CCL5-CCR5-mediated Apoptosis in T Cells

REQUIREMENT FOR GLYCOSAMINOGLYCAN BINDING AND CCL5 AGGREGATION*

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CCL5 (RANTES (regulated on activation normal T cell expressed and secreted)) and its cognate receptor, CCR5, have been implicated in T cell activation. CCL5 binding to glycosaminoglycans (GAGs) on the cell surface or in extracellular matrix sequesters CCL5, thereby immobilizing CCL5 to provide the directional signal. In two CCR5-expressing human T cell lines, PM1.CCR5 and MOLT4.CCR5, and in human peripheral blood-derived T cells, micromolar concentrations of CCL5 induce apoptosis. CCL5-induced cell death involves the cytosolic release of cytochrome c, the activation of caspase-9 and caspase-3, and poly(ADP-ribose) polymerase cleavage. CCL5-induced apoptosis is CCR5-dependent, since native PM1 and MOLT4 cells lacking CCR5 expression are resistant to CCL5-induced cell death. Furthermore, we implicate tyrosine 339 as a critical residue involved in CCL5-induced apoptosis, since PM1 cells expressing a tyrosine mutant receptor, CCR5Y339F, do not undergo apoptosis. We show that CCL5-CCR5-mediated apoptosis is dependent on cell surface GAG binding. The addition of exogenous heparin and chondroitin sulfate and GAG digestion from the cell surface protect cells from apoptosis. Moreover, the non-GAG binding variant, (44AANA47)-CCL5, fails to induce apoptosis. To address the role of aggregation in CCL5-mediated apoptosis, nonaggregating CCL5 mutant E66S, which forms dimers, and E26A, which form tetramers at micromolar concentrations, were utilized. Unlike native CCL5, the E66S mutant fails to induce apoptosis, suggesting that tetramers are the minimal higher ordered CCL5 aggregates required for CCL5-induced apoptosis. Viewed altogether, these data suggest that CCL5-GAG binding and CCL5 aggregation are important for CCL5 activity in T cells, specifically in the context of CCR5-mediated apoptosis.

Chemokines were originally identified for their selective chemoattractant and proadhesive effects. They are responsible for directing leukocyte migration by forming chemokine gradients and triggering firm arrest by activating integrins on the leukocyte cell surface. It is now apparent that chemokines exhibit critical functions in many diverse developmental and immunological operations (1–6). A member of the β-chemokine family, CCL5 is both a T cell chemoattractant and an immunoregulatory molecule. Interestingly, CCL5 is preferentially chemotactic for T cells of the Th1 and memory phenotype (7, 8). This may be due to CCL5 binding to CCR5, which is predominantly expressed on memory Th1 T cells (9, 10). Given the prevalence of memory Th1 T cells in inflammatory diseases and the coincident increased expression of CCL5 and CCR5, CCL5-CCR5-mediated events in T cells may be critical in disease pathogenesis (11, 12).

CCL5 is a T cell co-stimulatory molecule in the context of CD3 stimulation (6, 13). Mice deficient in CCL5 demonstrate impaired T cell proliferation and cytokine production in response to antigen or anti-CD3 stimulation (6). Anti-CD3 stimulation of T cells together with 0.1 ng/ml to 10 ng/ml (nanomolar) CCL5 treatment results in proliferation and cytokine production (13). At higher, 1 μM (micromolar) concentrations, CCL5 stimulates antigen-independent activation of T cells in terms of cell proliferation, increased CD25 membrane expression, and cytokine production, indicating that high doses of CCL5 can bypass T cell receptor recognition of antigen to activate T cells (14, 15). At these micromolar concentrations, CCL5 forms large oligomers with a mass greater than 100 kDa (16, 17). Mutation of the acidic amino acid residues glutamine 26 to alanine (E26A) or glutamine 66 to serine (E66S) in CCL5 results in CCL5 variants that are unable to form higher order aggregates at micromolar concentrations (16, 18). These mutants are unable to activate T cells, implying that the aggregating properties of CCL5 are important for T cell activation (16, 17). Notably, the nonaggregating mutants retain their ability to signal via classical G-protein-dependent pathways in vitro. CCL5, as well as other chemokines, can bind to glycosaminoglycans (GAGs)3 on the cell surface or the extracellular matrix to increase relative chemokine concentrations (19, 20). The predominant GAG binding site for CCL5 has been shown to be the BB motif (where B represents either of the basic amino acids arginine or lysine) in the 40 S loop (21, 22), and GAG binding in vivo has been shown to be critical for CCL5 function (23). Residues critical for GAG binding of other chemokines, including CCL3, CCL4, and MCP-1, have now been

