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# A Broad-Spectrum Caspase Inhibitor Attenuates Allergic Airway Inflammation in Murine Asthma Model<sup>1</sup>

Akiko Iwata,<sup>2\*</sup> Kazumi Nishio<sup>†</sup> Robert K. Winn,<sup>\*</sup> Emil Y. Chi,<sup>‡</sup> William R. Henderson, Jr.,<sup>†</sup> and John M. Harlan<sup>†</sup>

Asthma is characterized by acute and chronic airway inflammation, and the severity of the airway hyperreactivity correlates with the degree of inflammation. Many of the features of lung inflammation observed in human asthma are reproduced in OVA-sensitized/challenged mice. T lymphocytes, particularly Th2 cells, are critically involved in the genesis of the allergic response to inhaled Ag. In addition to antiapoptotic effects, broad-spectrum caspase inhibitors inhibit T cell activation *in vitro*. We investigated the effect of the broad-spectrum caspase inhibitor, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), on airway inflammation in OVA-sensitized/challenged mice. OVA-sensitized mice treated with z-VAD-fmk immediately before allergen challenge showed marked reduction in inflammatory cell infiltration in the airways and pulmonary blood vessels, mucus production, and Th2 cytokine production. We hypothesized that the caspase inhibitor prevented T cell activation, resulting in the reduction of cytokine production and eosinophil infiltration. Treatment with z-VAD-fmk *in vivo* prevented subsequent T cell activation *ex vivo*. We propose that caspase inhibitors may offer a novel therapeutic approach to T cell-dependent inflammatory airway diseases. *The Journal of Immunology*, 2003, 170: 3386–3391.

Airway hyperreactivity has been attributed to chronic airway inflammation and the severity of this hyperreactivity correlates with the number of Th2 cytokine-producing T lymphocytes and eosinophils in the airways (1). Th2 cells secrete IL-4, IL-5, and IL-13, which promote IgE production and eosinophil development, release, and survival (2).

It is known that eosinophils and T cells express very late Ag (VLA-4;<sup>3</sup>  $\alpha 4\beta 1$ ; CD49d/CD29) on their surface and bind to VCAM-1 (CD106) on endothelium and fibronectin in matrix. VLA-4-dependent adhesion has been shown to make an important role for eosinophil and T cell infiltration to inflammatory sites (3–5). In a previous study, we reported that intranasal administration of an anti-VLA-4 mAb blocked inflammatory cell infiltration, Th2 cytokine release, and hyperresponsiveness to methacholine in a mouse asthma model (6). Since VLA-4 signaling transduces survival signals as well (7–9), we hypothesized that VLA-4 blockade might trigger T lymphocyte or eosinophil apoptosis and diminish airway inflammation.

To test this hypothesis, we sought to prevent inflammatory cell apoptosis by administration of a broad-spectrum caspase inhibitor along with anti-VLA-4 mAb, reasoning that the caspase inhibitor would abrogate the inhibitory effect of VLA-4 blockade. We used

*N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), which prevents apoptosis in various animal models such as ischemia-reperfusion injury (10), sepsis (11), hepatitis (12), and fibrosis (13). Of great surprise, we found that z-VAD-fmk administration by i.v. injection before VLA-4 mAb treatment led to a further reduction in leukocyte infiltration. Most notably, pretreatment with the caspase inhibitor alone immediately before allergen challenge markedly attenuated eosinophil accumulation, mucus production, and Th2 cytokine release. This result was counterintuitive, since inhibition of apoptosis should exacerbate inflammation by preventing apoptosis of effector cells. Therefore, we investigated whether caspase inhibition (i.e., treatment with z-VAD-fmk) was directly anti-inflammatory, apart from modulation of apoptosis. We found that treatment of nonsensitized mice with i.v. z-VAD-fmk significantly inhibited subsequent *ex vivo* T lymphocyte activation induced by CD3 cross-linking. In summary, we report that allergen-induced airway inflammation in OVA-sensitized/challenged mice was reduced by the broad-spectrum caspase inhibitor z-VAD-fmk, consistent with an important role of caspases in T cell activation.

## Materials and Methods

### Animals

BALB/c mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). All animal use procedures were approved by the University of Washington Animal Care Committee (Seattle, WA).

