Colutellin A, an immunosuppressive peptide from Colletotrichum dematium

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Colletotrichum dematium is an endophytic fungus recovered from a Pteromischum sp. growing in a tropical forest in Costa Rica. This fungus makes a novel peptide antimycotic, colutellin A, with a MIC of 3.6 μg ml⁻¹ (48 h) against Botrytis cinerea and Sclerotinia sclerotiorum. Collutellin A has a mass of 1127.7 Da and contains residues of Ile, Val, Ser, N-methyl-Val and β-aminoisobutyric acid in nominal molar ratios of 3 : 2 : 1 : 1 : 1, respectively. Independent lines of evidence suggest that the peptide is cyclic and sequences of Val-Ile-Ser-Ile and Ile-Pro-Val have been deduced by MS/MS as well as Edman degradation methods. Collutellin A inhibited CD4⁺ T-cell activation of interleukin 2 (IL-2) production with an IC₅₀ of 167.3 ± 0.38 nM, whereas cyclosporin A in the same test yielded a value of 61.8 nM. Inhibition of IL-2 production by collutellin A at such a low concentration indicates the potential immunosuppressive activity of this compound. In repeated experiments, cyclosporin A at or above 8 μg ml⁻¹ exhibited high levels of cytotoxicity on human peripheral blood mononuclear cells, whereas collutellin A or DMSO (carrier) alone, after 24 and 48 h of culture, exhibited no toxicity. Because of these properties collutellin A has potential as a novel immunosuppressive drug.

INTRODUCTION

Endophytic microbes represent a relatively untouched reservoir of biologically active compounds that have potential uses in medicine and agriculture (Strobel et al., 2004). Some of these organisms make such notable compounds as the anticancer drug taxol, and a plethora of antibiotics, antioxidants and immunosuppressants. Each tropical rainforest seems to have its own unique suite of endophytes, which may be related to or dependent upon the composition of the flora they inhabit (Strobel & Daisy, 2003).

A collection of plant materials obtained from a rainforest in Costa Rica yielded a plethora of fungal endophytes. Colletotrichum dematium, an endophyte of Pteromischum sp., was of interest since it was strongly inhibitory to Botrytis cinerea, a common pathogen on the leaves and flowers of many plant species. This inhibitory activity was used as a guide for isolating a peptide antimycotic. Because this compound seemed to have certain chemical and biological similarities to cyclosporin A, an important immunosuppressive drug (Ruegger et al., 1976), it was studied in matched biological experiments with cyclosporin.

Since the discovery of cyclosporin A from Trichoderma polysporum in 1976, it has been the principal immunosuppressive...
agent used in medicine (Ruegger et al., 1976). Presently, cyclosporin A, along with tacrolimus (FK506) and sirolimus (rapamycin), are three immunosuppressants which act on CD4+ T-cells used in clinical practice. These compounds have gained widespread acceptance for use in organ and tissue transplantation, various autoimmune diseases and with some other non-autoimmune inflammatory diseases. Generally, all three drugs can cause nephrotoxicity (Daoud et al., 2007). In addition, cyclosporin A and tacrolimus can also cause neurotoxicity and beta-cell toxicity (Tanabe, 2003; Froud et al., 2006). Finally, cyclosporin A can cause more serious nephrotoxicity, hypertension and hyperlipidaemia in comparison to tacrolimus (Andoh et al., 1996). Novel compounds with low toxicity that act in an effective and useful manner will contribute to our arsenal of substances that act to suppress the immune system and will be especially helpful to those with autoimmune diseases and organ recipients.

This report describes the isolation and identification of a C. dematium strain from Pterosichum sp. growing in a tropical forest in Costa Rica. This fungus is an endophyte that was identified on the basis of its morphological and genetic characteristics. Extracts of the fermentation broth of C. dematium possess selective antifungal activity associated with a novel antifungal peptide that we have termed collutellin A. A description of the isolation and partial characterization of collutellin A is presented, along with the results of experiments comparing its antifungal and immunosuppressive activities with those of cyclosporin A, and their associated cellular toxicities. Collutellin A showed a relatively strong ability to inhibit the production of interleukin 2 (IL-2) from activated CD4+ T-cells, suggesting that it may have potential as a novel immunosuppressive drug. Surprisingly and importantly, in contrast to cyclosporin A, it possessed little or no cytotoxicity to human blood cells.

