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Granzyme B Expression Is Enhanced in Human Monocytes by TLR8 Agonists and Contributes to Antibody-Dependent Cellular Cytotoxicity

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FcγRs are critical mediators of mAb cancer therapies, because they drive cytotoxic processes upon binding of effector cells to opsonized targets. Along with NK cells, monocytes are also known to destroy Ab-coated targets via Ab-dependent cellular cytotoxicity (ADCC). However, the precise mechanisms by which monocytes carry out this function have remained elusive. In this article, we show that human monocytes produce the protease granzyme B upon both FcγR and TLR8 activation. Treatment with TLR8 agonists elicited granzyme B and also enhanced FcγR-mediated granzyme B production in an additive fashion. Furthermore, monocyte-mediated ADCC against cetuximab-coated tumor targets was enhanced by TLR8 agonist treatment, and this enhancement of ADCC required granzyme B. Hence we have identified granzyme B as an important mediator of FcγR function in human monocytes and have uncovered another mechanism by which TLR8 agonists may enhance FcγR-based therapies.

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Monoclonal Abs directed against tumor-expressed Ags have proved to be useful agents against a variety of tumor types including B cell lymphomas, colon carcinomas, and breast cancer. However, the low rates of complete remission and the relatively high relapse rate both suggest that there is still a need for improvement (1). Monocytes and macrophages are known for their ability to phagocyte IgG-opsonized infectious particles (2) and are also major mediators in the destruction of tumor cells (3, 4).

Responses to Ab-coated targets are largely mediated by the FcγRs (5). Human monocytes and macrophages express at least four different functional FcγRs: FcγRI, FcγRIIA, FcγRIIB, and FcγRIIIA (6). Of these, FcγRI, FcγRII, and FcγRIIIA are activating receptors that either contain a cytoplasmic ITAM, as in the case of FcγRIIa, or associate with the γ-subunit homodimer that has an ITAM (6). Association with the γ-subunit is critical for both the signaling and surface expression of FcγRI and FcγRIIa. For example, mice deficient in γ-subunit expression do not express any activating FcγR on their immune cells (7). Conversely, FcγRIIB is an inhibitory receptor with an ITIM rather than an ITAM (8). Signaling events downstream of activating FcγR lead to proinflammatory responses in addition to phagocytosis such as cytokine (9) and superoxide (10) production. Hence monocytes/macrophages play the dual role of fighting against opsonized targets and activating other immune cells.

Granzymes have been shown to be critical effectors of T and NK cell cytolytic immune responses (11). Within the context of antitumor immunity, granzyme B has been shown to elicit tumor cell apoptosis (12), although it is now known that many tumors evade granzyme-mediated death by expressing inhibitory factors such as Serpin B9 (13). The most well-known function of granzyme B is that of activating proapoptotic pathways within target cells via cleavage of BID, as well as procaspases 3, 7, 8, and 10, but this enzyme has also been found to cleave other targets such as cytoskeleton-related proteins, growth/survival receptors, and DNA Fragmentation Factor (12). In addition to these cell-damaging activities, granzyme B is also capable of enhancing proinflammatory responses by cleavage of pro–IL-18 (14) and IL-1α (15). Although cytotoxic T and NK cells are the predominant cell types that produce granzyme B, it has been shown that many other immune and nonimmune cells including activated macrophages can produce and secrete this enzyme (16).

TLRs are innate immune sensors located primarily either on the cell surface or within endosomes, where they detect components of bacteria and viruses such as LPSs, peptidoglycans, unmethylated DNA, dsRNA, and ssRNA (17). Within the context of cancer therapy, treatment with TLR agonists such as imidazoquinolines (18, 19) and unmethylated DNA (CpG) (20) has been studied. The potential of such TLR agonists to drive proinflammatory signaling within immune cells and potentially combat the immunosuppressive...
influences of tumors offers great promise, although it has also been shown that TLR-driven inflammatory signals may also promote tumor growth (21).

Previously we have shown that the TLR7/8 agonist R-848 could modulate monocyte FcγR expression and enhance the antitumor effects of tumor-directed mAbs (22). In an effort to understand in greater depth the biologic effects of TLR7 and TLR8 agonists on monocytes within the context of FcγR signaling, we found that TLR8, but not TLR7, agonists elicited the production of granzyme B. Furthermore, FcγR activation also led to granzyme B production, and the simultaneous activation of FcγR and TLR8 resulted in additive effects on monocyte granzyme B production. Finally, we also found that granzyme B was responsible for a significant portion of monocyte-mediated Ab-dependent cellular cytotoxicity (ADCC), and that inhibition of granzyme B nullified the increased ADCC seen with TLR8 agonist treatment. Hence granzyme B production represents a novel mechanism by which monocytes can target Ab-coated tumor cells, and this can be enhanced through the use of TLR8-selective agonists.

