Signaling Alterations in Activation-Induced Nonresponsive CD8 T Cells

Ee Loon Tham and Matthew F. Mescher

*J Immunol* 2001; 167:2040-2048; doi: 10.4049/jimmunol.167.4.2040
http://www.jimmunol.org/content/167/4/2040

---

**References**
This article **cites 46 articles**, 28 of which you can access for free at:
http://www.jimmunol.org/content/167/4/2040.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Signaling Alterations in Activation-Induced Nonresponsive CD8 T Cells

Ee Loon Tham*† and Matthew F. Mescher2*‡

Costimulation-dependent production and autocrine use of IL-2 by activated CD8 T cells results in initial clonal expansion, but this is transient. The cells quickly become anergic, unable to produce IL-2 in response to Ag and costimulation, irrespective of the form of costimulation. This activation-induced non-responsiveness (AINR) differs from “classical” anergy in that it results despite the cells receiving both signal 1 and signal 2. AINR cells can still proliferate in response to exogenous IL-2, but can no longer produce it. Other TCR-mediated events including cytolysis function and IFN-γ production are not affected in the AINR state. To characterize the mechanism(s) responsible for lack of IL-2 production in CD8 T cells in the AINR state, microspheres bearing immobilized anti-TCR Abs or peptide-MHC complexes, B7-1, and ICAM-1 were used to provide well-defined stimuli to the cells. Comparison of normal and AINR cells revealed that in AINR cells extracellular signal-regulated kinase (ERK) is upregulated more transiently, Janus kinase activation is substantially reduced, and activation of p38 is eliminated. PMA and ionomycin restored proliferation and IL-2 production in AINR cells, indicating a signaling defect upstream of Ras and protein kinase C.

We have demonstrated that CD8 T cells that were stimulated in vitro by microspheres bearing anti-TCR Abs and coimmobilized B7-1 and ICAM-1 or by Ag-bearing cells as APC become nonresponsive, or anergic, after an initial response (4). We have further shown that this activation-induced nonresponsiveness (AINR) occurs following an in vivo response to allogeneic tumor, even when the tumor cells express B7-1 (4) and following an in vivo response to a syngeneic tumor (5). AINR is characterized by a specific defect in IL-2 production since the activated cells can still respond to exogenous IL-2, and in response to TCR engagement can produce IFN-γ and lyse Ag-bearing target cells (4, 5). The surface levels of TCR, CD8, CD28, and LFA-1 on AINR cells are the same or higher than on naive cells. In addition, CTLA-4 does not appear to be involved in the induction or maintenance of AINR in CD8 T cells. Addition of anti-CTLA-4 mAb during primary in vitro stimulation did not prevent development of AINR, nor did it reverse nonresponsiveness when added to the cells following the primary response (4). Similarly, in vivo administration of anti-CTLA-4 mAb did not overcome CD8 nonresponsiveness following a response to syngeneic tumor, whereas the same treatment did overcome nonresponsiveness of CD4 Th cells in this system (6).

The AINR that develops in CD8 T cells is distinctively different from the classical anergy described for CD4 T cell clones. Both are characterized by impaired ability to proliferate and produce IL-2 upon rechallenge, but AINR develops after full stimulation with Ag and costimulation, whereas clonal anergy in CD4 T cells results when the cells are stimulated through just the TCR. Clonal anergy in CD4 T cells has been extensively studied but the physiological relevance of this phenomenon remains unclear. It may be a mechanism for rendering peripheral self-reactive T cells unresponsive (reviewed in Ref. 7).

CD8 T cells become AINR following an initial in vitro or in vivo response (4–6), and this appears likely to provide a regulatory checkpoint for converting an initially helper-independent CTL response to one that requires help in the form of IL-2 produced by CD4 T cells in order for the response to be maintained (6). The biochemical basis for this potentially important biological nonresponsiveness has not been defined and we therefore sought to understand how CD8 T cells in the AINR state selectively uncouple TCR and/or costimulatory-mediated signals involved in IL-2 production while leaving other TCR-mediated functions intact. Results of studies of anergic CD4 T cell clones suggested that this
might involve defects in activation of mitogen-activated protein kinases (MAPKs) that are important in signaling leading to IL-2 production (8–10). The results reported here demonstrate that AINR CD8 T cells are deficient in up-regulation of extracellular signal-regulated kinase (ERK), Janus kinase (JNK) and p38 MAPKs, and that at least ERK and p38 are critical for IL-2 production by CD8 T cells.