### Allergen sensitization and challenge

Mice were sensitized and later challenged with OVA (Pierce, Rockford, IL) as previously described (14). Mice were immunized with OVA (100  $\mu$ g) complexed with aluminum potassium sulfate in a 0.2-ml volume, administered by i.p. injection on day 0. On days 8, 15, 18, and 21, mice were anesthetized with 0.2 ml of ketamine (10 mg/ml) and xylazine (1 mg/ml) diluted in 0.9% saline. Mice received 250  $\mu$ g of OVA by intratracheal (i.t.) administration. Intratracheal challenges were performed as previously described (15, 16). Mice were anesthetized by i.p. injection of a 0.2 ml of a mixture of ketamine and xylazine (10 and 1 mg/ml, respectively) in normal saline and were placed on board in the supine position. Two hundred fifty micrograms (100  $\mu$ l of a 2.5 mg/ml) of OVA (on day 8) and 125  $\mu$ g (50  $\mu$ l of 2.5 mg/ml) of OVA (on days 15, 18, and 21) were placed on the back

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<sup>3</sup> Abbreviations used in this paper: VLA-4, very late Ag-4; z-VAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; y-VAD-fmk, Z-Tyr-Val-Ala-As(OMe)-fluoromethyl ketone; z-FF-fmk, Z-Phe-Phe-fluoromethyl ketone; P<sub>enh</sub>, enhanced pause; BAL, bronchoalveolar lavage; i.t., intratracheal; FADD, Fas-associated death domain.

of the tongue of each animal. The control group received normal saline with aluminum potassium sulfate by i.p. route on day 0 and 0.05 ml of 0.9% saline by i.t. route on days 8, 15, 18, and 21.

### Ab treatment

Mice were given 2  $\mu\text{g/g}$  weight of blocking CD49d mAb dose (BD PharMingen, San Diego, CA) by the i.t. route at 30 min before OVA challenge on days 15 and 21 (6). For control mice, CD16/CD32 mAb (BD PharMingen) was given.

### Administration of z-VAD-fmk

z-VAD-fmk (Bachem California, Torrance, CA) was dissolved in DMSO and diluted in 0.9% saline. Mice were given z-VAD-fmk at 1 or 5  $\mu\text{g/g}$  weight in 0.3 ml by tail vein injection at 60 min before OVA challenge on days 18 and 21. The z-VAD-fmk dose was determined from the report (11) that 1.0 and 10  $\mu\text{g/g}$  of body weight in mice significantly decreased sepsis-induced apoptosis of T lymphocytes almost equally. Z-Tyr-Val-Ala-As(Ome)-fluoromethylketone (y-VAD-fmk; Enzyme Systems Products, Livermore, CA) and Z-Phe-Phe-fluoromethylketone (z-FF-fmk; Enzyme Systems Products) were dissolved and given in the same manner as z-VAD-fmk. Control mice were given DMSO at 0.1  $\mu\text{l/g}$  weight in 0.3 ml of saline. In previous studies, in this mouse asthma model, we did not observe any direct anti-inflammatory effects of DMSO on allergen-induced airway inflammation (17) or on cell-based thoredoxin activation on nitrosylation of p65 in A549 cells stimulated with LPS (18).

### Pulmonary function testing

*In vivo* airway responsiveness to methacholine was measured 24 h after the last OVA challenge in conscious, freely moving, spontaneously breathing mice using whole-body plethysmography (model PLY 3211; Buxco Electronics, Sharon, CT) as previously described (16, 19). Mice were challenged with aerosolized saline or increasing doses of methacholine (5 and 20 mg/ml) generated by an ultrasonic nebulizer (DeVilbiss Health Care, Somerset, PA) for 2 min. The degree of bronchoconstriction was expressed as enhanced pause ( $P_{\text{enh}}$ ), a calculated dimensionless value, which correlates with the measurement of airway resistance, impedance, and intrapleural pressure in the same mouse.  $P_{\text{enh}}$  readings were taken and averaged for 4 min after each nebulization challenge.  $P_{\text{enh}}$  was calculated as follows:  $P_{\text{enh}} = [(T_e/T_r - 1) \times (\text{PEF}/\text{PIF})]$ , where  $T_e$  is expiration time,  $T_r$  is relaxation time, PEF is peak expiratory flow, and PIF is peak inspiratory flow  $\times$  0.67 coefficient. The time for the box pressure to change from a maximum to a user-defined percentage of the maximum represents the relaxation time. The  $T_r$  measurement begins at the maximum box pressure and ends at 40%.

### Bronchoalveolar lavage (BAL)

On day 22, after measurement of airway hyperreactivity, the mice were sacrificed by exsanguination by cardiac puncture and the left lung was isolated by tying off the left main stem bronchus. The right lung was lavaged with one wash of 1 ml of saline and then additional lavage of 1 ml in and out five times for a total of 2 ml. The total number of leukocytes per 0.05-ml aliquot was determined after methylene blue nuclear staining. The remaining BAL fluid was centrifuged at  $200 \times g$  for 10 min at  $4^\circ\text{C}$  and supernatants were stored at  $-80^\circ\text{C}$  until assay of cytokine protein levels. The cell pellets were resuspended in saline containing 10% BSA and smears were made on glass slides. Eosinophils were determined by Diff-Quick staining (Dade Behring, Newark, DE).

