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A Membrane Form of TNF-α Presented by Exosomes Delays T Cell Activation-Induced Cell Death

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In common with many other cell types, synovial fibroblasts produce exosomes. In this study, we show that the exosomes produced by synovial fibroblasts obtained from individuals with rheumatoid arthritis (RASF), but not exosomes produced by synovial fibroblasts obtained from individuals with osteoarthritis, contain a membrane bound form of TNF-α as demonstrated by colloidal gold immunostaining of TNF-α and confirmed by both Western blot and mass spectrometry. The RASF-derived exosomes, but not exosomes derived from fibroblasts obtained from individuals with osteoarthritis, are cytotoxic for the L929 cell, a TNF-α-sensitive cell line, and stimulate activation of NF-κB and induction of collagenase-1 in RASF. These effects are blocked by addition of soluble TNFR1 (sTNFbp), suggesting that a TNF-α-signaling pathway mediates these biological activities. sTNFbp also reduced the production of exosomes by RASF, suggesting the interruption of a positive amplification loop. Exosomes can transmit signals between cells, and RASF exosomes, effectively taken up by anti-CD3-activated T cells, activated AKT and NF-κB and rendered these activated T cells resistant to apoptosis. Neutralization of exosomal membrane TNF-α by sTNFbp partially reversed this resistance, suggesting that not only TNF-α but also additional exosomal proteins may contribute to the development of apoptosis resistance. The Journal of Immunology, 2006, 176: 7385–7393.

Exosomes are formed by reverse budding of the membrane of late endosomes or multivesicular bodies (MVB) and are released into the extracellular space by fusion of MVB with the plasma membrane. Although the function of exosomes is still poorly understood, recent studies on the fate and functions of exosomes, mostly in the immune system, have led to the hypothesis that exosomes represent a refined intercellular exchange device allowing transfer of proteins and lipids between cells (1). For example, exosomes produced by IL-10-treated dendritic cells can effectively prevent collagen II-induced arthritis in both rabbit and mouse models (2). Salivary gland epithelial cell exosomes packed with a variety of autoantigens might have Ag-presenting function capable of stimulating either induction of T cell tolerance or an autoimmune response (3). Indeed, exosomes have been shown to be a source of Ag for APCs and to participate in Ag presentation to T lymphocytes (4–6). The presence of biologically active molecules, such as TNF-α, within the exosomes might also affect the immune response, and recently, a number of TNF family proteins have been identified on exosomes derived from dendritic cells (7).

It is well recognized that TNF-α contributes to its pathogenesis of rheumatoid arthritis (RA), a chronic inflammatory disease characterized by destructive polyarthritis. Anti-TNF therapy for RA has proven remarkably effective in decreasing inflammation, improving patient function, and attenuating cartilage and bone erosions (8–10). In virtually all animal models of arthritis (e.g., adjuvant-induced, collagen-induced, and serum-induced arthritis), the symptoms are ameliorated by interference with the TNF-α-signaling pathways through the use of anti-TNF-α Abs, soluble TNFR fusion proteins or genetic manipulation (10, 11). TNF occurs in two forms: a type II membrane protein (mTNF) and a soluble form (sTNF) that is derived from the membrane form by proteolytic cleavage. TNF is initially expressed as the 26-kDa transmembrane protein, and after cleavage at the membrane by TNF-α-converting enzyme, the 17-kDa soluble protein is released. The relative roles of mTNF and sTNF in terms of function have not been determined, in large part due to the absence of physiologically relevant models that enable the two forms of TNF to be compared. To date, the main evidence for an in vivo role for mTNF has come from transgenic mice, in which nonleavable TNF is overexpressed in various organs (12). These studies have demonstrated that mTNF plays a role in tissue destruction and autoimmune inflammation.

CD4+ T cells are a major cell population in the inflammatory infiltrate in the joints of RA patients, and their topographic distribution in some of the histologically most typical cases is consistent with an involvement in a germinal center reaction comparable to the Ag-specific immune responses in secondary lymphoid organs (13, 14). However, the survival signals that protect the infiltrating CD4+ T cells from activation-induced cell death (AICD) are still not identified.

In this study, we demonstrate that exosomes produced by synovial fibroblasts obtained from individuals with rheumatoid arthritis...
(RASF) contain a membrane-bound form of TNF-α that is cytotoxic for L929 target cells, an effect eliminated by treatment with a soluble TNFRI (sTNFbp). CD4 T cells cocultured with RASF exosomes, develop apoptosis resistance, and blockade of exosomal TNF-α led to partial restoration of CD4 T cell AICD. Taken together, these observations suggest that RA synovial fibroblasts may communicate with infiltrating CD4 T cells through RASF exosomes in the joints and such communication may lead to blunting of AICD in these infiltrating T cells. The membrane-bound form of TNF-α on the RASF exosomes may mediate these effects on T cell AICD at least in part by sustaining the activities of both Akt and NF-κB in activated T cells, thus blocking some effects of caspase activation.

Materials and Methods

Cell culture

Primary synovial cell lines were established as described previously (15). In brief, small pieces of synovial tissue were digested with 1 mg/ml type I collagenase (Sigma-Aldrich). After dissociation of the fibroblasts, the digested cells were centrifuged at 1000 revolutions per minute for 5 min and plated in a 25-cm² flask in 8 ml of DMEM supplemented with 10% FBS. The cells were cultured to 80% confluence before they were used in the experiments. All experiments were performed using primary synovial cell lines. All of the synovial fibroblast cultures were prepared from fresh synovial tissue biopsy samples obtained through the Tissue Procurement Service of the University of Alabama at Birmingham (UAB) from patients with RA or osteoarthritis (OA) obtained at the time of total joint replacement according to protocols approved by the Institutional Review Board. The diagnosis of RA satisfied the clinical criteria of the American College of Rheumatology. All patients were female and between the ages of 45 and 73 years; the 10 patients with RA were age matched with 10 patients with OA.

