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TCR Activation Eliminates Glutamate Receptor GluR3 from the Cell Surface of Normal Human T Cells, via an Autocrine/Paracrine Granzyme B-Mediated Proteolytic Cleavage

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The majority of resting normal human T cells, like neuronal cells, express functional receptors for glutamate (the major excitatory neurotransmitter in the CNS) of the ionotropic α-aminooxy-5-methyl-4-isoxazoleproionic acid (AMPA)-receptor subtype 3 (GluR3). Glutamate by itself (∼10 nM) activates key T cell functions, including adhesion to fibronectin and laminin and chemotactic migration toward CXCL12/stromal cell-derived factor 1. In this study, we found by GluR3-specific immunostaining, flow cytometry, and Western blots that GluR3 cell surface expression decreases dramatically following TCR activation of human T cells. CXCR4, VLA-4, and VLA-6 also decrease substantially, whereas CD147 increases as expected, after TCR activation. Media of TCR-activated cells “eliminates” intact GluR3 (but not CXCR4 and VLA-6) from the cell surface of resting T cells, suggesting GluR3 cleavage by a soluble factor. We found that this factor is granzyme B (GB), a serine protease released by TCR-activated cells, because the extent of GluR3 elimination correlated with the active GB levels, and because three highly specific GB inhibitors blocked GluR3 down-regulation. Media of TCR-activated cells, presumably containing cleaved GluR3B peptide (GluR3 aa 372–388), inhibited the specific binding of anti-GluR3B mAb to synthetic GluR3B peptide. In parallel to losing intact GluR3, TCR-activated cells lost glutamate-induced adhesion to laminin. Taken together, our study shows that “classical immunological” TCR activation, via autocrine/paracrine GB, down-regulates substantially the expression of specific neurotransmitter receptors. Accordingly, glutamate T cell neuroimmune interactions are influenced by the T cell activation state, and glutamate, via AMPA-GluR3, may activate only resting, but not TCR-activated, T cells. Finally, the cleavage and release to the extracellular milieu of the GluR3B peptide may in principle increase its antigenicity, and thus the production, of anti-self GluR3B autoantibodies, which activate and kill neurons found in patients with various types of epilepsy. The Journal of Immunology, 2007, 178: 683–692.
“neurological” signals. Such neuroimmune dialogues may lead to augmented, suppressed, or entirely different T cell functions.

In general, T cells may encounter glutamate both in the periphery and in the brain and other parts of the CNS, under both physiological and pathological conditions. Within the CNS, both specific and nonspecific T cells can patrol and act either beneficially (7, 8) or detrimentally (9). For example, T cells in the brain are crucial and beneficial when they combat and clear encephalomyelitis-inducing viruses from the CNS (7, 8). Yet, autoreactive anti-myelin T cells invading the CNS are highly detrimental, as they lead to multiple sclerosis (9). In principle again, in all these conditions and others, T cells in the CNS may be exposed to relatively low levels of glutamate (<1 μM) present either in CSF or brain ECF (10). According to our previous study, such low physiological glutamate concentrations are optimal for activating resting normal peripheral human T cells and for triggering their adhesion to extracellular matrix glycoproteins and their chemotactic migration (5). In addition to the above, T cells within the brain could possibly encounter excess glutamate (~100 μM), present in a kaleidoscope of pathological conditions, among them traumatic brain injury, acute brain anoxia/ischemia, epilepsy, glaucoma, meningitis, brain neurodegeneration associated with different chronic diseases, amyotrophic lateral sclerosis and Alzheimer’s disease (reviewed in Ref. 11). In all these conditions, the very high/extra glutamate causes overactivation of its glutamate receptors on neurons and glia, leading to massive death of these glutamate cells (i.e., excitotoxicity (12)), which in turn leads to a further release of intracellular glutamate, thereby amplifying the excitotoxic neuronal death and brain damage. As to the periphery, T cells (resting and activated) may be exposed to glutamate (at various concentrations) in peripheral glutamate-rich organs, such as the liver, kidney, lung, muscle, bone, and blood (13).

In this study, we found that while resting normal human T cells express very high levels of functional GluR3, upon TCR-activation of such cells there is a dramatic down-regulation of GluR3 from the cell surface, as a result of an autocrine/paracrine proteolytic cleavage mediated by granzyme B (GB), a potent serine protease released by activated T and NK cells (14). These results reveal a completely novel GB-mediated mechanism by which the encounter of resting T cells with an Ag, leading to activation of their key “immunological receptor” (i.e., the TCR), leads to the elimination from the cell surface of an important neurotransmitter receptor (i.e., GluR3).