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3 The abbreviations used are: GAG, glycosaminoglycan; 7-AAD, 7-amino actinomycin D; PARP, poly(ADP-ribose) polymerase; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline.
identified (21, 24–30). Whether the interaction of CCL5 with GAGs induces cellular activation through a novel signaling mechanism is not clear. However, CCL5 and its interaction with GAGs facilitate oligomerization and probably contribute to efficient receptor presentation.

In this study, we examined CCL5 activity in T cells in the context of GAG binding, aggregation, and apoptosis. We present evidence that CCL5 aggregates form at high ligand concentrations to induce apoptosis in T cell lines and in primary human T cells in a CCR5-dependent manner. We show that CCL5-induced apoptosis involves the cytosolic release of the mitochondrial proapoptotic factor cytochrome c, the activation of caspase-9 and -3, and poly(ADP-ribose) polymerase (PARP) cleavage. Additionally, we provide evidence for the critical role of intracellular tyrosine residue 339 of CCR5 in mediating cell death that is independent of G-protein-mediated events. Finally, we show that CCL5-GAG interactions and CCL5 oligomerization are important prerequisites to initiate a cascade of events resulting in T cell death. Taken together, our data suggest a potential novel role for CCL5 in determining T cell fate during an immunological response.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—Human T cell lines PM1, PM1.CCR5, MOLT-4, and MOLT-4.CCR5 as well as the anti-CCR5 monoclonal antibody (2D7) were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. All cells were maintained in culture in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 100 units/ml penicillin, 100 mg/ml streptomycin (Invitrogen), and 25 µg/ml plasmocin (InvivoGen). Antibodies for cleaved caspase-3 (1:1000) and caspase-9 (1:1000) were purchased from Santa Cruz Biotechnology, Inc., and anti-PARP antibody (1:2000) was purchased from BD Biosciences. Murine monoclonal anti-human CCL5 antibody and anti-tubulin antibody (1:2000) was purchased from R & D Systems. Heparin sodium salt, chondroitin sulfate A, and chondroitinase ABC were from Sigma. JC-1 was purchased from Molecular Probes, Inc. (Eugene, OR). Wild type CCL5, CCL5 drotinase ABC were from Sigma. JC-1 was purchased from Molecular Probes, Inc. (Eugene, OR). Wild type CCL5, CCL5 drotinase ABC were from Sigma. JC-1 was purchased from Molecular Probes, Inc. (Eugene, OR).

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CAGG Binding and CCL5 Aggregation in T Cell Apoptosis

