Fas IgM CH-11 mAb was obtained from MBL (Nagoya, Japan). All other drugs or chemicals were purchased from Sigma-Aldrich (Milan, Italy).

Results

Effects of L-glutamate on T cell AICD

To study the effects of L-glutamate on AICD of human T lymphocytes, freshly isolated PBMC were treated with PHA ($1\,\mu g\,ml^{-1}$) in the presence of recombinant human IL-2 ($2\,U\,ml^{-1}$) for 8 days to activate and expand T cells and sensitize them to AICD (see Methods). Then, T cells were stimulated with anti-CD3 mAb ($10\,\mu g\,ml^{-1}$; 18 h) in the absence or presence of increasing concentrations (1×10^{-8}–1×10^{-4} M) of L-glutamate and the surviving cells were counted by cytofluorimetric analysis after staining with Annexin V and propidium iodide. CD3 stimulation induced a $44.5\pm2.2\%$ of AICD, expressed as percentage of relative cell loss (see Methods) in the L-glutamate un-treated cells (Figure 1a). When the cells were exposed to L-glutamate the percentage of relative cell loss was significantly ($P\leqslant0.01$; $n=10$) reduced in a concentration-dependent manner ($EC_{50}=6.3\times10^{-8}$ M). The maximum effect ($20.1\pm2.8\%$ of AICD) was obtained at 1×10^{-6} M of L-glutamate (Figure 1b). The protective effect of L-glutamate was due to AICD inhibition and not to increased cell proliferation since no cell count increase was detected in any culture condition during the 18 h of AICD assay (data not shown).

Effects of iGlu and mGlu receptor agonists on T cell AICD

To determine whether the L-glutamate-mediated inhibition of AICD of human T cells is mediated by specific activation of iGlu receptors, we evaluated the effect of increasing concentrations (1×10^{-8}–1×10^{-4} M) of NMDA, AMPA or kainate, selective iGlu receptor agonists. All selective iGlu receptor agonists were ineffective at all concentrations tested (1×10^{-8}–1×10^{-4} M) (Figure 1b). On the contrary, (1*S*,3*R*)-ACPD, the prototype agonist of mGlu receptors, significantly ($P\leqslant0.01$; $n=10$) reduced the percentage of relative cell loss in a concentration-dependent manner ($EC_{50}=3.2\times10^{-7}$ M) (Figure 1c). The maximum effect ($23.2\pm2.0\%$ of AICD) was measured at 1×10^{-4} M of (1*S*,3*R*)-ACPD.

As (1*S*,3*R*)-ACPD is not a subtype-specific agonist, to pharmacologically characterize the mGlu receptor subtype(s) involved in the L-glutamate-induced AICD inhibition, we evaluated the effects of increasing concentrations (1×10^{-8}–1×10^{-4} M) of quisqualate, L-CCG-I, and L-AP4, selective agonists of group I, II or III mGlu receptors, respectively. Quisqualate significantly ($P\leqslant0.01$; $n=10$) protected cells from AICD, whereas L-CCG-I and L-AP4 were ineffective at all concentrations tested (1×10^{-8}–1×10^{-4} M) (Figure 1c). Quisqualate was more potent and effective than (1*S*,3*R*)-ACPD (EC_{50}: 4.5×10^{-8} *versus* 3.2×10^{-7} M; maximum effects: 12.9 ± 3.0 *versus* $23.8\pm2.8\%$ of AICD at 1.0×10^{-5} M). We obtained similar results with both (*S*)-3,5-DHPG, a selective group I mGlu receptor agonist acting on both $mGlu_1$ and $mGlu_5$ receptors, and CHPG, a highly selective $mGlu_5$ receptor agonist (Figure 1d). (*S*)-3,5-DHPG was more potent and effective than CHPG (EC_{50}: 1.0×10^{-6} *versus* 2.0×10^{-5} M; maximum effects: $14.1\pm2.5\%$ *versus* $23.0\pm1.8\%$ AICD at 1.0×10^{-3} M).

