Supplementary Material

http://www.jimmunol.org/content/suppl/2011/06/27/jimmunol.1001670.DC1

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J Immunol published online 27 June 2011
http://www.jimmunol.org/content/early/2011/06/27/jimmunol.1001670
A Critical Role for Granzymes in Antigen Cross-Presentation through Regulating Phagocytosis of Killed Tumor Cells

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Granzymes A and B (GrAB) are known principally for their role in mediating perforin-dependent death of virus-infected or malignant cells targeted by CTL. In this study, we show that granzymes also play a critical role as inducers of Ag cross-presentation by dendritic cells (DC). This was demonstrated by the markedly reduced priming of naive CD8⁺ T cells specific for the model Ag OVA both in vitro and in vivo in response to tumor cells killed in the absence of granzymes. Reduced cross-priming was due to impairment of phagocytosis of tumor cell corpses by CD8α⁺ DC but not CD8α⁻ DC, demonstrating the importance of granzymes in inducing the exposure of prophagocytic “eat-me” signals on the dying target cell. Our data reveal a critical and previously unsuspected role for granzymes A and B in dictating immunogenicity by influencing the mode of tumor cell death and indicate that granzymes contribute to the efficient generation of immune effector pathways in addition to their well-known role in apoptosis induction. * The Journal of Immunology, 2011, 187: 000–000.

Tumor cell death induced by various agents can activate the immune system and generate an Ag-specific response against the tumor, a concept known as immunogenic cell death. The induction of immunogenic cell death is studied using chemotherapeutic drugs, Ab treatment, irradiation, or repeated freeze–thaw cycles (1). The in vivo clearance of dead cells is mainly mediated by macrophages, but their engulfment by dendritic cells (DC) and subsequent Ag processing is critical for cross-presentation of cellular Ags. In vitro and in vivo studies revealed a central role for the CD8α⁺ DC subset in phagocytosis and cross-presentation of cell-associated Ags to prime a specific CTL response (2–5). The molecular signals associated with various forms of tumor cell death are known to influence the subsequent immune response: uptake of apoptotic cells minimizes inflammation due to secretion of anti-inflammatory cytokines such as TGF-β or IL-10 and transcriptional repression of IL-12p35 in phagocytes, and uptake of necrotic cells is associated with induction of an immune response (6, 7). However, immunogenic cell death has been described to occur as a result of preapoptotic events in response to drug treatment or irradiation (8, 9). Similarly, the engulfment of primary or secondary necrotic material can lead to an immune response against cellular Ags in some but not all experimental settings (5, 10–12).

Various laboratories have shown that granzyme A (GrA) and granzyme B (GrB) activate important and independent cell death pathways and are instrumental in perforin-dependent killing of virus-infected and malignant cells (13, 14). The induction of cell death by CTL from GrB-deficient mice is marked by reduced DNA fragmentation accompanying target cell apoptosis, but this defect can be compensated by increasing the incubation time (15). In contrast, apoptosis is not defective in GrA-deficient mice (16). We recently showed that both NK cells and CTL from mice deficient in granzymes A and B (GrAB) induce a unique and phe-notypically distinct form of target cell death (17). The cell death morphology induced by GrAB-deficient CTL appeared similar to that induced by wild-type (WT) CTL. However, quantitative and kinetic live cell microscopy showed that cells dying in response to GrAB-deficient CTL failed to display phosphatidylserine (PS) on their surfaces. Accordingly, dying cells did not bind annexin V until their cell membranes had become permeable to propidium iodide (PI), indicative of secondary necrosis (17). However, the functional significance of this finding was not further explored.

Studies addressing the role of granzymes in priming an immune response have not previously been reported. In this study, we used Ag-specific killing of tumor cells by WT or granzyme-deficient CTL to examine the downstream effects of GrAB on generation of an immune response. For the first time to our knowledge, we show that cell death occurring in the absence of GrAB leads to reduced cross-presentation of a model tumor Ag in vitro and in vivo. In terms of mechanism, we demonstrate that engulfment of tumor cells by DC is critically dependent on the presence of granzymes. These data define a new and important role of gran-
zymes in influencing the quality and magnitude of an immune response. This shows that the mode of CTL-mediated cell death influences subsequent target cell phagocytosis and Ag cross-presentation and thus has far more profound effects on immunity than previously recognized.

