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Tumor-Associated a2 Vacuolar ATPase Acts As a Key Mediator of Cancer-Related Inflammation by Inducing Pro-Tumorigenic Properties in Monocytes

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Cancer-related inflammation profoundly affects tumor progression. Tumor-associated macrophages (TAMs) are known regulators of that inflammation, but the factors that initiate cancer-related inflammation are poorly understood. Tumor invasiveness and poor clinical outcome are linked to increased expression of cell surface-associated vacuolar adenosine triphosphatases. The a2 isoform vacuolar adenosine triphosphatase is found on the surface on many solid tumors, and we have identified a peptide cleaved from a2 isoform vacuolar adenosine triphosphatase called a2NTD. a2NTD has properties necessary to induce monocytes into a protumorigenic TAM phenotype. The peptide upregulated both pro- and anti-inflammatory mediators. These included IL-1β and IL-10, which are important in promoting inflammation and immune escape by tumor cells. The secretion of inflammatory cytokine IL-1β was dependent on ATP, K+ efflux, and reactive oxygen species, all mediators that activate the inflammasome. These findings describe a mechanism by which tumor cells affect the maturation of TAMs via a nontraditional cytokine-like signal, the a2NTD peptide. The Journal of Immunology, 2011, 186: 1781–1789.

Recent studies have pointed toward an inflammatory component that is present in tumor microenvironments, including neoplasms that do not have obvious inflammatory processes (1). A prominent feature of cancer-related inflammation (CRI) is leukocyte infiltration (2, 3). Key to understanding CRI is the cross-talk that occurs between cancer cells and leukocytes. Potential initiators of cellular cross-talk are vacuolar adenosine triphosphatases (V-ATPases) found on the cell surface of tumors (4, 5). However, their role in tumorigenesis has only recently been explored. Human melanoma cell lines demonstrated increased secretion of matrix metalloproteinases (MMPs) and angiogenic factors when cultured at a low pH (6). Overexpression of the V-ATPase caused induction of MMP-2 (7). Comparison of a more invasive to a less invasive breast cancer cell line found a positive correlation between high expression of plasma membrane-targeted V-ATPases and cell invasiveness (8).

Previous experiments that investigated whether V-ATPase played a role in CRI produced promising results (9, 10). The hypothesis generated from those experiments was that the N-terminal domain of the a2 isoform vacuolar ATPase (a2V) was cleaved from the a2 subunit. The resulting peptide (a2NTD) had inflammatory properties. Further experiments showed that monocytes responded to a2NTD stimulation (10).

Macrophages are programmed to respond to diverse environmental stimuli, and their plasticity can be classified into two extreme phenotypes that fall along a continuum. M1 cells are the classically activated macrophages that respond to microbial products and IFN-γ. In comparison, anti-inflammatory molecules such as glucocorticoids, IL-4, IL-13, and IL-10 induce the M2 or alternatively activated macrophages (11, 12). M1 macrophages are involved in type 1 reactions that include microbial elimination and oxygen and nitrogen radical formation. In contrast, M2 cells favor type 2 reactions and promote angiogenesis, tissue remodeling, and repair (13).

As noted before, leukocytes are found in solid tumors during CRI; most prominent are the tumor-associated macrophages (TAMs). TAMs are derived from circulating monocytes that are chemotactically recruited to tumor sites. There is strong evidence linking increased TAM cell density with poor prognosis (14, 15). The TAM activation state depends on the tumor microenvironment milieu and is described as the “macrophage balance” hypothesis (16). The tumor signals that affect the balance between M1 or M2 polarization are still unknown, although IL-10, TGF-β, and CSF1 have been shown to play a part (2). TAMs with the protumoral phenotype are functionally similar to the immunosuppressive, M2-polarized macrophages. To our knowledge, our study demonstrates for the first time the direct involvement of a V-ATPase by a cleaved peptide, a2NTD, in inducing monocytes into M2-like cells.