The L929 cell line was maintained in DMEM supplemented with 5% FCS at 37°C in a 5% CO₂ incubator. For most assays, the cells were plated at 4 × 10⁴ cells/ml for 24 h. After washing with PBS, the cells were resuspended in DMEM containing 2% FCS and returned to standard incubator conditions for 2 h before treatment.

Isolation of RASF and synovial fibroblasts obtained from individuals with OA (OASF) exosomes

RASF or OASF cells were grown to 80% confluence in DMEM supplemented with 10% FCS, washed with a large volume PBS (20 ml/10-cm dish), and cultured with sera-free CD293 medium (Invitrogen Life Technologies) for 48 h before the supernatants were harvested.

Exosomes were collected from the medium by differential centrifugation. In brief, cells were removed by centrifugation for 10 min at 200 × g. The supernatants were collected and centrifuged sequentially according to the following protocol: twice for 10 min at 500 × g, once for 15 min at 2,000 × g, once for 30 min at 10,000 × g, and once for 60 min at 70,000 × g using an SW28 rotor (Beckman Instruments). The exosomes pelleted during the final centrifugation step were resuspended in PBS and repelleted at 70,000 × g. The repelleted exosomes were resuspended in 5 ml of 2.6 M sucrose, 20 mM Tris-HCl (pH 7.2), and floated onto a linear sucrose gradient (2.0 – 0.25 M sucrose, 20 mM Tris-HCl (pH 7.2)) in an SW41 tube for 16 h at 270,000 g max, once for 15 min at 70,000 × g max, and once for 30 min at 10,000 × g max and then a second polyclonal Ab to MMP-1 proteinase 1 (MMP-1) Ab. The sample RASF supernatant was added at different dilutions, incubated for 1 h at 37°C in a petri dish. The aliquots were stored at −20°C for examination of the presence of exosomes by electron microscopy. Usually, 300 μg of exosomal proteins was obtained from 1 × 10⁶ RASF cells. The quality of each preparation was checked by electron microscopy.

Electron microscopy

Droplets of the sucrose-purified exosomes were placed directly on carbon-coated Formvar nickel-grids and left to adsorb onto the grids at 4°C for 1 h, then fixed with electrondimeroscopic-grade 2% paraformaldehyde (Fluka) and electronmicroscopy-grade 0.1% glutaraldehyde (Fluka) at 4°C for 1 h. After blocking with 2% cold fish gelatin (Sigma-Aldrich) and PBS (20 min), the grids were immunolabeled using a mouse monoclonal anti-TNF-α Ab (Santa Cruz Biotechnology) for 1 h at room temperature. The unbound Ab was removed by four washes, and then revealed by incubation with 12-nm anti-mouse IgG-gold particles (The Jackson Laboratory). After extensive washes with PBS, the grids were stained with 1% aqueous uranyl acetate before observation under a Hitachi H7000 electron microscope (Electronic Instruments).

Mass spectrometry (MS) identification of proteins

The protein bands of interest from the colloidal blue-stained SDS-PAGE gel were excised, destained, and then rehydrated and digested “in gel” with trypsin overnight at 37°C for liquid chromatography-tandem MS identification using a method as described previously (16). The MassLynx 3.5 software (Micromass) was used for instrument operation, data acquisition, and analysis. The search for amino acid sequence similarity was performed using the basic local alignment search tool (BLAST) and/or Scans available from ExPASy (at www.expasy.ch).

ATP-lite M assay to evaluate sTNFRI blockade of the TNF-α-mediated cytotoxic effect of exosomes against L929 cells

L929 murine fibrosarcoma cells were grown in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), and L-glutamine (2 mM) (DMEM/FCS). The cells were seeded in 96-well microtiter plates at 4 × 10⁴ cells/well and, the next day, serial dilutions of RASF exosomes in DMEM with or without sTNFbp was added to the cells in the presence of actinomycin D (1 μg/ml). After a 24-h incubation, the number of surviving cells was determined using the ATP-lite assay as described by the manufacturer (Packard Instruments).

Preparation of cell nuclear extracts and colorimetric NF-κB assay

The indicated numbers of RASF or OASF cells or CD4 T cells were stimulated with RASF or OASF exosomes alone or together with sTNFbp (1 μg/ml) for the indicated time periods. Nuclear extracts were prepared from the harvested cells using a Nuclear Extract kit (Active Motif) and the NF-κB DNA-binding activity was detected using the TransAM NF-κB Family Transcription Factor assay kit (Active Motif) according to the manufacturer’s protocol. Briefly, microwells coated with a double-stranded oligonucleotide containing the NF-κB consensus sequence were incubated with the nuclear extract for 1 h at room temperature and the washed three times. The captured active transcription factor was incubated for 1 h with Abs specific for the p65 or p50 NF-κB subunits, then for 1 h with anti-rabbit IgG-coupled HRP. After incubation with developing solution for 10 min, the OD was measured at 450 nm using a microtiter plate spectrophotometer.

Evaluation of the induction of collagenase production by exosomes

RASFs or OASFs were grown to 80% confluence in DMEM plus 10% FBS. The cells were then washed with PBS and cultured for 24 h in DMEM supplemented with 2% FBS. The cells were then stimulated with RASF or OASF exosomes, with or without sTNFbp, as described above for 24 h. The supernatants were then collected for ELISA. Levels of collagenase were determined by sandwich ELISA (Amersham Pharmacia). The assay is a two-site ELISA sandwich format. Standards and samples were incubated in microtiter wells precoated with rabbit anti-matrix metalloproteinase 1 (MMP-1) Ab. The sample RASF supernatant was added at different dilutions, incubated for 1 h, then a second polyclonal Ab to MMP-1 was added. The binding of the second Ab bound to the wells was detected with donkey anti-rabbit conjugated with HRP using tetramethylbenzidine-hydrogen peroxide, in dimethylformamide as the developing agent. The reaction was stopped by addition of an acid solution, and the absorbance of the reaction mixture read at 450 nm using a microtiter plate spectrophotometer. The concentration of MMP-1 in a sample was determined by interpolation from a standard curve. The sensitivity and linear range of this standard curve was 6.25–100 ng/ml.