Materials and Methods

Materials and Abs

We used the following: FCS, l-glutamine, penicillin/streptomycin/ampicillin/tetracyclerin (Biological Industries); BSA, protease inhibitor mix, l-glutamate, PMA (Sigma Aldrich); ECL (Amersham Biosciences); commercial GB and GB colorimetric substrate Ac-IEPD-pNA (Calbiochem); GB inhibitors Ac-IEPD-CHO (Calbiochem); Ac-IETD-CHO and Z-IETD-FMK (Alexis Biochemicals); laminin (ICN Biomedicals); anti-CD3 and anti-CD28 mAbs (BD Pharmingen); mouse anti-human CXCR4 (R&D Systems); VLA-4 and VLA-6 (R & D Systems); and CD147 (BD Pharmingen) mAbs; normal mouse serum, FITC-conjugated anti-mouse and anti-rat IgG, HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories).

Human T cells

Density gradient centrifugation was used to separate the lymphocytes from the erythrocytes, dead cells, polymorphonuclear leukocytes, and granulocytes. A “fresh” 50-ml sample of leukocytes, without plasma and without prior cooling, supplied by the blood bank, was diluted 1/1 in PBS and added to Uni-SEPmax™ tubes (Novanned) containing at their bottom a solution of 5.6% polysacrose and 9.6% sodium metrizoate. The tubes were centrifuged (1200 rpm, 30 min) and the resulting layer of lymphocytes (migrating to the interface between the plasma and polysacrose/sodium metrizoate) was removed by a 2-ml pipette. The lymphocytes were washed twice with PBS (1000 rpm, 10 min) and resuspended in 8 ml of PBS containing 5% FCS. Nylon wool columns were then used to separate the T cells from the other lymphocytes (i.e., B cells and NK cells). The cell suspension (2 ml/column) was loaded (by syringe injection) on nylon wool columns (Novamed) that had been preincubated for 30 min at 37°C with PBS/5% FCS. After this cell loading, the columns were further incubated, lying flat, for 1 h at room temperature. Following incubation, PBS (12 ml/column) was added to the columns to elute the nonadherent T cells. The eluted cells were collected in a clean tube and centrifuged (800 rpm, 15 min). The resulting cell population consisted of >90% T cells, as evaluated by TCR staining and flow cytometry, using FACSsort. The cells were maintained (37°C, humidified incubator, 5% CO2) in RPMI 1640 supplemented with 10% FCS, 1% glutamine, and 1% antibiotics.

Human cytotoxic TALL-104 cells and their target human B-lymphoma Raji cells

The two cell types were obtained from American Type Cell Culture and maintained (37°C, humidified incubator, 5% CO2) either in IMDM (TALL-104) or RPMI 1640 (Raji), supplemented with 10% FCS, 1% glutamine, and 1% antibiotics.

TCR activation

TCR activation was performed as previously described (15) with some modifications. Briefly, non-tissue-culture-treated 24-well plates (Falcon) were coated overnight at 4°C with anti-CD3 and anti-CD28 mAbs (1 μg/ml in PBS). The wells were then washed with PBS, blocked for 1 h at 37°C (PBS/1% BSA), and washed again. The T cells (either normal resting human T cells or cytotoxic human TALL-104 cells) were resuspended in their respective fresh medium and seeded in the anti-CD3/CD28-coated wells (1 × 105/ml), and the plates were incubated for 72 h (37°C, humidified incubator, 5% CO2). Then, the cells and their medium were collected from each well, transferred into 50-ml tubes, centrifuged (1200 rpm, 10 min), and both the TCR-activated cells and their culture medium were collected and transferred into clean separate tubes. This TCR-activation method (by anti-CD3 and anti-CD28 mAbs) commonly used worldwide, results in our hands in activation of >90% of the human T cells. This is often judged by a marked increase in cell size (i.e., increased forward scatter of the TCR-activated cells compared with the resting T cells, evident by flow cytometry). Also, each time TCR activation is performed, the TCR-activated cells are visualized microscopically, to confirm that they appear as large round blasts, easily distinguishable from the resting T cells. Finally, the TCR-activated cells have increased proliferation rate and markedly elevated cytokine secretion (tested occasionally by common procedures for cell proliferation and by ELISA).

Immunofluorescence staining and flow cytometry analysis

Normal human T cells (either resting or following 72 h TCR activation) were stained to single immunofluorescence staining, using rat polyclonal anti-GluR3, IgG Ab previously prepared and characterized in our laboratory (5) (25 μg/ml per 1 × 106 cells/100-μl tube; 30 min on ice), or normal rat IgG for control. The cells were then stained with FITC-conjugated anti-rat IgG (100 μl of 1/10 dilution). In addition, the cells were also stained with mouse anti-human CXCR4, VLA-4, VLA-6, or CD147 mAbs; normal mouse serum, FITC-conjugated anti-mouse and anti-rat IgG, HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories).