Materials and Methods

Abs and reagents

CL075 (TLR8-selective agonist) (23, 24) was purchased from Invivogen (San Diego, CA). The TLR7-selective agonist VTX-2337 (25) was provided by VentiRx Pharmaceuticals (Seattle, WA). 3M-055 (TLR7-selective agonist) was provided by 3M (Minneapolis, MN). Z-AAD-CMK (TLR2 agonist) was purchased from Enzo Life Sciences (Farmingdale, NY). Pam3CSK®4 (TLR2 agonist) was purchased from Invivogen (Carlsbad, CA). Polynosinic-polycytidylic acid (TLR3 agonist) and recFLA-ST (TLR5 agonist) were obtained from Invivogen and CPG oligonucleotide (TLR9 agonist) from Invivogen. LPS from Escherichia coli strain 0127:B8 (TLR4 agonist) was obtained from Sigma-Aldrich (St. Louis, MO). Brefeldin A (BFA) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The TLR8-selective agonist VTX-2337 (25) was purchased from Enzo Life Sciences (Farmingdale, NY). Pam3CSK®4 (TLR2 agonist) was purchased from Invivogen (Carlsbad, CA). Polynosinic-polycytidylic acid (TLR3 agonist) and recFLA-ST (TLR5 agonist) were obtained from Invivogen and CPG oligonucleotide (TLR9 agonist) from Invivogen. LPS from Escherichia coli strain 0127:B8 (TLR4 agonist) was obtained from Sigma-Aldrich (St. Louis, MO). Brefeldin A (BFA) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cross leukopacks via Ficoll centrifugation (Mediatech, Manassas, VA) were purchased from Invitrogen. Reverse transcriptase, random hexamers, and SYBR Green PCR mix were purchased from Applied Biosystems (Foster City, CA). pNF-κB Ab for Western blotting was purchased from Cell Signaling Technology (Beverly, MA), and anti-Serpin B9 was from Abcam (Cambridge, MA). Abs against actin and GAPDH, as well as all HRP-conjugated secondary Abs, were from Santa Cruz Biotechnology.

Peripheral blood monocyte isolation

Peripheral blood monocytes (PBMs) were isolated from deidentified Red Cross leukopacks via Ficoll centrifugation (Mediatech, Manassas, VA) followed by CD14+ selection using MACS (Miltenyi Biotec, Cambridge, MA). PBMs were resuspended in RPMI 1640 containing 10% heat-inactivated FBS (HyClone, Logan, UT), penicillin/streptomycin, and 1-glutamate (Invitrogen). The purity of monocytes obtained was >97%, as determined by flow cytometry with CD14 Ab.

Western blotting and ELISAs

Western blots were done as described previously (22). In brief, cells were lysed in TEN buffer (50 mM Tris [pH 8.0], 10 mM EDTA, 10 mM Na3P2O7, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM Na2VO4, 10 μg/ml each aprotinin and leupeptin). Postnuclear protein-matched lysates were boiled in Laemmli sample buffer and separated by SDS-PAGE, transferred to nitrocellulose membranes, probed with the Ab of interest, then developed with Pierce ECL 2 Western blotting Substrate (Thermo Scientific, Rockford, IL). Cell supernatants for ELISAs were collected and centrifuged at 16,000 × g to clear cellular debris; cell lysates were prepared by lysing cells with radioimmunoprecipitation assay buffer (20 mM EDTA, 20 mM Na3P2O7, 20 mM NaF, 0.5% C24H39NaO4, 0.1% SDS, 1% Triton X-100 in 1× TBS along with protease inhibitors), followed by centrifugation. ELISAs were done according to the respective manufacturer protocols: human TNF-α, IL-6, IL-12/IL-23 p40, and IFN-γ (R&D Systems, Minneapolis, MN); human granzyme B (eBioscience, San Diego, CA); and human Perforin-1 (Abcam, Cambridge, MA).

Microarrays

Microarray analysis was performed as previously described (26). In brief, PBMs (n = 3 donors) were isolated as described previously and treated overnight with or without TLR7- or TLR8-selective agonists (3M-055 and VTX-2337, respectively) at 1 μM. RNA was extracted from PBMs using TRIzol, purified using an RNeasy Mini Kit (Qiagen, Valencia, CA), then labeled and hybridized to Affymetrix (Santa Clara, CA) hgu133plus2 chips according to manufacturer instructions at The Ohio State University Wexner Medical Center Microarray-Genetics core facility. Resulting data

![FIGURE 1. TLR8 agonist treatment upregulates granzyme B in PBMs.](http://www.jimmunol.org qua.)
files were analyzed with R (27) and BioConductor (28), using the “limma” package (29) to identify differentially expressed genes. Array data have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE64480.