**Materials and Methods**

**Cells and reagents**

CD8 T cells used for experiments were obtained from either C57BL/6 mice (Charles River Breeding Laboratories, Raleigh, NC) or OT-1 TCR-transgenic mice specific for K\(^\text{b}\)/OVA\(_{264}\) peptide (11) (originally a gift from Dr. F. Carbone, Monash Medical School, Victoria, Australia). All animals were maintained in the specific pathogen-free facility at the University of Minnesota and were used at 6–12 wk. Lymph nodes were harvested, washed, and homogenized with a tissue homogenizer. The resulting single-cell suspension was treated with 11 mM KHC0/152 mM NH\(_4\)Cl to lyse RBC and then adherent cell depleted for 1 hr. CD8 T cell enrichment was carried out on negative selection columns (Cytovax Biotechnologies, Edmonton, Alberta, Canada) according to the protocol provided by the suppliers. The purity of CD8 T cells assessed by flow cytometry and was typically 90%–95% with <2% CD4 T cells.

Anti-TCR Abs 145-2C11 and B20.1.1, specific for CD3e and V\(_\alpha\)2 TCR chains, respectively, were purchased from BD Pharmingen (San Diego, CA). B7-1 and ICAM-1 proteins were purified as previously described (12, 13). H-2 \(^\text{K}^{\text{b}}\)/OVA\(_{264}\) peptide complexes were prepared using standard protocols (14–16). Briefly, constructs encoding H-2 \(^\text{K}^{\text{b}}\) H chain fusion protein with a biotinylation site and human \(\beta2\)-microglobulin were expressed in Escherichia coli. The H chain fusion protein and \(\beta2\)-microglobulin proteins were purified from inclusion bodies and refolded in the presence of excess OVA\(_{264}\) \(^\text{K}^{\text{b}}\) peptide to form complexes. Biotinylation of the complexes was then done using BirA ligase (AviDity, Denver, CO).

Recombinant murine IL-2 (R&D Systems, Minneapolis, MN) and human IL-2 were used at final concentrations of 50 and 2.5 U/ml, respectively. PMA (Molecular Probes, Eugene, OR) and ionomycin (Calbiochem, La Jolla, CA) were used at 10 ng/ml and 1 \(\mu\)M, respectively.

**Protein immobilization on latex microspheres**

Microspheres used for stimulating responder cells were prepared by incubating 10\(^5\)–50 \(\mu\)M sulfate polystyrene latex microspheres (Interfacial Dynamics, Portland, OR) with the relevant proteins in 1 ml PBS for 20 min at 4°C on a rotator. 2C11/B7-1/ICAM-1 microspheres were prepared by the sequential addition and incubation of 145-2C11, ICAM-1, and B7-1 for 20 min each. The resulting microspheres were subsequently cleared by centrifugation at 14,000 rpm for 10 min at 4°C. The pellets were resuspended in 1 ml PBS, washed once more, and were monitored using the FACsCalibur software (BD Biosciences, San Jose, CA). The Abs used were goat anti-Armenian hamster Ig-FITC (Jackson ImmunoResearch, West Grove, PA), anti-mouse IgG F(ab\(^\prime\))\(_2\) FITC or biotin anti-mouse IgG F(ab\(^\prime\))\(_2\) FITC (BD Pharmingen, Franklin Lakes, NJ). The plasmid encoding the GST-c-jun (aa 1–79) fusion protein was a kind gift from Dr. R. Hipskind (Institut de Genetique Moleculaire, Montpellier, France). Briefly, precipitation was carried out by incubating cell lysates in HEPES binding buffer (20 mM HEPES (pH 7.7), 300 mM NaCl, 1.5 mM MgCl\(_2\), 0.1 mM EDTA, 0.5% Triton X-100, 1 \(\mu\)g/ml aprotinin, 1 \(\mu\)g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) at 4°C for 14 h. The reaction mixtures were heated to 95°C for 10 min followed by 20 min each at 4°C. Inhibition of the MAPKs was examined using PD98059 and SB202190 (Calbiochem) dissolved in DMSO. Cells were preincubated in the presence of the inhibitor, or DMSO alone for controls, for 45 min at 37°C prior to stimulation with microspheres. Additional inhibitor, at the same final concentration, was added to the cell lysate before assaying enzyme activity.

**ERK and JNK assays**

For kinase assays, 1 \times 10\(^5\) normal or AINR CD8 OT-I cells were pelleted with 2 \times 10\(^5\) ligand-bearing microspheres by centrifugation at 1250 \(g\) for 15 min at 4°C. The pellets were then incubated in a 37°C water bath for the indicated times and the cells were then lysed by addition of ice-cold buffer containing 25 mM HEPES (pH 7.7), 300 mM NaCl, 1.5 mM MgCl\(_2\), 0.2 mM EDTA, 0.5% Triton X-100, 1 \(\mu\)g/ml aprotinin, 1 \(\mu\)g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride and 1% Triton X-100, 20 mM \(\text{MgCl}_2\), dNTP mix (10 mM each), and 1 U DNA polymerase (Bioline). The reaction mixtures were heated to 95°C for 10 min followed by 20 min each at 4°C. The pellets were then collected by centrifugation at 12,500 \(g\) for 10 min at 4°C. The supernatants were then diluted and assayed for enzyme activity.