These data indicate that L-glutamate-induced AICD inhibition is mediated by group I mGlu receptor activation.

Effects of group I mGlu receptor antagonists on T cell AICD

To asses the relative contribution of $mGlu_1$ and $mGlu_5$ receptor subtypes, we evaluated the effects of increasing concentrations (1×10^{-8}–1×10^{-3} M) of AIDA, a selective group I mGlu receptor antagonist, LY 367385, a selective $mGlu_1$ receptor antagonist, and MPEP, a selective $mGlu_5$ receptor antagonist (Kew & Kemp, 2005), on AICD inhibition mediated by L-glutamate (1×10^{-6} M) (Figure 2a), (1*S*,3*R*)-ACPD (1×10^{-4} M) (Figure 2b) or quisqualate (1×10^{-6} M) (Figure 2c). All antagonists significantly ($P\leqslant0.01$; $n=8$) antagonized the inhibitory effects of mGlu receptor agonists in a concentration-dependent manner: the IC_{50} values calculated on quisqualate effect were: 8.7×10^{-5}, 4.3×10^{-6} and 6.3×10^{-7} M for AIDA, LY 367385 and MPEP, respectively. AIDA (1×10^{-3} M) abolished the effects of L-glutamate, (1*S*,3*R*)-ACPD, and quisqualate; LY 367385 and MPEP, however, only partially antagonized the protective effects of the agonists (maximum effects calculated on quisqualate effect was 38.4 ± 0.5 and $37.8\pm1.2\%$ of AICD at 1×10^{-3} M).

These results demonstrate that both group I mGlu receptor subtypes contribute to the L-glutamate-mediated inhibition of AICD of human T lymphocytes.

Effects of L-glutamate on the Fas/FasL system

AICD is partly due to recruitment of the Fas/FasL system (Green *et al*., 2003). To assess whether L-glutamate acts on this