Isolation of human CD4⁺ T cells from peripheral blood

Peripheral blood obtained from healthy donors was diluted 1/1 with RPMI 1640 medium (Invitrogen Life Technologies) and layered onto Ficoll-Paque (Pharmacia). Samples were centrifuged at 300 × g for 30 min and the layer of mononuclear cells was collected and washed in RPMI 1640 before removal of adherent cells by a 1-h incubation at 37°C in a petri dish. Nonadherent cells were collected and CD4⁺ T cells were negatively isolated using the MACS CD4⁺ T cell isolation kit (Miltenyi Biotec) according to the manufacturer’s instructions. The resulting CD4⁺ T cell population (of >90% purity) was split for the in vitro culture and for analysis of cell purity by flow cytometry.
**T cell activation**

All T cells were cultured in RPMI 1640 supplemented with 10% heat inactivated normal human serum, 2 mM l-glutamine, 25 mM HEPES buffer, 100 U/ml penicillin, and 100 μg/ml streptomycin at a cell density of 1 × 10^6 T cells/ml. CD4+ T cells were activated with 0.05 μg/ml anti-CD3 mAb (OKT3) (BD Pharmingen). Twenty-four hours after stimulation RASF exosomes were added, and this was repeated daily for 5 days. Then, T cells were harvested for measurement of proliferation by [3H]thymidine incorporation and induction of IFN-γ. The proliferating assays were performed with a standard ELISA method as described previously. In brief, 18 h before harvesting the cells were pulsed with [3H]thymidine (1 μCi/ml), harvested onto filter paper, and [3H]thymidine incorporation determined by scintillation counting. The proliferation assays were performed in triplicate.

**FACS analysis of apoptotic T cells**

Induction of apoptosis in activated T cells as a result of anti-CD3 Ab cross-linking was determined by flow cytometry analyses with PI and Annexin V FITC staining (17). Briefly, anti-CD3-stimulated T cells were harvested at the indicated time point after treatment with RASF exosomes. Early apoptotic cells were defined as single positive (annexin V FITC) and dead cells as double positive (annexin V FITC). The cells were also stained with CD4-allophycocyanin and anti-Fas-FITC or with CD4-allophycocyanin and FasL-biotin plus streptavidin-FITC and analyzed by flow cytometry using a FACS Calibur flow cytometer and CellQuest software (BD Biosciences). Appropriate isotypic controls were used in every experiment.

**Western blot analysis**

CD4 T cells either treated or untreated with RASF exosomes were cultured and harvested at different time points. Cell pellets were washed once with PBS and lysed by SDS sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 50 mM DTT, and 0.1% (w/v) nonfat dry milk for 1 h at room temperature. Proteins were detected using an anti-Akt, anti-phospho-Akt (Cell Signaling Technology), anti-IκBα, Bcl-2, Bcl-xL, anti-β-Actin (BD Pharmingen), and FLIP (Alexis Biochemicals). The primary Ab was detected with an HRP-conjugated secondary Ab and visualized by chemiluminescent detection (Kirkegaard & Perry Laboratories).

For Western blot analysis of exosomal proteins, 10 μg of RASF or OASF exosomes or human TNF-α (eBioscience) as an additional molecular marker were then prepared in lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10% glycerol, Nonidet NP40 0.2%, supplemented with a protease inhibitor mixture; Roche Biochemicals). The proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes by electroblootting, and the nonspecific binding sites were blocked by incubation in TBS containing 0% Tween 20 and 5% (w/v) nonfat dry milk for 1 h at room temperature. Bound primary Abs were visualized with HRP-conjugated goat anti-mouse-IgG (Jackson ImmunoResearch Laboratories) and chemiluminescent detection (Kirkegaard & Perry Laboratories).

**Akt kinase assay**

CD4 T cells were harvested at specific times after treatment with exosomes and lysed in lysis buffer. After equalizing the protein to 1.0 mg/200 μl in a lysis buffer that contained a rabbit anti-mouse Akt Ab (Cell Signaling Technology) and proteinase inhibitor cocktail (Roche Biochemicals), the proteins were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes by electroblootting, and blocked. The blots were probed with phospho-Akt (Ser473) Ab, anti-Akt Ab, and anti-β-Actin Ab. RASF or OASF exosomes were purified on sucrose gradients from culture supernatants of primary synovial fibroblasts isolated from patients with either RA (n = 5) or OA (n = 5) as described in the text. The exosomes were then lysed using a protein lysis buffer (Roche). A total of 10 μg of total protein lysate was used for Western blot analysis.

**TaqMan RT-PCR**

An ABI PRISM 7700 sequence detection system (Applied Biosystems) was used for amplification and detection of the gene transcripts of interest. In each Taqman run, serial 5-fold dilutions of a single-stranded cDNA derived from a commercially available human positive control RNA (Applied Biosystems) were amplified to create a standard curve, and values of unknown samples were estimated relative to this standard curve. Standard curves showed a linear relationship between the copy number (defined as 1 ng of 1000-bp DNA = 9.1 × 10^{11} molecules) of the original internal standard and the number of PCR cycles that were required to exceed a preset threshold, according to the method described previously (18). PCRs for each sample were run in duplicate in three 5-fold serial dilutions. From these standard curves, the relative amount of cDNA for the 18S ribosomal RNA, GAPDH, and each gene was determined and expressed as a ratio of the amount of this material in cells treated or untreated with RASF exosomes after samples were standardized to the relative expression of the 18S ribosomal RNA and GAPDH.