In our present study, we describe a unique cancer-produced signaling molecule, which induces monocytes to express a TAM phenotype. Our data showed a2NTD to be in cancer cell line supernatants but not in nontumorigenic cell supernatants. We found several cytokines and chemokines that were upregulated both at the gene and protein levels that are important in TAM phenotype maturation. These included proinflammatory IL-1β and IL-6 and anti-inflammatory IL-10. a2NTD-induced IL-1β secretion required an activated inflammasome, a protein complex used in the processing of pro–IL-1β into its mature form. Mediators of inflammasomal activation included ATP, potassium efflux, and reactive oxygen species (ROS). Collectively, these studies provide
evidence of the key role of a2NTD as an effective signal from cancer cells that has the ability to modulate monocyte activity.

Materials and Methods

Reagents and Abs

Recombinant a2NTD was expressed and purified from Escherichia coli and subjected to endotoxin removal column chromatography (Proteome Res. Sources, Austin, TX). LPS (E. coli 055:B5), Ac-WEDD-CHO oxidized ATP (oATP), polyoxymine B (PMB), vitamin C, and N-acetylcysteine were purchased from Sigma-Aldrich (St. Louis, MO). Apyrase was from New England Biolabs (Ipswich, MA).

Monoconal anti-a2V-ATPase (2C1) Ab was generated as previously described (17), is specific for aa 488–510, the transmembrane region of the protein, and is conjugated to Alexa Fluor 680 (Covance, Denver, PA). Polyclonal anti-a2NTD was generated as previously described (10) and recognizes aa 142–344, the N-terminal region of the a2 subunit. Blocking mAbs against TLRL4, CD14, and isotype controls were from eBioscience (San Diego, CA). The NF-kB pathway sampler kit (no. 9936) and the NF-kB family member sampler kit (no. 4766) were from Cell Signaling Technology (Beverly, MA). Anti-actin, -heat shock protein 90, -Sp1, and -lamin A/C were from BD Biosciences (San Jose, CA). Anti-GAPDH was from Amblon (Austin, TX). Anti-tubulin was from Millipore (Billerica, MA). Donkey anti-mouse and donkey anti-rabbit Abs conjugated to infrared dyes 680 and 800CW were from Li-Cor Biosciences (Lincoln, NE).

Mononuclear isolation and cell culture

These studies were approved by the Rosalind Franklin University of Medicine and Science Institutional Review Board. After informed consent was obtained in accordance with the Declaration of Helsinki, peripheral blood was collected into sodium heparin vials (BD Biosciences, Franklin Lakes, NJ). The PBMC fractions were collected after differential density centrifugation over Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ). Monocytes were purified from PBMCs via magnetic separation using the Monocyte Isolation Kit II according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified PBMCs and monocytes were cultured in X-VIVO 15 medium with gentamicin (Lonza, Walkersville, MD). For human cell lines, TOV-112D (TOV), MCF-7 (MCF), MDA-MB-231 (MDA), and JEG-3 (JEG) cells were purchased from American Type Culture Collection and maintained in Eagle’s MEM. THP-1 cells were stimulated with a2NTD or media alone. Pellets were flash frozen and stored at −80°C until RNA isolation. Total RNA was isolated using the silica-based spin column method (Sigma-Aldrich). Reverse transcription and cDNA synthesis were prepared via the RT^{a} first-strand kit (SA-Biosciences, Frederick, MD). Equal concentrations of cDNA were loaded onto PCR arrays using SYBR Green chemistry from SABiosciences. The Human Inflammatory Response and Autoimmunity array contained 84 genes with three additional genes added: human IL-33, MMP-9, and TIMP-3. Arrays were run on an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA). After obtaining the threshold cycle (Ct) values, the 2^{−ΔΔCt} method was used to calculate fold differences in gene expression in cells cultured with media versus cells cultured with a2NTD after normalization to five housekeeping genes. Data analysis was done using a web portal provided by SABiosciences. The data discussed in this publication have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus under accession number GSE525490 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE525490).

Immunoblotting

THP-1 cytoplasmic lysates were generated from the nuclear extraction procedure (see below). Total cell lysates were made with RIPA buffer plus protease inhibitor mixture (Thermo Scientific, Rockford, IL). After BCA protein determination (Thermo Scientific), lysates were mixed with 2× sample buffer and heated at 95°C for 5 min. Equal amounts of protein were subjected to SDS-PAGE and transferred to 0.2 μm nitrocellulose. Abs from Cell Signaling Technology were used following supplier protocols. For all other Abs, membranes were blocked in protein-free PBS blocking buffer (Thermo Scientific) for 1 h at room temperature. Primary and secondary Abs were incubated with protein-free blocking buffer (5% skim milk in PBS) for 1 h at room temperature. For all membranes, protein signals were detected using an Odyssey imaging instrument and analyzed using instrument software (Li-Cor Biosciences).