**IL-2 protein and mRNA measurements**

The amounts of murine IL-2 in 50-\(\mu\)l culture supernatants were determined by sandwich ELISA using capture and detection Abs purchased from BD Pharmingen according to the protocol provided by the supplier. The Abs were specific for murine IL-2 and were not reactive toward the recombinant human IL-2 in the culture. Results are expressed as the average (±SD) of triplicate samples. The detection limit of the assay was 0.04 ng/ml.

For determination of IL-2 mRNA by RT-PCR analysis, normal or AINR CD8 T cells and 2C11/B7-1/ICAM-1 microspheres were pelleted in 96-well V-bottom plates (1.5 \times 10\(^5\) cells and 3 \times 10\(^3\) microspheres/well) by centrifugation at 2000 rpm for 10 min at 4°C. The pellets were subsequently incubated at 37°C for 8 h. No significant change in cell viability was observed at the end of the incubation period. Total RNA was isolated from the cells using the GlassMax RNA Microisolation kit (Life Technologies, Gaithersburg, MD). All RNA samples were treated with DNase I (Life Technologies) for 15 min. One microgram of RNA was reverse transcribed to cDNA using 2 \(\mu\)g oligo(dT)\(_{12–18}\) primers and 200 U SuperScript II following the protocol provided by the supplier (Life Technologies). The reverse transcriptase was subsequently heat inactivated by heating for 10 min at 95°C.

For each sample, PCRs were carried out in the presence of IL-2 primers (5’ primer, 5’-ATGTCACCAGATCCGTGACATCCTGTGTC-3’; 3’ primer, 5’-ATGCAAAATCCAGAAATCCTGCGAGGTTCC-3’) or \(\beta\)-actin primers (5’ primer, 5’-CCTAAGGCACACGGTAGAAG-3’; 3’ primer, 5’-CTCTCATGTTGCTGAGAAGGCA-3’), PCR buffer, 50 mM NaCl, 125 mM ATP, 1 mM MgCl\(_2\), and 1 U Taq polymerase (Amersham, Piscataway, NJ) and water. The PCR mixtures were heated to 95°C for 10 min followed by 27 cycles of the following conditions: 95°C for 1 min, 63°C for 2 min, and 72°C for 3 min. This number of cycles was determined to be in the linear amplification range under these conditions. PCR products were resolved on a 1% agarose and RNA quantitation was performed using IQMac software (Molecular Dynamics, Sunnyvale, CA).

**Proliferation and Induction of AINR in CD8\(^+\) T cells**

Unless indicated otherwise, 5 \times 10\(^3\) responder cells were cultured in triplicate in 96-well flat-bottom culture plates (Falcon; BD Biosciences) with 1 \times 10\(^3\) microspheres in a total volume of 200 \(\mu\)l. Microspheres were pulsed with 1 \(\mu\)Ci \([\text{H}]\)thymidine/well for the last 8 h of the indicated time and were subsequently harvested using a LKB-Wallac cell harvester (Wallac, Turku, Finland). Proliferation was assessed by measuring \([\text{H}]\)thymidine incorporation using a Betaplate liquid scintillation counter (Wallac). Results are expressed as the average (±SD) of triplicate samples.

When CD8 T cells were stimulated with 2C11/B7-1/ICAM-1 (or K\(^\text{b}\)/OVA/B7-1/ICAM-1) microspheres, proliferation peaked on day 2 or 3 and then rapidly declined as the cells lost their ability to respond to the initial stimulus. Between day 2.5 and day 3.5, cells become AINR; these cells were pooled, treated on Lymphocyte M (Cedarlane Laboratories, Hornby, Ontario, Canada) gradient centrifugation and subjected to further analyses. In each experiment, a secondary proliferation assay was set up in parallel to confirm that the cells were no longer responsive to the initial stimulus, 2C11/B7-1/ICAM-1 or K\(^\text{b}\)/OVA/B7-1/ICAM-1 microspheres.

