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The Cathepsin B Inhibitor, z-FA-FMK, Inhibits Human T Cell Proliferation In Vitro and Modulates Host Response to Pneumococcal Infection In Vivo

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The cathepsin B inhibitor, benzoylcarbonyl-phenyl-alanyl-fluoromethylketone (z-FA-FMK) at nontoxic doses was found to be immunosuppressive and repressed human T cell proliferation induced by mitogens and IL-2 in vitro. We showed that z-FA-FMK suppresses the secretion of IL-2 and IFN-γ as well as the expression of IL-2R α-chain (CD25) in activated T cells, whereas the expression of the early activated T cell marker, CD69, was unaffected. Furthermore, z-FA-FMK blocks NF-κB activation, inhibits T cell blast formation, and prevents cells from entering and leaving the cell cycle. z-FA-FMK inhibits the processing of caspase-8 and caspase-3 to their respective subunits in resting T cells stimulated through the Ag receptor, but has no effect on the activation of these caspases during Fas-induced apoptosis in proliferating T cells. When administered in vivo, z-FA-FMK significantly increased pneumococcal growth in both lungs and blood, compared with controls, in a mouse model of intranasal pneumococcal infection. Because host response to bronchopneumonia in mice is T cell dependent, our collective results demonstrated that z-FA-FMK is immunosuppressive in vitro and in vivo. The Journal of Immunology, 2006, 177: 3827–3836.

Peptidyl fluoromethylketones with amino acids phenylalanine and alanine in the P1 and P2 positions, respectively, have been shown to be irreversible inhibitors of some members of the cathepsin enzyme family (1, 2). In particular, benzoylcarbonyl-Phe-Ala-fluoromethylketone (z-FA-FMK) was found to be a potent inactivator of the human cathepsin B and binds tightly to the active site of the enzyme. Once bound to the active site, the fluoromethylketone group alkylates the cysteine residue in the active site and forms a covalent bond, which irreversibly blocks its proteolytic activity.

Cathepsin B is a cysteine protease found mainly in the lysosomes (3). However, in rheumatoid arthritis (RA), cathepsin B is secreted into the synovial fluid and adjacent inflamed tissues by leukocytes, synoviocytes, and chondrocytes (4, 5). This enzyme activity was found to be increased in the synovial fluid and synovial lining in RA patients. In addition, cathepsin B level increases progressively during the course of experimental arthritis in animal joint tissues (6–8). These studies suggest that cathepsin B may be a good target for therapeutic intervention for the treatment of RA using z-FA-FMK. Indeed, a number of early in vivo studies have shown that z-FA-FMK is very efficient in preventing the destruction of articular cartilage and bone in chronic inflammatory arthritis induced by adjuvant in mice (2, 8, 9).

Recently, z-FA-FMK was found to inhibit LPS-induced cytokine production in macrophages by blocking the transactivation potential of NF-κB (10). This suggests that the therapeutic action of z-FA-FMK in the treatment of RA may not be due to the inhibition of secreted cathepsin B alone, but may involve the inhibition of NF-κB dependent gene expression. In addition to the inhibition of cathepsin B, z-FA-FMK also inhibits the caspases during apoptosis and downstream apoptotic events such as phosphatidylserine externalization and DNA fragmentation in T cells treated with synthetic retinoid-related molecules (11). Although caspases play a pivotal role in apoptosis, recent studies clearly showed that these cysteine proteases, particularly caspase-8, play an important role in T cell activation and proliferation (12, 13). Inhibition of caspases using the peptidyl fluoromethylketone caspase inhibitor z-VAD-FMK has been shown to block T cell proliferation (12, 14, 15). Furthermore, T cells in mice that have germline deficiency in caspase-8 proliferate poorly upon stimulation and defective caspase-8 in human T cells display lymphocyte activation defects along with the expected defects in lymphocyte apoptosis and homeostasis (13, 16). The nonspecific inhibition of caspases and the blocking of NF-κB transactivation by z-FA-FMK suggest that this peptidyl fluoromethylketone may have potential immunosuppressive properties, which could have contributed to the remarkable efficacy of z-FA-FMK on RA in vivo. Moreover, immunosuppressive agents, such as steroids and cyclosporine, have been proven to be effective in the treatment of inflammatory diseases such as RA (17–19).