NF-kB transcription factor assay

THP-1 cells were stimulated with a2NTD for various times. Nuclear fractions were isolated using NE-PER extraction reagent (Thermo Scientific) with 1× protease/phosphatase inhibitors (Thermo Scientific). Cytoplasmic fractions generated from the lysis procedure were used in immunoblots. The TransAM NF-kB Chemi kit was used following the manufacturer’s instructions (Active Motif, Carlsbad, CA). Briefly, 1 μg nuclear lysate was incubated with biontinylated or nonbiontinylated consensus probe (5′-GGGAGACCTCC-3′) or a scrambled probe for 1 h in a 96-well plate coated with streptavidin. Primary Abs specific for NF-kB proteins and secondary Ab were incubated for 1 h. Chemiluminescent detection was via plate reader (BioTek, Winooski, VT). Raji nuclear lysate was used as a positive control.

Cytokine, ATP, and endotoxin determinations

Coculture experiments were conducted as previously described (9). PBMCs, monocytes, and THP-1 cells were stimulated with or without a2NTD in 96-well polypropylene plates at 2–3×10^6 cells/well in triplicate. For TLRL4 and CD14 blocking Ab assays, PBMCs were preincubated with anti-TLRL4, anti-CD14, or relevant mouse isotype Ab for 1 h at 37°C before adding a2NTD or media for an additional 24 h. For PMB cultures, a2NTD or LPS was resuspended with PMB at 50 μg/ml for 1 h at 37°C. Then, the a2NTD/PMB and LPS/PMB mixtures were added to PBMCs for 24 h. The following concentrations were used for the indicated experiments: 25 μM/ml apyrase, 300 μM/oATP, 25 and 100 mM KC1, 1 mM vitamin C, and 50 mM N-acetylcysteine. Cell-free supernatants were collected and stored at −80°C until assayed. IL-1β was measured using ELISA (R&D Systems, Minneapolis, MN). A Milliplex Human Cytokine 42-plex panel was conducted by Millipore (St. Charles, MO) on supernatants from stimulated monocytes and assayed on a Luminex instrument (Luminex, Austin, TX). The panel included 42 cytokines, chemokines, and growth factors. ATP was measured via CLS II kit (Roche Applied Science). The Pyro-Gene Recombinant Factor C assay kit was used to determine the endotoxin concentration in a2NTD preparations (Lonza).

Intracellular ROS measurements

Two dyes were used to detect ROS: 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA): excitation wavelength, 504 nm; emission wavelength, 524 nm) and dihydroethidium (DHE; excitation wavelength, 518 nm; emission wavelength, 605 nm) (Invitrogen). Both dyes were reconstituted with DMSO. Monocytes and THP-1 cells were incubated with dye at 37°C for 30 min. Cell pellets were resuspended in media and incubated for 15 min at 37°C to allow esters to cleave CM-H2DCFDA to trap it inside the cell. Cells were then incubated with a2NTD, PMA, or media alone with or without 100 μM diethyldithiocarbamic acid sodium salt trihydrate from Enzo Life Sciences (Plymouth Meeting, PA). Two methods were used to detect intracellular...
ROS. After a2NTD stimulation, cells were analyzed for fluorescence via flow cytometer on an LSR II (BD Biosciences) or via a microplate reader in a 96-well black plate (BioTek).

Cell viability and cytotoxicity assays
PBMCs, primary monocytes, and THP-1 cells were incubated with a2NTD for 24 h. Cell viability was measured via two assays: an ATP and an NADH assay. Cellular toxicity was measured by using a live versus dead cell probe assay. All three kits were conducted following the manufacturer’s instructions (Promega, Madison, WI).

Statistical analysis
A Student paired t test was used to compare two groups. If more than two groups were being compared, then a repeated measures ANOVA was applied. Results were reported as the mean ± SEM, and significance is defined in figure legends. Results were analyzed using Sigma Plot 11 (Systat Software, San Jose, CA).