T cell extraction and Western blots

Normal human T cells (3 × 106 cells, either resting or following 72 h TCR activation) were suspended in PBS, washed by centrifugation (1200 rpm, 10 min), and resuspended in homogenization buffer (50 mM Tris HCL (pH 7.6) 1 mM EDTA) containing protease inhibitors (1/250 dilution of mix). The cells were then disrupted by ice on sonication (5–10 × 20 s with 20 s rest intervals), until 80–90% of the cells appeared disrupted, as judged by trypan blue exclusion. Cell homogenates were centrifuged (1,000 × g, 5 min at 4°C), and the supernatants were collected and centrifuged again (14,000 × g, 45 min at 4°C). The resulting fractions of membranal proteins were subjected to protein determination, resolved by PAGE (10% SDS PAGE) and transferred to a nitrocellulose membrane. After transfer, blots were blocked (PBS/0.1% Tween 20/5% milk for 1 h at room temperature) washed extensively and hybridized with an anti-GluR3 mAb prepared and characterized recently in our laboratory (5 μg/ml, overnight at 4°C). After extensive washing, blots were incubated with HRP-conjugated

GB CLEAVES GLUTAMATE RECEPTOR FROM TCR-ACTIVATED T CELLS

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anti-mouse IgG secondary Ab (1/20,000 dilution, 1 h at room temperature). The targeted proteins were visualized by ECL.

**GluR3 cleavage assay**

Resting human T cells were exposed for 1 h at 37°C to different culture media derived either from resting or TCR-activated cells (1 × 10⁶ resting cells in 0.5-ml medium sample). The cells were then washed and subjected to single immunofluorescence staining for GluR3 as described above. In some experiments, GB inhibitors, which block GB itself either competitively or noncompetitively, were added to the culture medium 5 min before exposing the resting T cells to these GluR3 cleaving media.

**Killing human B-lymphoma Raji cells by human cytotoxic TALL-104 cells**

To induce killing of target Raji by effector TALL-104, the two types of cells were mixed in a 5:1 ratio (1 × 10⁶ effector:2 × 10⁵ target) in a 48-well plate (Nunc) at a total volume of 500 µl. This cell mixture was incubated for 24 h (37°C, humidified incubator, 5% CO₂) and the culture media were carefully collected from the upper portion of the wells. Culture medium of TALL-104 cells incubated alone served as negative control. To evaluate the magnitude of cytotoxicity exerted by the TALL-104 effector cells against the Raji target cells, we measured lactate dehydrogenase release with the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) according to the manufacturer’s instructions.

**Determination of GB levels in the culture medium**

To evaluate GB levels in the T cell medium, enzymatic reactions were conducted as previously described (16). In specific, all reactions were performed in a flat-bottom 96-well plate (Costar) at a final volume of 100 µl. The GB colorimetric substrate Ac-IEPD-pNA was diluted in PBS containing 10% DTT to a final concentration of 100 µM and was then mixed with GB-containing culture medium (that were diluted 1/10 in the same buffer). The substrate and culture medium were incubated at 37°C and the color that developed following cleavage of the colorimetric substrate by GB present in the culture medium was measured as the OD at 405 nm using a spectrophotometer. Serial dilutions (50–0 ng/ml) of commercial GB were used to create a standard curve and translate the OD values to actual levels of GB.

**Inhibition of anti-GluR3B monoclonal or polyclonal Ab binding to GluR3B peptide by preincubation with medium of TCR-activated T cells**

Mouse anti-GluR3B mAb (90 ng/ml), rat anti-GluR3B polyclonal Ab (140 ng/ml), or rabbit anti-GluR3B affinity-purified polyclonal Ab (170 ng/ml) were each preincubated (1 h at room temperature with shaking, at 300 µl total volume) with culture medium derived from either resting or TCR-activated human T cells, or with the culture medium alone. Then, all the different samples of anti-GluR3B Abs were tested by ELISA for their specific binding to GluR3B-coated microtiter plates or nonspecific binding to BSA-coated plates, as previously described (17, 18). Of note, the anti-GluR3B mAb was recently prepared and characterized in our laboratory.

**In vitro T cell adhesion assay**

Normal human T cells (either resting or TCR-activated) were resuspended in RPMI 1640/0.1% BSA, and treated (37°C, 30 min, 5% CO₂) with either 10 nM glutamate, PMA (positive control), or PBS. Microtiter flat-bottom 96-well plates were precoated with laminin (1 µg/ml, overnight incubation at 4°C). The purified T cells were then added (1 × 10⁵ cells/100 µl/well) to the laminin-coated plates, and then placed in a humidified incubator (37°C, 30 min, 5% CO₂). The plates were then washed several times with PBS to remove nonadherent T cells. After the final washing, the microtiter wells were supplemented with lysis-substrate solution (60 µl/well of 0.5% Triton X-100 in water mixed with an equal volume of 7.5 mM p-nitrophenol-N-acetyl-β-D-glucosaminide (Sigma-Aldrich) in 0.1 M citrate buffer (pH 5.0)). The plates were then transferred to a CO₂-free 37°C incubator for overnight incubation. The following day, the microtiter wells were supplemented with stop solution (90 µl/well of 50 mM glycine (Sigma-Aldrich) (pH 10.4) containing 5 mM EDTA), and the OD was measured at 405 nm.