**Western blot analysis to detect phosphorylated p38**

Normal (2 \times 10\(^5\)) and AINR CD8 OT-I cells were stimulated as described above for ERK and JNK assays and then lysed by addition of ice-cold 1% Triton X-100 lysis buffer (1% Triton X-100, 20 mM Tris (pH 7.6), 150 mM

**The Journal of Immunology**

By guest on April 11, 2017 http://www.jimmunol.org/ Downloaded from
NaCl, 1 mM Na2VO4, 1 mM PMSF, 1 μg/ml apro tinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). Laemmli loading buffer (6×) was then added, the samples were then boiled for 5 min, and resolved on a 12% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane (Amersham, Little Chalfont, U.K.) which was subsequently blocked with 5% milk powder. The membrane was probed with an Ab specific for phosphorylated p38 (Cell Signaling Technology, Beverly, MA) followed by HRP-conjugated sheep anti-mouse IgG (Jackson ImmunoResearch) and developed using the SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). In experiments examining inhibition of phosphorylation of p38, cells were preincubated in the presence of the inhibitor, or DMSO alone for controls, for 45 min at 37°C before stimulation with microspheres and preparation of the cell lysate for Western blotting.

Results

Induction of AINR in CD8 T cells is independent of the type of primary stimulation

Our previous work showed that coimmobilizing B7-1 and ICAM-1 on microspheres along with an anti-TCR mAb provided potent costimulation to CD8 T cells, stimulating IL-2 production and proliferation. However, within 3 days the cells stopped proliferating and became refractory to further costimulation. To determine whether this AINR involved CD28- or LFA-1-dependent signals, we examined whether AINR was induced when the individual costimulatory ligands were present along with the TCR stimulus. When OT-I CD8 lymph node T cells were stimulated with microspheres having B20 anti-Vα2 mAb immobilized along with B7-1 (B20/B) or ICAM-1 (B20/I), significant proliferative responses were observed (Fig. 1A). As expected (12), optimal cell proliferation was achieved when both B7-1 and ICAM-1 were coimmobilized with B20 (Fig. 1A). In all cases, the proliferative responses were transient (Fig. 1A), and proliferation and clonal expansion declined after day 2.

The decline in responses after day 2 suggested that the cells become AINR with either costimulatory ligand, and this was directly examined. Shortly after the peak of proliferation (at 60 h),
cells stimulated with B20/B/I, B20/B and B20/I were harvested, washed, and restimulated, and the secondary responses were assessed 2 days later. Cells from all three groups failed to respond when restimulated with either BSA-blocked microspheres or B20/B/I microspheres (Fig. 1B). In all three cases, however, the AINR cells were able to respond to exogenous IL-2 (Fig. 1B).

The requirement for B7-1 or ICAM-1 in the primary stimulation can be bypassed by the addition of IL-2 to cells stimulated with microspheres having just anti-TCR mAb on the surface. CD8 T cells that were activated in this way using B20 on microspheres and exogenously added IL-2 (Fig. 1C) also became nonresponsive to subsequent restimulation with B20/B/I microspheres, but still proliferated in response to exogenous IL-2 in the secondary stimulation (Fig. 1D). These results demonstrate that AINR develops in CD8 T cells within 3 days of initial stimulation, irrespective of the nature of the primary stimulus. Thus, stimulation through just the TCR and IL-2R are sufficient; signaling through CD28 and/or LFA-1 is not required to induce AINR (Fig. 1D), nor does it prevent establishment of AINR (Fig. 1B).

In other experiments, we have stimulated CD8 T cells from OT-I TCR-transgenic mice with microspheres having immobilized with H-2 K\(^{b}\)/OVA\(_{257-264}\) peptide complexes, B7-1, and/or ICAM-1 and have observed the induction of AINR with a similar time course. The differences between using anti-TCR mAb or K\(^{b}/\)OVA\(_{257-264}\) for signal 1 were quantitative, rather than qualitative, with the latter giving stronger proliferative response and enhanced biochemical signals (data not shown). RNAs isolated from naive and AINR cells, both stimulated and unstimulated, were reverse transcribed and amplified by PCR. In unstimulated naive and AINR cells, IL-2 mRNA was not detectable (Fig. 2). Upon stimulation, a large increase in IL-2 mRNA was observed in the naive cells whereas the amount of IL-2 mRNA in AINR cells was minimal (5-fold less than in normal cells) (Fig. 2). In a similar experiment, a 10-fold difference in IL-2 mRNA was observed between naive and AINR cells stimulated with K\(^{b}/\)OVA/B7-1/ICAM-1. Thus, cells that have undergone initial stimulation and become AINR display a profound defect in their ability to increase levels of IL-2 mRNA in response to TCR engagement and costimulation.