In the present study, we examined the potential immunosuppressive properties of z-FA-FMK on primary human T cells. Our results demonstrated that z-FA-FMK is immunosuppressive and blocks T cell proliferation induced by mitogens as well as IL-2 in vitro. The peptidyl fluoromethylketone also blocked NF-κB activation and downstream cytokine production in T cells as well as blocking cell cycle progression. We showed that z-FA-FMK blocks caspase-8 and caspase-3 processing to their respective subunits during T cell activation but had no effect on caspase-mediated cell death. When administered in vivo, z-FA-FMK significantly exacerbated both pulmonary and systemic pneumococcal pneumonia in mice treated with adjuvant in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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infection in mice. The host immune response to pneumococcal infection has recently been shown to be T cell dependent (20). The data presented in this paper are in keeping with a protective role for T cells during pneumococcal infection. Collectively, our results demonstrate that z-FA-FMK is immunosuppressive in vitro and in vivo.

Materials and Methods

Reagents

Benzyloxy carbonyl-valyl-alanyl-aspartic acid-(O-methyl)-fluoromethylketone (z-VDAM-FMK) and benzyloxy carbonyl-phenyl-alanyl-acid-fluoromethylketone (z-FA-FMK) were purchased from ICN, mAb against CD3 (clone OKT3) was purified from hybridoma (American Type Culture Collection) culture supernatants and anti-CD28 mAb was purchased from R&D Systems. Rabbit anti-caspase-3 was a gift from Xiao-Ming Sun (Medical Research Council Toxicology Unit, Leicester, Leicester, U.K.). FITC-conjugated anti-CD25 and RPE-conjugated anti-CD69 were acquired from Transduction Laboratories and DakoCytomation, respectively. Recombinant Fas ligand (Fasl), anti-Fas, and anti-poly(ADP-ribose) polymerase (PARP) were obtained from Alexis Biochemicals. Goat-anti-caspase-8 and rabbit anti-NE-kB p65 (C-20) were from Santa Cruz Biotechnology. Wallac BetaPlate scintillation reagent (PerkinElmer) was added to the glass fiber filter mats and, and the radioactivity was determined on a 1450 Microbeta liquid scintillation counter (PerkinElmer). T lymphocyte division following mitogen stimulation was determined using CFSE labeling of the cells (22). In brief, PBMCs were suspended in PBS at a density of 5×10^5 per ml and incubated with 5 μM CFSE at 37°C for 10 min. Following incubation with CFSE, the labeled PBMCs were washed twice in RPMI 1640 medium to remove excess CFSE. The CFSE-labeled cells were treated with mitogens as described previously in the presence or absence of z-FA-FMK. As the T lymphocytes divide, CFSE is sequentially diluted, resulting in a decreased fluorescence intensity in the cells, which can be followed by flow cytometry.

Determination of cell surface CD25 and CD69 expression using flow cytometry

Following treatments, PBMCs (5×10^5 cells) were centrifuged down and the supernatants discarded. The cell pellets were resuspended in 50 μl of staining buffer (2% BSA in PBS). FITC-conjugated anti-CD25 (10 μl), RPE-conjugated anti-CD69 (10 μl), or the appropriate fluorochrome-conjugated mouse IgG (isotype control) were added to the cells and incubated on ice for 30 min in the dark. The cells were then washed twice in staining buffer before analyzed by flow cytometry.

Measurement of secreted IL-2 and IFN-γ in culture supernatants

Following treatments, the cells were removed by centrifugation and the supernatants collected and kept frozen. The secreted IL-2 and IFN-γ in the supernatants were detected using the DuoSet ELISA kits from R&D Systems, according to the manufacturer’s instructions.