**Statistical analysis**

Statistical significance was analyzed by the Student t test.

**Results**

**TCR activation of normal human T cells cause marked decrease in the cell surface expression of the glutamate receptor of the ionotropic AMPA subtype 3 (GluR3)**

In this study, we focused on GluR3 and asked whether the high GluR3 expression, evident on the vast majority of resting normal peripheral human T cells (5), is modified after TCR activation. Of note, the exact level of GluR3 surface expression in freshly purified normal peripheral human T cells is not constant. In T cells of most human individuals tested thus far, GluR3 expression was on the higher side: 75–90% of the cells were GluR3 positive, but there were few occasional extremes on the lower side (e.g., 30%). In line with this, we found (and reported in several of our previous studies) variations between different human individuals, in respect to the exact levels of many other T cell expressed and secreted molecules, and in regards to the exact level of a given T cell function. Although a given trend can be clear, constant, and statistically significant, the exact values may vary markedly from one individual to the other. For this reason, we normally study a large number of different individuals and subject all the results to statistical evaluation.

Two mAbs, anti-CD3 and anti-CD28, were used for achieving TCR activation in vitro, like those commonly used worldwide. Thereafter, the levels of the membranal GluR3 protein expressed in the resting and TCR-activated normal human T cells were determined by GluR3-specific immunofluorescence staining and flow cytometry. For the GluR3-specific staining, we used an Ab directed against the GluR3B peptide (aa 372–388 of the GluR3 extracellular domain), used successfully for several purposes in our previous studies (5). Now we found that following the TCR activation (72 h), GluR3 expression on the T cell surface decreased dramatically (Fig. 1A). Importantly, the reduction in GluR3 protein levels following TCR activation was confirmed also by GluR3-specific Western blotting (Fig. 1F).

For comparison, we asked whether TCR activation affects similarly other receptors expressed on the cell surface of T cells. For testing that, we measured in parallel the expression levels of four additional key T cell proteins, which play important roles in T cell function. These included CXCR4, the receptor of the CXCL12/SDF-1 chemokine; VLA-4, the α₅β₁ integrin receptor for fibronectin, and VLA-6, the α₆β₄ integrin receptor for laminin; and CD147, a cell surface regulator of matrix metalloproteinase production and secretion, known to be highly expressed in activated T cells (19). Interestingly, we found that following TCR activation the surface levels of CXCR4 also decreased drastically (from ~90 to ~5% positive, Fig. 1B), in line with previous demonstrations (20), and so did the levels of VLA-4 (from ~85 to ~38%) and VLA-6 (from ~88 to ~15%) (Fig. 1, C and D). In contrast, and as expected from previous studies (19), the surface levels of CD147 increased following TCR activation (Fig. 1E). Thus, TCR activation caused significant down-regulation of GluR3, CXCR4, VLA-4, and VLA-6 and a marked up-regulation of CD147.

The very low GluR3 cell surface expression after TCR activation is restored within the next ~48 h

With time, as the T cells reverted in vitro from the TCR-activation phase back to the “resting” phase, GluR3 cell surface expression was restored (Fig. 2A). Thus, GluR3 cell surface expression, which was minimal at day 3 (i.e., immediately after TCR activation), increased gradually thereafter, until reaching again, within the next ~48 h, the very high GluR3 expression starting levels (Fig. 2A). Of note, the cell surface expression of CXCR4, VLA-4, and...
A–D. Normal human T cells were either left untreated or activated (72 h) via their TCR, using anti-CD3 and anti-CD28 mAbs. Then, the cells (both the resting and the TCR activated) were subjected to single immunofluorescence staining specific for GluR3 (A), CXCR4 (B), VLA-4 (C), VLA-6 (D), and CD147 (E), using rat anti-GluR3 polyclonal Ab or mouse mAbs to CXCR4, VLA-4, VLA-6, and CD147 (black lines in A–E), followed by the appropriate FITC-conjugated anti-rat or anti-mouse secondary Abs. Isotype control staining (dotted lines) was performed with normal rat IgG or normal mouse serum, respectively. One representative experiment is shown of at least five independent experiments performed for each marker. The numbers represent the percentage of positive cells in the M1 region (A–D) or the geometric mean reflecting the staining intensity (E). F. Normal human T cells were either left untreated or activated (72 h) via their TCR with anti-CD3 and anti-CD28 mAbs. Following such TCR activation, the membranal proteins were purified and examined by Western blotting for GluR3 content, using mouse anti-GluR3 mAb. One representative experiment of two is shown.

VLA-6 were also restored with time but with faster kinetics (data not shown).