### Lung histology

The left lung tissue was fixed in Carnoy's solution at  $20^\circ\text{C}$  for 15 h. The tissues were embedded in paraffin and cut into 5- $\mu\text{m}$  sections. A minimum of 10 fields was randomly examined by light microscopy by a blinded observer. The intensity of the cellular infiltration around pulmonary blood vessels and airways was assessed on a semiquantitative scale ranging from 0 to 4+ (20). Airway mucus (i.e., mucin and sulfated mucosubstances) was identified after staining with methylene blue, H&E, and Alcian blue as previously described (17). Occlusion of the airway diameter by mucus was assessed on a semiquantitative scale ranging from 0 to 4+. Each airway section was assigned a score for airway diameter occlusion by mucus based on the following criteria: 0, 0–10% occlusion; 1, 10–30% occlusion; 2, 30–60% occlusion; 3, 60–90% occlusion; and 4, 90–100% occlusion (17). Airway edema was assessed on a 0–4+ scale (16). An investigator blinded to the protocol design performed the morphometric analysis.

### Assay of T lymphocyte activation *ex vivo*

Two hours after z-VAD-fmk or DMSO administration, spleens were aseptically removed and mechanically dissociated in cold PBS, followed by depletion of erythrocytes with lysis buffer containing  $\text{NH}_4\text{Cl}$ . Splenocytes were suspended at a concentration of  $1 \times 10^7$  cells/ml in RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu\text{g/ml}$  streptomycin, 10 mM HEPES (Life Technologies), and 20  $\mu\text{M}$  2-ME (Sigma-Aldrich, St. Louis, MO).

Splenocytes were incubated at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  atmosphere and stimulated for 48 h in anti-mouse CD3 T cell activation 96-multiwell plates (BD Biocoat; BD PharMingen). Briefly,  $5 \times 10^5$  cells/200  $\mu\text{l}$  were seeded to each well. After 48 h of incubation, cell-free culture supernatants were collected and stored at  $-70^\circ\text{C}$  until cytokine analyses were performed.

### ELISA for Th2 cytokines

IL-4 and IL-5 protein levels in BAL fluid and stimulated splenocytes medium were assayed using BD PharMingen OptEIA assays according to the manufacturer's protocol. The OD were read on a microplate reader (EL 340; Bio-Tek Instruments, Winooski, VT) at  $\lambda = 510$  nm. Cytokine levels were determined by comparison with standards.

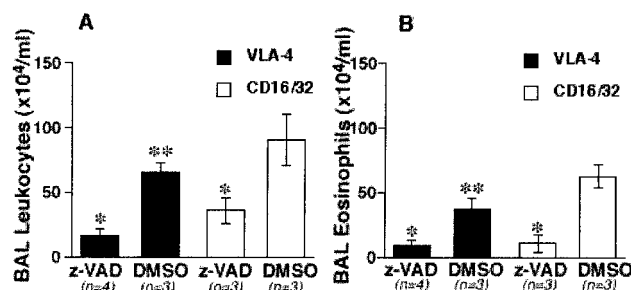
### Statistical analysis

Results are reported as the means  $\pm$  SE of the combined experiments. Differences were analyzed for significance ( $p < 0.05$ ) by Student's two-tailed  $t$  test for independent means. Differences in pulmonary function data were analyzed by linear regression followed by the Fisher's protected least-significant difference test. Statistical analyses were performed using Statview 4.1 and Super ANOVA software (Abacus Concepts, Berkeley, CA).

## Results

### z-VAD-fmk inhibits allergen-induced leukocyte infiltration

Mice were sensitized with OVA i.p. on day 0 and challenged on days 8, 15, 18, and 21 by the i.t. route. Mice treated with DMSO and control mAb at days 18 and 21 before OVA challenge showed a marked infiltration of total leukocytes and eosinophils into BAL fluid 24 h after the last i.t. challenge (Fig. 1). Treatment with anti-VLA-4 mAb reduced eosinophil infiltration in BAL fluid by 40% compared with CD16/CD32 ( $p = 0.043$ ). z-VAD-fmk administration (5  $\mu\text{g/g}$  weight) 30 min before anti-VLA-4 mAb treatment further inhibited eosinophil infiltration, with a 74% reduction in BAL fluid eosinophils compared with mice treated with DMSO and anti-VLA-4 mAb ( $p = 0.045$ , by Student's two-tailed  $t$  test). Mice treated with z-VAD-fmk and control mAb had an 82% reduction in eosinophils compared with animals treated with DMSO and control mAb ( $p = 0.003$ ).