**Real-time RT-PCR**

Cells were lysed in TRIzol reagent (Invitrogen/Life Technologies, Carlsbad, CA), and RNA isolation was completed according to the manufacturer’s instructions. Reverse transcription was done with 10–100 ng total RNA. The cDNA was run in triplicate for each donor on an Applied Biosystems Step One Plus system, with automatically calculated thresholds. Relative expression was calculated as 2^{\Delta\Delta Ct}, with \Delta Ct calculated by subtracting the average Ct of two housekeeping controls (CAP-1 and GAPDH) from the Ct of the transcript in query (30). Primer sequences used to amplify cDNA from human PBMs were as follows: TNF-α (forward 5'-GCT TGT TCC TCA GCC TCT TCT-3', reverse 5'-GTT TTG GTA TCG TGG AAG GAC T-3'); IL-6 (forward 5'-CAC AGA CAG CCA CTC ACC TC-3', reverse 5'-TCT GCT GCC AGT GCC TCT TT-3'); IL-12p40 (5'-GTA GAG GCA GGG ATG ATG TTC T-3') and GAPDH (forward 5'-AGC CAT CAC TTG GAT GAG G-3', reverse 5'-TCC TTT AAG GGG CTA-3'). Primer sets for granzyme B (forward 5'-TAC ACC AGC GGG GCC TTA GGT TG-3', reverse 5'-AGC GCA CAG CCA ACT T-3'), Perfomin-1 (forward 5'-TGT ACG AGT GCC TCA GAG AGC-3', reverse 5'-GAT ATG CAC CAG GCA AAA AAT TC-3'), and Serpin B9 (forward 5'-GGT TTG CTA CAA CAT GGG G-3', reverse 5'-AAA GAC AGT GCC TCT AG-3') were designed by the Ohio State University Wexner Medical Center. Primer sets for granzyme B (forward 5'-TAC ACC AGC GGG GCC TTA GGT TG-3', reverse 5'-GAT ATG CAC CAG GCA AAA AAT TC-3'), and Serpin B9 (forward 5'-GGT TTG CTA CAA CAT GGG G-3', reverse 5'-AAA GAC AGT GCC TCT AG-3') were designed using National Center for Biotechnology Information’s primer BLAST and ordered from Invitrogen.

**Flow cytometry**

R-PE-labeled anti–granzyme B (clone GB12) and its isotype control were purchased from Invitrogen. FITC-labeled anti-CD14 and its isotype control were purchased from BD Pharmingen. Three hours before staining, 10 μl/ml BFA was added to the cells. Intracellular staining was performed using fixation and permeabilization buffer from BD Biosciences (San Jose, CA) as per the manufacturer’s instructions. In brief, cells were harvested after BFA treatment and counted to 3 × 10^6 cells/sample. Then cells were washed and blocked with 10% goat serum + 1% FBS for blocking FcRs. Anti-CD14 and its isotype were added and incubated for 30 min at 4°C; then cells were washed with PBS. Cells were fixed and permeabilized by incubating in Cytofix/Cytoperm solution for 20 min at 4°C, followed by washing two times with Perm/Wash buffer. Cells were incubated with anti-granzyme B and its isotype in Perm/Wash buffer for 1 h at 4°C, washed two times in Perm/Wash buffer, and resuspended in 1% paraformaldehyde.

**Fixed Ab-coated target cells**

The MDA-MB-468 breast cancer cell line was obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin/streptomycin, and L-glutamate. These cells were incubated on ice for 2 h with or without clinical-grade cetuximab or rituximab (negative control). After three washes in ice-cold PBS, Ab-coated cells were fixed with 1% paraformaldehyde at room temperature for 20 min. Repeating three washes in ice-cold PBS, Ab-coated fixed cells were resuspended in RPMI 1640 supplemented with 10% FBS, penicillin-streptomycin, and L-glutamate. Target cells were added to the PBMs at an E:T cell ratio of 3:2 and incubated at 37°C for 16 h. Cell-free supernatants were collected for subsequent experiments and analyses.