**Activation of MAPKs is defective in AINR CD8 T cells**

MAPKs are involved in the regulation of transcription factors required for IL-2 gene expression in CD4 T cells. Anergized CD4 T cells clones show a marked reduction in ERK and JNK activities upon stimulation and are unable to produce IL-2 (8–10). ERK is fully activated by TCR engagement whereas the activation of JNK requires the engagement of both TCR and CD28 (18). These observations suggested that the AINR state induced in CD8 T cells might involve one or more defects in activation of MAPKs.

Using microspheres having K\(^{b}/\)OVA alone, or coimmobilized B7-1, ICAM-1, or both, we assessed the contributions of TCR engagement and costimulation to the activation of ERK and JNK in naive and AINR CD8 T cells. We also examined a third MAPK, p38, since recent data have indicated that it is involved in signal integration during costimulation of primary murine T cells (19). ERK and JNK activities were determined in lysates of stimulated cells using GST fusion proteins as substrates. Initial experiments showed that ERK activity in normal cells peaked between 2.5 and 5.0 min and JNK activity at 10.0 min (data not shown). The costimulation requirement for ERK and JNK activation in CD8 T cells was therefore determined at 2.5 and 10 min, respectively (Fig. 3A, B). As shown in Fig. 3A, ERK activity in both naive and AINR cells increased in response to K\(^{b}/\)OVA alone and the presence of B7-1 and/or ICAM-1 did not further increase the activity, demonstrating that costimulation plays a minimal role in the activation of ERK in CD8 T cells. Unlike ERK activation, optimal activation of JNK in naive cells required B7-1 and/or ICAM-1 (Fig. 3B). Furthermore, AINR cells showed a significant defect in activating JNK in response to either B7-1 or ICAM-1, although substantial up-regulation of JNK was seen when both costimulatory ligands were provided (Fig. 3B). The levels of ERK and JNK protein were found to be essentially identical in naive and AINR cells, as assessed by Western blotting using specific Abs (data not shown).

Initial experiments showed that p38 enzymatic activity in cell lysates was too low to be reproducibly measured. Therefore, activation of p38 was assessed by Western blotting with a phosphospecific anti-p38 Ab to determine the amount of the active phosphorylated form of the enzyme in the cells upon stimulation. The costimulation requirement for p38 was determined at 10 min since p38 phosphorylation is maximal at this time when naive CD8 T cells are stimulated with K\(^{b}/\)OVA/B7-1/ICAM-1 (data not shown). Stimulation of naive cells with K\(^{b}/\)OVA alone caused a modest increase in p38 phosphorylation (1.4-fold more than unstimulated naive cells). In the presence of B7-1 and/or ICAM-1, p38 phosphorylation in naive CD8 T cells was further enhanced by about 2.5-fold (Fig. 3C). In contrast to naive cells, little or no increase in phospho-p38 was detected in AINR cells in response to any of the stimuli (Fig. 3C). Western blotting using an Ab that recognizes both active and inactive forms of p38 showed that naive and AINR cells did not differ significantly in total p38 protein levels (data not shown).

**ERK and p38 are required for costimulation-dependent IL-2 production and proliferation by CD8 T cells**

To examine the requirement for the MAPKs in stimulating production of IL-2, specific inhibitors of ERK and p38 were examined for their effects on up-regulation of IL-2 mRNA. PD98059 is a specific inhibitor of mitogen-activated protein/ERK kinase 1, the upstream kinase required for ERK activation, and does not inhibit...
JNK or p38 (20). Treatment of cells with PD98059 before stimulation with K<sup>b</sup>/OVA/B7-1/ICAM-1 inhibited up-regulation of ERK activity (Fig. 3D) and blocked IL-2 mRNA up-regulation (Fig. 3E) with the same dose response.

Recent studies have shown that pyridinylimidazole inhibitors such as SB202190 compete for the ATP binding site and inhibit the phosphorylation of p38 that is required to activate the enzyme (21), but do not inhibit ERK (22). Treatment of cells with SB202190 before stimulation with K<sup>b</sup>/OVA/B7-1/ICAM-1 inhibited p38 phosphorylation in the 10–50 μM range (Fig. 3F) and blocked IL-2 mRNA up-regulation in the same concentration range (Fig. 3G). In contrast, SB202190 inhibited JNK activity only at high concentrations (~100 μM) and at lower concentrations potentiated JNK activity (Fig. 3F). Thus, at 10 and 50 μM concentrations where IL-2 mRNA up-regulation was substantially reduced, JNK activity was as high or higher than in cells that were not treated with the inhibitor. Together these results suggest that the activation of ERK and p38 is required for IL-2 mRNA transcription in CD8 T cells stimulated via the TCR and costimulatory receptors. Whether JNK activity is required cannot be determined from these experiments, but activation of this enzyme does not appear to be sufficient for IL-2 mRNA up-regulation.