**METHODS**

**Isolating, culturing, identifying and storing of Colletotrichum dematium.** The culture of C. dematium used in this study was obtained as an endophyte from a small cutting taken from an immature *Pterosichum* sp. plant collected in a Caribbean coastal Costa Rican rainforest. A number of other plant samples, including *Dipteryx sp., Monstera sp., Cercopia* sp. and others were collected at the same time and in the same area. Endophytes were recovered from each plant sample in the collection using the standard methods of surface treatment, tissue removal and plating on water agar (Strobel & Daisy 2003). One fungus, designated CR-12, was recovered from *Pterosichum* sp. and when grown on potato dextrose agar (PDA) was initially shown to have antifungal activity by virtue of a bioassay test (Castillo et al., 2007). The organism was examined for its morphological and spore-forming features as described below for taxonomic purposes. In addition, molecular biological studies were performed on this fungus. It was grown on PD broth for 7 days and the mycelium was harvested and the DNA was extracted using the DNeasy Plant and Fungi Mini kit (Qiagen) according to the manufacturer’s directions. The ITS regions of the fungus were amplified using PCR and the universal ITS primers ITS1 (5’-TCC GTA GGT AAG CCG G-3’) and ITS4 (5’-TGC GGC TGC GAT CTA TAT TGA TAT GC-3’). All other procedures were carried out as described by Ezra et al. (2004). The DNA was sequenced at the W. M. Keck Facility at Yale University. The sequence data are deposited in GenBank (accession no. EU330193).

Plugs (PDA) containing the mycelium were placed in 15% (v/v) glycerol and stored at −70 °C. However, the best storage conditions for the fungus were obtained by growing it on sterilized barley and placing the infested grains at −70 °C. The fungus was deposited as No. 2341 in the living mycological culture collection at Montana State University.

**Test fungi and bacteria.** All plant-pathogenic fungi used in the bioassay test system were obtained from Drs Don Mathre and Nina Zidak of the Montana State University Department of Plant Sciences. All fungi were grown on PDA at 23 °C and only freshly transferred cultures (4–7 days old) were used in the fungal bioassay tests.

**Scanning electron microscopy (SEM).** Isolate CR-12 was grown on PDA before processing for SEM. Many agar pieces containing the fungus were placed into filter paper packets and suspended in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2–7.4) with Triton X (a wetting agent). Tissues were aspirated for 5 min and incubated overnight as previously described (Ezra et al., 2004). For SEM some of the fungal material was critical-point dried, coated by gold sputtering and images were recorded with a Philips model FEI XL30 ESEM FEG microscope in high-vacuum mode using the Everhart–Thornley detector. Freshly prepared wet specimens were examined by environmental scanning electron microscopy (ESEM) and images were recorded with the FEI XL30 ESEM FEG instrument in the environmental mode as described by Castillo et al. (2005). A gaseous electron detector was used with a spot size of 3, at 15 kV. The temperature was 40 °C with a chamber pressure which ranged from 5 to 6 Torr (−665–800 Pa) providing humidity up to 100% at the sample. Conidia were measured using ImageJ software (http://rsb.info.nih.gov/ij/).

**Minimum inhibitory concentrations (MICs).** Assays were performed in sterile 24-well plates with each well containing 500 μl potato dextrose broth. The plates were incubated for 48–288 h at 25 °C. The MIC was defined as the minimum concentration of compound resulting in no visible growth of the test organism. The compounds were dissolved in methanol, which represented less than 0.5% total methanol in each well test. Several small plugs of agar 3 × 3 × 3 mm containing actively growing test fungi were then placed into each well, with some wells serving as controls. The experiments were repeated at least twice with essentially the same results.