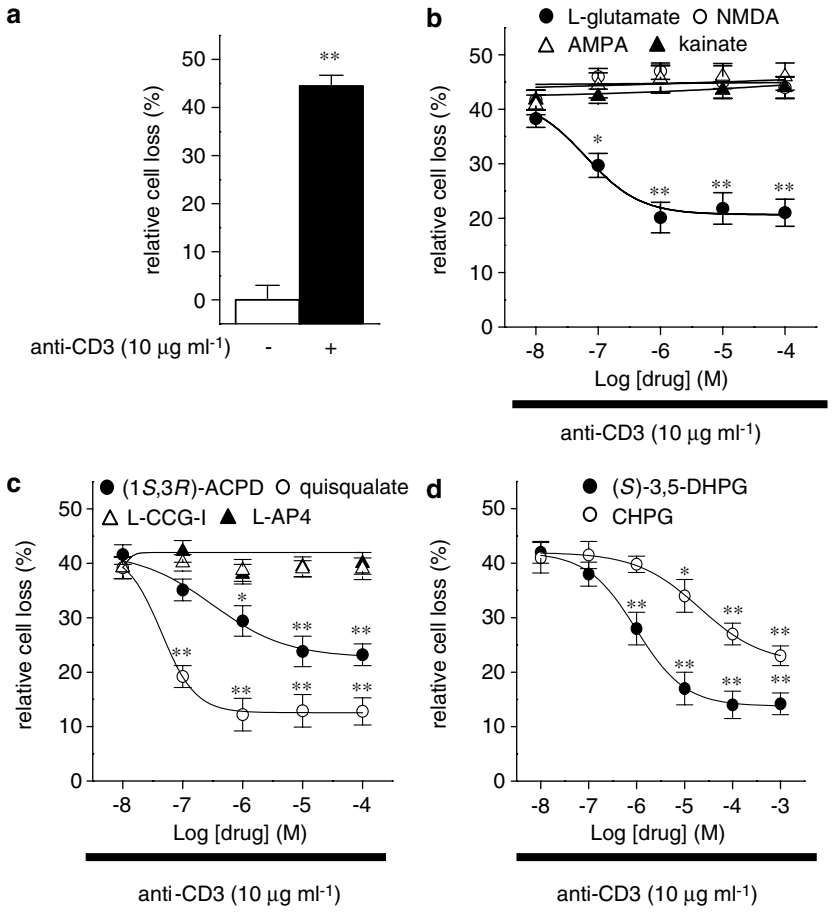

Figure 1 Concentration–response curves for the L-glutamate or iGlu/mGlu receptor agonists inhibitory effects on AICD of human T lymphocytes. Activated T cells were stimulated with anti-CD3 mAb ($10\,\mu g\,ml^{-1}$; 18 h) (a); in the presence of increasing concentrations (1×10^{-8}–1×10^{-3} M) of L-glutamate, NMDA, AMPA or kainate (b), (1*S*,3*R*)-ACPD, quisqualate, L-CGG-I or L-AP4 (c), (*S*)-3,5-DHPG or CHPG (d), and the surviving cells were counted by cytofluorimetric analysis after staining with Annexin V plus propidium iodide. CD3 stimulation induced a 44.5±2.2% of AICD in comparison to anti-CD3-untreated cells. The EC_{50} values were: 6.3×10^{-8} M for L-glutamate, 3.2×10^{-7} M for (1*S*,3*R*)-ACPD, 4.5×10^{-8} M for quisqualate, 1.0×10^{-6} M for (*S*)-3,5-DHPG and 2.0×10^{-5} M for CHPG. The white and dashed bars show the relative cell loss in anti-CD3-untreated and -treated cells in the absence of other drugs (a). The results are expressed as the mean±s.e.m. of at least ten experiments. $*P\leqslant0.05$; $**P\leqslant0.01$ *versus* anti-CD3 mAb-treated cells.

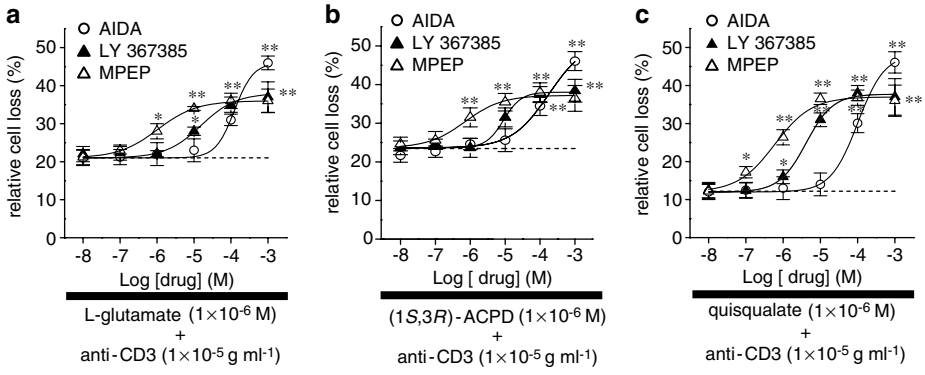

Figure 2 Inhibition of the L-glutamate, (1*S*,3*R*)-ACPD or quisqualate protective effects on AICD of human T lymphocytes. Activated T cells were stimulated with anti-CD3 mAb ($10\,\mu g\,ml^{-1}$; 18 h) in the presence of (1×10^{-6} M) L-glutamate (a), (1×10^{-4} M) (1*S*,3*R*)-ACPD (b), (1×10^{-6} M) quisqualate (c) and increasing concentrations (1×10^{-8}–1×10^{-3} M) of AIDA, LY 367385 or MPEP. Surviving cells were counted by cytofluorimetric analysis after staining with Annexin V plus propidium iodide. The IC_{50} values, calculated when quisqualate was used, were: 8.7×10^{-5} M for AIDA; 4.3×10^{-6} M for LY 367385 and 6.3×10^{-7} M for MPEP. Dot lines represent the relative cell loss in anti-CD3 mAb-treated cells in the presence of L-glutamate (1×10^{-6} M) (a), (1*S*,3*R*)-ACPD (1×10^{-4} M) (b) quisqualate (1×10^{-6} M) (c). The results are expressed as the mean±s.e.m. of cell death of at least eight experiments. $*P\leqslant0.05$, $**P\leqslant0.01$ *versus* anti-CD3 mAb-treated cells in the presence of mGlu receptor agonists.

system, we evaluated its effect on FasL expression and Fas-induced T-cell death.