Materials and Methods
Mice and reagents
C57BL/6 and C57BL/6.6ml mice were obtained from the Walter and Eliza Hall Institute of Medical Research (Melbourne, VIC, Australia). C57BL/6, GrAB mice were originally obtained from M. Simon [Max Planck Institut für Immunobiologie, Freiburg, Germany (18)] and were maintained at the Peter MacCallum Cancer Centre and crossed with C57BL/6.OT-1 mice (GrAB.OT-1). Genotype of GrAB.OT-1 was confirmed by PCR. Perforin-deficient C57BL/6.OT-1 mice (Pfp.OT-1) were generated as described previously (19). Expression of the transgenic TCR on in-house-bred C57BL/6.OT-1 (WT.OT-1), GrAB.OT-1, and Pfp.OT-1 was confirmed by staining for CD8α and Vε2-TCR by FACS analysis. Mice were used at 6-10 wk of age and were handled according to the ethical guidelines of the Peter MacCallum Cancer Centre and the University of Melbourne.

Complete RPMI 1640 medium containing 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 μM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 10 mM HEPES supplemented with 10% FBS was used in all experiments with primary cells. The Ab clones used in this study were 53-6.7 (CD8α), N418 (CD11c), PC61.5 (CD25), MEL-14 (CD62L), H1.2F3 (CD69), GL1 (CD86), eBio25-D1.16 (SIINFEKL on H-2k* b) (all eBioscience), 3/23 (CD40), 16-10A1 (CD80), 1D4B (CD107a), AF6-88.5 (H-2kb), 2G9 (I-A/E), and B20.1 (Vε2-TCR) (all BD Pharmingen). Viability of cells was determined by the addition of PI during analysis on CantoII or LSRII analyzers (Becton Dickinson). Data analysis was performed by FlowJo software (Tree Star).

Target cell culture
All tumor lines were maintained in complete DMEM medium containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES. The different MC38 lines expressing membrane-bound OVA (MC38ova) or the matching empty vector control (MC38ev) were generated using retroviral transfection (20). For functional assays, the different MC38 lines were seeded in 12- or 24-well plates at least 24 h before starting cytotoxic assays to ensure a stable monolayer before addition of effector cells. Cells of MC38 lines were seeded in increasing numbers to allow selection of monolayer with comparable confluence at the time of effector cell addition.

Activation of effector cells
Total splenocytes from WT.OT-1, GrAB.OT-1, and Pfp.OT-1 mice were activated in vitro after red cell lysis in 0.83% NH4Cl. C57BL/6 splenocytes were pulsed with 1 μg/ml SIINFEKL peptide (Auspep), irradiated (15 Gy), and used as stimulator cells. Effector cells (2.5 × 10^7/ml) were seeded at a 1:1 ratio with stimulators in 6-well plates for 3 d in presence of 200 ng/ml SIINFEKL peptide (Auspep) and 0.01% DNase and stopped after 20 min by addition of 0.1 M EDTA solution. Splenocytes were loaded onto Nycodenz gradients and centrifuged at 1700 × g for 10 min at 4°C. Light density fraction was collected, and CD11c+ cells were enriched using a commercial DC isolation kit (Invitrogen). After bead enrichment, preparations that contained plasmacytoid and conventional DC populations were further purified by FACS (FACS Vantage–Diva or Aria II; BD) into total CD11c+ and purity of final DC preparation was routinely >98%.

In vitro cross-presentation assays
For cytotoxic assays, 2 × 10^5 effector cells in complete RPMI medium was added onto the MC38ova target cell monolayer in 12-well plates with comparable confluence (70–80%, equivalent of ∼10^6 tumor cells) for 4 and 18 h to ensure comparable amount of Ag present in all cytotoxic cultures. Freshly sorted CD11c+ DC were added at a DC to tumor cell ratio of 1:1 to these cultures for 3 h, after which CD8α+ as well as CD8α- DC subpopulations were re-sorted subsequently. Labeling of DC was performed using CD11c–allophycocyanin and CD8α–PE–Cy7 Ab, and purity of the final DC subpopulations was routinely >98%. Finally, 2.5 × 10^5 DC were incubated with 5 × 10^5 CD8α+ CFSE+ cells. Activation markers on viable, total FCSE+ OT-I populations were characterized using FACS analysis by simultaneous staining with CD8, CD69, CD25, and CD62L Abs.

In vivo cross-presentation assays
After washing in PBS, cellular content of cytotoxic assays was injected i.p. into C57BL/6 recipient mice (three mice per group). Animals had received 10^6 enriched CFSE-labeled, naive OT-I i.v. shortly prior to being challenged. Spleens were harvested after 3 d, and numbers of proliferating OT-I cells were determined as previously published (23). Briefly, 2.5 × 10^5 blank Sphero beads (BD Pharmingen) were added to each tube, and stopping gate was set to 5 × 10^3 beads consistently for all samples. Numbers of proliferating OT-I cells were determined from CD8+CFSElow cells. Activation markers on viable, total FCSE+ OT-I populations were characterized using FACS analysis by simultaneous staining with CD8, CD69, CD25, and CD62L Abs.