**Statistical analysis**

Data are presented as arithmetic means of at least three independent experiments ± SEM. Statistical analysis was performed using the Student-Newman-Keuls multiple range test and Duncan’s multiple range test. A p value of <0.05 was considered to be statistically significant.

**Results**

The membrane-bound form of TNF-α is detected on exosomes produced by RASFs, but not OASFs

Many types of normal cells and tumor cells have now been shown to produce exosomes (19-26). Electron microscopic analysis of the supernatants of RASFs and OASFs that had been cultured for 48 h indicated that these cells produce exosomes.

The function of exosomes remains largely unknown, but recently, a number of TNF family proteins have been identified on exosomes derived from dendritic cells. Western blotting of the exosomes produced by RASFs and OASFs indicated that TNF-α is associated with the exosomes isolated from the supernatants of RASFs but not OASFs (Fig. 1A). The presence of TNF-α on

![FIGURE 1. Identification of a membrane form of TNF-α on the RASF exosomes. A. Exosomes were analyzed by Western blotting with a monoclonal anti-TNF-α Ab. RASF or OASF exosomes were purified on sucrose density gradients from culture supernatants of primary synovial fibroblasts isolated from patients with either RA (n = 5) or OA (n = 5) as described in the text. B. The exosomes were then lysed using a protein lysis buffer (Roche). A total of 10 μg of total protein lysate was used for Western blot analysis.](http://www.jimmunol.org/doi/10.1182/jimn.2009.10.50001)
exosomes is also evident with TNF-α colloidal gold immunostaining (Fig. 1C). Comparison of the molecular mass of TNF-α from the RASF exosomes with soluble TNF-α indicates that exosomes express the membrane-bound form and not the soluble form of TNF-α (Fig. 1B). The 14-3-3-β protein is present in exosomes produced by many different types of cells including RASF and OASF, and similar amounts of 14-3-3-β protein were detected in both RASF and OASF exosomes (bottom panel, Fig. 1A). This finding suggests that the absence of detectable membrane TNF-α on OASF exosomes is not due to lesser amounts of OASF exosomes loaded on SDS-PAGE and that, therefore, the association of TNF-α with RASF exosomes is a specific effect.

RASF exosomal TNF-α induces cytotoxicity of L929 cells

To determine whether the TNF-α associated with the RASF exosomes is biologically active and to further confirm the association, we evaluated the ability of the exosomes to induce cytotoxicity of L929 cells. Stimulation with TNF-α in L929 cells leads to cell death and has been used for evaluation of the cytotoxic activity of TNF-α (27, 28). Using the ATPLite assay, we found that RASF exosomes had a cytotoxic effect on L929 cells, but that OASF exosomes had only a minimal effect (Fig. 2A). The cytotoxic effect of the RASF exosomes correlated with their concentration (Fig. 2A). To further determine whether the cytotoxic effect of the RASF exosomes on L929 cell was dependent on TNF-α, the RASF or OASF exosomes were preincubated with sTNFbp at different concentrations for 1 h at 37°C before addition to the L929 cell cultures. Addition of sTNFbp blocked the cytotoxic effect of the RASF exosomes on the L929 cells in a concentration-dependent manner (Fig. 2B), further suggesting that the RASF exosome-induced cytotoxicity of L929 is mediated by a TNF-α-signaling mechanism. The addition of sTNFbp has no effect on the low levels of cytotoxic activity of the OASF exosomes.

**FIGURE 2.** TNF-α associated with RASF exosomes are cytotoxic to L929 cell. A total of 4 × 10⁴ cells/ml murine fibroblast cells L929 were seeded in supplemented DMEM in 96-well microtiter plates. After 24 h, cells were treated with RASF or OASF exosomes at the concentrations indicated (A) or cells were treated with RASF or OASF exosomes (50 ng/ml) that had been preincubated with sTNFbp for 1 h at 37°C at the concentrations indicated (B). The viability of the cells was assessed after 24 h of incubation by the ATPLite assay as described in Materials and Methods. Data are presented as percentage of cytotoxicity for L929 cell line induced by the exosomes isolated from each RASF (n = 10) or OASF (n = 10). Sample size n = 10 with triplicates, mean ± SEM. If the p value is >0.05, indicating that these groups are significantly different, α = 0.05, Duncan’s multiple range test.

RASF exosomes induce activation of the NF-κB-signaling pathway in RASF

TNF-α-mediated activation of the NF-κB pathway has been shown to play an important role in the resistance of RASF to apoptosis and may possibly drive the hyperplasia of rheumatoid synovium through up-regulation of the NF-κB-associated proto-oncogenes. Therefore, we analyzed whether NF-κB is activated in RASFs by treatment with RASF exosomes in the presence or absence of sTNFbp (1 μg/ml). Nuclear proteins were extracted at different time points after treatment, and their ability to bind the NF-κB consensus sequence was quantitated by ELISA. OASF exosomes, or the exosome diluent (PBS), were used as controls. Stimulation of RASF cells with RASF exosomes (50 ng/ml) resulted in a marked increase in the binding activities of NF-κB subunits p50 (Fig. 3A) and p65 (Fig. 3B), with the effect being more pronounced in the case of p50 subunit (Fig. 3A). The activation of NF-κB was detected within 30 min after addition of RASF exosomes. This activity was not observed on treatment with OASF exosomes, or PBS (Fig. 3A), and the activation was completely neutralized by addition of sTNFbp, suggesting that activation of NF-κB is a specific effect of RASF exosomes and is mediated by TNF-α. Similarly, stimulation of OASF cells with RASF exosomes also induced NF-κB activity similar to that in RASF (data not shown),