As predicted by the results examining IL-2 mRNA up-regulation, PD98059 and SB202190 also inhibited proliferation of CD8 T cells stimulated with K<sup>b</sup>/OVA/B7-1/ICAM-1. Lymph node OT-I CD8 T cells were stimulated with K<sup>b</sup>/OVA/B7-1/ICAM-1 microspheres in the presence of the inhibitors and cell proliferation was determined after 48 h by [3H]thymidine incorporation. PD98059 inhibited proliferation with a dose dependence that correlated closely with its capacity to inhibit ERK activation and IL-2 mRNA up-regulation (Fig. 4A). Similarly, the ability of SB202190 to inhibit proliferation also correlated closely with its inhibition of phosphorylation of p38 and IL-2 mRNA up-regulation, but not with JNK activation (Fig. 4B). These results further confirmed that up-regulation of ERK and p38 is required for IL-2 gene expression and proliferation in CD8 T cells.

**Up-regulation of ERK, JNK, and p38 activities is reduced in AINR cells at all times**

The above results strongly suggested that the inability of AINR cells to respond to TCR engagement and costimulation involves, at least in part, a defect in up-regulation of activity of the MAPKs. To confirm that up-regulation of these enzymes is defective over the entire course of the response, we examined the time course for up-regulation of ERK and JNK activities and p38 phosphorylation in normal and AINR cells. ERK kinase activity was activated early (within 1 min) in both naive and AINR cells but decreased much more rapidly in AINR cells than in naive cells at later time points (Fig. 5A). In four experiments, at the time that ERK activity peaked in the naive cells, AINR cells had 41–53% less activity. In contrast to ERK, JNK activity increased more slowly upon stimulation, peaking between 10 and 15 mins, and activity in AINR cells was substantially reduced at all time points in comparison to naive cells (Fig. 5B). In four experiments, at the time that JNK...
Cell proliferation was assessed by [3H]thymidine incorporation during the expressed as a percentage of the control activity in the absence of inhibitor.

**FIGURE 4.** Dose responses for inhibition of MAPKs, IL-2 mRNA, and proliferation. A, PD98059 inhibition of ERK substrate phosphorylation (GST-Erk) and IL-2 mRNA were determined by densitometry of the results shown in Fig. 3, D and E, and expressed as a percentage of the control activity in the absence of inhibitor. Cell proliferation was assessed by [3H]thymidine incorporation during the final 8 h of a 48-h culture of cells with K<sup>0</sup>/OVA/B7-1/ICAM-1 microspheres and the indicated concentration of inhibitor; results are expressed as a percentage of the incorporation in the absence of inhibitor.

**Discussion**

We found that ERK and JNK activities and p38 phosphorylation were restored when AINR cells were treated with PMA and ionomycin (Fig. 5), although appearance of phospho-p38 was consistently lower in AINR cells than in naive cells despite comparable amounts of total p38 protein in both types of cell. We therefore examined the ability of these agents to restore proliferation and IL-2 production in AINR cells. When used at 10 ng/ml and 1 μM, respectively, PMA and ionomycin provided potent stimulation for proliferation and IL-2 production of naive CD8 T cells, and the responses were greater than those obtained using 2C11/B7-1/ICAM-1 microspheres as the stimulus (Fig. 6, A and C). Naive cells stimulated with PMA and 2C11 proliferated to the same extent as those treated with PMA and ionomycin, although substantially less IL-2 was produced (Fig. 6, A and C). As shown previously, AINR cells did not proliferate in response to 2C11/B7-1/ICAM-1 (Fig. 6B). However, when these cells were stimulated with PMA and ionomycin, their ability to proliferate and produce IL-2 was restored (Fig. 6, B and D), and PMA alone or along with 2C11 microspheres stimulated some response. Naive and AINR CD8 T cells did not respond to 2C11 or ionomycin alone or in combination (data not shown).

Thus, treatment of AINR cells with PMA and ionomycin activates ERK, JNK, and p38 (Fig. 5) and restores proliferation and IL-2 production (Fig. 6). To confirm that the proliferative response of the PMA/ionomycin-treated cells was dependent on activation of the MAPKs, AINR cells were stimulated with PMA/ionomycin in the presence of various concentrations of PD98059 and SB202190. As expected, both PD98059 and SB202190 inhibited proliferation of the cells in a dose-dependent manner (Fig. 7).