Nuclear translocation of NF-κB RelA, p65

This is essentially as described previously (23). Purified T cells (3×10^6 cells) were costimulated with anti-CD3 and anti-CD28 for 2 h, washed with cold PBS, and fixed with 1 ml of paraformaldehyde (4%) for 20 min at room temperature. The cells were permeabilized with PBS containing 3% BSA and 0.2% Triton X-100 for 2 min in room temperature. The permeabilized cells were washed twice and resuspended in 100 μl of PBS with 3% BSA and rabbit anti-p65 Ab (1/50 dilution) for 45 min at room temperature. The cells were then washed and incubated with anti-rabbit Ab conjugated with AlexaFluor 1/2000 dilution) in a final volume of 200 μl for 30 min in the dark. Following this, the cells were washed twice and resuspended in 10 μl PBS: glycerol (50/50, v/v). The cells were mounted onto slides and viewed using confocal microscopy. Images were randomly acquired from each sample, and cells with NF-κB p65 nuclear localization were counted. A minimum of 500 cells was analyzed for each sample.

Western blotting

Following treatments, a total of 2×10^6 cells was washed in PBS and resuspended in 30 μl of lysis buffer (0.1 M NaCl, 1 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1% Triton-X-100, and 1 mM PMSF). The cells in lysis buffer were taken through 3×freeze/thaw cycles on dry ice. Protein concentration was measured using the Bradford assay (BioRad). Protein (20 μg) from whole-cell lysates was diluted in loading buffer (2% SDS, 10% glycerol, 50 mM Tris-HCl (pH 6.8), 0.2% bromophenol blue, and 100 mM DTT) and resolved using 15% SDS-PAGE. The separated proteins were transferred onto Hybond C membrane (Amersham Biosciences) and probed with Abs to caspase-8, –3, PARP, and β-actin. Detection was conducted using chemiluminescence (Amersham).

Bacteria

Streptococcus pneumoniae serotype 2, strain D39 was obtained from National Collection of Type Cultures (London, U.K.). Bacteria were identified as pneumococci before experiments by Gram stain, catalase test, α-hemolysis on blood agar plates and by optochin sensitivity. To standardize virulence of pneumococci, bacteria were passage through mice as described previously (24) and subsequently recovered and stored at −70°C. When required, suspensions were thawed at room temperature and bacteria harvested by centrifugation before resuspension in sterile PBS.

Infection of mice with S. pneumoniae

Eight- to 10-wk-old female MFI outbred mice weighing 30–35 g (Harlan Breeders) were anesthetized slightly with 2.5% (v/v) fluothane (AstraZeneca) over oxygen (1:5–2 L/min). As described previously (20), 50 μl of

with [methyl-3H]thymidine (0.037 MBq). The cells were harvested onto glass fiber filter mats using a Tomtec automated multwell harvester (PerkinElmer), Wallac BetaPlate scintillation reagent (PerkinElmer) was added to the glass fiber filter mats, and the radioactivity was determined on a 1450 Microbeta liquid scintillation counter (PerkinElmer). T lymphocyte division following mitogen stimulation was determined using CFSE labeling of the cells (22). In brief, PBMCs were suspended in PBS at a density of 5×10^5 per ml and incubated with 5 μM CFSE at 37°C for 10 min. Following incubation with CFSE, the labeled PBMCs were washed twice in RPMI 1640 medium to remove excess CFSE. The CFSE-labeled cells were treated with mitogens as described previously in the presence or absence of z-FA-FMK. As the T lymphocytes divide, CFSE is sequentially diluted, resulting in a decreased fluorescence intensity in the cells, which can be followed by flow cytometry.

Determination of cell surface CD25 and CD69 expression using flow cytometry

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Following T cell activation, the cytokine IL-2, which is a T cell growth factor, acts in an autocrine and paracrine fashion to drive T cell proliferation (25). To understand the underlying mechanisms of z-FA-FMK-induced inhibition of T cell proliferation, we examine whether z-FA-FMK has any effect on the production of IL-2 in activated T cells. As illustrated in Fig. 2A, control unstimulated T cells do not produce any IL-2. However, when the T cells were costimulated with anti-CD3 and anti-CD28 for 24 h, there was a huge increase in the concentration of IL-2 in the culture supernatants as detected using ELISA. The presence of 50 μM of z-FA-FMK in the cell culture inhibited the production of IL-2 by ~65%, and higher concentration of z-FA-FMK (100 μM) further reduced the amount of IL-2 secreted into the culture supernatants. DMSO has no effect on IL-2 secretion in activated T lymphocytes. The inhibition of IL-2 production by z-FA-FMK suggests that gene transcription dependent processes may be inhibited by z-FA-FMK. To this end, we examined whether z-FA-FMK has any effect on IFN-γ secretion following T cell activation. As shown in Fig. 2B, resting T cells produced low levels of IFN-γ, but upon stimulation for 24 h through the Ag receptor, the concentration of this cytokine in the culture supernatants was markedly increased. The presence of z-FA-FMK (50 and 100 μM) markedly inhibited the production of IFN-γ in these activated T cells.