Results
a2V-ATPase utilizes microvesicles in IL-1β induction
a2V protein levels were determined in four human cancer cell lines: choriocarcinoma JEG, breast adenocarcinomas MDA and MCF, and ovarian adenocarcinoma TOV. HaCat cells, a spontaneously immortalized human keratinocyte line (19), were used as non-tumorigenic controls. a2V was expressed in all cell lines tested, but a2NTD could not be detected in any cell lysate (Fig. 1A). Both MCF and HaCat cells expressed relatively low concentrations of a2V, whereas MDA expressed the highest (Fig. 1B, Supplemental Table 1).

Because cell surface-localized V-ATPases correlated with invasive potential (8), we next examined the cell lines for surface expression of a2V. All cell lines expressed surface a2V to varying degrees (Fig. 1C). JEG had the highest and HaCat cells had the lowest a2V surface expression.

Earlier studies associated a number of immunological effects with a2V expression (20, 21) as well as with the recombinantly expressed peptide (10). In similar experiments, MDA cocultured with PBMCs induced the most IL-1β compared with the other cell lines; this correlated with the relatively high a2V expression in MDA cells (Fig. 1D, 1E). MCF induced the least amount of IL-1β, which correlated with its relatively low a2V expression (Fig. 1D, 1E). When anti-a2NTD Ab was added, IL-1β levels decreased significantly (Fig. 1E). HaCats induced relatively low levels of IL-1β, and this effect was not reduced significantly in the presence of anti-a2NTD, suggesting involvement of other secreted factors.

The inability to detect a2NTD in total cell lysates was inconsistent with the experiments that demonstrated the neutralization potential of anti-a2NTD Ab in cocultures. This led us to examine culture supernatants for a2NTD more closely. Our previous work suggested that recombinant a2NTD had limited solubility. We investigated whether a2NTD might be found in microvesicles, a form of extracellular trafficking comprised of plasma membrane blebs.
and exosomes (22, 23) that has been shown to regulate immunological activities (24) and tumor growth (25, 26). Microvesicles from all the cancer cell lines were positive for α2V and α2NTD (Fig. 1G, Supplemental Table 2). The cells lines that expressed higher levels of α2V (MDA, TOV, and JEG) had higher levels of α2NTD in their microvesicles, and the cell lines that had low expression of α2V (MCF and HaCat) had little to no α2NTD in microvesicles (Fig. 1H).

α2NTD upregulates gene expression and protein secretion of pro- and anti-inflammatory cytokines and chemokines

To further understand the types of genes affected by α2NTD, THP-1 cells, a human monocytic leukemia cell line commonly used as a model for monocytes, were tested via real-time PCR array after stimulation with α2NTD or media alone. Primary human monocytes were then used to confirm these changes in gene regulation at the protein level. To prove the changes in gene expression translated into secreted proteins, culture supernatants were collected from human monocytes stimulated with α2NTD or media alone and quantified via Luminex xMAP technology.

α2NTD triggered an appreciable fold induction of proinflammatory cytokine genes as early as 2 h for IL-1α (1.8-fold), IL-1β (2.5-fold), and IL-6 (3.5-fold) (Fig. 2A). In supernatants, α2NTD treatment induced a 19.6-fold increase in IL-1α, an 11.9-fold increase in IL-1β, and an 8.5-fold increase in IL-6 at 2 h (Fig. 2B).

**Figure 2.** Induction of cytokine and chemokine genes/proteins in THP-1 cells/monocytes treated with α2NTD. Cells were stimulated with or without α2NTD (100 ng/ml) and tested for differences in cytokines and chemokines after 2, 6, 12, and 24 h. Gene expression: expression levels were determined by real-time PCR array in THP-1 cells (2.5 x 10⁶). Fold change was calculated by dividing treated samples by media control samples for each time point (n = 3). Protein secretion: protein levels were measured in supernatants from monocytes (1 x 10⁶). Concentrations were calculated from standard curves, and fold changes were calculated by dividing concentrations from treated samples by media control samples for each time point (n = 3). A and B, Proinflammatory cytokines. C and D, Anti-inflammatory cytokines. E and F, Chemokines. IL1RA, IL-1R antagonist.

Similar to its respective PCR array data, IL-1β secretion increased over time. With regard to cytokines involved with a TAM-specific phenotype, there were no changes in IL-12 and IL-23 (Fig. 2B).