The “elimination” of intact GluR3 from the cell surface following TCR activation is mediated by a soluble factor, secreted by the TCR-activated cells

What could be the mechanism responsible for the substantial down-regulation of GluR3 cell surface expression following TCR activation? One possibility is that soluble factors secreted from the TCR-activated T cells may act in an autocrine/paracrine fashion to cleave GluR3 from the T cell surface. To test this hypothesis, we incubated resting human T cells, which express high levels of GluR3 (see Fig. 1A), with medium of either TCR-activated cells or resting cells, or with PBS for further control (1 h, 37°C). Following these treatments, the resting T cells were washed and their cell surface GluR3 levels were examined by GluR3-specific immunofluorescence staining and flow cytometry. In parallel and for comparison, following all these treatments, we also measured the expression levels of CXCR4 and VLA-6 on the cell surface of the resting T cells. We found that the short-term incubation of the resting human T cells with culture medium of TCR-activated T cells induced an extensive reduction of GluR3 cell surface expression (Fig. 2B), while the incubation with medium derived from resting T cells had no effect. Interestingly, whereas the incubation with the TCR-activated medium reduced markedly the membranal levels of GluR3 in resting T cells, it had no effect on the levels of CXCR4 and VLA-6, which remained high (data not shown). Together, these results suggest that a soluble factor released by TCR-activated T cells cleaves GluR3, but not CXCR4 and VLA-6, from the T cell membrane. Hence, the reduction of the latter receptors following TCR activation (Fig. 1, B and D) must be mediated by a different mechanism, which is beyond the scope of this study.

Human cytotoxic T cells, following encounter with their target cancer cells, release to the medium a soluble factor, probably granzyme B, which cleaves GluR3 from the cell surface of resting human T cells

Upon TCR activation, T cells secrete numerous soluble factors, such as cytokines and proteolytic enzymes. Among these is the key serine protease GB, known to be released from activated immune cells, primarily T cells and NK cells, and responsible for killing virus-infected cells and tumor cells (14). Because GluR3 has an extracellular GB cleavage site (21), we hypothesized that GluR3 “disappears” from the T cell outer membrane after TCR activation due to an autocrine/paracrine cleavage mediated by soluble GB released to the medium by the TCR-activated cells.

To test this hypothesis and find out whether GB can indeed cleave GluR3 from the T cell surface, we used the medium of the human cytotoxic T cell line TALL-104 (22) as a natural source for GB, as these CTLs were previously reported to kill cancer target cells in a GB-dependent mechanism (22). To achieve maximal release of GB to the conditioned medium by the TALL-104 cells, we exposed these cells either to one of their target cancer cells, i.e., the human B cell lymphoma line Raji, or to TCR-activating anti-CD3 and anti-CD28 mAbs. Twenty four hours after activation, we collected the respective TALL-104 culture medium and incubated (1 h at 37°C) fresh resting human T cells, which express high levels of GluR3, with the medium of the cancer-activated, TCR-activated, or nonactivated TALL-104 cells. We found that the culture medium of either the Raji-activated (Fig. 3A) or the TCR-activated (Fig. 3B) TALL-104 cells induced a substantial decrease in the levels of GluR3 expressed in resting human T cells, while the medium
of the untreated (i.e., nonactivated) TALL-104 cells failed to do so (Fig. 3). Importantly, in the above experiments, we confirmed that the TALL-104 human cytotoxic cells indeed killed their target (LDH) Raji cells, as evident by highly elevated lactate dehydrogenase release (data not shown). Together, these results suggest that human CTLs secrete a soluble factor, suspected to be GB, which can cleave GluR3 from the surface of resting human T cells.

The extent of GluR3 elimination from the T cell membrane correlates with the concentration of active GB in the medium of TCR-activated cells

For further testing our hypothesis that GB may be the enzyme responsible for the disappearance of GluR3 from the cell surface of TCR-activated T cells, we determined the actual amounts of functional GB in the extracellular medium of TCR-activated and resting human T cells. Again, the T cells were purified from fresh blood samples of three human blood donors and each T cell population was either cultured as such, or underwent TCR activation. Using a specific GB colorimetric substrate Ac-IEMP-D-pNA (as previously described (16)), we evaluated the levels of active GB, and found that the medium of two samples of resting T cells contained 6.4 and 15.4 ng/ml GB (Fig. 4, points resting 1 and 2, mean GB concentration: 10.9 ng/ml; the third sample could not be tested due to technical reasons), while the three TCR-activated medium contained 79.6, 101.1, and 245.7 ng/ml GB, respectively (Fig. 4, points TCR-act1-3, mean GB concentration: 142.1 ng/ml). These results revealed that, as expected, TCR activation led to marked increase in the levels of active GB secreted to the culture medium.

Next, we tested the ability of each of these T cell culture media to cleave GluR3 from the cell surface of resting human T cells. We found (Fig. 4, expressing the results as the percent of GluR3 reduction) a linear correlation between the concentration of active GB in these T cell media and their GluR3 cleaving potency. The higher GB concentration, the higher magnitude of GluR3 reduction it induced (Fig. 4).