**A**

Figure 1. Micromolar concentrations of CCL5 induce apoptosis in PM1.CCR5 T cells. A, 2 x 10^5 PM1.CCR5 cells/ml were treated with varying doses of CCL5 for 24 h, and the percentage of apoptotic cells was detected by staining with annexin V-FITC and 7-AAD. Data are representative of three independent experiments. B, 2 x 10^5 PM1.CCR5 cells/ml were either left untreated (control) or treated with CCL5 (10 μg/ml) for either 24 or 48 h. A DNA fragmentation assay was performed as described under “Experimental Procedures.” Cells treated with phorbol 12-myristate 13-acetate and ionomycin (P + I) for 24 h served as a positive control. Data are representative of five independent experiments. C, cell surface CCR5 expression was determined for native PM1, PM1.CCR5, native MOLT-4, and MOLT-4.CCR5 cells by FACS. The dotted line represents the fluorescence intensity using an FITC-labeled isotype control IgG antibody. The thick solid line and the gray solid line represent the fluorescence intensity using primary anti-CCR5 antibody in conjunction with the secondary anti-mouse FITC. Data are representative of three independent experiments. D, cell surface CCR5 expression was determined for native PM1, PM1.CCR5, native MOLT-4, and MOLT-4.CCR5 cells by FACS. The dotted line represents the fluorescence intensity using an FITC-labeled isotype control IgG antibody. The thick solid line and the gray solid line represent the fluorescence intensity using primary anti-CCR5 antibody in conjunction with the secondary anti-mouse FITC. Data are representative of three independent experiments. E, 2 x 10^5 native PM1 and native MOLT-4 cells/ml were either left untreated or treated with 10 μg/ml CCL5 for 24 h. Apoptotic cells were detected by staining with annexin V-FITC and 7-AAD. The percentage of the cell population in each quadrant is indicated to the right of each FACS blot. Data are representative of five independent experiments.

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**RESULTS**

Micromolar Concentrations of CCL5 Induce Apoptosis in CCR5-expressing T Cells—Chemokines and their receptors have been implicated in determining survival (33) and death (34–37) of various cell types. To investigate the biological consequences of CCL5-CCR5 interactions on T cell survival or death, PM1.CCR5 T cells were treated with different doses of CCL5, and the viability of cells was assessed using the apoptosis marker annexin V and the permeability indicator 7-AAD. At 10 ng/ml to 1 μg/ml (nanomolar) doses, CCL5 treatment did not affect viability, but at 10 μg/ml (micromolar) doses, CCL5 induced apoptosis (Fig. 1A). Classical apoptotic cell death may be distinguished by DNA fragmentation, revealed when cells were treated with phorbol 12-myristate 13-acetate and ionomycin or 10 μg/ml of CCL5 (Fig. 1B). Additionally, we confirmed that 10 μg/ml CCL5 induced apoptosis in PM1.CCR5 and another CCR5-expressing T cell line, MOLT-4.CCR5 (Fig. 1C). By contrast, native PM1 and MOLT-4 cells lacking CCR5 expression were not susceptible to CCL5-inducible apoptotic cell death (Fig. 1, D and E). Notably, the PM1 and
MOLT-4 cell lines do not express CCR1, an alternate receptor for CCL5 in T cells.

**CCL5-induced Cell Death Is Mediated by the Mitochondrial/Apoptosome Pathway**—At nanomolar doses, CCL5 acts as a costimulatory signal for T cells. Indeed, costimulation through CD28 in the context of CD3 protects cells from activation-induced cell death (38, 39). Perhaps at micromolar doses, CCL5 bypasses the T cell receptor to induce apoptosis. The CD95/CD95L apoptotic pathway has been studied extensively in CD4^+^/H11001 T cells. We did not observe any change in CD95 or CD95L expression following CCL5 treatment in extensive time course studies (data not shown). Moreover, pretreatment of cultures with the anti-CD95L monoclonal antibody, NOK1, did not prevent CCL5-mediated apoptosis (data not shown).

Changes in mitochondrial membrane permeability (ΔΨm) led to the efflux of apoptotic factors, the release of cytochrome c, apoptosis formation, and finally chromatin clumping and DNA fragmentation. Accordingly, we examined CCL5-inducible changes in mitochondrial membrane potential and observed a time-dependent reduction in ΔΨm (Fig. 2A). Indeed, the results in Fig. 2B reveal a CCL5-inducible and time-dependent cleavage of caspase-9 (37 kDa) at 2 h that is maximal at 8 h. These data confirm that CCL5 induces apoptosis in T cells in a CCR5- and mitochondrial pathway-dependent manner.