There was a peak in IL-10 gene (4.5-fold) and protein expression (16.0-fold) levels at 12 h (Fig. 2C, 2D). IL-1R antagonist showed no significant change in gene expression and was not found to be secreted to appreciable amounts in supernatants (Fig. 2C, 2D).

α2NTD induced the upregulation of many chemokines that mediate leukocyte recruitment to sites of inflammation and tumorigenesis. Most chemokines that showed increased gene expression levels came from two families: MCP and MIP (Fig. 2E). While the PCR array data indicated upregulation of both MCP and MIP genes, only the MIP-1 proteins were found in high concentrations after α2NTD stimulation (Fig. 2F). All fold induction values are listed in Supplemental Tables 3 and 4.

**Canonical NF-κB pathway is activated upon α2NTD stimulation**

Several genes from the PCR array are regulated by the transcription factor NF-κB, including IL-1R antagonist, IL-6, IL-10, and CCL2/3/4 (27). To determine whether α2NTD activated the NF-κB pathway, THP-1 cytoplasmic and nuclear lysates (Supplemental Fig. 1) were examined for NF-κB-related proteins after α2NTD stimulation.

Phosphorylated IκB kinase (IKK)β could be detected as early as 30 min poststimulation and decreased by 4 h (Fig. 3A). Phosphorylated IκBα was not detected. Phosphorylated IKKα could not be detected. Phosphorylated IκBo was...
and p100 are proteolytically processed to produce p50 and p52, respectively. Phosphorylation of RelA enhances its transcriptional activity (28), and there was an appreciable increase in phosphorylated RelA starting at 30 min (Fig. 3C). There were only minor increases in total protein levels for p100 and c-Rel. p52 could only be detected faintly at 24 h.

The induction of IkBα degradation by a2NTD facilitates the migration of NF-κB members into the nucleus where they can activate transcription. There was a statistically significant increase in p50 (2 h), p65 (1 h), and RelB (4 h) in nuclear lysates when compared with the 0 time point (Fig. 3D, Supplemental Fig. 2). These time points correlated with the array data where increased gene expression was seen at 2 h. The data showed a2NTD signaling used the canonical NF-κB pathway due to the increased levels of active RelA and p50. Whereas active RelB was upregulated to the nucleus, p52 was not, which indicated that noncanonical NF-κB pathway (29) was not participating in a2NTD signaling.

**FIGURE 3.** NF-κB is involved in a2NTD-induced gene upregulation. THP-1 cells (4 × 10⁶/8 ml) cultured for 24 h under serum-free conditions were stimulated with a2NTD (100 ng/ml) for indicated times. Cytoplasmic fractions were immunoblotted for (A) IKKα, IKKβ, and phosphorylated IKKα/β, (B) IκBα and phosphorylated IκBα, and (C) NF-κB members RelA, phosphorylated RelA, RelB, c-Rel, p105/p50, and p100/p52. Representative blots from n = 3. D, Nuclear fractions were tested for activated NF-κB members, which are capable of binding a DNA probe. Lysate/probe mixtures were loaded onto 96-well plates and incubated with Abs for specific NF-κB proteins. Positive wells were detected by chemiluminescence (n = 3, mean ± SEM). *p < 0.005; **p < 0.001 compared with the 0 time point for each respective NF-κB member.

detected at ~1 h, which increased until 4 h (Fig. 3B). Total IκBα protein expression levels dropped by 30–40% after 5 min of a2NTD stimulation (Fig. 3B).

The NF-κB family is comprised of five proteins: RelA/p65, RelB, c-Rel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52). p105 and p100 are proteolytically processed to produce p50 and p52, respectively. Phosphorylation of RelA enhances its transcriptional activity (28), and there was an appreciable increase in phosphorylated RelA starting at 30 min (Fig. 3C). There were only minor increases in total protein levels for p100 and c-Rel. p52 could only be detected faintly at 24 h.

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**TLR4 and CD14 are involved with a2NTD-induced IL-1β secretion**

To further investigate the mechanism of how a2NTD induces IL-1β secretion, monocytes were cultured with increasing concentrations of a2NTD and for different time points. a2NTD increased IL-1β secretion in a dose-dependent (Fig. 4A) and time-dependent manner (Fig. 4B). The induction of IL-1β secretion could be abrogated by blocking mAbs against TLR4 and CD14 (Fig. 4C). TLR4 and CD14 are well-characterized receptors for endotoxin, and thus several experimental controls were conducted to demonstrate the IL-1β inducing activity was specific to a2NTD.