**PMA and ionomycin restore proliferation and IL-2 production in AINR cells**

Activation of p21<sup>ras</sup> and protein kinase C (PKC) leads to activation of the MAPKs and the active form of p21<sup>ras</sup> participates in a variety of situations including in vitro stimulation with TCR and B7 and/or ICAM-1 ligand (Ref. 4 and Fig. 1, A and B), TCR, and IL-2 (Fig. 1, C and D), and in vivo responses to B7-bearing tumor (4) and syngeneic tumor (5, 6). The AINR cells can no longer produce IL-2 upon restimulation, but could still lyse target cells. AINR in CD8 T cells is distinctly different from these previously described forms of anergy, however, in that it results following full stimulation by TCR engagement, costimulation, and autocrine IL-2-driven proliferation. AINR develops in CD8 T cells following responses in a variety of situations including in vitro stimulation with TCR and B7 and/or ICAM-1 ligand (Ref. 4 and Fig. 1, A and B), TCR, and IL-2 (Fig. 1, C and D), and in vivo responses to B7-bearing tumor (4) and syngeneic tumor (5, 6). We would suggest that development of AINR following full stimulation is an inherent response to CD8 T cells and serves to provide an important regulatory checkpoint, converting an initially helper-independent response to a helper-dependent response. Thus, development of AINR limits an initial CD8 response, and even when Ag persists a sustained response is only obtained if IL-2 provided by CD4 helper T cells is available to support further expansion.

The AINR state that develops in CD8 T cells is characterized by an inability of the cells to increase IL-2 mRNA (Fig. 2) or protein upon restimulation and a resulting failure to proliferate. Development of AINR does not require signals through CD28, LFA-1, or
other costimulatory receptors, in that it also occurs when the initial stimulus is just TCR engagement and exogenous IL-2 (Fig. 1, C and D). Failure to increase IL-2 mRNA levels might result from defective activation of transcription, decreased mRNA stability, or both (31).

MAPK signaling cascades are activated upon stimulation of T cells and are involved in the regulation of numerous transcription factors, including those required for IL-2 mRNA transcription (32, 33). In anergic CD4 clones, the failure to activate p21ras upon TCR and CD28 engagement leads to a profound block in ERK and JNK up-regulation (8–10) and failure to transactivate AP-1, a transcription factor required for optimal IL-2 production (34). Both ERK and JNK signaling cascades act synergistically to regulate the level and activity of AP-1. There is considerable evidence in support of an important role(s) for JNKs in activating T cell proliferation (35, 36), although there is also some evidence to indicate that they may not be essential in all cases (37). Mutational analysis of CD28-mediated costimulation has shown that optimal IL-2 production requires CD28-mediated signals in addition to those for JNK activation (38). A recent report demonstrated that coengagement of TCR and CD28 also acts synergistically to activate a third member of the MAPK family, p38, in splenic T cells and suggested that activation of this enzyme is necessary for IL-2 production (19).

Examination of the MAPKs in normal resting CD8 T cells showed that ERK is activated in response to stimulation through just the TCR, with little or no further increase when costimulation is provided (Fig. 3A). In contrast, JNK activation required costimulation (Fig. 3B), as is seen for Jurkat T cells (18) and CD4 T cell clones (8). Our results with CD8 T cells differ from those of Zhang...
et al. (19) who found that stimulation of resting murine T cells with anti-TCR and anti-CD28 mAbs did not result in JNK activation. Cross-linking CD28 with an Ab may not provide an identical set of signals to that generated when CD28 binds its native B7 ligand, as was done in our experiments. TCR engagement alone caused a significant increase in the active phosphorylated form of p38 and a further substantial increase occurred in response to costimulation (Fig. 3C), as has been reported for mouse splenic T cells (19). Consistent with their abilities to costimulate CD8 T cell proliferation (13) either B7-1 or ICAM-1 could provide costimulation to up-regulate JNK and p38. Activation of all three MAPKs was defective in AINR cells, but the extent of the defect varied for the different enzymes. ERK was activated to comparable levels in normal and AINR cells at very early times (1 min), but at longer times (2.5 min) was greatly reduced in comparison to normal cells (Fig. 5A). In contrast, JNK activity was substantially reduced in AINR cells at all time points in comparison to normal cells (Fig. 5B). The block in p38 activation was most profound, with little or no phosphorylated p38 being detectable in the AINR cells at any time (Fig. 5C).