FasL expression was evaluated by direct immunofluorescence and cytofluorimetric analysis on activated T cells (day 8) cultured for 18 h in the presence of GM6001 (3×10^{-5} M), a broad spectrum matrix metalloproteinase inhibitor used to maximize FasL expression (Meng *et al*., 2004) and in the absence or presence of anti-CD3 mAb ($10\,\mu g\,ml^{-1}$) and L-glutamate (1×10^{-6} M). Results showed that stimulation of activated T cells with anti-CD3 mAb increased FasL expression, but the upregulation was significantly higher in the absence than in the presence of L-glutamate (MFI-R were 7.1 ± 0.3 *versus* 4.2 ± 0.8, respectively, $P \leqslant 0.05$; $n = 4$) (Figure 3a and b). In the same experiments, L-glutamate inhibited AICD induced by anti-CD3 mAb at levels comparable to the inhibition of FasL expression (cell loss in the absence and presence of L-glutamate was 43.2 ± 1.2 *versus* $21.1 \pm 2.4\%$, $P \leqslant 0.01$; $n = 4$) (Figure 3c).

Fas-induced T-cell death was then evaluated by treating activated T cells (day 8) with an anti-Fas mAb ($1\,\mu g\,ml^{-1}$) in the absence or presence of L-glutamate (1×10^{-6} M) and counting surviving cells after 18 h as in the AICD assay. L-Glutamate did not inhibit Fas-induced cell death (Figure 4), which indicates that it does not affect Fas signalling.

These results suggest that L-glutamate protects T cells from AICD by inhibiting FasL expression.

Expression of group I mGlu receptors by human T lymphocytes

To verify whether L-glutamate-mediated inhibition of AICD is directly exerted on T cells, we evaluated the expression of $mGlu_1$ and $mGlu_5$ mRNA in resting and activated PBMC, purified T cells, MDM (see Methods), several T cell lines (FRO, SUP-T1, H9, HuT-78 and Jurkat), and the THP-1 monocytic cell line by RT–PCR (Figure 5). $mGlu_1$- and $mGlu_5$-specific amplification fragments were obtained from all samples, except for $mGlu_1$ in the H9 line and $mGlu_5$ in the SUP-T1 line. Identity of the amplified fragments was confirmed by direct sequencing (data not shown).

Discussion

This work shows that L-glutamate at low concentration range (10^{-7}–10^{-4} M) protects T cells from AICD by acting on group I mGlu receptors ($mGlu_1$ and $mGlu_5$ receptor subtypes). In fact: (i) the protective effect was induced by (1*S*,3*R*)-ACPD, the prototype agonist of mGlu receptors, but not by NMDA, AMPA and kainate, specific iGlu receptor agonists; (ii) it was induced by quisqualate, (*S*)-3,5-DHPG, and CHPG, group I-selective mGlu receptor agonists, but not by L-CCG-I and L-AP4, group II- or III-selective mGlu receptor agonists; (iii) the protective effects of (1*S*,3*R*)-ACPD and quisqualate were antagonized by AIDA, LY 367385, and MPEP, group I-selective mGlu receptor antagonists (Kew & Kemp, 2005). These results are in line with those of other authors showing that T cells express $mGlu_1$ and $mGlu_5$ receptors (Pacheco *et al*., 2004). Moreover, stimulation of group I mGlu receptors protects neuronal and non-neuronal cells from apoptosis induced by different insults (Copani *et al*., 1998; Allen *et al*., 2000; Lin & Maiese, 2001; Pizzi *et al*., 2000; Rong *et al*., 2003).