The inhibition of IL-2 production in activated T cells by z-FA-FMK suggests that the lack of IL-2 in the extracellular milieu may be the reason for the poor level of cell proliferation. To examine this possibility, exogenous rIL-2 (25 U/ml) was added to T cells costimulated with anti-CD3 and anti-CD28 in the presence of z-FA-FMK. As illustrated in Fig. 3, the addition of rIL-2 to the cell cultures was unable to reverse the inhibition of T cell proliferation induced by z-FA-FMK. The results suggest that z-FA-FMK may be targeting an upstream signaling event leading to gene transcription, which also may block the up-regulation of the high-affinity IL-2R α-chain (CD25). To determine this, PBMCs costimulated with anti-CD3 and anti-CD28 in the presence or absence of z-FA-FMK were stained for the cell surface expression of CD25. As illustrated in Fig. 4, z-FA-FMK inhibited the expression of CD25 in activated T cells, with >50% inhibition observed with 100 μM of peptidyl fluoromethylketone. However, z-FA-FMK has little effect on an early T cell activation marker, CD69 (Fig. 4), which is stored preformed in the cytoplasm before expression on the T cell surface (26). The results suggest that z-FA-FMK blocks processes that are dependent on gene transcription, such as IL-2, IFN-γ, and CD25.

We next examined whether z-FA-FMK has any effect on IL-2 driven T cell proliferation. To this end, purified T cells were activated with PHA for 7 days, washed, and the proliferating cells cultured in medium supplemented with rIL-2 (25 U/ml) in the presence or absence of z-FA-FMK. In this approach, the expression of CD25 on the cell surface remained highly expressed in the cycling T cells and the presence of rIL-2 should maintain the T cell proliferation. As illustrated in Fig. 5, the cycling T cells in the presence of supplemented rIL-2 continue to proliferate as indicated by the incorporation of [3H]thymidine. In the presence of z-FA-FMK, the uptake of [3H]thymidine was inhibited in a dose-dependent manner. These results suggest that IL-2-driven T cell proliferation was inhibited by z-FA-FMK.

z-FA-FMK inhibits the IL-2 autocrine system in T lymphocytes

z-FA-FMK prevents T cells from entering and leaving the cell cycle

To further characterize the antiproliferative property of z-FA-FMK, we examined whether the cell cycle progression in these

Statistical analysis of the data

The experimental data were analyzed using one-way ANOVA followed by Dunnet’s test and the Mann-Whitney U test was used for analysis of the pneumococcal virulence studies.
cells was being blocked. The DNA content of human T lymphocytes costimulated with CD3 and CD28 Abs in the presence or absence of z-FA-FMK was labeled with PI and analyzed using flow cytometry. As illustrated in Fig. 6, after 72 h of costimulation with anti-CD3 and anti-CD28, a large number of the T cells have entered the cell cycle and were distributed in the S/G2/M phases. In the presence of 50 μM z-FA-FMK, the number of cells at G2/M was slightly increased, suggesting that cells are being prevented from leaving the cell cycle. At higher concentration of z-FA-FMK (100 μM), the number of T cells entering S/G2/M phase was markedly reduced. Our results suggest that z-FA-FMK at high concentration blocked the cells from entering the cell cycle, whereas lower concentration of z-FA-FMK may prevent cells from leaving the cell cycle.