Phagocytosis assays
Staining of MC38ova target cells with 5 μM CFSE was performed 24 h prior to starting the assays with different OT-I effectors to allow optimal adherence of tumor cells in 24-well plates. For cytotoxic assays, 10^6 effector cells were added in complete RPMI medium for the indicated times onto MC38ova monolayer selected for comparable confluence at the time of effector cell addition (70–80%). Subsequently, total CD11c+sorted DC were added for 3 h to allow phagocytosis. Combined cytotoxic and phagocytosis assays were performed, stained with CD8α Ab, and analyzed by flow cytometry. Gating was performed on the CD8α+ or CD8α- subpopulations to assess CFSE uptake within the different subpopulations.
For PS-blocking experiments with purified, recombinant annexin V (final 25 μg/ml; BD Biosciences), cytotoxic cultures were performed in complete DMEM medium to provide sufficient Ca\(^{2+}\) to allow annexin V binding.

Expression of activation markers and cytokine production by DC

DC were isolated from FLT3 ligand tumor-bearing mice (24) and finally retrieved from cytotoxic cultures of WT.OT-I or GrAB.OT-I (over 4 or 18 h, respectively) by FACS as described for cross-presentation assays. DC (20,000) were cultivated overnight in complete RPMI medium supplemented with 10 ng/ml recombinant GM-CSF (MBL). Staining for SIIN
day, CD80, CD86, and I-A/E (all biotinylated, together with CD11c-allophycocyanin and CD8α–PE–Cy7 to define DC subpopulations) was performed after Fc receptor blocking with 2.4G2 Ab clone. Finally, DC were incubated with eFluor 450 conjugated to streptavidin (eBioscience) for analysis. Geometric mean of eFluor 450 fluorescence was assessed from duplicate (CD8\(^{+}\) DC) or triplicate (CD8\(^{+}\) DC) values for each Ag. Supernatants from DC cultures were analyzed for IL-12p70 and TNF-α by Cytokine Bead Array (BD Biosciences) according to the manufacturer’s instructions.

Confocal microscopy

The CD8\(^{+}\) DC were re-sorted via FACS from cytotoxic cultures of CFSE-labeled MC38ova target cells incubated either with GrAB.OT-I or WT.OT-I target cells over 4 or 18 h. Cells were attached to slides by centrifugation (250 rpm, 10 min) and then fixed with 3.7% (w/v) paraformaldehyde in 100 mM PIPES, 5 mM MgSO\(_4\), 10 mM EGTA, and 2 mM DTT (10 min, room temperature) (25). All DC were imaged in Prolong Gold anti-fade reagent (Invitrogen). Cell samples were examined using an Olympus Fluoview FV10-ASW1.7 viewer attached to an MRC-1024 Bio-Rad laser-scanning confocal microscope or a Zeiss Axioskop2 microscope at room temperature. All data shown are raw and unaltered.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 5.0a (GraphPad Software).

Results

Target cell death mediated by CTL in the absence of GrAB

Our experiments aimed to determine whether tumor cells undergoing cell death in response to either granzyme-deficient or -sufficient CTL would impact on the induction of an immune response to a tumor Ag. To enable the study of MHC-restricted responses, we generated C57BL/6 TCR-transgenic mice (WT.OT-I, responding to the OVA peptide257–264 H-2K\(_{b}\)-SIINFEKL) that also lacked expression of GrAB (GrAB.OT-I). Consistent with previous reports (13, 14), there was minimal killing of OVA-expressing EL-4 (E.G7ova) target cells by GrAB-deficient CTL over a standard 4-h assay; however, the deficiency was partly rescued by extending the incubation time (Fig. 1A). Cytotoxicity was Ag-restricted as no lysis occurred in target cells devoid of peptide (EL-4). For subsequent experiments, it was important to identify assay conditions where equivalent numbers of dead target cells could be generated in response to WT or GrAB-deficient CTL. For example, the experiment of Fig. 1A carried out with E.G7ova cells showed that equivalent release of \(^{51}\text{Cr}\) could be achieved at an E:T ratio of 10 if the incubation time was extended from 4 up to 22 h. The results of many (n = 7–19) cytotoxic assays are summarized in Fig. 1B, demonstrating the strong reproducibility of these killing kinetics.