**ADCC**

ADCC assays were done as previously described (22). In brief, PBMs were isolated and incubated overnight with or without 1 μM VTX-2337. The

![FIGURE 2](http://www.jimmunol.org)  Concentration response and time course of TLR8-induced granzyme B production. (A–C) PBMs (n = 3) were incubated overnight with 0, 0.01, 0.1, 1.0, or 10.0 μM of the TLR8-selective agonist CL075. Plotted are transcript levels as measured by RT-PCR (A), and protein levels measured by ELISA in cell lysates (B) and supernatants (C). (D–F) PBMs (n = 3) were incubated for 0, 1, 3, 6, 16, or 24 h with 1 μM CL075; then granzyme B expression was measured by RT-PCR (D) and by ELISA in cell lysates (E) and supernatants (F). Error bars represent SD.
next day, monocytes were counted and plated in V-bottom 96-well plates with
51Cr-loaded MDA-MB-468 cells incubated with no Ab, negative-control rituximab or with cetuximab, and with or without 100 μM Z-AAD-CMK for each condition. The ratio of effector monocytes to target MDA cells was 25:1. After 20 h of coincubation, levels of 51Cr in supernatants were measured using a gamma counter. The percent cytotoxicity was calculated as (sample − minimum)/(maximum − minimum) × 100, where minimum was measured as target cells incubated with no Ab or effector cells, and maximum was measured as target cells that had been lysed with 10% SDS. Cytotoxicity percentages for rituximab-treated samples (negative-control Ab treatment) were subtracted from their corresponding cetuximab-treated samples to represent Ab-dependent cytotoxicity.

Statistical analyses

Data were analyzed by mixed-effect model, incorporating observational dependencies within a donor (31). Hypothesis testing of synergistic (Fig. 6) and inhibitory effects (Figs. 4 and 5) was tested by interaction contrasts. Multiplicities were adjusted by Holm’s method (32). For other experiments with only two groups involved, Student t tests (paired or unpaired, as appropriate) were used to test for statistically significant differences.

Results

**TLR7 and TLR8 agonists elicit differential responses**

It has previously been shown that human PBMCs respond differently to TLR7, TLR8, and TLR9 agonists (33). In this study, we examined the effects of TLR7 versus TLR8 agonists on monocyte activation. We treated human PBMs overnight with 3M-055 (TLR7-selective agonist) or with VTX-2337 (TLR8-selective agonist) and compared transcriptional responses with untreated monocytes using microarrays. Results showed that both agonists led to significant changes in inflammatory response transcripts, but that the TLR8 agonist elicited quantitatively and qualitatively greater responses (Supplemental Figs. 1A, 1B). Further, to verify that the differences seen were qualitative and quantitative, we identified differential expression of transcripts upregulated uniquely by TLR7 agonist treatment in PBMs from additional donors including protein kinase C α, kinase suppressor of Ras 1, and MAPK 1 (Supplemental Fig. 2A–C).

Of particular interest, granzyme B was among the genes up-regulated by TLR8 but not TLR7 agonist treatment (Fig. 1A), which we verified at the transcript and protein levels (Fig. 1B, 1C). Next, to use an independent measure of TLR8-mediated granzyme B production, we treated monocytes overnight with or without TLR8 agonist, incubated the cells for an additional 3 h with BFA to prevent secretion, and then performed intracellular staining for granzyme B. To verify that the cells were monocytes, we costained and gated for CD14 expression. Results from flow cytometry showed a distinctive shift in granzyme B in monocytes treated with the TLR8-selective agonist (Fig. 1D, 1E).

**TLR8-mediated granzyme B production is concentration and time dependent**

Our original choice of TLR8 agonist dosage stemmed from our earlier study with the TLR7/8 dual-agonist R-848, where we found that 1 μM was sufficient to elicit changes in FcγR (22). To identify the optimal concentration for inducing the expression of granzyme B, we treated monocytes overnight with concentrations from 0.01 to 10.0 μM TLR8 agonist. RT-PCR showed that 1 μM was the optimal concentration (Fig. 2A). ELISAs showed similar results, with 1 μM leading to increases in both secreted and intracellular granzyme B (Fig. 2B, 2C).

Next, we examined the kinetics of granzyme B production at both the RNA and the protein levels. We treated monocytes for 1, 3, 6, 16, and 24 h with 1 μM TLR8 agonist and measured both

**FIGURE 3.** NF-κB is required for TLR8-mediated granzyme B production. PBMs (n = 3) were pretreated with or without the NF-κB inhibitor, BAY 11-7085 (BAY) for 30 min or with DMSO control, then treated for 16 h with or without 1 μM CL075. Granzyme B transcript levels were measured by RT-PCR (A), and protein levels in cell lysates (B) and supernatants (C) measured by ELISA. Western blotting was performed using cell lysates to measure phospho-p65. Levels of TNF-α were measured by ELISA. *p < 0.05.
transcript and protein of granzyme B. Transcript reached a peak in as little as 3 h and then decreased, but remained well greater than basal levels at 16 and 24 h (Fig. 2D). However, granzyme B protein was still low at 3 h and steadily increased to the 24-h time point for both intracellular (Fig. 2E) and secreted (Fig. 2F) enzyme.