The disappearance of intact GluR3 from the cell surface of TCR-activated cells is blocked by GB inhibitors

To gain direct proof that GB is indeed responsible for GluR3 down-regulation following TCR activation, we tested a commercially available GB instead of medium of TCR-activated

FIGURE 2. Culture medium of TCR-activated cells eliminates GluR3 from the cell surface of resting human T cells. A, GluR3 cell surface expression was determined by GluR3-specific immunostaining and flow cytometry, in resting normal human T cells at day 0 (representing the day the T cells were purified from a fresh human blood sample), and at days 3–7 after in vitro TCR-activation. *, p < 0.05 vs day 0 (Student’s t test). B, Normal resting human T cells were exposed (1 h, 37°C) to either medium of TCR-activated cells (■), medium of resting T cells (□), or PBS (□). Following all treatments, the resting T cells were washed and subjected to GluR3-specific immunofluorescence staining and were analyzed by flow cytometry, as described previously. One representative experiment of six is shown. Numbers represent the percent of GluR3-positive cells ± SD. *, p = 0.0131 vs PBS (Student’s t test).

FIGURE 3. Following encounter with their target cancer cells, human activated cytotoxic T cells secrete to the medium a soluble factor, which in turn reduces GluR3 surface expression in resting T cells. Normal resting human T cells were exposed (1 h, 37°C) to either medium of Raji-activated TALL-104 cells (■ in A), medium of TCR-activated TALL-104 cells (■ in B), medium of resting TALL-104 cells (□ in A and B), or PBS (□ in A and B). Following treatment, cells were washed and subjected to single immunofluorescence staining for GluR3, as described previously. One representative experiment of three performed (for either Raji- or TCR-activated TALL-104 cells) is shown. Numbers represent the percent of GluR3-positive cells ± SD. *, p = 0.0144 (A) and 0.0078 (B) vs PBS (Student’s t test).
cells, but unfortunately could not achieve reliable results because the commercial GB substrate buffer (5–10 mM DTT, as suggested by the manufacturer and previously used (21)) exerted detrimental nonspecific effects on the normal human T cells (data not shown).

Having to choose an alternative methodology, we used three highly specific GB inhibitors, i.e., Ac-IEPD-CHO, Ac-IETD-CHO, and Z-IETD-FMK, and tested their ability to prevent GluR3 down-regulation upon TCR activation. We found that all these GB inhibitors blocked the ability of TCR-activated medium to eliminate GluR3 from the cell surface of resting human T cells (Fig. 5). Although the blocking was clearly significant for all three GB inhibitors, they differed in the extent of block they induced (Fig. 5). Finally, adding one of these GB inhibitors (i.e., Ac-IEPT-CHO) to resting T cells during the 72 h TCR-activation process blocked GluR3 down-regulation following TCR-activation (data not shown).

Preincubation with medium of TCR-activated but not with medium of resting T cells inhibits the specific binding of anti-GluR3B mAb to GluR3B peptide

Previous studies showed that GluR3 contains a GB cleavage site in its extracellular portion (aa 385–388) (21). GB cleavage at this site is expected to remove a ~400-aa-long portion of GluR3 that contains an extracellular epitope termed the GluR3B peptide, corresponding to aa 372–388 of GluR3 that contains the GB cleaving site at its C-terminal region (17, 18, 23). This GluR3B peptide is the autoantigen recognized by highly specific anti-GluR3B Abs that are found in ~25% epilepsy patients (24–26) and that activate their Ag the GluR3 receptor, induce ion currents, kill neurons, and glia in vitro and in vivo (17, 27–30) and cause brain damage (18, 28, 31).

Herein, aiming to gain evidence for the presence of a soluble GluR3B-containing GluR3 fragment in the extracellular milieu of TCR-activated cells, we performed competition assays for binding of the GluR3B peptide by anti-GluR3B-specific Abs. Specifically, in these assays we tested whether medium of TCR-activated human T cells, expected to harbor the GluR3B-containing portions, can compete with purified GluR3B peptide for the binding of anti-GluR3B mAb (prepared and characterized recently in our laboratory, study in preparation). For control, we used medium of resting normal human T cells (derived from the very same T cells as above, yet without undergoing TCR activation), and thus not expected to harbor the GluR3B-containing fragments. The results (Fig. 6) show that preincubation of anti-GluR3B mAb (1 h, room temperature) with the medium of TCR-activated T cells decreased significantly (36% inhibition) its specific binding to GluR3B-coated ELISA plates, while the medium of the resting T cells failed to do so. The two latter media did not affect the low nonspecific binding of the anti-GluR3B mAb to control BSA-coated plates, showing that the competition was indeed specific for GluR3B binding. Finally, prior incubation with the medium of TCR-activated cells also significantly decreased the binding of the GluR3B peptide by

**FIGURE 4.** The concentrations of active GB in the medium of TCR-activated T cells correlate with the degree of GluR3-cleavage activity exerted by the respective medium. The amounts of active GB present in medium of TCR-activated or resting human T cells were measured using a specific GB colorimetric substrate. The ability of the same respective samples to reduce GluR3 surface expression was evaluated by 1-h incubation of resting human T cells with these medium samples followed by GluR3-specific immunofluorescence staining and flow cytometry. Results are presented as nanograms per milliliter GB vs the percent of reduction in GluR3 surface expression and are derived from three independent experiments.