To ensure that genuine target cell death (as observed with \(^{51}\text{Cr}\) release) was induced by GrAB-deficient CTL over the extended incubation time, CFSE-labeled E.G7ova were cocultured with either WT or GrAB-deficient CTL and analyzed for their scatter profile by flow cytometry (Fig. 1C). Unlike cells that undergo necrosis, cells killed through the granule pathway characteristically undergo both a reduction in size (reduced forward scatter) and increased granularity (increased side scatter) as they die (26).

FIGURE 1. Target cell death induced by GrAB.OT-I. A, Cytotoxicity of GrAB.OT-I is delayed. \(^{51}\text{Cr}\)-labeled E.G7ova or Ag-negative EL-4 target cells were incubated with varying numbers of WT.OT-I or GrAB.OT-I effector cells incubated for 4 h (short term) or 22 h (long term). Graphs show means ± SEM of triplicates of one representative experiment. B, Delayed killing phenotype induced by GrAB-deficient effector cells is highly reproducible. Summary of all \(^{51}\text{Cr}\) release assays with E.G7ova or SIINFEKL-pulsed EL-4 at a E:T ratio of 10:1 given as means ± SEM. Black bars, WT.OT-I; white bars, GrAB.OT-I. Statistical analysis by one-way ANOVA including Bonferroni posttests.

C, Long-term killing assay with GrAB.OT-I results in comparable morphology of dead target cells as that of short-term killing assay with WT.OT-I. CFSE-labeled E.G7ova target cells were incubated with WT.OT-I or GrAB.OT-I at an E:T ratio of 2:1 for 4 or 22 h. Morphology of target cells was analyzed by categorizing morphology as live cells (FSC<sub>high</sub>SSC<sub>low</sub>), dead/dying cells (FSC<sub>low</sub>SSC<sub>high</sub>), and debris (FSC<sub>low</sub>SSC<sub>low</sub>). *p < 0.05, **p < 0.001. n.s., not significant.
As seen with \[^{51}Cr\] release assays, few dead target cells were observed at 4 h in response to GrAB.OT-I CTL. With prolonged incubation time, however, the extent of target cell death approached that seen with WT.OT-I CTL. Minimal killing was observed using OT-I CTL deficient in perforin (Pp.OT-I) even under prolonged incubation (Supplemental Fig. 1), showing that killing through other death pathways was minor in this setting.

To show that GrAB-deficient cells exhibit the same level of activation as WT CTL, we compared the phenotype of both effector populations. Stimulation with syngeneic SIINFEKL-pulsed splenocytes resulted in similar expression patterns of the activation markers CD69, CD25, and CD62L by GrAB.OT-I and WT.OT-I cells over the activation period (Fig. 2A). Reduced cytotoxicity was not due to impairment of granule exocytosis, as both CTL populations showed exposure of the lysosomal marker CD107a in response to cognate target cells (exemplified in Fig. 2B, and data pooled over four independent assays in Fig. 2C). Both CTL populations also showed equivalent secretion of IFN-γ upon encountering E.G7ova target cells (Fig. 2D). Differences in target cell death in response to GrAB.OT-I or WT.OT-I CTL could therefore not be put down to inadvertent changes in activation status, exocytosis of granule contents, or induction of effector cytokines.

**Cell death mediated by GrAB-deficient CTL causes impaired cross-presentation in vitro**

Our next aim was to determine whether our observations with E.G7ova could be generalized to other OVA-expressing tumor target cells. Consistent with our results on E.G7ova target cells, killing of OVA-expressing MC38ova mouse adenocarcinoma target cells (MC38ova) was also delayed in response to GrAB.OT-I CTL (Fig. 3A). Following similar kinetics to dying E.G7ova cells, incubation of MC38ova with GrAB.OT-I over 18 h resulted in comparable cytotoxicity to incubation with WT.OT-I over 4 h and was confirmed in several similar assays (n = 4–5; summarized in Fig. 3B).