**NF-κB activation is required for monocyte granzyme B production**

Because TLR8 stimulation activates NF-κB, we next tested whether inhibition of NF-κB would prevent TLR8-mediated granzyme B production in monocytes. We pretreated monocytes with the NF-κB inhibitor BAY-11-7085 and then incubated them overnight with TLR8 agonist. Results showed that blockage of NF-κB prevented induction of transcript (Fig. 3A) and protein (Fig. 3B, 3C) of granzyme B. Western blotting was done to verify inhibition of NF-κB by analyzing p65 phosphorylation (Fig. 3D). NF-κB inhibition by BAY-11-7085 was also confirmed by measuring the production of TNF-α, a known NF-κB–dependent cytokine (Fig. 3E).

**TLR4 and TLR8 both drive granzyme B production in human monocytes**

It has previously been shown that *E. coli* LPS, as well as a bacterial lysate, could increase transcript of granzyme B in monocytes (34). In addition, granzyme B and Perforin-1 have been found in Kupffer cells of hepatitis patients (35). Hence it was important to identify which other TLRs apart from TLR8 were capable of driving its expression. We treated monocytes overnight with agonists for TLRs 2, 3, 4, 5, 7, 8, and 9 and measured granzyme B production by ELISA. Results showed that agonists for TLR4 and TLR8 led to the greatest levels (Fig. 4A), which closely corresponded to levels of TNF-α production (Fig. 4B). We did not observe any additive or synergistic effects from combining TLR4 and TLR8 agonists, because granzyme B levels did not increase beyond what was seen with TLR8 agonist treatment alone (Supplemental Fig. 3). Because of the close correspondence between TNF-α and granzyme B, however, we hypothesized that TNF-α and perhaps other secreted factors were acting in an autocrine/paracrine fashion to drive granzyme B production in monocytes.

**Granzyme B induction is indirect and requires the secretion of TLR8-induced factors**

Because TLR8 agonist treatment leads to the production and secretion of numerous chemokines/cytokines (23), we next tested whether the secretion of such factors was required for granzyme B induction. For this, we used BFA, which blocks secretion but not production of proteins by disrupting the Golgi complex (36, 37). Monocytes were pretreated with BFA, followed by incubation overnight with TLR8 agonist. As shown in Fig. 5A, BFA blocked the transcription of granzyme B in response to TLR8 agonist. Likewise, granzyme B protein in both supernatant (Fig. 5B) and cell lysate (Fig. 5C) was reduced. We also examined secretion of TNF-α as a control and found that, as expected, BFA blocked secretion but not transcription of TNF-α after incubation with TLR8 agonist (Fig. 5D, 5E, respectively). This suggests that one or more factors act in an autocrine/paracrine fashion to elicit granzyme B production after TLR8 agonist treatment.

**IL-12 is required for TLR8-mediated granzyme B production**

Although several cytokines were upregulated by TLR7 and TLR8 agonists, microarray analysis showed that many were significantly higher with TLR8 activation. These included TNF-α, IL-6, IFN-γ, and IL-12 p40 (Fig. 6A, 6D, 6G, 6I, respectively). Array results were verified using RT-PCR (Fig. 6B, 6E, 6H, 6K, respectively) and with ELISAs (Fig. 6C, 6F, 6L, respectively).

**TLR8-mediated granzyme B production enhances monocyte ADCC**

We have previously reported that the TLR7/8 dual agonist R-848 could enhance the ability of monocytes to lyse Ab-coated tumor cells in vitro, and microarray results from that study (22) showed an increase of 7.3-fold in granzyme B transcript. Results in this study suggest that it was the TLR8-activating quality of R-848 that led...
to granzyme B production, so we next asked whether granzyme B may be a mechanism by which TLR8 agonists could enhance ADCC. To test this, we treated monocytes with or without TLR8 agonist and concurrently incubated them with paraformaldehyde-fixed MDA-MB-468 tumor cells coated with no Ab, rituximab (negative-control Ab), or cetuximab. After overnight incubation, we measured granzyme B in cleared supernatants and found that both cetuximab (positive-control Ab) and TLR8 agonist treatment significantly enhanced the secretion of granzyme B (Fig. 8A). The combination of cetuximab plus TLR8 agonist led to an additive enhancement of granzyme B production (Fig. 8A). This suggests that monocytes can produce granzyme B upon contact with Ab-coated tumor target cells, and that TLR8 agonist treatment can strengthen this response.