**FIGURE 5.** GB inhibitors block the elimination of GluR3 from the cell surface. Normal resting human T cells were incubated (1 h, 37°C) with either medium of TCR-activated cells alone (■ in A and B), medium of TCR-activated cells plus 1 μM Ac-IEPD-CHO (□ in A), or medium of TCR-activated cells plus 20 μM Ac-IETD-CHO or Z-IETD-FMK (□ in B). Following these incubations, the resting T cells were washed and subjected to GluR3-specific single immunofluorescence staining and flow cytometry. Shown are representative experiments of three performed for each GB inhibitor. Numbers represent the percent of reduction in GluR3 expression ± SD. **, p = 0.0288 (+Ac-IEPD-CHO), 0.0118 (+Ac-IETD-CHO), and 0.0245 (+Z-IETD-FMK) vs medium TCT-act (Student’s t test).
the rat and rabbit GluR3B-specific polyclonal Abs tested in parallel (data not shown).

In view of all the other findings revealed in this study, a logical interpretation of these results (Fig. 6) would be that a GluR3 soluble extracellular fragment, cleaved from TCR-activated T cells and containing the GluR3B peptide, can bind to highly specific anti-GluR3B Abs, and by doing so reduces the binding of these Abs to purified GluR3B peptide. Parallel experiments, done with polyclonal rat and rabbit anti-GluR3B Abs (rather than the monoclonal anti-GluR3B Ab), yielded similar results (data not shown). *p = 0.0034 vs medium (Student’s t test).

In parallel to losing intact GluR3 from the cell surface, TCR-activated cells lose glutamate-induced adhesion to laminin

Does the decrease in GluR3 surface expression following TCR activation have functional consequences? As mentioned earlier, we previously found that glutamate on its own, at physiological concentration and following 30-min exposure, elevates the adhesion of resting normal human T cells to glycoproteins of the extracellular matrix, among them laminin and fibronectin (5). These effects were mediated by glutamate/AMPA receptors, and most probably by the GluR3 subtype of AMPA receptors highly expressed on these cells (Ref. 5 and confirmed herein). Fig. 7 shows that while resting T cells indeed respond to glutamate (10 nM) and increase their adhesion to laminin, TCR-activated cells do not. Interestingly, while the TCR-activated T cells could not be driven to adhesion by glutamate, they retained a profound responsiveness to the nonspecific proadhesive signal delivered by PMA (used as a routine positive control) and in that respect resembled the resting T cells (data not shown). Together, these results show that in parallel to the loss of GluR3 from the T cell surface following TCR activation, the activated T cells lose their responsiveness to the glutamate-induced (but not PMA-induced) proadhesive effect.

Discussion

The core finding of this study is that the classical immunological activation of normal resting human T cells, via their TCR (induced normally in vivo by a specific Ag, and herein by anti-CD3 and anti-CD28 mAbs), leads to the elimination of intact glutamate receptors from the cell surface. This elimination of the intact glutamate receptor (of the AMPA GluR3 subtype) upon TCR activation occurs due to an autocrine/paracrine proteolytic cleavage by GB, a protease secreted by TCR-activated cells. This glutamate receptor down-regulation in TCR-activated cells was observed by GluR3-specific flow cytometry and Western blots. With time, as the T cells reverted in vitro from the TCR-activation phase back to the resting phase, GluR3 cell surface expression was restored. We focused herein on GluR3, as we previously found that GluR3 is expressed at very high levels on resting (i.e., peripheral nonactivated) normal human T cells, human T leukemia cell lines, and a mouse anti-MBP87–99 encephalitogenic T cell line (5), and that glutamate, by activating AMPA receptors (the family of glutamate receptors to which GluR3 belongs), triggers on its own T cell...
adhesion to fibronectin and laminin and chemotactic migration to CXCL12/SDF-1 (5).