**Expression of Intact CCR5, but Not CCR5Y339F, Renders PM1 Cells Susceptible to CCL5-inducible Apoptosis**—CCL5-mediated CCR5 activation results in the exchange of GTP for GDP by the Gα subunit, the dissociation of heterotrimeric G-proteins into Gα and Gβγ subunits, and subsequent signal transduction. Additionally, we and others have provided evidence for the CCL5-CCR5-dependent recruitment and activation of distinct protein-tyrosine kinases (reviewed in Ref. 40). Pertussis toxin catalyzes ADP-ribosylation of the α subunit of heterotrimeric G-proteins into Goα and Goβγ subunits, and subsequent signal transduction. Accordingly, to determine the contribution of G-protein-coupled events to CCL5-inducible apoptosis, in dose-response experiments we examined the consequence of pertussis toxin treatment and observed no effects (data not shown). To investigate whether phosphorylation of CCR5 on intracellular tyrosine residues influences CCR5-CCR5-mediated apoptosis, the intracellular residue Tyr^339^ was mutagenized to phenylalanine. Vectors for intact CCR5 and 19-kDa fragments) at 8 h that is sustained for 24 h (Fig. 2C). The activation of caspase-3 was further confirmed by intracellular FACS analysis using the anti-active caspase-3 antibody; at 10 h post-CCL5 treatment, active caspase-3 was detected (Fig. 2D). Finally, we examined the cleavage of the endogenous caspase-3 substrate PARP in similar time course studies. The 85-kDa cleavage fragment of PARP was detected at 8 h post-CCL5 treatment and to a greater extent at 16 h (Fig. 2E).

**Micromolar Concentrations of CCL5 Induce Apoptosis in CCR5-expressing Primary T Cells**—Human primary T cells were isolated from peripheral blood from healthy donors and activated as described under “Experimental Procedures.” Cells were subsequently sorted based on cell surface CCR5 expression and were >95% CD3 positive (Fig. 3A). To further investigate the biological consequences of CCL5-CCR5 interactions in primary T cells, CCR5^+^ and CCR5^-^-primary T cells were treated with CCL5 for 24 h. Consistent with our data for PM1.CCR5 cultures, CCL5-inducible apoptosis was dependent on CCR5 expression and did not occur at 100 ng/ml to 1 μg/ml (nanomolar) CCL5 doses but required 10 μg/ml (micromolar) doses (Fig. 3B). Fig. 3C reveals a CCL5-inducible and time-dependent cleavage of caspase-9 (37 kDa) at 2 h that is maximal at 8 h. These data confirm that CCL5 induces apoptosis in T cells in a CCR5- and mitochondrial pathway-dependent manner.
CCR5Y339F cDNA were constructed (as described under “Experimental Procedures”) and introduced into native PM1 cells. Each transfectant was analyzed for cell surface CCR5 expression using an anti-human CCR5 antibody (Fig. 4A), which does not distinguish among the intact or mutant receptors, and clones exhibiting similar ectopic expression lev-
GAG Binding and CCL5 Aggregation in T Cell Apoptosis

**FIGURE 2.** Micromolar concentrations of CCL5 induce cytochrome c release, caspase-9 and -3 activation, and PARP cleavage. A, $1 \times 10^6$ PM1.CCR5 cells were treated with 10 µg/ml CCL5 for the indicated times. Cells were collected and stained with 2 µM JC-1 and analyzed by FACS. Mitochondrial depolarization was measured by decrease of JC-1 accumulation in the mitochondria (thus an increase in JC-1 monomers) due to loss of membrane potential. CCCP was used as positive control and gating correction (data not shown). B, Data are representative of two independent experiments (mean ± S.D.). *, $p < 0.05$; **, $p < 0.01$. C, PM1.CCR5 cells were either left untreated or treated with 10 µg/ml CCL5 for the indicated times. Cells were harvested, and lysates were resolved by SDS-PAGE and immunoblotted with anti-cytochrome c antibody. Membranes were stripped and reprobed for tubulin as loading control. The relative -fold increase of protein level is shown as signal intensity over loading control. Data are representative of two independent experiments. WB, Western blot.