**FIGURE 3.** TNF-α associated with RASF exosomes induce activation of NF-κB in RASF cells. A total of 1 × 10⁶ cells of RASFs or OASFs were stimulated with RASF or OASF exosomes (50 ng/ml), respectively, in the presence of sTNFbp (1 μg/ml) or PBS as a control. The nuclei were then extracted at the time points indicated. NF-κB DNA-binding activity was measured using a colorimetric NF-κB assay (A and B) and the induction of MMP-1 in the culture supernatants was measured by ELISA at hours 6, 12, and 24 after exosome stimulation (C). Values represent the means of triplicate determinations (n = 5). Data are presented as means ± SEM from three independent experiments. *, p < 0.05; **, p < 0.01, calculated as untreated cells vs stimulated cells. The data at each time point were derived using exosomes isolated from RASF or OASFs from five patients analyzed individually.
suggested that activation of NF-κB activity mediated by RASF exosomes is not cell-type specific, but is specific to RASF exosomes.

As NF-κB is a well-characterized activator of MMP-1 (29–31), we next examined the effect of RASF exosomal TNF-α on induction of MMP-1. The levels of MMP in the supernatants of cells treated as described above were assayed by ELISA. In agreement with the RASF exosome-mediated activation of NF-κB, we found that RASF exosomes induce the expression of MMP-1 in RASF in a time-dependent manner (Fig. 3C). The production of MMP-1 in the cells treated with RASF exosomes plus sTNFbp was significantly lower \( (p < 0.0018) \) (Fig. 3C) and treatment with OASF exosomes or exosome diluent had little effect on induction of MMP-1.

**Production of RASF exosomes is enhanced by TNF-α stimulation**

As the production of exosomes is regulated by the proteasome degradation pathway, and the TNF-α-signaling pathway involves the ubiquitination pathway, we next examined whether the production of RASF exosomes is enhanced by TNF-α stimulation. RASFs were starved by culturing in DMEM medium with 2% FBS for 16 h, and then stimulated with TNF-α at the different concentrations for an additional 48 h. The exosomes in the supernatants were purified by differential centrifugation, and the production of exosomes was quantified using the Bradford protein assay. The effect of TNF-α on the production of exosomes by OASF was analyzed similarly. We found that the production of RASF exosomes was enhanced significantly by addition of TNF-α-stimulated RASFs (Fig. 4A, \( p < 0.0011 \)) although TNF-α only enhanced the yield of OASF exosomes slightly. As the membrane-bound form of TNF-α is detected on the RASF exosomes, the effect of sTNFbp on the production of RASF exosomes was also examined. The yield of RASF exosomes was reduced in the RASFs cultured in the presence of sTNFbp in a concentration-dependent manner (Fig. 4B). In contrast, there was no effect on OASF exosome production. Therefore, the production of RASF exosomes is regulated by a TNF-α pathway through both paracrine and autocrine mechanisms.

**Coculture of RASF exosomes with CD4 T cells results in sustained T cell proliferation**

The infiltration of autoreactive CD4+ T cells into synovium has been considered the major instigator of joint inflammation, and both hyperproliferation and decreased AICD of infiltrating T cells have been proposed as mechanisms underlying the pathogenesis of RA (13–15, 32). Therefore, we investigated whether incubation of RASF exosomes with anti-CD3 Ab-activated CD4 T cells might have effects on CD4 T cell proliferation and subsequent AICD. RASF exosomes or OASF exosomes were added daily, starting with the second day of culture of CD4 T cells that had been stimulated with anti-CD3 Ab. The effects of RASF exosomes treatment on T cell activation were determined by \(^{[3]}\)Hthymidine incorporation assay. IL-2 and IFN-γ induction in the cell culture supernatants. RASF exosomes exhibited no significant effects on T cell proliferation or on induction of IL-2 or IFN-γ by CD4 T cells during the initial phases of culture in comparison with the T cells stimulated with anti-CD3 alone. However, by day 6, the T cells cocultured with RASF exosomes maintained proliferation (Fig. 5A), induction of IFN-γ (Fig. 5B), and IL-2 (Fig. 5C) in a RASF-exosome dose-dependent manner whereas equal concentrations of the OASF exosomes did not have a comparable effect (data not shown). Preincubation of RASF exosomes with

![FIGURE 4. TNF-α associated with RASF exosomes and exogenous TNF-α enhance the production of exosomes. RASF or OASF cells grown at 80% confluency were treated with a TNF-α at different concentrations (A) or with a elevated concentrations of sTNFbp (B) for 48 h, then exosomes were isolated and purified as described in Materials and Methods. The production of exosomes in the cell culture supernatants was quantified by the Bradford assay. Data are representative of at least three independent experiments performed in triplicate. The data were derived using exosomes isolated from RASF or OASFs from 10 patients analyzed individually. There was a statistically significant enhancement of production of RASF exosomes in the TNF-α-stimulated RASF compared with the OASF. **, \( p < 0.01 \).](http://www.jimmunol.org/)