**z-FA-FMK inhibits the nuclear translocation of NF-κB RelA (p65) in activated T lymphocytes**

A previous study has shown that z-FA-FMK blocks LPS-induced cytokine production in monocytes by interfering with NF-κB signaling (10). Because NF-κB is required for IL-2, IFN-γ, and CD25

**FIGURE 1.** z-FA-FMK inhibits T cell proliferation and blasts formation at nontoxic concentrations. T cell proliferation in PBMCs was stimulated with PHA, costimulated with anti-CD3 and anti-CD28, or with PMA and ionomycin in the presence or absence of various concentrations of z-FA-FMK. T cell proliferation was assayed using [3H]thymidine incorporation (A). B, The toxicity of z-FA-FMK was assessed after 24 h in resting T cells and after 72 h in activated T cells using PI uptake. C, Activated T cells undergoing cell division are measured using CFSE labeling, and T cell activation was determined by the formation of T cell blasts (D). The results are means ± SEM from five (A) or three (B and D) experiments. C, The results are one representative of three experiments. D, Region 1 (R1) consists of T cell blasts, whereas region 2 (R2) consists of mostly dead cells and debris. * Significantly decreased (p < 0.05) from control with mitogens alone.
gene transcription, IL-2 signaling as well as T lymphocyte activation (27), we examined the effect of z-FA-FMK on the signaling of this transcription factor. The nuclear translocation of Rel A (p65) in T lymphocytes activated through the Ag receptor was examined as reported previously using immunohistochemistry to localize p65 (23). Using this approach, we showed that the translocation of Rel A into the nuclei was detected in 65% of the T lymphocytes when costimulated with anti-CD3 and anti-CD28 for 2 h (Fig. 7), indicating that NF-κB signaling was activated (23). In the presence of z-FA-FMK (50 and 100 μM), there was a significant decrease in nuclear translocation of p65 in the stimulated T lymphocytes, suggesting that NF-κB signaling was suppressed by this peptidyl fluoromethylketone. Because NF-κB plays a pivotal role in gene transcription and T cell activation, its inhibition by z-FA-FMK could account for the decreased in cytokine (IL-2 and IFN-γ) production, CD25 up-regulation in the activated T cells, and the inhibition of IL-2 signaling during T cell proliferation.

z-FA-FMK inhibits caspase-8 and caspase-3 activation during T cell activation, but not during FasL-induced apoptosis

In recent years, a number of studies (12–16) have shown that various caspases were activated during T cell proliferation. However, to date, only caspase-8 has been shown to play an important role in T cell activation and proliferation (13, 16). Because z-FA-FMK has been shown previously to block caspases (11), we examined its effect on the activation of caspases during T cell activation through the Ag receptor. Primary T cells were costimulated with anti-CD3 and anti-CD28 in the presence of z-FA-FMK for 24 h.

As illustrated in Fig. 8, both caspase-8 and caspase-3 were activated to their respective subunits, p43/41 and p20 in T cells following 24-h stimulation, which confirmed previous reports (14, 15). In the presence of z-FA-FMK (50 and 100 μM), the processing of caspase-8 and caspase-3 to their subunits in activated T cells was completely abolished, whereas the caspase inhibitor, z-VAD-FMK, at 50 and 100 μM has no effect (Fig. 8). This suggests that

FIGURE 2. z-FA-FMK inhibits the production of IL-2 and IFN-γ in activated purified T cells. Purified CD4+ and CD8+ T cells were costimulated with anti-CD3 and anti-CD28 for 24 h in the absence or presence of z-FA-FMK. The amount of IL-2 (A) and IFN-γ (B) secreted into the culture supernatants were determined using ELISA as outlined in Materials and Methods. Results are the means ± SEM from three experiments. * Significantly reduced (p < 0.05) from control.