To assess the importance of up-regulation of the MAPKs in signaling for IL-2 production and proliferation, we examined the effects of PD98059, a specific inhibitor of mitogen-activated-protein/ERK kinase (and thus ERK), and SB202190, an inhibitor of p38 and at higher concentrations, JNK. PD98059 decreased ERK activity in a dose-dependent manner (Fig. 3D), and IL-2 mRNA up-regulation (Fig. 3E) and proliferation (Fig. 4) were inhibited in the same concentration range. Thus, a TCR-dependent increase in ERK activity appears to be critical for IL-2 production and proliferation. Similarly, SB202190 inhibited p38 phosphorylation, IL-2 mRNA up-regulation, and proliferation, with substantial inhibition at 10 μM and almost complete inhibition at 50 μM concentrations. SB202190 also inhibited JNK activation, but only at high concentration (100 μM). At lower SB202190 concentrations that effectively inhibited p38 activation, IL-2 mRNA up-regulation, and proliferation, JNK activity was substantially enhanced over the level of activation seen in cells not treated with the drug (Fig. 4). These results strongly suggest that activation of p38, like ERK, is critical for IL-2 production and proliferation. Whether JNK activation is also required cannot be determined from these experiments. It does appear that, however, that JNK activation is not sufficient in the absence of p38 up-regulation since JNK activity remains high at SB202190 concentrations where p38 activation and IL-2 production are inhibited. Furthermore, JNK can be up-regulated in AINR cells when potent costimulation is provided by B7-1 and ICAM-1 together (Fig. 3B), but IL-2 production and proliferation do not occur. The conclusion that p38 is critical for the CD8 T cell response is consistent with the recent report of Zhang et al. (19) providing evidence for the importance of p38 in activating murine splenic T cells.

Defective activation of the MAPKs in AINR cells suggested a defect in a common upstream signaling pathway, most likely at the level of p21ras (39, 40). To address this possibility, we examined the effects of treating AINR cells with PMA, an activator of ras and PKC, and ionomycin, a calcium ionophore. PMA- and ionomycin-restored ERK, JNK, and p38 activation (Fig. 5), IL-2 production (Fig. 6D) and proliferation (Fig. 6B) in the AINR cells. Proliferation of AINR cells in response to PMA/ionomycin was blocked by both PD98059 and SB202190 (Fig. 7), indicating that these pharmacological agents were acting, at least in part, by restoring activation of the MAPKs. An increase in intracellular Ca2+ concentration occurs upon stimulation through the TCR and results from release of Ca2+ from intracellular stores followed by the influx of extracellular Ca2+ (41). We have not directly examined whether AINR cells flux Ca2+ in response to TCR ligation, but would predict that they do given that they retain lytic effector function, an activity that is dependent on Ca2+ signaling (42). PMA in conjunction with TCR ligation, but not PMA alone, could restore IL-2 production (Fig. 6D) and proliferation (Fig. 6B) by the AINR cells. Thus, some of the TCR-dependent signals needed to activate these functions appear to remain intact in the AINR cells.

There are a number of reports of CD4 T cells becoming unresponsive following in vitro or in vivo responses to a variety of stimuli including Mls-1 (43), enterotoxin (44), and mitogenic anti-CD3 (45). In experiments examining in vivo and in vitro stimulation with anti-CD3 mAb Andris et al. (45) showed that unresponsiveness developed even when the initial response included a contribution from CD28-dependent costimulation. Unresponsiveness has also been demonstrated in CD4 Th clones following an initial response to Ag in the presence of adequate costimulation, but naive cells did not appear to be sensitive to this form of unresponsiveness (46). Thus, it remains unclear whether CD4 T cells enter a state of AINR comparable to that of CD8 T cells following a primary in vitro or in vivo responses to Ag and costimulation.

AINR develops in CD8 T cells following an initial response to a full stimulus, both in vitro and in vivo, and appears likely to be an integral part of the developmental program that a CD8 T cell progresses through following TCR engagement and costimulation. There are numerous reports in the literature from a variety of virus and tumor model systems where CD8 T cells are observed to become nonresponsive. Rather than this being a defect in the response or a nonresponsiveness induced in some manner by the disease agent, it may in most cases simply be the result of the normal progression of the CD8 response. AINR would provide a critical checkpoint to convert a helper-independent CTL response to a helper-dependent response, requiring provision of IL-2 by CD4 helper T cells to sustain and further expand the Ag-specific CD8 cells. Understanding the biochemical basis for the AINR state has the potential to suggest ways of reversing the nonresponsiveness to sustain a CTL response in cases where an effective helper response by CD4 T cells cannot be induced. As a first step in developing such an understanding, we have demonstrated in this report that the defect in AINR cells results, at least in part, from an inability to up-regulate MAPK signaling pathways through either the TCR or costimulatory receptors.

Acknowledgments
We would like to thank Caridad Rosette and Wei Li for helpful advice and discussion, Debra Lins for expert technical assistance, and Yoji Shimizu and Daniel Mueller for critical reading of this manuscript.

References

Downloaded from www.jimmunol.org by guest on April 11, 2017