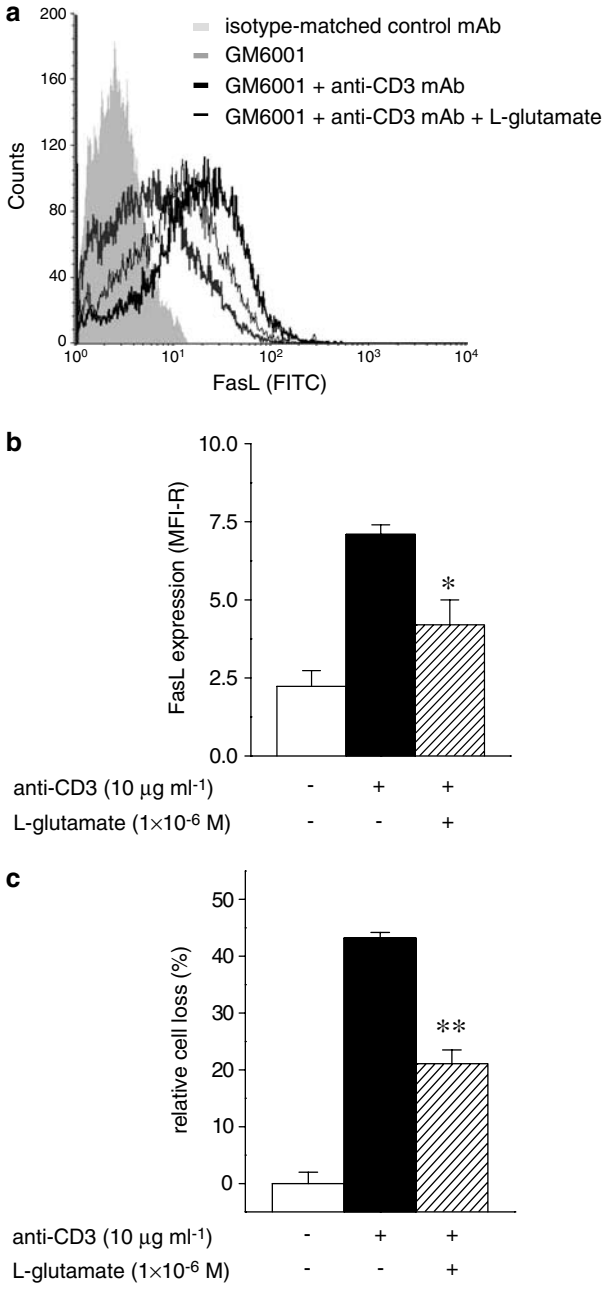

Figure 3 FasL expression in human T lymphocytes. Activated T cells were stimulated with anti-CD3 mAb ($10\,\mu g\,ml^{-1}$; 18 h), in the presence of GM6001 (3×10^{-5} M), and in the absence or presence of L-glutamate (1×10^{-6} M). FasL expression evaluated by direct immunofluorescence and cytofluorimetric analysis using a fluorescein isothiocyanate (FITC)-conjugated anti-FasL mAb (a). (b) FasL expression of L-glutamate (1×10^{-6} M) untreated or treated T cells expressed as MFI-R (see Methods) of total T lymphocytes. Activated T cells were stimulated with anti-CD3 mAb ($10\,\mu g\,ml^{-1}$; 18 h) in the presence of GM6001 (3×10^{-5} M) and in the absence or presence of L-glutamate (1×10^{-6} M). Surviving cells were counted by cytofluorimetric analysis after staining with Annexin V plus propidium iodide (c). The results are expressed as the mean ± s.e.m. of cell death of at least four experiments. $*P \leqslant 0.05$, $**P \leqslant 0.01$ *versus* anti-CD3 mAb-treated cells in the absence of L-glutamate.