**FIGURE 1.** Anti-VLA-4 mAb and z-VAD-fmk reduce inflammatory cell infiltration in BAL fluid of OVA-sensitized/challenged mice. BAL fluid was obtained from mice treated with z-VAD-fmk (5  $\mu\text{g/g}$  weight) or DMSO (0.1  $\mu\text{l/g}$  weight) by i.v. administration at 1 h before OVA challenge on days 18 and 21, followed 30 min later by i.t. administration of anti-VLA-4 mAb or control CD16/CD32 mAb (2  $\mu\text{g/g}$  weight). Total leukocytes (A) and eosinophils (B) per milliliter of lavage fluid are shown (mean  $\pm$  SEM). \*,  $p < 0.05$  vs DMSO in anti-VLA-4 or anti-CD16/CD32 mAb treated-groups and \*\*,  $p < 0.05$  vs CD16/CD32 mAb-treated control mice using Student's two-tailed  $t$  test.



In additional experiments, z-VAD-fmk was administered i.v. at 1 or 5  $\mu\text{g/g}$  weight on days 18 and 21 at 1 h before the OVA challenge, and total leukocyte and eosinophil cell counts were determined in BAL fluid (Fig. 2). On day 22, 24 h after the last OVA challenge, total leukocytes and eosinophils were reduced by 62 and 65%, respectively, in mice receiving z-VAD-fmk at 5  $\mu\text{g/g}$  compared with the control group treated with DMSO ( $p < 0.001$ ). z-VAD-fmk at 1  $\mu\text{g/g}$  weight also reduced leukocyte infiltration (total leukocytes and eosinophils in z-VAD-fmk vs DMSO groups;  $p = 0.02$  and  $p = 0.007$  by Student's two-tailed  $t$  test, respectively).

#### IL-4 and IL-5 levels in BAL fluid

Th2 cytokine levels in BAL fluid were measured by ELISA (Fig. 3). IL-4 and IL-5 levels in lavage fluid were reduced in z-VAD-fmk-treated mice vs DMSO-treated animals by 57.0% ( $p = 0.001$ , by Student's two-tailed  $t$  test) and 65.0% ( $p = 0.013$ ), respectively.

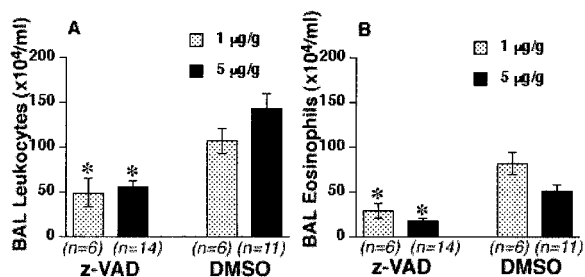
#### Histology

Twenty-four hours after the final i.t. OVA challenge, BAL was performed on the right lung and left lung tissue was obtained to assess the effect of z-VAD-fmk on airway inflammation histologically. Mice treated with z-VAD-fmk at 5  $\mu\text{g/g}$  weight, 1 h before OVA challenge on days 18 and 21, showed a dramatic reduction of both inflammatory cell infiltration of the airway parenchyma and surrounding blood vessels and airway mucus production (Fig. 4A). In contrast, an intensive infiltration of eosinophils and other leukocytes in the lung parenchyma and blood vessels was observed in OVA-sensitized/challenged, DMSO-treated mice (Fig. 4B).

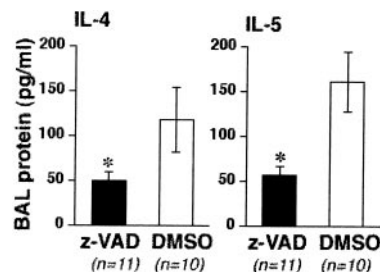
The number of infiltrated cells in the airways and blood vessels, mucus occlusion of airway diameter, edema, Alcian blue-positive mucus cells in the airway epithelium, and eosinophils per unit area ( $2200 \mu\text{m}^2$ ) were determined by morphometric analysis (Fig. 5). z-VAD-fmk treatment significantly reduced the total inflammatory cell and eosinophil infiltration into the airways and perivascular area of pulmonary blood vessels by 69% ( $p < 0.001$ , by Student's two-tailed  $t$  test) and 75% ( $p < 0.001$ ), respectively (Fig. 5, A and B). Mucus occlusion of the airway diameter and the number of mucus-secreting cells in airway epithelia were reduced by z-VAD-fmk treatment (Fig. 5, C and D). The degree of edema was also attenuated by z-VAD-fmk (Fig. 5E).