FIGURE 3. The inhibition of T cell proliferation induced by z-FA-FMK is not reversed by exogenous rIL-2. T cell proliferation was induced by costimulation with anti-CD3 and anti-CD28 in the presence or absence of z-FA-FMK (50 μM) for 72 h. rIL-2 (25 U/ml) was added to the cell cultures where indicated at the beginning of the experiments. The incorporation of [3H]thymidine was determined as in Materials and Methods. The results (inset) are the means ± SEM from triplicate samples from one representative experiment of four.
the inhibition of caspase-8 and caspase-3 processing by z-FA-FMK was not due to the blocking of caspase activity per se. To confirm that z-FA-FMK is not blocking caspase activity, we examined its effect on FasL-induced apoptosis in proliferating T cells, where caspases play a pivotal role in the apoptotic-signaling pathway (28). To this end, T cells that have been proliferating for 7 days were exposed to FasL, followed by cross-linking with anti-Flag. As shown in Fig. 9A, proliferating T cells readily undergo apoptosis when treated with FasL and was unaffected by z-FA-FMK (up to 100 \( \mu \)M). However, z-VAD-FMK at 50 \( \mu \)M effectively blocked FasL-induced apoptosis in these cells. Western blot analysis (Fig. 9B) revealed that some caspase-8 was processed in control proliferating T cells. However, as the cells undergo apoptosis following FasL treatment, there was a further increased in the activation and processing of caspase-8 as shown by the loss in the proenzyme and a concomitant increased in the cleaved p43/41 subunits above control levels. All these were abolished in the presence of z-VAD-FMK but not with z-FA-FMK. Interestingly, we could not detect any cleaved caspase-3 fragments in control proliferating T cells. This could be due to rapid degradation of the cleaved caspase-3 fragments in these proliferating cells because resting T cells activated for 24 h retained some of the cleaved caspase-3 fragments (Fig. 8). However, when the proliferating T cells were exposed to FasL, caspase-3 was readily processed to its p17 subunit. Similar to caspase-8, z-FA-FMK has no effect on the activation of caspase-3, although it did prevent the processing of the p20 fragments to the p17 subunits. In contrast, z-VAD-FMK completely blocked the processing of caspase-3 (Fig. 9B).

To further confirm the above findings, we examined the effect of z-FA-FMK on the activation of caspase-8 and caspase-3 during Fas-induced apoptosis using a different model system. As illustrated in Fig. 10A, Jurkat T cells readily undergo apoptosis when stimulated with agonistic anti-Fas for 6 h. Similar to FasL-induced cell death in proliferating T cells, z-VAD-FMK (50 and 100 \( \mu \)M) blocked Fas-induced apoptosis in Jurkat T cells. In contrast, the
presence of z-FA-FMK (50 and 100 μM) significantly increased Fas-induced apoptosis. Western blot analysis showed that both caspase-8 and caspase-3 were not processed in untreated Jurkat T cells, but were cleaved to its respective subunits, p41/43 and p17 during Fas-induced apoptosis (Fig. 10B). As expected, z-VAD-FMK completely blocked the activation of both caspase-8 and caspase-3. Surprisingly, z-FA-FMK, which is often used as a control for peptidyl fluoromethylketones, has a small inhibitory effect on the processing of caspase-8. The activation of caspase-3 during Fas-induced apoptosis was unaffected by z-FA-FMK in these cells, but there was a marked accumulation of the p17 subunits and its p20 precursors. This suggests that the degradation of these fragments may be inhibited by z-FA-FMK. With the accumulation of the cleaved caspase-3 fragments, the cleavage of PARP, a preferred substrate of caspase-3 was found to increase markedly in the presence of z-FA-FMK. This further confirmed that z-FA-FMK does not inhibit caspase-3 activity.

Effects of z-FA-FMK on the growth of pneumococci in blood and lung tissue of MF1 outbred mice

Our in vitro results demonstrated that z-FA-FMK have immunosuppressive effects on T cells. To determine whether this peptidyl fluoromethylketone has similar effects in vivo, we examined the effect of z-FA-FMK in a mouse model of pneumococcal disease. Previous studies had shown that CD4+ T cells played an important early role in the protective host response to pneumococcal infection, and mice deficient in CD4+ T cells were more susceptible to pneumococcal bronchopneumonia and septicemia, compared with their wild-type parent mice (20). Therefore, we proposed that if z-FA-FMK inhibits T cell function in vivo, mice administered with this peptidyl fluoromethylketone would have less protection...
During T cell proliferation, z-FA-FMK suppresses the activation of T cells. It inhibits the suppression of interleukin-2 (IL-2) and interferon-γ (IFN-γ) production, which is crucial for T cell proliferation induced by mitogens and IL-2 in vitro. The molecular mechanism underlying the inhibition of T cell proliferation mediated by z-FA-FMK involves pleiotropic effects, including the suppression of IL-2 and IFN-γ production, marked reduction in CD25 expression, inhibiting NF-κB signaling, impeding cells from entering and leaving the cell cycle and the inhibition of the activation of caspase-8 and caspase-3. When administered to mice infected with *S. pneumoniae* in vivo, z-FA-FMK was found to exacerbate the disease, as pneumococcal numbers significantly increased in both blood and lungs of infected animals, compared with controls. These results are supportive of previous publications, which demonstrate the key role T cells play in the host response to pneumococcal infection. Taken together, our results demonstrate that z-FA-FMK is capable of inducing immunosuppression in vivo.