Cell protection from AICD could be mediated by direct activation of glutamate receptors expressed on T cells or on bystander cells, such as, macrophages. However, the relative

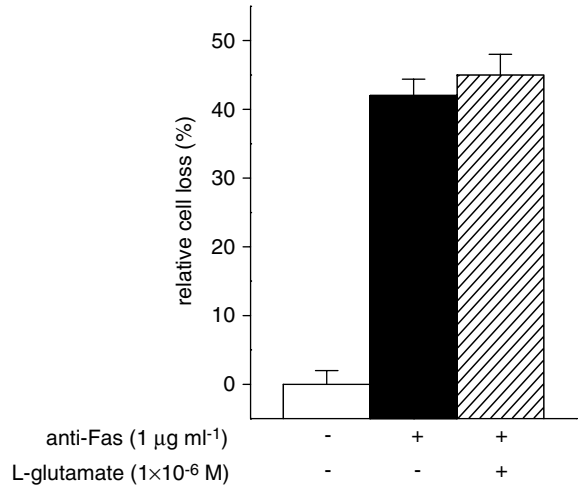

Figure 4 Fas-induced T-cell death. Activated T cells were stimulated with anti-Fas mAb (1 μg ml^{-1}; 18 h), in the absence or presence of L-glutamate (1×10^{-6} M). Surviving cells were counted by cytofluorimetric analysis after staining with Annexin V plus propidium iodide. The results are expressed as the mean $\pm$ s.e.m. of cell death of at least four experiments.

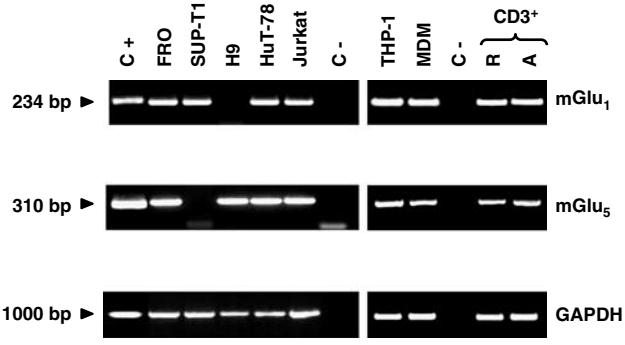

Figure 5 Expression of $mGlu_1$ and $mGlu_5$ receptors in human lymphoid and myeloid cells. RT–PCRs were performed on total RNA isolated from mouse fibroblasts L(tk$^-$) cells stably transfected with human $mGlu_1$ or $mGlu_5$ receptor cDNA (C+), FRO, SUP-T1, H9, HuT-78, Jurkat, THP-1 cell lines, human MDM, human resting T cell (R-CD3$^+$) or human PHA (1 μg ml^{-1}) plus IL-2 (2 U ml^{-1})-activated T cells (A-CD3$^+$) using specific primers; in the negative control (C-) reverse trascriptase was omitted. GAPDH was used as house keeping gene to normalize cDNA amount. The results are representative of at least five experiments.

contribution of macrophages should be low in our system, since AICD assays contained $<0.5\%$ residual macrophages.

AICD is a complex death response involving upregulation of both Fas and FasL, and sensitization of Fas connection to caspase cascades activating the apoptotic response. However, direct inhibition of Fas signalling was not involved in the L-glutamate-mediated antiapoptotic effects, since L-glutamate did not inhibit T-cell death induced by direct triggering of Fas. By contrast, the L-glutamate effects may be mediated by inhibition of FasL expression, which is supported by the observation that L-glutamate induced comparable inhibition of AICD and FasL expression. However, our data do not rule out the possibility that L-glutamate may also act on other receptors, such as those of TNF, TRAIL or possibly IFN-γ, whose role in AICD has been postulated (Sytwu *et al*., 1996; Tucek-Szabo *et al*., 1996; Martinez-Lorenzo *et al*., 1998; Refaeli *et al*., 2002).