Next, we performed ADCC assays to test whether granzyme B was involved in monocyte-mediated killing of Ab-coated targets. Monocytes were treated overnight with or without TLR8 agonist, followed by incubation for 20 h with Ab-coated MDA-MB-468 cells in the absence or presence of the granzyme B inhibitor Z-AAD-CMK. Results showed that the TLR8 agonist enhanced ADCC and that the granzyme B inhibitor reduced the amount of killing in untreated monocytes (Fig. 8B). More importantly, the inhibitor significantly reduced the ADCC induced by TLR8 agonist (Fig. 8B). Granzyme B inhibition led to a 59% reduction in ADCC by TLR8 agonist-treated monocytes, which roughly corresponds to the efficacy of granzyme B inhibition we observed after Z-AAD-CMK treatment (65% as measured by ELISA; Supplemental Fig. 4). To show that the reduction in ADCC was not due to an effect of granzyme B inhibition on monocyte viability, we performed parallel experiments using Annexin V/propidium iodide staining. Results showed that viability was unaffected by granzyme inhibitor treatment (data not shown). Collectively, these results suggest that TLR8 agonists can elicit granzyme B production by monocytes and that granzyme B is directly involved with monocyte-mediated ADCC.

**TLR8 upregulates Perforin-1 and SERPIN B9 in monocytes**

Another protein commonly associated with granzyme B is Perforin-1, which permits entry of granzyme B into the target cell cytosol. Perforin-1 has been shown to trigger a membrane repair response that results in the formation of large vesicles in the target cells that contain both granzyme B and Perforin-1, where Perforin subsequently forms pores that permit the movement of granzyme B into the cytosol (41, 42). Detailed time-lapse experiments have also revealed that Perforin-1 can create large, transient pores in the target cell membrane, which permits diffusion of granzyme B directly into the target cell cytosol (43). To determine whether TLR8 agonist treatment induced Perforin-1, we examined our microarray results and saw that Perforin-1 was significantly increased in monocytes treated with TLR8, but not TLR7, agonists (Fig. 9A), which we confirmed using RT-PCR and ELISA (Fig. 9B, 9C, respectively). Hence it is likely that Perforin-1 plays a role in the delivery of granzyme B from monocytes after TLR8 agonist treatment.

It is known that NK cells, T cells, and even several cancer cells produce the serine protease inhibitor Serpin B9, which serves to neutralize and protect against granzyme B (12). Interestingly, it has also been found that monocytes express Serpin B9 and that certain types of infection such as by EBV can lead to its upregulation (44). Hence we asked whether monocytes produced Serpin B9 in parallel to granzyme B after TLR8 agonist treatment. We examined our microarray results and found that TLR8 agonist treatment led to a significant upregulation of Serpin B9.
of 4-fold (Fig. 9D). We confirmed this using RT-PCR and Western blotting (Fig. 9E, 9F, respectively). These results suggest that TLR8 agonist-treated monocytes not only express granzyme B, but also express SerpinB9 to prevent killing of self by the protease.

Discussion

Monocytes and macrophages have long been known as effectors of ADCC (45–51), and they are second only to NK cells in this capacity among mononuclear cells (52). However, the mechanisms by which monocytes perform this function have not been fully elucidated but were likely to be different from that used by NK cells. For example, it has been shown that adherent phagocytic cells began lysing Ab-coated targets after 8 h and completed this process by 24 h, whereas nonadherent lymphoid cells began and finished this process at 2 and 8 h, respectively (51). Other findings support this, showing that NK cells lyse Ab-coated targets within 4 h (25, 53), but measurable levels of ADCC by monocytes/macrophages might be seen only after as long as 18 h (22, 54). Despite this difference in time required for ADCC between cell types, we have shown that monocytes, much like NK cells, also use granzyme B for ADCC. Monocyte FcγR activation leads to granzyme B production and this granzyme B is required for monocyte-mediated ADCC. In addition, we have also found that TLR8, but not TLR7, agonists could induce this protease. TLR8-mediated production of granzyme B was shown to depend on NF-κB and IL-12, which would be in agreement with an earlier study showing that TLR8 agonists led to stronger NF-κB activation and IL-12 production than TLR7 agonists (23).