**Bioassay-guided isolation and purification of collutellin.** Isolate CR-12 (17 l) was grown in shake culture for 28 days at 25 °C. The fungal mycelium was removed from the fermentation broth by filtration and extracted three times with equal volumes of n-butanol. The bioactivity of the extract/fraction was evaluated by placing a small amount of material on a PDA plate and challenging with several plugs of agar supporting the growth of Botrytis cinerea (bioassay-guided fractionation). Approximately 40 g (dry wt) of the extract was fractionated by liquid chromatography on a 5.0 × 28.0 cm column of silica gel (Selecto Scientific; 32–63 mesh) using 700 ml of each solvent system in a stepwise gradient of increasing polarity: (A) methylene chloride 100%; (B) chloroform 100%; (C) chloroform: ethyl acetate 50:50 (v/v); (D) ethyl acetate 100%; (E) ethyl acetate: ethanol 50:50 (v/v); (F) ethanol 100% and (G) methanol 100%. Fractions B, C and D were the most active in the antifungal assay at a concentration of 100 μg ml−1. These active fractions were flash evaporated to dryness and were again chromatographed on a 3.0 × 58.0 cm silica column (same material and same solvent programme) eluted with 1 l of each
of the solvent systems A, B, C, D to obtain eight active subfractions (~400 ml each) using the same elution profile as before. The subfraction at 1.3–1.6 l was selected by virtue of its bioactivity against B. cinerea. It was further purified by semipreparative HPLC on a Waters 600E HPLC with a Phenomenex Spherelcclone column (5 µODS (250 x 10 mm)) under gradient conditions (flow: 5 ml min⁻¹, 0 min H₂O: methanol 50:50, v/v; 20 min methanol 100%; 40 min acetonitrile 100%). Detection was at 220 nm and the most biologically active product eluted in a distinct single peak at 24.6 min; it yielded only one compound, with a mass of 1127.7 Da (collutellin A). This compound was used for all biological assays and chemical analysis. However, other biological activity remained before and after this peak (referred to as the broad peak in the Results) at 1.1–1.9 l as subfractions of the second silica column and it too was subjected to HPLC; the main peak had a retention time of 23.9 min and it contained collutellin A and three other collutellin A-like derivatives. This peak was subjected to LC/MS analysis.

**General instrumental procedures.** UV spectra were recorded in 100% methanol using a Beckman DU-50 UV–visible spectrophotometer. NMR spectra were recorded on a Varian INOVA AS-600 MHz spectrometer, using the signals of the residual solvent (δH 3.3 and δH 4.9 p.p.m. for deuterated MeOH) at 23 °C. Masses of the compounds were obtained by transform ion cyclotron resonance mass spectrometry (FTICR) on an LTQ FT ULTRA instrument (Thermo Scientific). Samples were suspended in 50% (v/v) acetonitrile/0.1% (v/v) formic acid at a concentration of 10 pmol µl⁻¹, and introduced into the instrument by nanoelectrospray at a flow rate of 50 nl min⁻¹. Tandem mass spectrometry and desalting experiments were done on a QSTAR system from Applied Biosystems (QqTOF). For desalting, measurements were taken before and after sample application to a ZipTip C18 (Millipore). MALDI-TOF experiments were performed on a Voyager DESTR MALDI-TOF instrument (Applied Biosystems). Samples and matrix (α-cyano-4-hydroxycinnamic acid) were mixed at a ratio of 1:1 (v/v) before being spotted onto the MALDI plate and air-dried. Electrospray mass spectral data were acquired using a Micromass LCT TOF mass spectrometer.

**Amino acid analysis and Edman sequencing methods.** Samples for amino acid analysis were dissolved in 50% (v/v) methanol/water and subjected to hydrolysis and analysis, essentially as described by Castillo et al. (2006). Automated Edman sequencing was performed on an Applied Biosystems CLC system.

**Immunosuppression and toxicity tests.** Collutellin A, in matched experiments with cyclosporin A, was examined for its ability to inhibit the activation of CD4⁺ T-cells for the production of IL-2 at various concentrations (Umland et al., 1999; Clark et al., 1999). This test is commonly taken as an indication of the potential that a compound may act as an immunosuppressant (Umland et al., 1999; Clark et al., 1999). Total spleen cells were isolated from C57/B6 mice and then preactivated with concanavalin A (ConA; 1 µg ml⁻¹, Sigma) for 2 days. These cells were then treated for 4 h with cross-linked hamster anti-mouse CD3/CD28 (1 µg ml⁻¹, BD Bioscience) antibodies in Bruf’s medium (Irvine Scientific) containing 5% fetal bovine serum and 1 x brefeldin A. After activation, cells were fixed and analysed for IL-2 production in the activated CD4⁺ T-cells using alloglycycocin (APC)-conjugated anti-IL-2 and PE-conjugated anti-mouse CD4 antibodies by flow cytometry.