#### Comparison of z-VAD-fmk with other fluoromethylketone inhibitors

To control for potential nonspecific effects of the fluoromethylketone moiety, we tested z-FF-fmk, which is a cathepsin-B and -L



**FIGURE 2.** z-VAD-fmk reduces inflammatory cell infiltration in BAL fluid of OVA-sensitized/challenged mice. BAL fluid was obtained from OVA-sensitized mice treated with z-VAD-fmk (5  $\mu\text{g/g}$  weight) or DMSO (0.1  $\mu\text{l/g}$  weight) by i.v. administration at 1 h before OVA challenge on days 18 and 21. The number (mean  $\pm$  SEM) of leukocytes (A) or eosinophils (B) per milliliter of lavage fluid was determined. \*,  $p < 0.05$  vs DMSO in 1  $\mu\text{g/g}$  (□) or 5  $\mu\text{g/g}$  (■) groups using Student's two-tailed  $t$  test.

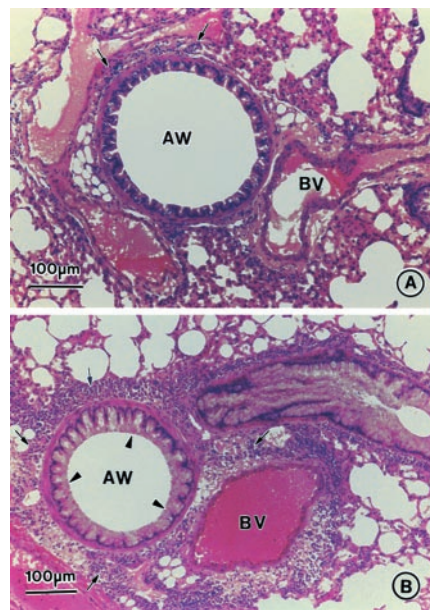


**FIGURE 3.** z-VAD-fmk reduces Th2 cytokine protein levels in BAL fluid of OVA-sensitized/challenged mice. IL-4 and IL-5 protein levels in BAL fluid from OVA-sensitized mice treated with z-VAD-fmk (5  $\mu\text{g/g}$  weight) or DMSO (0.1  $\mu\text{l/g}$  weight) by i.v. administration at 1 h before OVA challenge on days 18 and 21. \*,  $p < 0.05$  vs DMSO using Student's two-tailed  $t$  test.

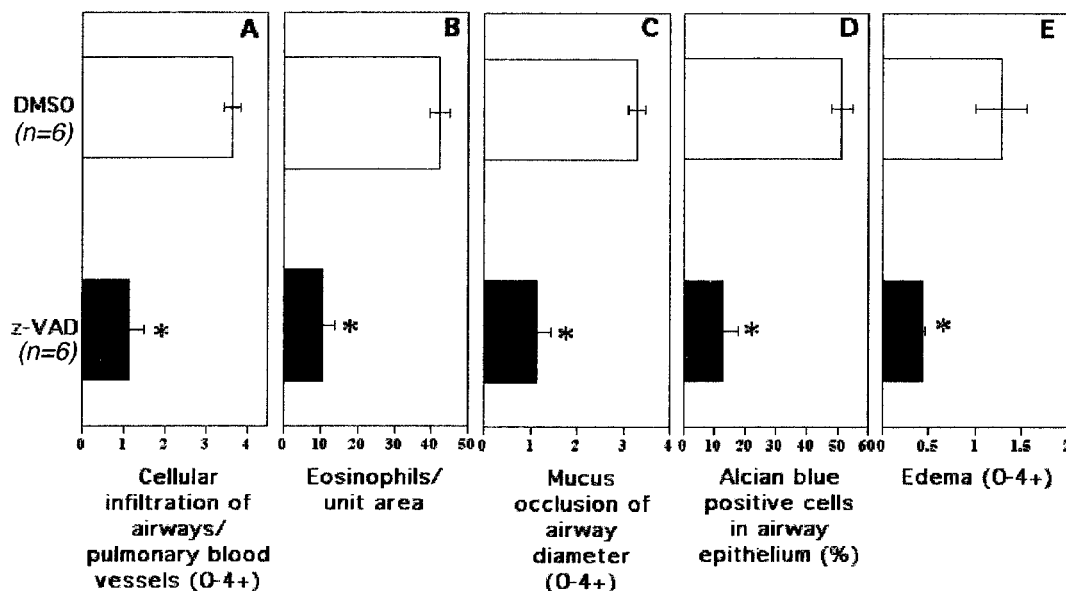
inhibitor. We also examined y-VAD-fmk, a more selective caspase inhibitor, which is directed primarily at caspase-1 and caspase-4. In comparison to z-VAD-fmk, y-VAD-fmk and z-FF-fmk did not reduce leukocyte infiltration in BAL fluid (Fig. 6) or improve methacholine-induced airway hyperreactivity (Fig. 7). y-VAD-fmk reduced the level of IL-4, but not IL-5, protein in BAL fluid, whereas z-FF-fmk was without any effect on Th2 cytokine protein levels (Fig. 6).