The endotoxin content of a2NTD preparations was found to be 5.2 ± 0.12 milli-endotoxin units/μg a2NTD or 0.52 ± 0.012 pg/μg a2NTD by the Limulus assay. This is within the reported range of commercially available recombinant proteins. Monocytes stimulated with LPS at a concentration that far exceeded that found in a2NTD doses induced far less IL-1β secretion (Fig. 4D). To further address endotoxin contamination, monocytes were cultured with PMB, an antibiotic whose actions include binding and inactivating LPS. PMB-treated LPS no longer induced secretion of IL-1β from monocytes, but PMB-treated a2NTD induced the same amount of IL-1β as did untreated a2NTD (Fig. 4E).

Previous data had already shown that depletion of a2NTD by immunoadsorption on anti-a2NTD beads removed the activity from the solution (10). Finally, there was a complete reduction of IL-1β secretion after a2NTD stimulation. a2NTD demonstrated a dose-dependent release of ATP after 4 h, which correlated with IL-1β concentrations in the culture supernatants (Fig. 5A, 5B).

Extracellular ATP has been shown to be a key player in IL-1β secretion (30). A study by Ferrari et al. (31) demonstrated that primary human monocytes have the ability to release endogenous ATP. We wanted to determine whether this endogenous release of ATP was responsible for IL-1β secretion after a2NTD stimulation. Monocytes and THP-1 cells stimulated with increasing doses of a2NTD demonstrated a dose-dependent release of ATP after 4 h, which correlated with IL-1β concentrations in the culture supernatants (Fig. 5A, 5B).

Two inhibitors were employed to determine whether ATP was required for a2NTD-induced IL-1β secretion: apyrase, an enzyme that degrades ATP to ADP/AMP, and oATP, an antagonist of P2X7 receptors. After 4 h, supernatants from cells treated with apyrase,
oATP, and a combination of the two showed a significantly decreased amount of IL-1β, demonstrating the importance of ATP (Fig. 5C).

Binding of ATP to the P2X7 ion channel allows potassium efflux down its concentration gradient (32). Monocytes and THP-1 cells were cultured in media that contained high concentrations of potassium, levels that reached intracellular potassium stores. After 4 h, there was decreased IL-1β in culture supernatants (Fig. 5D).

The relationship between ATP and IL-1β has been shown to involve the activation of the NLRP3 inflammasome in monocytes and macrophages (33). Activated NLRP3 inflammasome cleaves caspase-1 into its active form, which then cleaves pro-IL-1β into mature IL-1β. With this in mind, monocytes and THP-1 cells were cultured with different doses of a caspase-1 inhibitor for 1 h prior to the addition of a2NTD. After 24 h, we saw a dose-dependent decrease in IL-1β in both cell types (Fig. 5E). These findings

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**FIGURE 4.** a2NTD induces IL-1β secretion in a TLR4- and CD14-dependent manner. IL-1β was assayed in culture medium by ELISA after the following treatments: (A) monocytes isolated by negative selection were treated with increasing concentrations of a2NTD and supernatants collected after 24 h or (B) a dose of 100 ng/ml and supernatants collected over time. C, Monocytes were pretreated with blocking Abs TLR4 or CD14 or isotype control (20 μg/ml) for 1 h before addition of 10 ng/ml a2NTD for 24 h. Concentrations were normalized to a2NTD treatment alone. D, Monocytes were treated with a2NTD or LPS for 24 h. E, a2NTD (20 ng/ml) or LPS (40 pg/ml) were preincubated with PMB for 1 h before stimulating monocytes for 24 h. F, Monocytes were treated with 100 ng/ml a2NTD or heat-inactivated a2NTD (15 min at 80°C) for 24 h (n = 4 for A–C, E, F; n = 10 for D; mean ± SEM). *p ≤ 0.05, **p ≤ 0.001 compared with a2NTD treatment alone.