To assess cross-presentation of the OVA Ag, purified CD11chigh DC were added to simultaneously completed cytotoxic cultures comprising MC38ova cells together with either WT or GrAB-deficient TCR transgenic CTL (for overview of the experimental schedule, see Supplemental Fig. 2A). After 3-h incubation, CD8α+ and CD8α− DC subpopulations were reisolated via FACS, incubated with CFSE-labeled, naive WT.OT-I lymphocytes, and T cell proliferation was assessed by quantifying the number of responding T cells showing CFSE dilution (Fig. 3C for individual histograms and quantified in Fig. 3D). Marked T cell proliferation was seen in response to the CD8α− DC exposed to target cells killed by WT CTL in cytotoxic assays. However, cross-presentation was greatly reduced when CD8α+ DC were exposed to target cells killed by GrAB.OT-I from long-term cultures. OT-I T cell proliferation represented genuine DC-dependent cross-presentation, as CD8α− DC failed to induce OT-I proliferation under the same conditions. Live tumor cells cocultured with DC induced minimal cross-presentation of the OVA Ag (Fig. 3C, 3D), most likely due to the small number of dead cells in those populations. Further, irradiated MC38ova target cells alone did not induce proliferation of naive OT-I cells (see Supplemental Fig. 2B), excluding the possibility that SIINFEKL presented by tumor cells could directly activate OT-I T cells.

The reduction in cross-presentation in response to target cells killed by GrAB.OT-I CTL did not occur simply as a result of...
the prolonged incubation, as extending the length of the cytotoxicity assay performed with WT.OT-I to 18 h did not diminish the proliferation of naive responder T cells (Fig. 4A). Also, cross-presentation was clearly dependent on target cell death, as prolonged incubation of MC38ova target cells with Pfp.OT-I CTL also greatly impaired subsequent cross-presentation (Fig. 4B),

FIGURE 3. Cross-presentation of target cells killed by GrAB-deficient CTL is reduced in vitro. A, Delayed killing of MC38ova by GrAB.OT-I. \[^{[3]}\text{Cr}\] release of MC38ova incubated with increasing numbers of WT.OT-I or GrAB.OT-I over 4 and 18 h (short or long term, respectively). Means \(\pm\) SEM are shown from triplicates of one representative experiment. B, Summary of all \[^{[3]}\text{Cr}\] release assays with MC38ova at an E:T ratio of 10:1 given as means \(\pm\) SEM. Statistical analysis by one-way ANOVA including Bonferroni posttests. C and D, Target cell death induced by WT.OT-I results in cross-presentation. Cytotoxic cultures of MC38ova with WT.OT-I or GrAB.OT-I were established over 4 and 18 h, respectively, and subsequently total CD11chigh DC were added for 3 h. In addition, CD11chigh DC were also added to wells for 3 h with MC38ova without any CTL to assess influence of tumor cells alone. CD8\(^+\) and CD8\(^+\) DC were re-sorted thereafter and subsequently cultured with CFSE-labeled, naive WT.OT-I for 3 d. Representative histograms are shown in C and quantification showing means \(\pm\) SEM, performed using beads for calibration gated on PI \(^{2}\text{CD8^+CFSElow}\) cells, is depicted in D. Graphs shown are representative from four independent repeats, and statistical analysis was performed using \(t\) test. \(*p < 0.05, **p < 0.01, ***p < 0.001. n.s., not significant.

FIGURE 4. In vitro cross-presentation of target cells killed by GrAB-deficient CTL is dependent on cell death. A, Prolonged tumor cell incubation with WT.OT-I in cytotoxic cultures does not impair cross-presentation. The experimental setup as described for Fig. 3C with cytotoxic cultures over 4 and 18 h for both CTL types. B, Cell death mediated in the presence of perforin and granzymes is necessary for the induction of cross-presentation. Cytotoxic cultures, including Pfp.OT-I as effector cells (long term), were set up as described for Fig. 3B. Graphs depicted in A and B are representative of at least three independent experiments (black bars, CD8\(^+\) DC; white bars, CD8\(^+\) DC) and represent means \(\pm\) SEM. C, Long-term incubation with perforin-deficient CTL does not result in cell death. Cytotoxic cultures were established with MC38ova tumor cells and various effector cells over 18 h. Specific \[^{[3]}\text{Cr}\] release was determined as means + SD from triplicates of one representative of three independent experiments. Statistical analysis using \(t\) test: \(*p < 0.05, **p < 0.01. n.s., not significant.
as a result of reduced target cell death in the absence of perforin in long-term cytotoxic assays. Again, the impact of other cell death pathways induced by CTL could be excluded, as killing of MC38ova tumor cells was minimal upon incubation with perforin-deficient CTL (Fig. 4C). Despite the marked reduction in T cell proliferation in the absence of perforin or GrAB, the expression of activation markers CD69, CD25, and CD62L on the few T cells that did undergo cell division was similar to T cells responding to MC38ova target cells killed by WT CTL (Supplemental Fig. 3A).