**FIGURE 3.** Micromolar concentrations of CCL5 induce apoptosis in human primary T cells. A, human peripheral T cells were isolated as described under "Experimental Procedures." FACS analysis shows cell surface CCR5 expression of CCR5- and CCR5+ T cell populations. B, $2 \times 10^7$ CCR5- or CCR5+ T cells/ml were incubated with varying doses of CCL5 for 24 h. The percentage of apoptotic cells was detected by annexin V-FITC and 7-AAD. Data are representative of three independent experiments (mean ± S.D.). ***, $p < 0.001$. C, CCR5+ T cells were either left untreated or treated with 10 µg/ml CCL5 for the indicated times. Cells were harvested, and lysates were resolved by SDS-PAGE and immunoblotted with anti-caspase-9 antibody. Membranes were stripped and reprobed for tubulin as a loading control. The relative -fold increase of protein level is shown as signal intensity over loading control. Data are representative of two independent experiments. WB, Western blot.

**FIGURE 4.** Introduction of CCR5 but not CCR5Y339F into PM1 T cells renders them susceptible to CCL5-inducible apoptosis. A, cDNA for intact CCR5, CCR5Y339F, or vector alone was introduced by retroviral transduction into native PM1 cells. Cell surface CCR5 expression of all transfectants was examined by FACS. The dotted line represents the fluorescence intensity using FITC-labeled isotype control IgG antibody. The thick solid line represents the fluorescence intensity using primary anti-CCR5 antibody in conjunction with the secondary anti-mouse FITC. B, $2 \times 10^5$ PM1 vector, PM1.CCR5, and PM1.CCR5Y339F cells/ml were either left untreated or treated with 10 µg/ml CCL5 for 24 h. The percentage of apoptotic cells was detected by staining with annexin V-FITC and 7-AAD. Data are representative experiment of five independent experiments (mean ± S.D.). ***, $p < 0.001$.

PM1.CCR5, and PM1.CCR5Y339F cells were either left untreated or treated with 10 µg/ml CCL5 for 24 h. The percentage of apoptotic cells was detected by annexin V-FITC and 7-AAD. Data are a representative experiment of five independent experiments (mean ± S.D.). ***, $p < 0.001$.

els were selected for use. In subsequent experiments, we examined whether 10 µg/ml (micromolar) doses of CCL5 would induce apoptosis in PM1 cells expressing tyrosine 339-deficient mutant CCR5. The data in Fig. 4B show that CCL5 induced apoptosis in PM1 cells expressing intact CCR5 but not in cells expressing CCR5Y339F.

**CCL5-induced Cell Death Is Dependent on GAG Interactions**—In addition to the interaction of chemokines with their cognate cell surface receptors, chemokines bind to heparin-like GAGs normally expressed on proteoglycan components of the cell surface and extracellular matrix, thereby creating a concentration gradient for cells to migrate along via a haptotactic mechanism (41–46). Different studies suggest that chemokine-
GAG interactions enhance the functional activities of chemokines by a mechanism that involves sequestration onto the cell surface (19, 20, 47). Binding studies with immobilized heparin and human umbilical vein endothelial cells revealed that the affinity interaction of CCL5 with GAGs can be ablated by the addition of heparin, heparin sulfate, chondroitin sulfate, and dermatan sulfate (45). Cell surface CCL5 binding was observed in both native PM1 and PM1.CCR5 cells, although consistently higher CCL5 binding was seen in PM1.CCR5, presumably due to expression of its high affinity receptor (Fig. 5A). The data suggest that PM1 T cells have GAGs on the cell surface that are able to bind and sequester CCL5. To address the role of GAG interactions in CCL5-induced cell death, PM1.CCR5 cells were treated with CCL5 and varying doses of heparin or chondroitin sulfate. The addition of heparin or chondroitin sulfate rescued PM1.CCR5 cells from CCL5-induced cell death; 10 μg/ml heparin or 100 μg/ml chondroitin conferred complete protection (Fig. 5B). Subsequently, PM1.CCR5 cells were treated with chondroitinase ABC to enzymatically remove the GAGs from the cell surface. Chondroitinase treatment signifi-