Two major subsets of blood monocytes have been identified, consisting of CD14⁺CD16⁻ and CD14⁻CD16⁺ in humans (CX₃CR1⁻CCR2⁺Gr1⁺ and CX₃CR1⁺CCR2⁻Gr1⁻ in mice) (55). These subsets have been found to be involved with different immune activities. For example, it has been shown by Biburger et al. (56) that among multiple immune cell types and subtypes, Ly6C⁺ monocytes were critical mediators of Ab function in mouse models of platelet depletion and B cell depletion. Interestingly, they found that other FcγR-expressing cells including mast cells, basophils, eosinophils, and neutrophils were dispensable for Ab-mediated platelet depletion. This highlights the importance of

FIGURE 6. TLR8 elicits greater cytokine production in PBMs. PBMs isolated from healthy donors (n = 3) were treated overnight with 1 μM 3M-055 (TLR7) or VTX-2337 (TLR8), or left untreated (UT). Affymetrix microarray analysis was performed, and expression values for IL-12/IL-23 p40 (A), IL-6 (D), IFN-γ (G), and TNF-α (J) are plotted. PBMs (n = 3) were then isolated and treated as above, and RT-PCR was done to measure transcript levels of IL-12/IL-23 p40 (B), IL-6 (E), IFN-γ (H), and TNF-α (K). PBMs (n = 3) were treated as above, and protein levels of IL-12/IL-23 p40 (C), IL-6 (F), IFN-γ (I) and TNF-α (L) were measured in cleared supernatants using ELISAs. Error bars represent SD. *p ≤ 0.05.
monocytes in such IgG-mediated activities, as only the absence of a monocyte subpopulation affected this depletion (56). Regarding the monocyte subpopulations themselves, they found that Ly6Clo monocytes expressed FcγRIV, and that IgG2a Ab-mediated depletion of both platelets and B cells depended on Ly6Clo and not Ly6Chi (which do not express FcγRIV) monocytes (56).

With regard to this study, findings such as those by Biburger et al. (56) raise the possibility that one of the two monocyte subtypes may have been predominantly responsible for FcγR- and TLR8-induced granzyme B production. Indeed, the monocyte isolation protocol we use has consistently yielded both CD16- and CD16+ monocytes as measured by cytometry with the 3G8 Ab (data not shown), leaving this possibility open. However, we have also found that TLR8 agonists significantly reduce monocyte CD16 expression as measured by both microarray analysis and PCR (data not shown), suggesting that monocytes were likely to be CD16- after TLR8 agonist treatment. In addition, we observed a whole-population shift in granzyme B rather than a shift within only a proportion of the monocytes after agonist treatment (Fig. 1E). Hence although we cannot rule out the production of granzyme B by nonclassical monocytes, it is likely that classical CD14+CD16-Clo monocytes

FIGURE 7. IL-12 is required for TLR8-mediated granzyme B production. PBMs (n = 3 for IL-12, IFN-γ, and TNF-α; n = 4 for IL-6) were treated overnight with or without 1 μM CL075 along with neutralizing Abs against IL-12 (A and B), IL-6 (D and E), IFN-γ (G and H), or TNF-α (J and K), or treated with recombinant human IL-12 (C), recombinant human IL-6 (F), recombinant human IFN-γ (I), or recombinant human TNF-α (L), using concentrations listed in Materials and Methods. Granzyme B was measured by ELISA in cleared supernatants after treatment with the respective neutralizing Abs against IL-12, IL-6, IFN-γ, and TNF-α (A, D, G, and J). As controls, ELISAs were also done to measure IL-12 (B), IL-6 (E), IFN-γ (H), and TNF-α (K). Granzyme B was also measured in cleared supernatants after overnight treatment with recombinant human IL-12 (C), recombinant human IL-6 (F), recombinant human IFN-γ (I), or recombinant human TNF-α (L). Error bars represent SD. *p ≤ 0.05. N.D., not detected.

FIGURE 8. TLR8-induced granzyme B enhances monocyte ADCC. (A) PBMs (n = 3) were treated overnight with or without 1 μM CL075 and with fixed MDA-MB-468 cells that had been incubated with no Ab (no Ab), rituximab (Rit; negative-control Ab), or cetuximab (Cet). ELISAs were done to measure granzyme B in cleared supernatants. (B) PBMs (n = 4) were incubated overnight with or without 1 μM VTX-2337, then also incubated with or without 100 μM Z-AAD-CMK (Z-AAD) and tested in an ADCC assay against MDA-MB-468 cells as described in Materials and Methods. Graph depicts percent Ab-mediated cytotoxicity, with negative-control Ab values subtracted out. Error bars represent SD. *p ≤ 0.05.
produced the bulk of TLR8-induced granzyme B within our experimental conditions.

Our results show that NF-κB and IL-12 are implicated in monocyte granzyme B production, but the precise pathways downstream of monocyte FcγR (or TLR8) responsible for this are not fully known. In NK cells, contact with IgG-coated cells has been shown to increase granzyme B production in a Ca^{2+}-independent manner (40); perhaps it is similar within the monocyte system. One candidate signaling pathway may be PI3K, because it is activated by FcγR clustering (6) and has been shown to be required for IL-15-mediated priming of NK cells (57).