In like manner, in matched experiments, both collutellin A and cyclosporin A were examined for their toxicity profiles. Blood was collected from healthy adult donors and peripheral blood mononuclear cells (PBMCs) were purified using Histopaque 1077 (Sigma Aldrich) according to the manufacturer’s instructions. The PBMCs were cultured at 1 x 10⁵ cells ml⁻¹ in X-vivo 15 medium (Cambrex) with varying concentrations of cyclosporin A, collutellin A or equivalent amounts of the carrier DMSO for 24 or 48 h. Cells were washed twice with PBS followed by staining with Annexin V directly conjugated to phycoerythrin (PE) or FITC and 7-aminoactinomycin D (7-AAD) using the Annexin V Apoptosis Detection kit I (BD Biosciences) according to the manufacturer’s instructions. Cells were then subjected to flow cytometry on a FACS Calibur equipped with an HTS loader (BD Biosciences). This staining procedure allowed for differentiation of viable, necrotic and apoptotic cells. The experiment was repeated at least three times and the variation between the dead cells detected was recorded as a function of concentration over 24 and 48 h test periods.

**RESULTS AND DISCUSSION**

**Fungal endophytes**

Each of the plants collected in the Costa Rican rainforest yielded a large collection of endophytic fungi. The stem tissues of *Pteromischum sp.*, however, supported the growth of a number of fungal colonies that proved to be identical to each other and were labelled CR-12. No other plant in the same area yielded this fungus. The colonies were brownish, round and discrete and each produced a yellow pigment. Multiple sporodochia were located throughout the surface of the fungal colonies and no sclerotia were produced until the cultures had aged for several days. Each sporodochium had several large setae possessing echinulate surfaces. The conidiophores were located close together and were macronematous to mononematous and irregularly branched. The conidia were aggregated in slimy masses. Each conidium was cylindrical and slightly curved (falcate) and rounded or slightly tapered at the ends. For critical-point-dried specimens the spores averaged 19.1 x 2.1 µm. Images obtained by ESEM were approximately the same length, but the spores averaged 2.68 µm in diameter (Fig. 1). Thus, it is apparent that the methods used to prepare specimens for regular SEM caused some shrinkage of the spores. On the basis of these morphological characteristics, this dematiaceous hyphomycetous fungus was identified as *Colletotrichum* sp. (Barnett & Hunter, 1999; Sutton, 1980). Most commonly, in temperate zones of the world this fungal genus is known for a wide diversity of plant-pathogenic species (Sutton, 1980). However, recently, many members of this genus have been found that are endophytic, especially from tropical plants, as exemplified by the work of Lu et al. (2004).

Further identification of CR-12 was done by an ITS-5.8S rDNA analysis followed by a BLAST search, confirming that the closest relatives (at the 98% level) of this fungus are various isolates of *Colletotrichum* spp., including *Colletotrichum graminicola*, and *Colletotrichum capsici*. Since *C. graminicola* is a fungal species designated for isolates of *Colletotrichum* that are pathogenic on corn (*Zea mays*), CR-12 cannot be given this species designation (Sutton, 1980). However, CR-12 is also genetically related to *Colletotrichum capsici*. It turns out that *C. capsici* is also a
pathogenic fungus, but some authors prefer to place non-pathogenic *C. capsici*-like fungi as a form of *C. dematium*, which is usually considered as a non-pathogenic taxon with slightly narrower conidia than *C. capsici* (Sutton, 1980). Certainly the conidial shape and size of CR-12 are also consistent with the assessment that this isolate be designated *C. dematium* (Sutton, 1980). The rDNA sequences of CR-12 are deposited in GenBank under accession number EU330193. Because of its ability to inhibit a number of pathogenic fungi, using a simple assay test, this endophytic *C. dematium* isolate was selected for further study of its extracellular bioactive components.