FIGURE 5. CCL5-GAG interactions are important for apoptosis. A, native PM1 and PM1.CCR5 cells were either left untreated or treated with 10 μg/ml CCL5 for 1 h on ice. CCL5 binding to the cell surface was determined by FACS analysis. The solid line represents staining with the FITC-labeled anti-CCL5 antibody, and the dotted line shows staining with the FITC-labeled isotype control antibody. Mean fluorescence intensity is indicated in each FACS histogram. Data are representative of two independent experiments. B, 2 × 10^5 PM1.CCR5 cells/ml were either left untreated or treated with 10 μg/ml CCL5 in the presence or absence of increasing doses of heparin or chondroitin sulfate A for 24 h. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are representative of three independent experiments (mean ± S.D.). **, p < 0.01. C, PM1.CCR5 cells were either pretreated with PBS or chondroitinase ABC prior to CCL5 treatment. CCL5 binding to the cell surface was determined by FACS analysis. Data are representative of three independent experiments. D, PM1.CCR5 cells were either pretreated with PBS or chondroitinase ABC, and cell surface CCR5 expression was determined by FACS analysis. E, PM1.CCR5 cells were either pretreated with PBS or chondroitinase ABC prior to 24 h of CCL5 treatment. Apoptotic cells were detected by staining with annexin V-FITC and 7-AAD. Data are representative of three independent experiments (mean ± S.D.). **, p < 0.01. F, PM1.CCR5 cells were either left untreated or treated with 10 μg/ml CCL5 or (44AANA47)-CCL5 for 24 h. Additionally, equal concentrations of (44AANA47)-CCL5 and wild type CCL5 were preincubated for 4 h at room temperature, and then PM1.CCR5 cells were stimulated for 24 h (1:1 (44AANA47)-CCL5/CCL5 heterodimer). Apoptotic cells were detected by staining with annexin V-FITC and 7-AAD. Data are representative of three independent experiments (mean ± S.D.). **, p < 0.01.
cantly reduced the ability of CCL5 to bind to the cell surface (Fig. 5C) without altering CCR5 expression itself (Fig. 5D). We show in Fig. 5E that chondroitinase treatment protected PM1.CCR5 cells from CCL5-induced death. Similarly, when PM1.CCR5 cells were treated with 10 μg/ml (micromolar) (44ANAA47)-CCL5, a non-GAG-binding variant of CCL5 (23), we did not observe apoptosis (Fig. 5F). Moreover, when PM1.CCR5 cells were treated with a mixture of equal concentrations of (44ANAA47)-CCL5 and intact CCL5, which had been premixed for 4 h at room temperature, the resulting heterodimer did not induce apoptosis (Fig. 5F). The data suggest that in the absence of CCL5-GAG interactions on the cell surface, CCL5-inducible CCR5 activation that leads to apoptosis does not occur.

**Aggregation of CCL5 Is Required for CCL5-induced Cell Death**—At micromolar concentrations, CCL5 forms higher order oligomers/aggregates (16, 18). Certainly, CCL5 oligomerization is necessary for CCR1-mediated arrest of leukocytes on activated epithelium during leukocyte recruitment, although subsequent CCR5-mediated transmigration does not require CCL5 aggregation (42). To address the importance of CCL5 aggregation in CCL5-induced PM1.CCR5 cell death, experiments were conducted using the E26A and E66S CCL5 nonaggregating mutants. Importantly, at 10 μg/ml (micromolar) concentrations, where native CCL5 forms large oligomers, E26A and E66S form tetramers and dimers, respectively (18). The results in Fig. 6 show that the E66S mutant did not induce cell death even at 100 μg/ml doses, in contrast to both the intact CCL5 and the mutant E26A. The data suggest that CCL5 tetramers are the minimal higher order aggregates required for inducing T cell death.