It is well established that the NF-κB family of transcription factors play a central role in the coordination of a wide variety of genes that control immune responses. NF-κB complexes are normally localized in the cytoplasm, where they are bound to inhibitory proteins known as IκBs. In T lymphocytes, the predominant form of NF-κB complexes activated is a heterodimer of the p65 subunit associated with either p50 or p52 subunit, although c-Rel/p50 is also present. Following T cell activation, the inhibitory IκB proteins are rapidly phosphorylated and degraded via ubiquitination through the proteasome pathway. This release of NF-κB transcription factors and their translocation into the nuclei and, together with the activator protein 1, regulates the transcription of cytokine and immunoreceptor genes. We observed that a large number of primary T cells activated through the Ag receptor were stained positive for p65 in the nucleus, which is in good agreement with previous study. In the presence of z-FA-FMK, the nuclear translocation of p65 in the activated T cells was markedly suppressed, suggesting that NF-κB signaling induced by Ag receptor stimulation is blocked. This confirms a previous finding where z-FA-FMK inhibits the activation of NF-κB in LPS-stimulated macrophages. It also accounts for the inhibition of IL-2 and IFN-γ production and as well as the expression of CD25 because NF-κB-regulated gene transcription is known to be required for these processes. In the presence of z-FA-FMK, the nuclear translocation of p65 in the activated T cells was markedly suppressed, suggesting that NF-κB signaling induced by Ag receptor stimulation is blocked. This confirms a previous finding where z-FA-FMK inhibits the activation of NF-κB in LPS-stimulated macrophages (10). It also accounts for the inhibition of IL-2 and IFN-γ production and as well as the expression of CD25 because NF-κB-regulated gene transcription is known to be required for these processes (29, 30). However, CD66 expression was unaffected by z-FA-FMK because it is constitutively stored in intracellular pools and can be expressed in the absence of gene expression (26). In addition, Mortellaro et al. showed that IL-2 signaling also requires the activation of NF-κB, which explains the lack of cell proliferation in activated T cells driven by supplemented rIL-2 in the presence of z-FA-FMK. Besides blocking NF-κB signaling, z-FA-FMK also affect cell cycle progression by impeding cells from leaving and entering the cell cycle. This correlates with the CFSE studies where cell division was markedly inhibited by z-FA-FMK. It would be interesting to see whether z-FA-FMK have any effect on the cell cycle genes during T cell proliferation.

In recent years, a number of studies have shown that the caspases, besides promoting cell death via apoptosis also play an important role in T cell activation. A number of peptidyl fluoromethylketone caspase inhibitors, which efficiently blocked apoptosis through the inhibition of caspases have been shown to block T cell proliferation induced by mitogens (12, 14, 15). Although z-FA-FMK was often used as a control for fluoromethylketone caspase inhibitors in apoptosis studies (33–35), it was reported to block effector caspases during T cell apoptosis induced by retinoid-related molecules (11). We confirmed that both caspase-8 and caspase-3 were processed following T cell activation via the Ag receptors in primary T cells and z-FA-FMK completely inhibited the processing of both caspases. Surprisingly, the caspase inhibitor, z-VAD-FMK has virtually no effect on the processing of caspase-8 and caspase-3 during T cell activation, suggesting that the activation of these two enzymes is not caspase dependent. This is in contrast to Fas-induced apoptosis where both caspase-8 and
caspase-3 were effectively blocked by z-VAD-FMK. These results suggest that the inhibition of caspase-8 and caspase-3 processing by z-FMK in activated T cells was not due to the inhibition of caspase activity per se. In addition, z-FMK has no effect on caspase-8 and caspase-3 activation or their activity during Fas-induced apoptosis, which involves the sequential activation of these enzymes (36). These results suggest that the processing of caspase-8 and caspase-3 during T cell activation is regulated differently from Fas-induced apoptosis. It is possible that both caspase-8 and caspase-3 are cleaved by the same enzyme during T cell activation, because the processing of both caspases was inhibited by z-FMK and not z-VAD-FMK. Although granzyme B has been shown to cleave both caspase-8 and caspase-3 and initiate apoptosis in target cells during cytotoxic killing (37, 38), cell death triggered by granzyme B is not inhibited by z-FMK (39, 40).