In relevance to our findings, several additional studies have shown in recent years that other types of GluRs are expressed by human T cells (32–35), and that TCR activation up-regulates the expression of the ionotropic glutamate/NMDA subunits NR1 and NR2A,B,D, and of the metabotropic glutamate receptor mGluR1 and 5 (33, 35). The present study is the first demonstration of an opposite effect, i.e., a marked down-regulation of a glutamate receptor (the AMPA GluR3) upon TCR activation. Currently, it is still unknown why are some GluRs up-regulated, while others down-regulated, following an encounter of T cells with a specific Ag and the subsequent TCR activation. Yet, in Table I, we integrate findings revealed by other groups (32–35), as well as ours (5), which together suggest that there may be a correlation between the activation status of the T cells (resting vis-à-vis TCR activation). The findings revealed herein are probably not only relevant to the neuroimmune interactions between glutamate and T cells in health and disease, but also to an entirely different, although potentially connected field. This is so, because the very same glutamate receptor subtype studied herein, i.e., GluR3, is not only a functional glutamate receptor, but also an autoantigen toward which pathogenic Abs are produced in some human epilepsy patients (24–27, 31). Such highly specific anti-GluR3 Abs, recently reported in patients with a unique type of epilepsy (24–26), and previously reported in patients with a unique type of epilepsy (i.e., Rasmussen’s encephalitis) (25, 26, 31), can be very detrimental to the CNS because they can mimic glutamate and activate their Ag (i.e., GluR3), evoke ion currents, and kill neurons and glia in various brain regions via excitotoxicity and/or complement activation (17, 27–30). For example, our recent study using a rat model for GluR3 autoimmunity showed that highly specific anti-GluR3 Abs, recently found in ~25% of patients with different types of epilepsy (24–26), and previously reported in patients with a unique type of epilepsy (i.e., Rasmussen’s encephalitis) (25, 26, 31), can be very detrimental to the CNS because they can mimic glutamate and activate their Ag (i.e., GluR3), evoke ion currents, and kill neurons and glia in various brain regions via excitotoxicity and/or complement activation (17, 27–30).

Table I. GluRs in human T cells and the effective glutamate concentration range

<table>
<thead>
<tr>
<th>Glutamate receptor expression in human T cells</th>
<th>Resting Normal Human T Cells</th>
<th>TCR-Activated Normal Human T Cells</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionotropic AMPA GluR3</td>
<td>Yes</td>
<td>No</td>
<td>5 and the present study</td>
</tr>
<tr>
<td>Ionotropic NMDA R1 and R2A,B,D</td>
<td>No/Low</td>
<td>Yes/High</td>
<td>35</td>
</tr>
<tr>
<td>Metabotropic mGluR1 and 5</td>
<td>No/Low</td>
<td>Yes/High</td>
<td>33</td>
</tr>
<tr>
<td>Responsiveness of T cells to different glutamate concentrations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low/physiological conc. (≥10 nM)</td>
<td>Yes</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>High/excess conc. (≥100 μM)</td>
<td>No</td>
<td>Yes</td>
<td>33</td>
</tr>
</tbody>
</table>

Table I. GluRs in human T cells and the effective glutamate concentration range

GB CLEAVES GLUTAMATE RECEPTOR FROM TCR-ACTIVATED T CELLS
TCR and driven to robust release of GB to the extracellular milieu. In turn, GB, acting in an autocrine/paracrine fashion, cleaves GluR3 from their cell surface, resulting in the release of a soluble GluR3-derived protein that contains the GluR3B peptide (see Fig. 6). This GluR3B peptide, shown to be the most antigenic epitope of GluR3 (17, 23), can under certain conditions be recognized by the humoral immune system as foreign (as in all cases of autoreactivity), leading to the production of anti-GluR3 Abs. In line with this hypothesis, the presence of GB cleavage sites has been suggested recently as a predictor of novel Ags (47) and GB sites have been found in numerous autoantigens (e.g., M2 in dermatomyositis, topoisomerase-I in diffuse scleroderma, and La in Sjögren’s syndrome (47)). Furthermore, the acetylcholine receptor α7 subunit, serving as the target autoantigen in myasthenia gravis, has GB cleavage sites (21). Of note, this acetylcholine receptor is expressed both in the neuromuscular junction as well as in T cells (48). Having said that, it is absolutely clear that further studies are required to test our working hypothesis and to determine whether in vivo, GB-mediated cleavage of GluR3 from TCR-activated T cells indeed augments the antigenicity/immunogenicity of the GluR3B peptide, increasing thereby the probability of the subsequent production of neuromopathic anti-GluR3 Abs. Upon gaining access to the CNS, such anti-GluR3 Abs can be highly detrimental.

In summary, the present study shows four novel and potentially very important T cell features: 1) the functional neuron-immune dialogues between glutamate and T cells may be diverse in different T cell activations states. Thus, glutamate, via AMPA GluR3 receptors, may have the ability to activate only resting, but not TCR-activated T cells; 2) activation of a key immunological receptor (the TCR) can cause the elimination of a functional neurotransmitter receptor from the T cell surface (restored after ~48 h); 3) GB secreted by TCR-activated cells can operate in an autocrine/paracrine manner, resulting in cleavage of a functional receptor from the T cell surface; 4) activation of T cells via a foreign Ag can operate in an autocrine/paracrine manner, resulting in cleavage of a functional receptor and release into the extracellular milieu of a portion of a self receptor (GluR3), which in its cleaved and soluble form, may be viewed as a non-self Ag, thereby potentially leading to detrimental autoimmune responses.

Disclosures

The authors have no financial conflict of interest.

References


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