Group I mGlu receptor stimulation leads to signalling through several pathways that may interfere with TCR signalling, such as the phospholipase C/protein kinase C (Pin & Duvoisin, 1995; Hermans & Challiss, 2001; Miglio *et al*., 2005b), the mitogen-activated protein kinase (MAPK) (Karim *et al*., 2001; Thandi *et al*., 2002), the phosphoinositide 3-kinases (PI3K) (Rong *et al*., 2003), and the cAMP/protein kinase A pathways (Reid *et al*., 1996; Balazs *et al*., 1998). It is worth noting that similar pathways are used by T-cell costimulatory receptors that can deliver antiapoptotic signals. For instance, CD28 triggers the PI3K/MAPK pathways and inhibits AICD by decreasing FasL expression in some experimental systems (Collette *et al*., 1998). However, it is relevant that, differently from CD28-mediated costimulation, group-I mGlu receptors display a specific effect on AICD without influencing cell proliferation (Lombardi *et al*., 2004) or response to other apoptotic signals, such as Fas triggering.

The antiapoptotic effect is detectable at low concentrations of L-glutamate with maximal effect at concentrations $>10^{-6}$ M and with the EC_{50} at 6.3×10^{-8} M. It is difficult to hypothesize physiological significance for these concentrations, since L-glutamate plasma concentrations are physiologically between 3 and 10×10^{-5} M (i.e. in the maximal effective range). One possibility is that glutamate receptor activity is strictly regulated during the T-cell response by controlling their surface expression (Miglio *et al*., 2005b) or connection with the signalling pathways; this would allow a temporal specificity of L-glutamate-mediated signalling and modulate T-cell sensitivity to AICD in different activation conditions. A second possibility is that mGlu receptors are located in specialized regions of the T-cell membrane, such as the immunological synapse between T cells and antigen-presenting cells, and are sensitive to small changes of L-glutamate concentrations in this narrow space. In this regard, the immunological synapse may be similar to the nervous synapse, where Na^+-dependent high-affinity L-glutamate transporters may reuptake L-glutamate and reduce the extracellular concentrations to the levels required for a high signal-to-noise ratio (Rothstein *et al*., 1994; Danbolt, 2001). It is intriguing that macrophages express glutamate transporters, similar to those present in the CNS, able to take up high amounts of L-glutamate under stimuli (Rimaniol *et al*., 2000). A third possibility is that mGlu receptor activation is responsible for a tonic survival signal that decreases cell sensitivity to AICD in the common body microenvironments where L-glutamate concentrations are high ($>1 \times 10^{-6}$ M); by contrast T cells would be more sensitive to AICD in specific microenvironments where L-glutamate concentrations are low ($<1 \times 10^{-6}$ M), such as in the CNS. This model could imply that L-glutamate plays a role in the immunological privilege of these tissues, but this is contradicted by the mean extracellular L-glutamate concentrations present in the CNS ($\sim 1 \times 10^{-6}$ M), which is effective in protecting T lymphocytes from AICD (on the border of the maximal effect plateau). However, the experimental L-glutamate concentrations may be higher than the physiological CNS extracellular levels, because of the release of intracellular L-glutamate by either apoptotic or damaged cells.

Together with previous reports, these data depict a complex role of L-glutamate in regulation of the immune response. Micromolar concentrations may have a receptor-mediated

effect capable to modulate TCR signalling by acting on both iGlu and mGlu receptors. Stimulation of mGlu receptors may sustain the immune response by decreasing cell susceptibility to AICD, whereas stimulation of non-NMDA or NMDA iGlu receptors may trigger cell adhesion to extracellular matrix glycoproteins, chemotactic migration (Ganor *et al*., 2003) and T-cell activation (Miglio *et al*., 2005b), respectively. By contrast, millimolar concentrations, reached in damaged tissues during acute and chronic inflammation, may have a metabolic effect possibly by modification of the intracellular levels of thiol compounds, resulting in modulation of the immune response with decreased cell proliferation and increased secretion of IFN-γ and IL-10 (Lombardi *et al*., 2004).

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