**Isolation and characterization of collutellin A**

The endophytic *C. dematium* yielded about 0.45 mg l⁻¹ of collutellin A (eluting from the HPLC column at 24.6 min), which possessed antmycotic activity. The compound had a mass of 1127.7 Da and a sole millimolar absorption at 210 nm with ε=1014. This absorptivity is associated with the peptide bond, and since no absorption bands appeared at 280 nm, the compound was presumed not to possess any aromatic amino acids (Silverstein *et al.*, 1991). The ¹H-NMR spectrum was characteristic of a peptide having aliphatic carbons along with more downfield resonances occurring with carbon atoms bearing protons with adjoining nitrogen and oxygen atoms (Fig. 2) (Silverstein *et al.*, 1991). The compounds found in the broad silica gel/HPLC peak at 23.9 min were subjected to ESI-QqTOF and LTQ-FT mass spectrometry, revealing both singly and doubly charged molecular ions of masses 1081.7, 1095.7, 1111.7 and 1127.7, of which the 1095.7 peak was the most prominent (Fig. 3). Sodium adducts of these molecules, all of which were 23 amu higher in mass (Fig. 3), also were observed, the intensities of which were substantially reduced by desalting (data not shown). Mass differences of 14 and 16 amu among the ions suggest that the compounds are related by the presence or absence of oxygen or methylene groups, the latter of which would be a common variation among lipopeptides with the lipid component being modified. These compounds were not further examined. Quantitative amino acid analysis of collutellin A revealed the presence of Ile, Val, Ser, N-methyl-Val and β-aminoisobutyric acid in nominal molar ratios of 3:2:1:1:1, respectively. Both Edman and mass spectrometric sequencing revealed the N-terminal tetrapeptide sequence Val-Ile-Ser-Ile and a tripeptide sequence Ile-Pro-Val. Signal levels of the first four amino acids were significant during Edman sequencing; thus the lack of...
those of cyclosporin A (a cyclic peptide with antifungal activity) all biological assays were conducted in matched experiments with these two compounds. Most interestingly, both of the compounds possess strong inhibitory activity against Botrytis cinerea and Sclerotinia sclerotiorum which remains stable up to 288 h (Table 1). The result with S. sclerotiorum is in close agreement with that of Rodriguez et al. (2006), who reported that cyclosporin A possessed an MIC of 0.1 µg per disc. Harel et al. (2006) showed that calcineurin plays a major role in both sclerotal development and pathogenesis of S. sclerotiorum. The calcineurin pathway may be involved in the pathogenic potential of this major fungal pathogen (Steinbach et al., 2007). It has been suggested that cyclosporin A may affect S. sclerotiorum by inhibiting the calcineurin pathway. This may also be true of collutellin A, since they have comparable patterns of antimycotic activity (Table 1). Furthermore, it will be interesting to investigate the possibility that B. cinerea and S. sclerotiorum can be used as a quick initial system for screening organisms for the production of immunosuppressants.

Both cyclosporin A and collutellin A possess a relatively narrow spectrum of antimycotic activity, with some organisms such as Pythium ultimum and Trichoderma viride not being affected and others being quite sensitive (Table 1). Overall, it is also worth noting that although the test fungi are nicely matched with respect to their sensitivities to the two compounds examined, the MIC values of cyclosporin A and collutellin A are, in some cases, more than 50 times different (Table 1). This suggests that the compounds may have different molecular targets within some of the test organisms.

Both collutellin A and cyclosporin A were examined for their ability to inhibit IL-2 production by activated CD4+ T-cells. Generally, the inhibition of IL-2 production is directly related to the ability of a compound to act in a whole biological system as an immunosuppressant (Umland et al., 1999; Clark et al., 1999). Using all of the appropriate controls (unstimulated cells and cells with DMSO + anti-CD3/28 antibodies) and various concentrations of collutellin A and cyclosporin A, IL-2 production for each compound was plotted and then calculated in mouse spleen cells activated with ConA. The IC50 for cyclosporin A was 61.8 nM and for collutellin A it was 167.5 ± 0.38 nM. Thus the IC50 of collutellin A is in the same range as cyclosporin A, giving an indication that collutellin A possesses immunosuppressive properties.