#### Effect of z-VAD-fmk on OVA-induced airway hyperreactivity to methacholine

Airway hyperreactivity to aerosolized methacholine was determined by noninvasive *in vivo* plethysmography 24 h following the



**FIGURE 4.** z-VAD-fmk reduces airway inflammation in OVA-sensitized/challenged mice. Lung tissue was obtained from mice treated with z-VAD-fmk or DMSO. The tissue was stained with H&E and examined by light microscopy. A, OVA-sensitized mice receiving z-VAD-fmk (5  $\mu\text{g/g}$  weight) by i.v. administration at 1 h before OVA challenge on days 18 and 21. z-VAD-fmk treatment showed a remarkable reduction of inflammatory cell infiltration of the airway parenchyma (arrows) and the surrounding of the blood vessels (BV) and of airway (AW) mucus release. Bar, 100  $\mu\text{m}$ . B, OVA-sensitized mice receiving DMSO (0.1  $\mu\text{l/g}$  weight) by i.v. administration at 1 h before OVA challenge on days 18 and 21. Airway (AW) mucus plugging is observed (arrowheads). An intensive leukocyte infiltration of the lung parenchyma (arrows) and blood vessels (BV) by eosinophils and other inflammatory cells is evident. Bar, 100  $\mu\text{m}$ .



**FIGURE 5.** z-VAD-fmk attenuates cellular infiltration of airways and pulmonary blood vessels, mucus occlusion of airways, and edema in OVA-sensitized/challenged mice. Lung tissue was obtained from mice receiving z-VAD-fmk (5  $\mu$ g/g weight) or DMSO (0.1  $\mu$ l/g weight) by i.v. administration at 1 h before OVA challenge on days 18 and 21. The infiltration by eosinophils and other inflammatory cells around airways and pulmonary blood vessels (A), the number of eosinophils per unit area (2200  $\mu$ m<sup>2</sup>; B), mucus occlusion of airway diameter by mucus (0–4+ scale; C), percentage of airway cells positives for mucus glycoproteins by Alcian blue staining (D), and degree of edema (scale 0–4+) were determined by morphometric analysis as described in *Materials and Methods*. \*,  $p < 0.01$  vs DMSO by Student's two-tailed  $t$  test.

last i.t. challenge with OVA. Airway hyperreactivity was observed in the OVA-treated mice after challenge with methacholine at 5, 10, and 25 mg/ml, with significant increases in  $P_{enh}$  (percentage of air). By regression analysis, z-VAD-fmk reduced airway hyperreactivity in OVA-treated mice compared with z-FF-fmk and y-VAD-fmk ( $p < 0.05$ ), although there was no statistical difference between z-VAD-fmk and DMSO (Fig. 7).

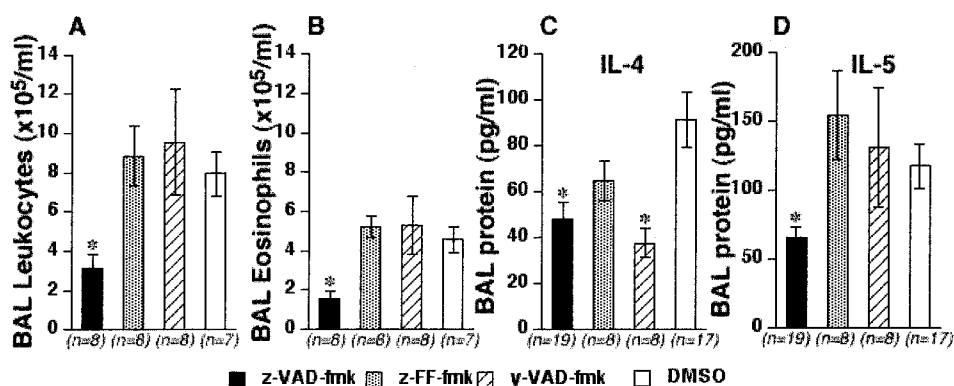
#### Effect of z-VAD-fmk on T lymphocyte activation *ex vivo*