Reduced in vivo cross-priming in response to target cells killed by GrAB.OT-I CTL

Subsequently, we wanted to examine whether in vivo priming of the OVA response was also abnormal when tumor cells were killed in the absence of GrAB. For this purpose, the cellular content of in vitro cytotoxic assays with MC38ova exposed to either WT.OT-I (short term) or GrAB.OT-I (long term) was injected i.p. into naive C57BL/6 mice that had also received naive, CFSE-labeled OT-I T cells i.v. prior to Ag challenge. The number of proliferating OT-I responder cells in the spleen was determined after 3 d (Fig. 5A). Consistent with our previous in vitro results, the number of proliferating T cells was significantly reduced in recipients of MC38ova target cells killed by GrAB.OT-I (Fig. 5B). Although differing quantitatively, the quality of the T cell response within both groups was comparable as indicated by similar numbers of cell divisions and expression of activation markers (Supplemental Fig. 3B).

Administration of increasing numbers of MC38ova cells killed by GrAB.OT-I (long term) or WT.OT-I (short term) revealed a strong dose dependence of T cell activation in vivo (Fig. 5C). This effect was only apparent using MC38ova killed by WT.OT-I; however, priming efficacy of tumor cells killed in the absence of granzymes was strongly impaired even at high doses of MC38ova.

Tumor cell death in the absence of granzymes results in impaired phagocytosis by CD8α+ DC

A critical event in Ag cross-presentation is the acquisition of tumor Ag through phagocytosis of the cell-associated Ag by DC. Therefore, we determined the capacity of CD8α+ and CD8α− DC to phagocytose dead/dying, CFSE-labeled MC38ova target cells after their incubation with either GrAB.OT-I or WT.OT-I CTL. Purified CD11c+DC were added to simultaneously finished cytotoxic cultures and analyzed for CFSE content 3 h later (Fig. 6A, 6B). Bright CFSE + CD8α+ DC became evident when the DC were added to MC38ova killed by WT.OT-I CTL over 4 h and were even more pronounced after 18 h, indicating phagocytic uptake, but not for CD8α− DC (see Supplemental Fig. 4A). However, far less phagocytosis was seen when MC38ova were incubated with GrAB.OT-I CTL or with no CTL at all. Despite the fact that similar levels of target cell death occurred in response to GrAB.OT-I CTL after 18 h, no significant phagocytosis of the dead cells was observed on prolonged incubation. No phagocytosis whatever occurred when Ag-negative MC38ev cells were used as targets in the cytotoxicity assays (Fig. 6B and Supplemental Fig. 4A). Fluorescence microscopy also confirmed that the flow cytometry findings represented authentic target cell engulfment rather than nonspecific cell adhesion (Fig. 6C; single-channel images and histograms of the CD8α+ DC population can be found in Supplemental Fig. 4B).

Analysis of CD8α+ DC upon engulfment of MC38ova killed in the presence or absence of granzymes revealed a marked reduction of SIINFEKL peptide reaching the cell surface bound to H-2Kb (Fig. 6D) when tumor cells were killed by GrAB.OT-I. However, expression of activation markers and cytokine production were similar in CD8α+ DC exposed to short-term cytotoxic cultures of target cells and WT.OT-I (Fig. 6E, 6F). This indicated that even when CD8α+ DC were incubated with equal amounts of tumor Ag, their reduced capacity to phagocytose tumor cells killed in the absence of granzymes resulted in reduced Ag cross-presentation but comparable expression of activation markers.