**DISCUSSION**

Chemokines are both chemotactic and immunoregulatory molecules. In addition to their roles in the recruitment of T cells to sites of inflammation and in triggering their adhesion and diapedesis, accumulating evidence implicates specific chemokines in antigen-independent T cell activation. Clearly, activated chemokine receptors are able to invoke many different signaling cascades that determine the functional outcome of the target cell. Herein we report on CCL5 activity in T cells in the context of GAG binding, oligomerization, and CCR5-mediated apoptosis. Certainly, CCL5-induced T cell death has been implicated as a potential immune escape mechanism in melanoma progression, associated with CCR5-mediated cytochrome c release and caspase-9 and -3 activation (48). CCL5-CCR5-mediated caspase-3 activation and cell death have been reported in neuroblastoma cells, and there is also evidence that HIV-1 virion-mediated apoptosis of bystander uninfected CD4+ T cells, which leads to T cell depletion in infected individuals, is CCR5-dependent (49).

The cell suicide machinery can be induced by several fac-
Tyr307 is conserved among CC chemokine receptors, Tyr339 of signaling effectors in mediating cell death. Dong investigate its contribution as a potential site for recruitment defect in the kinetics of CCL5 binding or internalization in binding is unaffected. In agreement, we do not observe a reported that whether HEK293 cells express intact CCR5 or and not Tyr307 (62). Accordingly, we focused on Tyr339 to sine phosphorylation signaling events mediated by Tyr339 dence that vaccinia virus activation of CCR5 results in tyro-
is unique to CCR5 and CCR4. In other studies, we have evi-
trations (16, 18). We have provided evidence that different non-
aggregating mutants variably affected cell death. Specifically, the E66S mutant did not induce cell death, even at concentrations reaching 100 μg/ml, in contrast to both the native aggregating CCL5 and the mutant E26A. The data suggest that CCL5

GAG Binding and CCL5 Aggregation in T Cell Apoptosis

FIGURE 6. The CCL5 aggregation mutant E66S does not induce PM1.CCR5 cell death. 2 × 10⁷ PM1.CCR5 cells/ml were either left untreated or treated with 10 μg/ml CCL5, E26A, E66S, or 100 μg/ml E66S. Apoptotic cells were detected by staining with annexin V-FITC and 7-AAD. Data are representative of three independent experiments (mean ± S.D.). **, p < 0.01.

PM1.CCR5Y339F cells in response to CCL5 (data not shown). However, as described herein, CCL5 induced apoptosis in PM1 cells expressing intact CCR5 but not in those expressing CCR5Y339F. The data suggest that Tyr339 may be a critical target for effector recruitment after CCR5 dimerization, an obligatory step to trigger signaling in response to CCL5 (54). Certainly, Tyr339 in the DRY motif of CCR2 has been identified as the primary target for Jak2-mediated CCR2b receptor phosphorylation after MCP-1 binding (55). Furthermore, CCR2bY139F acts as a CCR2b dominant negative mutant, blocking chemokine responses by forming nonfunctional dimers with intact CCR2b.

We investigated the role of CCL5-GAG interactions in mediating T cell apoptosis. The addition of exogenous heparin and chondroitin sulfate completely rescued PM1.CCR5 cells from CCL5-induced cell death in a dose-dependent manner. We infer that heparin and chondroitin sulfate compe-
eters, which then converge to activate caspases via two pathways: one involving caspase-8 recruitment to death recept-
tors (tumor necrosis factor or CD95) and the other involving the mitochondrial/apoptosome pathway (reviewed in Ref. 50). Our studies show that CCL5 induced dissipation of mitochondrial membrane potential and cytochrome c release into the cytosol in a time-dependent manner, with no involvement of CD95/CD95L. This was accompanied by increased cleavage of caspase-9, caspase-3, and PARP. Taken together, the data indicated that CCL5-inducible apoptosis in CCR5-expressing T cells is mediated by activation of the mitochondrial/apoptosome pathway.