It has been previously shown that monocytes express granzyme B transcript, and that stimuli such as E. coli LPS and M. leprae lysates could modestly increase its expression (34). In this study, induction of granzyme B at both the transcript and the protein levels appeared to be specific to certain TLR-activating stimuli, as only agonists for TLR4 and TLR8 showed efficacy. This selectivity is in agreement with a previous finding that LPS, but not other selected TLR agonists, led to granzyme B production by monocytes (58). These findings may have implications regarding which TLR agonists might be most effective within the context of Ab therapy, because monocytes/macrophages are one of the major cell types at and within tumors (59, 60).

Although known for its proapoptotic effects on target cells, granzyme B has been shown to affect a wide range of processes, including many pathologic conditions (16). As such, the inducible nature of monocyte-derived granzyme B may be optimal, as it would help ensure that the monocytes were in direct contact with the tumor targets before releasing this protease. For Ab therapy, monocytes would presumably produce and release this protease only upon conjugate formation with opsonized tumor cells, thereby minimizing potentially harmful nonspecific effects.

However, augmentation of FcγR responses with a TLR8 agonist would likely induce monocytes to secrete granzyme B while in circulation as well. Hence, within this context, a more localized administration of TLR8 agonist may be better. Results from a recent phase I dose-escalation trial using s.c. administration of a TLR8 agonist showed good tolerance, although some degree of systemic circulation was seen (61). Perhaps when used as an adjudant for Ab therapy, an Ab–drug conjugate would offer the best solution, where the TLR8 agonist would be linked to the therapeutic Ab. This might help ensure that monocytes/macrophages produce granzyme B primarily at the tumor sites.

In conclusion, we have found that human monocytes perform ADCC in a granzyme B–dependent manner, and that TLR8 agonists can enhance this. These results help answer the longstanding question regarding how monocytes destroy Ab-coated tumor cells. In addition, these findings also uncover a mechanism by which TLR8 agonists confer antitumor properties.

Disclosures
G.N.D. and R.M.H. are employees of VentiRx Pharmaceuticals; J.P.V. is an employee of 3M. All other authors have no financial conflicts of interest.

References

FIGURE 9. TLR8 upregulates Perforin-1 and Serpin B9 in monocytes. (A) Expression values of Perforin-1 found in the microarray analysis. (B and C) PBM (n = 3) were treated overnight with either 1 μM 3M-055 or CL075, or left untreated. (B) Transcript levels of Perforin-1 were measured using RT-PCR. (C) Perforin-1 protein was measured in cleared supernatants using ELISA. (D) Microarray expression data for Serpin B9. (E and F) PBM (n = 3) were treated overnight with either 1 μM 3M-055 or CL075, or left untreated. (E) RT-PCR was used to measure transcript levels of Serpin B9. (F) Western blotting was done to measure Serpin B9 protein. Blots shown are representative of three independent experiments. Error bars represent SD. *p ≤ 0.05.
Supplemental Figure 1. TLR7 versus TLR8 agonists lead to distinct expression profiles. Human PBM (n=3) were treated overnight with 1 µM of either 3M-055 (TLR7) or VTX-2337 (TLR8) and compared to untreated samples using Affymetrix microarray analysis. The heatmap (A) and Venn diagram (B) were generated by selecting genes with log$_2$ fold-changes of 3 or more. Within the heatmap, blue represents low expression and red high expression.
Supplemental Figure 2. Transcripts differentially upregulated by TLR7 and not TLR8 agonists in monocytes. Human PBM (n=3) were treated overnight with either 3M-055 (TLR7) or VTX-2337 (TLR8) and compared to untreated samples using Affymetrix microarray analysis. Graphs represent expression values of 3 genes upregulated with TLR7 but not TLR8 agonist. A. Protein kinase C alpha (PRKCA). B. Kinase suppressor of Ras 1 (KSR1). C. Mitogen-activated protein kinase 1 (MAPK1).
Supplemental Figure 3. Lack of additive effects of TLR4 and TLR8 activation on Granzyme B production in monocytes. Human peripheral blood monocytes (PBM) were isolated and treated overnight with vehicle (UT), 500 ng/ml LPS (TLR4), 1 µM CL075 (TLR8) or the combination of LPS and CL075. Cleared supernatants were collected and assayed for Granzyme B via ELISA.
Supplemental Figure 4. Inhibition of Granzyme B by Z-AAD-CMK.

Human PBM (n=2) were pretreated for 30 minutes with vehicle or with either 10 or 100 µM Z-AAD-CMK, then incubated overnight with either vehicle or with 1 µM CL075. Granzyme B was measured via ELISA.