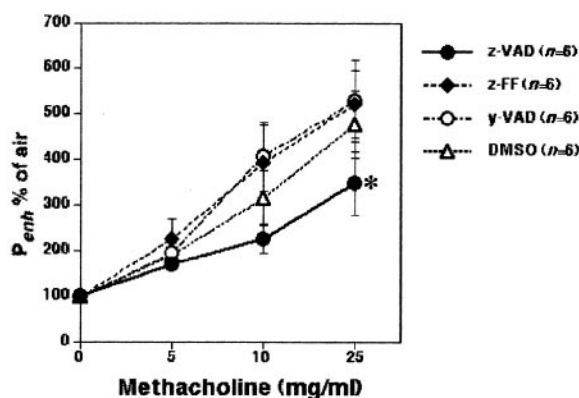
To investigate the potential mechanism(s) of action of z-VAD-fmk, we examined isolated splenocytes *ex vivo*. Mice were given z-VAD-fmk by i.v. injection at 2 h before sacrifice and splenocytes were isolated. T lymphocytes in the mixture of splenocytes were activated by incubating with anti-CD3 for 48 h, and cytokine levels were then measured in supernatant medium (Fig. 8). Levels of the Th2 cytokines IL-4 and IL-5, which were released into supernatant medium, were reduced in splenocytes obtained from z-VAD-fmk-treated animals vs DMSO-treated animals by 45.2% ( $p = 0.049$ , by Student's two-tailed  $t$  test) and 59.5% ( $p = 0.001$ ), respectively.

#### Discussion

We anticipated that inhibition of apoptosis by administration of a broad-spectrum caspase inhibitor would exacerbate allergen-induced lung inflammation by prolonging survival of T lymphocytes or eosinophils. Surprisingly, systemic injection of the pan-caspase inhibitor z-VAD-fmk immediately before OVA challenge reduced inflammatory cell accumulation, mucus hypersecretion, and Th2 cytokine release in OVA-sensitized/challenged mice. The effects of the caspase inhibitor in this model are likely due to its anti-inflammatory activities, rather than inhibition of apoptosis. As a broad-spectrum caspase inhibitor, z-VAD-fmk may inhibit the processing of pro-IL-1 and pro-IL-18 to the mature cytokines by caspase-1 (21). IL-1 $\beta$  activates NF- $\kappa$ B, which, in turn, induces inflammatory gene expression and leads to eosinophil recruitment (22, 23). In allergic lung responses IL-18 has been reported to enhance eosinophil infiltration, accompanied by an elevation of Th2 cytokine expression in the lungs (24–26). Caspase-3 is involved in the processing of pro-IL-16 to the mature cytokine (27)

**FIGURE 6.** z-VAD-fmk but not z-FF-fmk or y-VAD-fmk reduces leukocyte accumulation and Th2 cytokine production in OVA-sensitized/challenged mice. z-VAD-fmk but not z-FF-fmk or y-VAD-fmk reduces infiltration of leukocytes (A) and eosinophils (B) in BAL from OVA-sensitized/challenged mice. \*,  $p < 0.05$  vs z-FF-fmk, y-VAD-fmk, and DMSO by Student's two-tailed  $t$  test. y-VAD-fmk reduced IL-4 (B) but not IL-5 (C) protein level in BAL, whereas z-FF-fmk was without any effect on Th2 cytokine protein levels. \*,  $p < 0.05$  vs DMSO by Student's two-tailed  $t$  test.

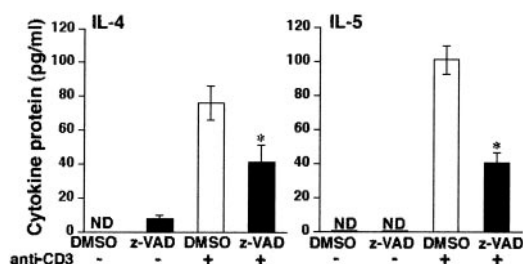




**FIGURE 7.** Effect of z-VAD-fmk on airway hyperreactivity to methacholine in OVA-sensitized/challenged mice. The degree of bronchoconstriction expressed as  $P_{enh}$  (percentage of air) to aerosolized methacholine (5, 10, and 25 mg/ml) was determined in OVA-sensitized/challenged mice treated with z-VAD-fmk, z-FF-fmk, y-VAD-fmk (at 5  $\mu$ g/g weight), and DMSO (0.1  $\mu$ l/g weight) by i.v. administration at 1 h before OVA challenge on days 18 and 21. \*,  $p < 0.05$  vs z-FF-fmk and y-VAD-fmk by linear regression followed by Fisher's protected least-significant difference test.

and IL-16 has been implicated in allergen-induced airway hyperreactivity and up-regulation of IgE (28). It is possible that these effects of caspases on cytokine processing and secretion contribute to the anti-inflammatory effects of z-VAD-fmk in the OVA-sensitized/challenged mice. In addition to cytokine production and apoptosis, caspases play a role in cell differentiation. Treatment with z-VAD-fmk blocked terminal differentiation of lens epithelial cells (29) and keratinocytes (30), the differentiation of monocytes into macrophages (31), and the differentiation of erythroid progenitors (32). However, it is unlikely that modulation of cell differentiation contributes to the inhibitory effects of z-VAD-fmk in this acute model.