CCL5-inducible apoptosis was not sensitive to pertussis toxin treatment, implying a Goi-independent mechanism. Accordingly, we focused on tyrosine residues in the intracell-
ular portion of CCR5. CCR5 contains three intracellular tyrosine residues, at positions 127, 307, and 339. Tyr127 lies in the second intracellular loop of the receptor in the DRY motif, highly conserved among CC chemokine receptors and implicated in mediating chemokine receptor signal trans-
duction. Mutation of the DRY motif in CCR5 results in a nonfunctional receptor with reduced surface expression incapable of Go subunit binding and signaling (51, 52). The other two intracellular tyrosine residues of CCR5, Tyr307 and Tyr339, reside in the C-terminal tail of the receptor. Whereas Tyr307 is conserved among CC chemokine receptors, Tyr339 is unique to CCR5 and CCR4. In other studies, we have evidence that vaccinia virus activation of CCR5 results in tyro-
sine phosphorylation signaling events mediated by Tyr339 and not Tyr307 (62). Accordingly, we focused on Tyr339 to investigate its contribution as a potential site for recruitment of signaling effectors in mediating cell death. Dong et al. (53) reported that whether HEK293 cells express intact CCR5 or the tyrosine mutant variant, CCR5Y339F, CCL5-receptor binding is unaffected. In agreement, we do not observe a defect in the kinetics of CCL5 binding or internalization in

![Graph](image_url)
tetramers are the minimal higher order aggregates required for inducing T cell death, in agreement with evidence that tetramers are the minimal order aggregates required to recruit cells \textit{in vivo} (23).

The ability of CCL5 to induce at least two distinct biological outcomes, chemotaxis and apoptosis, is an important feature of this chemokine. At nanomolar concentrations, CCL5-CCR5 interactions induce a pertussis toxin-sensitive signaling cascade responsible for the activation of integrins and chemotaxis. CCL5 at micromolar concentration triggers distinct tyrosine phosphorylation signaling events, leading to prolonged calcium influx, hyperphosphorylation, and generalized T cell activation (15). The effects of micromolar CCL5 in T cells have been well documented, including influencing proliferation, cytokine production, and permissiveness for HIV-1 infection (14–17, 57–60). As an extension of these, the present study describes a potential novel mechanism by which high concentrations of CCL5 determine T cell fate through activation of the mitochondrial/apoptosome pathway. Because micromolar concentrations of CCL5 are required to invoke this outcome, the important question is whether these concentrations of CCL5 are achievable or likely \textit{in vivo}. Certainly, unusually high CCL5 concentrations may be realizable at sites of acute infection or inflammation through the sequestration of CCL5 by cell surface and/or extracellular matrix GAGs. In addition, the unique ability of CCL5 to form aggregates, facilitated through GAG binding, may also lead to an increase in local CCL5 concentration (16–18, 20–23, 45). We therefore infer that the CCL5-CCR5-induced apoptosis of T cells we observe is not likely to be an \textit{in vitro} artifact but is attainable \textit{in vivo}. We argue against the possibility of CCL5 aggregates blocking the interaction of growth factors with their receptors and indirectly inducing apoptosis, since the viability of native PM1 and MOLT-4 cells lacking CCR5 expression yet able to sequester CCL5 aggregates by GAG binding was not affected by micromolar CCL5.

This study describes a potential mechanism by which CCL5-CCR5 interactions determine T cell fate. Apoptosis of T lymphocytes is critical in maintaining both central and peripheral tolerance and homeostasis. Activation-induced cell death in T cells is certainly a major mechanism of clonal deletion in the immune system. Death receptors, especially CD95/CD95L interactions, have been described as an important inducer of activation-induced cell death in T cells, although different effectors, including c-Myc and TRAIL, have also been identified. Recently, Tyner \textit{et al.} (61) described an anti-apoptotic signaling pathway in macrophages mediated by nanomolar CCL5-CCR5 interactions. Although apparently contradicting our findings, the lineage of the cell type studied and the lower dose of CCL5 employed may explain these different observations. In the present study, we describe a potential novel mechanism by which high concentrations of the CCL5 determine T cell fate through activation of the mitochondrial/apoptosome pathway. Our results suggest that CCL5-induced cell death, in addition to CD95/CD95L-mediated events, may contribute to clonal deletion of T cells during an immunological response. The identification of specific CCR5-mediated signaling effectors critical for apoptosis is currently under investigation.

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