![FIGURE 5. RASF exosomes sustain the proliferation of CD4 T cells at the late phase of T cell activation. A total of \(10^5\) cells sorted of MACS-purified CD4 T cells were incubated in complete medium with 1 µg/ml plate-bound anti-CD3 in 96-well U-bottom plates (Nunc) for 1 day and then different amounts of RASF exosomes were daily added to the anti-CD3-activated CD4 T cell culture. The proliferative response was determined at days 2, 4, 6 of coculture by \(^{[3]}\)Hthymidine incorporation. \(^{[3]}\)Hthymidine was added for the remaining 18 h of culture. Proliferation was measured using a liquid scintillation counter. Data are presented as mean cpm ± SD of triplicate cultures (A). Supernatants were harvested at day 6 after culture and secreted IFN-γ (B) and IL-2 (C) were measured by ELISA. The proportion of early apoptotic (annexin V+PI−) plus dead cells (annexin V+PI+) (percentage) was determined by flow cytometric analysis following annexin V and PI staining 6 days after the culture. One representative of five independent experiments is shown (D). Results obtained from CD4 T cells isolated five individuals of health subjects, in three independent experiments, were pooled and expressed as mean ± SD (E). The asterisk above the bar indicates exosomes treatment groups that were significantly different from the control group. *, \( p < 0.05; **, p < 0.01. \) The percentages of CD4 T cell death at day 2, 4, and 6 after anti-CD3 stimulation were determined (F). Data are presented as percentage of death of the CD4 T cell isolated from each health individuals \((n = 5)\) with triplicates, mean ± SEM, *, \( p < 0.05; **, p < 0.01. \)](http://www.jimmunol.org/)
sTNFbp significantly reversed the effect of RASF exosomes in sustaining T cell proliferation, suggesting that RASF exosome-associated TNF-α plays a role in sustaining T cell proliferation. To further define the nature of sustained T cell proliferation after treatment with RASF exosomes, we used FACS analysis to assess the effect of RASF exosomes on T cell AICD at day 6 after anti-CD3 stimulation (Fig. 5D). Treatment with RASF exosomes at concentrations of 100 ng/ml significantly reduced the cell death of activated T cells up to 42% (Fig. 5E), while the equivalent concentration of OASF exosomes did not. Further, the reduction in cell death varied in a dose-dependent fashion (Fig. 5F) and as the reciprocal of the effect on cell proliferation as shown in Fig. 5A. These results suggest that RASF exosomes may play a role in the induction of resistance to AICD in T cells and that the cross-talk between T cells and RASF via exosomes might reduce apoptosis of anti-CD3-activated T cells, leading to CD4 T cell continuation of proliferation.

Up-regulated NF-κB and Akt activities are associated with an RASF exosome-mediated reduction in T cell AICD

Previous investigations showed that TNF-α stimulation of T cells leads to the activation of both NF-κB and Akt. The activation of NF-κB and Akt plays a critical role in cell survival signaling pathways and development of autoimmune diseases in both human autoimmune diseases and mouse models (33, 34). We examined activities of NF-κB and Akt at day 6 after T cells were exposed to RASF exosomes. The results of Western blot analyses of Akt indicate that levels of phosphorylated Akt were increased by stimulation of primary CD4 T cells with RASF exosomes in a dose-dependent manner (Fig. 6A, top panel). This induction effect was specific for phosphorylated Akt, because there were no changes detected in total amount of Akt between treatments (Fig. 6A, second panel from the top). We also evaluated the phosphorylation status of GSK-3β, a known substrate of Akt. The phosphorylation...
of GSK-3 β correlated directly with the Akt phosphorylation status, supporting the view that the Akt pathway is functionally active (Fig. 6A, fourth panel from the top). Similarly, NF-κB activity of 6-day cultured CD4 T cells was increased at 60 min after CD4 T cells were stimulated with RASF exosomes (Fig. 6, B and C). The activity of NF-κB increased in a manner dependent on the dose of RASF exosomes. Amounts of IκB-α, which is an NF-κB-specific inhibitor, was also reduced as the concentration of RASF exosomes increased (Fig. 6A, third panel from the top), which is correlated with increased activation of NF-κB. These results suggest that a significant activation of both NF-κB and Akt were observed in CD4 T cells after 6 days of anti-CD3 stimulation in the presence, but not absence, of RASF exosomes. It is unlikely that differences in activity of either Akt or NF-κB resulted from the changes of global protein expression because the levels of β-actin loaded on each lane were not affected by RASF exosome treatments (Fig. 6A, bottom panel).

Because activation of Akt or NF-κB has been shown to up-regulate a number of apoptosis-associated proteins (35–37), we examined the effects of RASF exosomes on the expression of FLIP, Bcl-2, and on the cleavage of both caspase-8 and caspase-3. CD4 T cells stimulated with anti-CD3 and treated with RASF exosomes have more FLIP (Fig. 6D, third column) but similar Bcl-2 detected in the cell lysates compared with lysates from CD3-stimulated cells not treated with exosomes (second column). This result paralleled partially blocked cleavage of both capase-8 and -3. Addition of sTNFpβ restored this cleavage process (Fig. 6D, fourth column), suggesting that RASF exosome-associated membrane TNF-α may play a role in blocking caspase activation through up-regulation of the FLIP pathway. RASF exosome-mediated blockade of caspase-8 and -3 activation was seen in the context of TCR signaling as non-CD3-stimulated cells did not have either caspase-8 or caspase-3 activated (Fig. 6D, first column). To determine whether the RASF-exosome-mediated induction of FLIP protein had a corresponding increase in mRNA, the expression of FLIP mRNA was quantitated by TaqMan PCR analysis. FLIP mRNA was significantly up-regulated after CD4 T cells were treated with RASF exosomes (Fig. 6E). This result was not due to the amount of mRNA used for the TaqMan PCR analysis as there were no differences in the amount of other apoptosis-related genes amplified (Fig. 6E).