When we first described membrane changes during tumor cell killing in the absence of GrAB using live cell microscopy, the most obvious difference in cell death was the lack of early PS exposure on the dying target cell (17), a hallmark of generic apoptosis but also a key event for engulfment by phagocytes (27). Therefore, we aimed to investigate phagocytosis in the presence of PS blockage by annexin V and cross-presentation of target cell from cytotoxic assays with WT.OT-I (which induce early PS exposure). Cytotoxic cultures were established with CFSE-labeled MC38ova target cells and activated WT.OT-I effector cells over 4 h, and recom-
FIGURE 6. Impaired phagocytosis of tumor cells killed in the absence of GrAB. CD11c+ DC were added to cytotoxic cultures of CFSE-labeled MC38ova tumor cells and GrAB-sufficient and -deficient effectors to allow phagocytic uptake. After 3 h, DC were identified by CD11c–allophycocyanin staining, and DC subpopulations were defined by CD8α–PE–Cy7 expression by flow cytometry. A, CFSE uptake was analyzed for each subpopulation as depicted schematically. B, Tumor cell death in the presence of GrAB leads to phagocytosis by CD8α+ DC. CFSE-labeled MC38ova or MC38ev target cells were incubated with either GrAB.OT-I or WT.OT-I in cytotoxic assays for 4 or 18 h (short term and long term). Gray, DC alone; orange, tumor cells without CTL; red, GrAB.OT-I; green, WT.OT-I. Histograms are shown from one representative of four independent experiments. CFSE histograms of CD8α2 DC are shown in Supplemental Fig. 4A. C, CD8α+ DC incubated with tumor cells from cytotoxic assays with WT.OT-I show uptake of CFSE-labeled particles. DC from the above-described experiment were re-sorted and fixed on slides for confocal microscopy (×60 oil immersion); scale bars, 20 μm. Images are shown from one of two independent experiments; single-channel images can be found in Supplemental Fig. 4B. D, Impaired phagocytosis results in reduced presentation of SIINFEKL on H-2Kb molecules of CD8α2 DC. CD8α+ and CD8α2 DC were re-sorted from cytotoxic cultures of CFSE-labeled MC38ova tumor cells together with GrAB.OT-I or WT.OT-I (over 18 or 4 h, respectively). DC (20,000) were seeded overnight and labeled with CD11c–allophycocyanin, CD8α–PE–Cy7, and biotinylated eBio25-D1.16, recognizing SIINFEKL on H-2Kb visualized by eFluor 450 bound to streptavidin. Gray, tumor cells without CTL; red, GrAB.OT-I; green, WT.OT-I. Histograms are representative of five experiments, and graph summarizes results from the corresponding experiment with triplicate values. E and F, Activation status of CD8α+ DC from cytotoxic cultures with GrAB.OT-I and WT.OT-I is similar. In parallel to H-2Kb expression as shown in D, we analyzed activation markers CD40, CD80, CD86, and MHC class II (I-A/E) expression and the production of IL-12p70 and TNF-α from supernatants by CD8α+ and CD8α2 DC using CBA. One representative experiment of three independent repeats is depicted as means + SEM in D–F. Data were analyzed using t test: **p < 0.01.
binant annexin V (25 μg/ml) was added to these cultures shortly before addition of DC. The addition of soluble annexin V had no effect on phagocytosis by CD11c<sup>hi</sup>CD8α<sup>+</sup> DC and subsequent cross-presentation (Fig. 7A, 7B). By comparison, this amount of annexin V was sufficient to inhibit macrophage-mediated uptake of MC38ova tumor cells killed by WT.OT-I under the same conditions (Supplemental Fig. 4C). The data indicate that other phagocytic signals in addition to PS exposure are important for target cell uptake by DC and that these signals are lacking in target cells killed in the absence of GrAB.

Discussion

Many studies during the past years have addressed the question of the immunogenicity of target cell death associated with various death stimuli such as gamma- or UV-irradiation, treatment with agonistic Abs or chemotherapeutic drugs, or repeated freeze–thaw cycles. However, cell death mediated by granule exocytosis via perforin-dependent granyme delivery has not been studied to date. In this study, we used GrAB-sufficient and -deficient CTL to kill tumor cells expressing the model Ag OVA to examine the role of granzymes in cross-presentation. Our results clearly demonstrate in vitro and in vivo that granzymes induce an immunogenic form of cell death by influencing the phagocytic uptake of cell-associated Ag.

Despite DC being exposed to equal numbers of dead/dying tumor cells (Fig. 3A, 3B), the cross-presentation of OVA Ag was markedly impaired when MC38ova cells were killed by GrAB-deficient effectors (Fig. 3C, 3D). Our in vitro studies focused on the ability to prime a specific CTL response by CD8α<sup>+</sup> DC, the most effective cross-presenting cells within the immune system. As an internal control, we used CD8α<sup>−</sup> DC reisolated from the same cytotoxic cultures and exposed to the same amount of Ag as stimulators of naive OT-I T cells. This allowed us to determine genuine cross-presentation rather than nonspecific carryover of tumor cell Ag from the cytotoxic cultures. In addition, MC38ova cells alone did not activate OT-I T cells directly, either when presented on live cells or in response to irradiation (Fig. 3 and Supplemental Fig. 2B). The observed effect was also independent on the DC to T cell ratio used in our assay system (Supplemental Fig. 2C). Finally we showed that on CD8α<sup>+</sup> DC, far fewer H-2K<sup>b</sup> molecules were occupied by SIINFEKL peptide after being exposed to tumor cells killed in the absence annexins (Fig. 6D), whereas levels of activation signals were comparable with those DC exposed to tumor cells killed by WT.OT-I (Fig. 6E, 6F). Therefore, we could demonstrate that granzyme-mediated cell death was necessary for phagocytosis and subsequent cross-presentation of cell-associated Ag.