**FIGURE 5.** P2X7 channel is important in a2NTD-induced IL-1β secretion. A, Monocytes and (B) THP-1 cells were stimulated with indicated a2NTD concentrations for 4 h. ATP (by chemiluminescence) and IL-1β (by ELISA) were assayed in culture medium. C–E, IL-1β was assayed by ELISA after treating monocytes and THP-1 cells for 4 h with media or a2NTD (100 ng/ml) alone or with the following: (C) a2NTD plus apyrase, oATP, or a combination of apyrase/oATP; (D) a2NTD plus KCl (25 mM) or KCl (100 mM), or (E) a2NTD plus increasing concentrations of caspase-1 inhibitor Ac-WEHD-CHO. Values are represented as a percentage of a2NTD treatment alone (C–E) (n = 6 for both monocytes and THP-1 cells, mean ± SEM). *p ≤ 0.01, **p ≤ 0.001 compared with a2NTD treatment alone.
indicate ATP and the P2X7 channel are involved in activating the inflammasome and a2NTD-induced IL-1β secretion.

**ROS generation upon a2NTD stimulation is important for IL-1β secretion**

How the activation of the NLRP3 inflammasome leads to the production of IL-1β is unknown. One theory is ATP signaling through the P2X7 ion channel, where pore formation is the means by which NLRP3 activators gain access into the cytoplasm. A second model suggests signaling via a common metabolite, specifically ROS, in response to inflammasome activators that interact with a cell surface receptor (33).

We wanted to know whether a2NTD induced ROS production. There was an increase in super oxide anion (Fig. 6A) and hydrogen peroxide/hydroxyl radicals (Fig. 6B) after a2NTD treatment in monocytes. Fluorescence increased over time starting at 30 min and decreased in the presence of antioxidants, N-acetylcyesteine, and vitamin C (Fig. 6C). There was a significant decrease in IL-1β secretion (Fig. 6D) when monocytes were treated with antioxidants in combination with a2NTD. For reasons that are not clear, we could not detect ROS in THP-1 cells (Supplemental Fig. 3), although the cells did respond to antioxidant treatments in a similar manner to monocytes (Fig. 6D). These data suggest that a2NTD could have a direct effect on free radical formation and that ROS are linked to a2NTD-induced IL-β secretion.

**Discussion**

CRI is driven mainly by cells of the innate immune system, predominantly TAMs (2, 34). Previous reports point to the importance of V-ATPases in tumor progression and migration (6, 8, 35). Other investigators have described the fundamental role of V-ATPase during cytokine trafficking and secretion (36–38). Our studies bridge the gap between these two research areas by showing that a peptide signal of V-ATPase origin participates in the induction of an inflammatory response from monocytes.

We show that incubation of monocytes with a2NTD leads to upregulation of several genes/proteins involved in M2 polarization. a2NTD induces an M2-like phenotype in monocytes (Fig. 2) described as IL-12low, IL-23low, and IL-10high (16). The increased levels of IL-10 correspond to the significant levels of p50 in the nucleus after a2NTD stimulation (Fig. 3D). Whereas p50 homodimers are traditionally associated with transcriptional repression (39), p50 induces IL-10 transcription by binding the IL-10 promoter in conjunction with another transcription factor, CREB-binding protein (40). In contrast, the transcription of IL-12p40 and p35 is activated by c-Rel (40). Both IL-12 and c-Rel were not upregulated in response to a2NTD (Fig. 3D). Additionally, p50 homodimers are responsible for the IL-10high/IL-12low M2 phenotype (41).

The low levels of IL-12 and IL-23 and the increased levels of IL-6 after a2NTD stimulation suggest that a2NTD is skewing monocyte differentiation toward a TAM phenotype at the expense of dendritic cell activation and maturation (42, 43). Additionally, the high levels of IL-10 can produce a tumor-specific anergy in dendritic cells (44). Immunological rescue of blocked dendritic cells has been shown with cytokines IL-4 and IL-13 (45). a2NTD did not significantly upregulate these cytokines.