The most likely target of the caspase inhibitor in the OVA-sensitized/challenged mice is the T lymphocyte. CD4 T lymphocytes play a critical role in the response to Ag challenge (33), and z-VAD-fmk treatment markedly reduced production of the Th2 cytokines IL-4 and IL-5. Attenuated T cell activation with decreased elaboration of these cytokines could account for reduced cellular infiltration and mucous production (34). This possibility was supported by studies in which we investigated the effect of z-VAD-fmk administered *in vivo* on cultured splenocytes stimulated *ex vivo*. IL-4 and IL-5 production, induced by cross-linking of CD3 on T lymphocytes, was significantly reduced in spleno-



**FIGURE 8.** Treatment with z-VAD-fmk *in vivo* reduces subsequent Th2 cytokine release from CD3-stimulated T lymphocytes *ex vivo*. Mice were given z-VAD-fmk (5  $\mu$ g/g weight) by i.v. injection at 2 h before sacrifice. Splenocytes were isolated and then activated by incubating with anti-CD3 for 48 h. IL-4 and IL-5 protein levels in supernatant medium were measured. \*,  $p < 0.01$  vs DMSO by Student's two-tailed  $t$  test ( $n = 4$ ). ND, Not detected.

cytes isolated from mice treated with z-VAD-fmk 2 h before. This result is consistent with z-VAD-fmk preventing T cell activation by inhibiting caspase activity. Furthermore, since any free z-VAD-fmk was removed when splenocytes were isolated and since z-VAD-fmk binds to active caspases not to procaspases (35), the inhibitory effect on subsequent CD3-triggered T cell activation *ex vivo* suggests that its target was an active caspase (or other cysteine protease) in resting T cells.

Caspases are involved in activation of several effector cell functions of T lymphocytes. In Jurkat cells, caspase-mediated cleavage of calcineurin contributed to IL-2 production during T cell activation by PHA (36). Notably, Kennedy et al. (37) showed that CD3 activation of T cells led to processing of caspase-8 but not caspase-3 and that z-VAD-fmk blocked CD3-induced T cell proliferation and IL-2 production. Similarly, Alam et al. (38) reported that z-VAD-fmk blocked proliferation, MHC class II expression, and blastic transformation during stimulation of PBL. Finally, Fas-associated death domain (FADD) is required for recruitment of caspase-8 and activation of downstream caspase-3. T lymphocytes from FADD-deficient mice (39) and from transgenic mice overexpressing a FADD-dominant negative (40) exhibited a defect in proliferation to mitogens.

Our study shows that the broad-spectrum caspase inhibitor z-VAD-fmk reduced cell infiltration in BAL and airway hyperresponsiveness in OVA-sensitized/challenged mice, but two other fluoromethylketone inhibitors, y-VAD-fmk and z-FF-fmk, did not show inhibition at equivalent dosing. Although y-VAD-fmk is a more selective caspase-1 and caspase-4 inhibitor and z-FF-fmk is a cathepsin-B and -L inhibitor, the *in vivo* results do not determine which specific caspase(s) (or cysteine protease(s)) is involved. Further studies are required to determine how caspases are activated in T cells without inducing apoptosis and the mechanism by which they regulate T cell effector functions.

By regression analysis, there was a significant difference between z-VAD and two other fluoromethylketone inhibitors in terms of an inhibitory effect on methacholine-induced airway responsiveness. However, there was no statistical difference between z-VAD and DMSO. Previously, we observed that reagents (i.e., leukotriene synthesis inhibitors, soluble IL-4R and CD49 mAb administered i.p.), which prevent eosinophil infiltration into the lungs, did not always prevent airway hyperreactivity to methacholine in OVA-sensitized/challenged mice (6, 17, 20). Thus, caspase inhibitors might be also a class of drugs that prevent eosinophil infiltration into lung but do not prevent airway hyperreactivity. In summary, the broad-spectrum caspase inhibitor z-VAD-fmk attenuated allergen-induced airway inflammation and hyperreactivity. Treatment with z-VAD-fmk *in vivo* also prevented subsequent T cell activation *ex vivo*. Together, these results suggest that caspase inhibitor treatment prevents T cell activation, resulting in reduction of Th2 cytokine production, and inflammatory cell infiltration. Although further investigation is needed to elucidate the detailed mechanisms of action, caspase inhibitors may represent a new class of drugs to treat allergic airway inflammation.

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