### Discussion

RASFs and OASFs produce exosomes with a size distribution similar to that seen for exosomes from other sources and consistent with the size criterion for exosomes proposed by Ther, et al. (26). The array of associated proteins identified by using the liquid chromatography-tandem MS analysis (Table I) is consistent with those identified in exosomes from other cell types, such as B lymphocytes and dendritic cells (5, 38, 39) and indicates that the RASF exosomes are derived from the MVB. Notably, however, RASF exosomal proteins include TNF-α which is absent from OASF exosomes. The presence of TNF-α was of particular interest as TNF-α plays a pathogenic role in RA, and the membrane form of TNF-α has been recognized as playing an important role in the inflammatory response and induction of arthritis. Here, we show that the membrane form of TNF-α is associated with exosomes produced by RASF. Three lines of evidences support this conclusion. First, TNF-α was detected by direct immunocolloidal gold staining and Western blot analysis of RASF but was absent in OASF exosomes. Second, RASF exosomes induced the cytotoxicity of TNF-α-sensitive L929 cells, with soluble TNF-R blocking this cytotoxic effect. Finally, RASF exosomes were potent inducers of NF-κB activation in the RASF that led to the degradation of IκB-α and induction of MMP-1. Importantly, RASF exosomes not only stimulated TNF-α signaling, leading to induction of MMP-1 and activation of NF-κB, but they also induced resistance to apoptosis of activated T cells in a paracrine manner.

It is well-recognized that activation of both Akt and NF-κB are regulated by TNF-α-signaling pathway and that activated Akt and NF-κB have pathogenic effects in the development of rheumatoid arthritis (15). Our data indicate that the effects of RASF exosomes on induction of MMP-1 by RASFs and on AICD defects of CD4 T cells are mediated by TNF-α with activation of Akt and NF-κB pathways. Furthermore, blockade of the activation of caspase-8 and caspase-3 and the up-regulation of anti-apoptosis gene FLIP may play a role in AICD defect.

The important role of TNF-α signaling in T cell survival through activation Akt was shown using TNFR knockout mice (40–43). Akt phosphorylation is defective in TNFR-deficient T cells and the activation of Akt during T cell activation is an important regulatory point for initiation and progression of the T cell

### Table I. Identities of proteins found in synovial fibroblast-derived exosomes

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Acc. No.</th>
<th>RASF</th>
<th>OASF</th>
<th>Unique Peptides</th>
<th>Coverage (%)</th>
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<tr>
<td>TNF-α</td>
<td>NP_038721</td>
<td>+</td>
<td>−</td>
<td>6</td>
<td>25</td>
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<tr>
<td>Myosin</td>
<td>CAB05105</td>
<td>+</td>
<td>+</td>
<td>4</td>
<td>8</td>
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<tr>
<td>Heat sock protein-90</td>
<td>AAA36026</td>
<td>+</td>
<td>+</td>
<td>4</td>
<td>25</td>
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<tr>
<td>Ezrin</td>
<td>Q96CU8</td>
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<td>−</td>
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<tr>
<td>Moesin</td>
<td>Moes_human</td>
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<td>+</td>
<td>3</td>
<td>15</td>
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<td>Annexin VI</td>
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<td>+</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>Heat shock protein 70</td>
<td>A27077</td>
<td>+</td>
<td>+</td>
<td>4</td>
<td>18</td>
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<tr>
<td>Actin</td>
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<td>+</td>
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<td>MHC class I Ag</td>
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<td>Enolase 1</td>
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<td>Aldose reductase</td>
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<td>P08107</td>
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<tr>
<td>14-3-3-β</td>
<td>2BQ0_B</td>
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<td>+</td>
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<td>Syntenin</td>
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<td>+</td>
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<tr>
<td>Complement C1q chain C</td>
<td>C1HuQC</td>
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<td>+</td>
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<td>5</td>
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<td>15</td>
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<tr>
<td>Kinesin-like protein</td>
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<td>−</td>
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<tr>
<td>Fibronectin precursor</td>
<td>CAA26536</td>
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<td>+</td>
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The Journal of Immunology 7391

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response. Overexpression of Akt resulted in the development of autoimmune diseases (44, 45). Therefore, RASF exosome-mediated activation of Akt in anti-CD3 activation of CD4 T cells points to one mechanism that may play a contributing role in development of RA.

Our data suggest that RASF exosomes not only activate Akt but also sustain NF-κB activity. Numerous lines of evidence have strongly supported TNF-α-mediated activation of NF-κB as one of key regulators of T cell survival and of MMP induction of RASF cells. Activation of NF-κB has also been proposed as a pathogenic factor contributing to hyperplasia of rheumatoid synovium. Our data support significant activation of NF-κB induced in T cells and RASF treated with RASF-derived exosomes, whereas OASF exosomes caused only slight time-dependent NF-κB activation in CD4 T cells. Our data also show that the rapid reduction of IκB-α correlated directly with the NF-κB activation status, supporting the view that the NF-κB pathway is functionally active. The observation that MMP induction in RASFs was blocked by addition of sTNFbp further supports the notion that NF-κB is also activated in RASFs. Other data in the literature suggest that the activation of NF-κB in T cells leads to up-regulation of both FLIP and Bcl-2 (46–49). However, our data show that more FLIP but not Bcl-2 was detected after RASF exosome treatment, suggesting that this effect may be due to the net result of multiple proteins packed in the exosomes interacting with the NF-κB pathway. Further investigation of which exosomal protein(s) play a role in the preferential activation of FLIP is underway.

Data generated by other investigators suggest that membrane-associated TNF-α is biologically active, and it has been presumed that the cytotoxic and inflammatory effects of TNF-α are through cell-to-cell contact (12). In contrast, our findings suggest that membrane-associated TNF-α may exercise its role through exosomes, instead of, or at least in addition to, cell-cell contact. Exosomal membrane proteins including TNF-α may play distinct role in comparison with soluble TNF-α. TNF-α is first produced as a 26-kDa transmembrane molecule (mTNF) which is cleaved by the metalloproteinase-disintegrin TNF-α-converting enzyme (50) to generate a soluble 17-kDa molecule (sTNF). Synovial inflammation and joint erosion appear to be dependent, at least in part, on cell-associated TNF-α signaling (12, 32, 51). Using a novel transgenic mouse that expresses only a membrane-associated form of TNF-α, Kollias and coworkers (12) demonstrated that expression of the 26-kDa form of TNF-α was sufficient in itself to induce arthritis. These animals spontaneously develop a pattern of arthritis similar to those of human RA at 6–8 wk of age (12). Although sTNF is regarded as the main ligand for TNFR1, mTNF is considered to be more effective than sTNF in activating TNFR2 (52). TNFR2 engagement leads to binding of TNFR-associated factors 1 and 2, leading to NF-κB activation and cell survival (53). Synovial hyperplasia and joint erosion appear to be dependent, at least in part, on cell-associated TNF-α signaling, with involvement of TNFR2 (54–56). We have demonstrated that RASF exosomal TNF-α strongly stimulates the activation of NF-κB in RASFs. Whether the preferential binding affinity of TNFR2 for the 26-kDa form of TNF-α on cell membranes is also true for TNF-α on RASF exosomes is currently being addressed through studies of TNFRI- or TNFR2-deficient cell lines.