Interestingly, inflammatory cytokine production is associated more with the M1 phenotype, whereas the M2 cells show differential regulation of members of the IL-1 system (2). The typical M2 macrophage phenotype of IL-12low/IL-12high also includes a lack of proinflammatory cytokine (IL-1, IL-6, and TNF) expression. An exception is found in macrophages exposed to immune complexes and TLR ligands, which retain their ability to secrete inflammatory cytokines in combination with high IL-10 and low IL-12 production (46). In our system, the release of a2NTD molecules from tumor cells is associated with an upregulation in IL-1α/β gene and protein expression with no change in expression of IL-1R antagonist (Fig. 2A–D). To further elaborate on this finding, our experiments demonstrate monocytes responding in a dose-dependent manner to a2NTD by secreting IL-1β, which was not due to contaminating LPS (Fig. 4D, 4E). IL-1β secretion has also been linked to a form of inflammatory cell death termed pyroptosis (47, 48). a2NTD does induce inflammatory cytokines, but there is no change in cell viability and cytotoxicity after a2NTD stimulation (Supplemental Fig. 4).

Several reports suggest that TNF-α and IL-1β have roles in tumorigenesis (49–53). In the case of TNF-α, low levels have been shown to trigger prosurvival signals in neoplastic cells (54), whereas high doses of TNF-α are being used to treat solid tumors (55). IL-1β is highly expressed in primary tumors, including
colorectal adenocarcinoma and melanoma, as well as being constitutively expressed in tumor cell lines (52). One of the effects of upregulated IL-1 expression is to increase endothelial cell permeability, an early sign of angiogenesis (52). a2NTD upregulated both of these inflammatory cytokines. Whereas there is an increase in TNF-α 24 h after a2NTD stimulation, IL-1β is more than twice the level of gene induction and three times the level of protein secretion compared with TNF-α (Fig. 2A, 2B). The effect of a2NTD on cytokine production to include both pro- and anti-inflammatory cytokines indicates that there is a fine balance between protumoral versus antitumoral signals expressed by TAMs in response to a2NTD. This is additional evidence of a2NTD shifting the focus of monocytes away from creating a tumoricidal environment and instead inducing monocytes to create a low-grade smoldering inflammation that benefits tumor survival.

We also show the importance of ATP, potassium efflux, and ROS in relation to a2NTD-dependent IL-1β secretion (Figs. 5, 6). Taken together, a2NTD most likely activates the IL-1β processing machinery, the inflammasome (33). a2NTD is a host-derived molecule that upregulates cytokines, and therefore would be characterized as containing a danger-associated molecular pattern. We believe a2NTD activates the NLRP3 inflammasome because NLRP3 responds to an array of structurally different molecules and is the most characterized inflammasome that responds to danger-associated molecular patterns (33). Receptors that detect danger-associated molecular patterns include the family of TLRs (56); we demonstrate TLR4 and CD14 to be possible receptor(s) for a2NTD (Fig. 4C). We are investigating further the specific receptors for a2NTD on monocytes.

There are no detectable levels of a2NTD in cell lysates, but a2NTD is found in microvesicular fractions isolated from culture supernatants (Fig. 1F). The trafficking of a2NTD into microvesicles coincides with the finding of increased lysosomal enzymes in the extracellular environment (57). We speculate that these enzymes, specifically the cathepsins, are responsible for cleaving a2NTD from a2V. Although we are not discounting other proteases such as MMPs, it is interesting that V-ATPases are responsible for acidifying lysosomal contents (58) and may direct lysosomal trafficking to the surface. Further studies are under way to characterize the physical properties of a2NTD that are responsible for this pattern of cleavage and trafficking and what enzyme(s) participate in cleaving the peptide.

The regulation of V-ATPase trafficking to the plasma membrane in tumors is also unknown. We postulate that the tumor microenvironment, which is described as acidic and hypoxic (59), plays a role in V-ATPase localization. Under these stressful conditions, a2V is sorted to the cell surface at the same time tumors upregulate cytokine secretion and microvesicle generation (60, 61). Consequently, a2NTD is cleaved from the protein into a microvesicle that is recognized by TAMs in the surrounding tissue. The outcome of TAM signaling by a2NTD is in preventing dendritic cells from recognizing the tumor and creating a proinflammatory environment to help in tumor growth.

V-ATPases have already been described as novel targets for anticancer therapeutics (62). In these models, V-ATPases are inhibited directly with drugs that block proton pumping activity. We suggest intervening at another target by blocking V-ATPase induction of an immune tolerogenic state during oncogenesis via a2NTD. More excitingly, a2NTD represents a new biomarker that has the potential to be linked to clinical prognosis and drug development.

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Disclosures

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References


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