Another protease that plays a role in the activation of caspases is cathepsin B from the lysosomes (41, 42), which has been shown to activate the proinflammatory caspase-11 in vitro (43). Although z-FMK was originally designed to block cathepsin B, z-VAD-FMK also blocks this cysteine protease activity and yet has no effect on the processing of caspase-8 and caspase-3 during T cell activation (44). This effectively rules out the involvement of cathepsin B in cleaving the caspases.

Although z-FMK has no effect on the activation of caspase-8 and caspase-3 during Fas-induced apoptosis, we consistently observed an accumulation of both cleaved caspase-3 p20 and p17 subunits in the Jurkat cells. The caspase-3 p20 fragment is an intermediate, formed during the activation of the proenzyme and undergoes autoprocessing to yield the p17 subunit (36). The accumulation of both fragments during Fas-induced apoptosis in the presence of z-FMK, suggests that the autoprocessing of p20 subunits may be suppressed. However, this is unlikely because only p20 will accumulate when autoprocessing is inhibited. It is more likely that z-FMK is blocking the degradation of both caspase-3 subunits (p20 and p17), presumably by inhibiting the ubiquitin-proteasome system. Similar accumulation of cleaved caspase-3 fragments have been reported previously with the proteosome inhibitor, lactacystin, where both caspase-3 activity and apoptosis were also increased (45). This is in good agreement with our results where PARP cleavage (caspase-3 activity) and apoptosis were increased in the presence of z-FMK.

The immunosuppressive effect of z-FMK was confirmed in vivo in an intranasal mouse model of pneumococcal infection. Previous studies have shown that mice, which are devoid of CD4+ T cells are significantly more susceptible to pneumococcal infection, with higher bacterial loads in both lungs and blood, compared with wild-type parents (20). In keeping with this, we have now shown that mice administered with z-FMK exhibited 40- to 50-fold higher pneumococcal numbers in both lungs and blood, compared with control mice, suggesting that the host T cell response to pneumococcal infection was significantly compromised by the peptidyl fluoromethylketone. Indeed, previous in vitro studies have shown that up-regulated CD25 expression in CD4+ T cells is a consequence of interactions with pneumococci (20). The immunosuppressive effect of z-FMK may account for the remarkable therapeutic effect in suppressing articular cartilage and bone destruction in chronic inflammatory arthritis in mice, where T cells play an indispensable role (2, 8, 9). In addition, z-FMK also was found to increase the mortality and parasitemia in mice infected with Trypanosoma cruzi (46). This parasitic disease affects a wide variety of cell types and tissues and cell-mediated immunity, particularly the CD8+ T cell plays an important role in the immune response to T. cruzi (47, 48). The increased in mortality induced by z-FMK treatment in mice infected with T. cruzi is likely to be mediated by its immunosuppressive properties.

In summary, we have shown that the caspase B inhibitor z-FMK is an immunosuppressive agent and inhibits pleiotropic processes involved in T cell activation and proliferation in vitro. When administered in vivo using a mouse model of pneumococcal disease where T cells play an important role in the protective host response to pneumococcal infection, z-FMK significantly increases pneumococcal loads in both lungs and blood. Taken together, our results demonstrated that z-FMK is immunosuppressive in vitro and in vivo.

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