In addition to TNF-α, other proteins packed in the RASF exosomes may play a role modulating infiltrating immune cells. RASF exosomes can be endocytosed by autoreactive T cells and B cells, and by serving as a shuttle between cells (1, 2, 7, 19), they may deliver proteins and/or lipids to the targeted cells. Thus, RASF exosomes are poised to alter the biological functions of multiple cell types, and one might consider therapeutic targeting of exosomes with additional agents beyond those binding and blocking TNF-α.

Evidence indicates that the ubiquitin pathway regulates the formation and fate of exosome budding through the cellular membrane, and TNF-α signaling plays a role in regulation of ubiquitin-mediated protein degradation (57–63). The observation that TNF-α can regulate the production of exosomes has not been previously recognized, and one might speculate that TNF-α-mediated enhancement of the production of RASF exosomes may also enhance the ubiquitination and immunogenicity of self proteins, as has been proposed by other investigators (64). The production of RASF exosomes may be also accelerated by the activation of pathways that regulate assembly of ubiquinated proteins into exosomes. Soluble N-ethylmaleimide-sensitive factor attachment receptor-mediated fusion of a TNF-α-contained vesicular-like structure is a requirement of this trafficking pathway (5). Our data indicate that TNF-α stimulation leads to enhanced production of RASF exosomes, and identification of specific pathways that regulate induction of RASF exosomes may lead to new therapeutic targets for RA treatment.

Acknowledgments

We thank Dr. Fiona Hunter for editorial assistance, Landon Wilson at UAB Mass Spectrometry Shared Facility for the MALDI-TOF-MS mass fingerprinting analysis.

Disclosures

The authors have no financial conflict of interest.

References


CORRECTIONS


In Results, in the penultimate sentence of the second paragraph under the heading A Gly-Gly motif is conserved within the CDR3 of lymph node T cell hybridomas, reference to Figure 7a and 7b are reversed. The corrected sentence is shown below.

The lymph node TCR contains a rigid loop with an extended planar surface (Fig. 7b), whereas the splenic Th1 TCR presents a round shape with a less extended surface (Fig. 7a).


The tenth author’s last name is incorrect. The correct name is Guillaume Darrasse-Jeze.


In Table II, the data reported for GA2–3b in column six (ΔmlID spores) should be negative (−) not 2-log shift (++). The corrected table is shown below.

Table II. mAbs raised against irradiated B. anthracis spores or purified B. anthracis exosporium

<table>
<thead>
<tr>
<th>Western</th>
<th>FACS</th>
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<tr>
<td>mAbs</td>
<td>Anti-E. coli Bc1A</td>
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<tr>
<td>AB2</td>
<td>ND*</td>
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<tr>
<td>AF10*</td>
<td>+</td>
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<tr>
<td>AH8</td>
<td>+</td>
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<tr>
<td>BD8*</td>
<td>+</td>
</tr>
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<td>BE12*</td>
<td>+</td>
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<td>BF1-4</td>
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<td>BF12</td>
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</tr>
<tr>
<td>BG11</td>
<td>+</td>
</tr>
<tr>
<td>CA3*</td>
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</tr>
<tr>
<td>DE3-1b</td>
<td>+</td>
</tr>
<tr>
<td>DE12</td>
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<tr>
<td>FD3-4b</td>
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<td>EF12</td>
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<tr>
<td>AA2-1</td>
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<tr>
<td>BA10-1b</td>
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<tr>
<td>DH4-1b</td>
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<tr>
<td>EA2-1</td>
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<tr>
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<tr>
<td>EA4-10</td>
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<tr>
<td>EG4-4b</td>
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<tr>
<td>FH6-1b</td>
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<tr>
<td>GA2-3b</td>
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<tr>
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<tr>
<td>HB2-2</td>
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<tr>
<td>IC801</td>
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<tr>
<td>JB5-1b</td>
<td>+</td>
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<tr>
<td>JC8-5b</td>
<td>+</td>
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</table>

*ND, Not determined, −, negative by Western or FACS; +, positive by Western or 1-log shift by FACS; ++, 2-log shift by FACS ABS-EF12, mAbs raised against spores; AA2-1-JC8-5, mAbs and raised against exosporium.

*Included in Ref. 9.
In **Results**, in the last sentence of the paragraph under the heading **CCL18 is up-regulated in BAL and sera from AA patients**, and in Figure 4C, the concentration of serum CCL18 is expressed incorrectly as “pg/ml” instead of “ng/ml.” The corrected sentence and figure are shown below.

CCL18 was significantly elevated in AA (73.9 ± 11.2 ng/ml) compared with NA (31.7 ± 5 ng/ml) subjects (Fig. 4C).

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The third author’s first name is incorrect. The correct name is Kaihong Su.


During production, the figure from an unrelated article was inadvertently inserted as the image for Figure 8. The correct figure is shown below. The error has been corrected in the online version, which now differs from the print version as originally published.