As a result of the enormous importance of cyclosporin A as the first widely used immunosuppressive compound, other natural products with similar activity have been sought. Although a large number of natural products have demonstrated immunosuppressant activity most studies have not included the corresponding cytotoxic activity of the reported compounds (Mann, 2001). Interestingly, the use of cyclosporin A in clinical settings has to be carefully monitored because of its cytotoxic activity. In this regard, its toxicity was compared with collutellin A in experiments...
with human peripheral blood mononuclear cells (PBMCs). Cyclosporin A exhibited a higher level of cytotoxicity on human PBMCs than collutellin A or DMSO alone after 24 and 48 h of culture (Fig. 4). Specifically, in repeated tests, at concentrations at or above 8 \( \mu g \) ml\(^{-1}\), cyclosporin induced significant levels of both necrosis and apoptosis, whereas collutellin A did not induce significant cell death above the DMSO controls at any concentration tested (Fig. 4).

Other organisms producing cyclosporin A and/or its derivatives have also been sought. At least 28 natural cyclosporins have been discovered, being produced by 25 different fungal taxa (Lawen et al., 1994; Jegorov et al., 1995; Traber & Dreyfuss, 1996). These include Acremonium luzulae, Aphanocladium sp., Beauveria brongniarti, Chaunopycnis alba, Cylindrotrichum oligospermum, Cylindrocarpon lucidum, Fusarium oxysporum, Fusarium solani, Isaria felina, Neocosmospora africana, Paecilomyces spp., Stachybotrys chartarum and Tolypocladium spp. (Sakamoto et al., 1993; Dreyfuss & Chapela, 1994; Jegorov et al., 1995; Traber & Dreyfuss, 1996). In addition, about 800 semisynthetic or synthetic analogues have been produced and tested in vitro, but only a few of them were worth testing in vivo (Rehacek, 1995). Most of the immunosuppressant compounds isolated from nature are lipopeptides, cyclic peptides or cyclic lipopeptides, but few have low cytotoxicity accompanied with high immunosuppressive activity. This fact makes the prospects for collutellin A, as a potential drug, seem promising since it has little or no toxicity and reasonable immunosuppressive potential.

### Table 1. MICs of collutellin A and cyclosporin A on common fungal pathogens

The MIC tests were performed at least twice with virtually the same results. The compounds were varied in concentration and placed into 0.5 ml PD broth along with a small agar plug (3 x 3 x 3 mm) supporting the growth of the test organism.

<table>
<thead>
<tr>
<th>Fungus tested</th>
<th>Cyclosporin A MIC (( \mu g ) ml(^{-1})) after time shown</th>
<th>Colutellin A MIC (( \mu g ) ml(^{-1})) after time shown</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>48 h</td>
<td>144 h</td>
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<tr>
<td>Pythium ultimum</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>Trichoderma viride</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>Sclerotinia sclerotiorum</td>
<td>0.07</td>
<td>0.1</td>
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<tr>
<td>Botrytis cinerea</td>
<td>0.07</td>
<td>0.1</td>
</tr>
<tr>
<td>Fusarium solani</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>Rhizoctonia solani</td>
<td>1.2</td>
<td>10.8</td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>1.2</td>
<td>3.6</td>
</tr>
<tr>
<td>Geotrichum candidum</td>
<td>&gt;100</td>
<td>&gt;100</td>
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</table>

**Fig. 4.** Collutellin A is less toxic to human PBMCs than is cyclosporin A. Human PBMCs were treated with varying concentrations of collutellin, cyclosporin A or equivalent amounts of DMSO (carrier) for 24 and 48 h. The cells (PBMCs) were then stained with 7-AAD and Annexin V directly conjugated to FITC or PE and subjected to flow cytometry. The percentages of necrotic (7-AAD\(^+\)/Annexin V\(^+\)), apoptotic (7-AAD\(^-\)/Annexin V\(^+\)) and viable (7-AAD\(^-\)/Annexin V\(^-\)) cells were calculated using CellQuest software (BD Biosciences). The graphs show the total necrotic+apoptotic cells as a percentage of the cell population. Data are representative of three experiments; error bars represent standard deviations between triplicate treatment groups.
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