There is strong consensus that GrB potently induces cell death through direct activation of procaspases and/or proteolytic processing of Bid (26). Although GrA has been shown to kill target cells when applied with perforin in vitro (28), it was also recently shown to induce the secretion of proinflammatory cytokines such as IL-1β from activated macrophages in a perforin-dependent manner (29). Of interest, IL-1β has lately been shown to be critical in tumor Ag presentation in vivo (30). Our findings are therefore consistent with an emerging body of work identifying previously unsuspected functions of granzymes in the induction of an immune response in addition to their well-described role in cytotoxicity.

The intriguing mechanistic observation of our study is that the reduced Ag cross-priming was brought about by defective Ag uptake by CD8α<sup>+</sup> DC. We found that only tumor cell death induced by granzyme-sufficient CTL resulted in strong phagocytosis, as confirmed by both flow cytometry and confocal microscopy (Fig. 6). By marked contrast, CTL-mediated killing in the absence of granzymes (GrAB.OT-I) resulted in only minor engulfment of tumor cells. As described previously (17), the lack of PS exposure on dying cells characterized the altered mode of tumor cell death induced by GrAB-deficient CTL compared with the “classic” apoptotic morphology induced by WT CTL, which features early exteriorization of PS. Exposure of PS is the major “eat-me” signal for uptake of apoptotic cells by phagocytes (31). However, studies using blockade of PS exposure by recombinant annexin V on apoptotic RMA lymphoma cells demonstrated the impact of PS on engulfment by macrophages but not DC, indicating that other molecular signals are required for uptake by DC (32). Using annexin V to block PS-mediated recognition or uptake by CD8α<sup>+</sup> DC, we could confirm that PS is not critical for uptake and subsequent cross-presentation by CD8α<sup>+</sup> DC (Fig. 7A, 7B). In turn, this would suggest that during cell death induced by GrAB-deficient CTL, factors other than PS exposure on the outer leaflet of the plasma membrane are necessary to initiate uptake and eventual cross-presentation by CD8α<sup>+</sup> DC. Various receptors are described that may be involved in phagocytosis and cross-presentation of Ags by DC, which include Tim-3 and Tim-4, various integrins and complement receptors; however, this topic is still quite controversial and debated (33–36). In this context, one of the potentially interesting signals might be the unidentified
ligand for Clec9A, a C-type lectin-like receptor expressed on CD8α+ DC shown to be critical for Ag cross-presentation of necrotic cells (5). Furthermore, another intriguing aspect is the impact of granzymes on various intrinsic danger signals such as calreticulin, high-mobility group box 1 molecules, heat shock proteins, ATP, or uric acid exposed or released during cell death that are known to enhance immunogenicity (8, 9, 30, 37, 38). There have been publications to date showing that tumor cells killed by CTL can release high-mobility group box 1 molecules or are able to induce calreticulin exposure on tumor cells (39, 40); however, the impact of granzymes in these particular reports is still unclear.

Our findings are likely to be particularly relevant where tumor cells are resistant to receptor-mediated (Fas, TNF, TRAIL) cell death, resulting in a reliance on the granzyme/perforin pathway to kill the cell. It is well known that cell death through Fas, TNF, or TRAIL results in classic apoptosis, with early PS exposure leading to efficient phagocytosis. It is also well recognized that tumors can reduce their sensitivity to these death mechanisms, for example by downregulating receptor levels, or through the expression of cytoplasmic inhibitors such as dominant-negative Fas-associated death domain or cellular FLIP (41). In such circumstances, the lack of expression of GrAB by CTLs or the additional expression of intrinsic granzyme inhibitors (serpins) by the tumor would be predicted to result in a marked deficiency in phagocytosis and therefore reduced cross-presentation of tumor Ag. Together with our previous studies (17, 42), we have now shown that PS fails to be displayed on a variety of cells attacked by GrAB-deficient CTLs, including RMA-S and M9H11, and that reduced phagocytosis of dead/dying cells can be demonstrated for both E.G7ova and MC38ova (this study). Our studies extend to both class I-restricted CTLs and NK cells, suggesting our observations will have impact when either the innate or the adaptive immune system is engaged to respond to a potentially immunogenic tumor. Finally, the magnitude of the immunogenicity induced by GrAB in comparison with other types of cell death via TNFR superfamily may be determined to sensitize for necrotic cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J. Exp. Med. 191: 432–434.


