CD169-Positive Macrophages Dominate Antitumor Immunity by Crosspresenting Dead Cell-Associated Antigens

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SUMMARY

The generation of tumor-directed cytotoxic T lymphocytes is considered crucial for the induction of antitumor immunity. To activate these CD8+ T cells, antigen-presenting cells (APCs) must initially acquire tumor cell-associated antigens. The major source of tumor antigens is dead tumor cells, but little is known about how APCs in draining lymph nodes acquire and crosspresent these antigens. Here we show that CD169+ macrophages phagocytose dead tumor cells transported via lymphatic flow and subsequently crosspresent tumor antigens to CD8+ T cells. Subcutaneous immunization with irradiated tumor cells protects mice from syngenic tumor. However, tumor antigen-specific CD8+ T cell activation and subsequent antitumor immunity are severely impaired in mice depleted with CD169+ macrophages. Neither migratory dendritic cells (DCs) nor lymph node-resident conventional DCs are essential for the crosspresentation of tumor antigens. Thus, we have identified CD169+ macrophages as lymph node-resident APCs dominating early activation of tumor antigen-specific CD8+ T cells.

INTRODUCTION

During tumor growth, tumor cells accumulate genetic mutations and acquire features that enable them to escape from the host immune system in various ways. In addition to these intrinsic properties of tumor cells that allow them to elude immune surveillance, the environment within and around the tumor often has a detrimental effect on the host immune system, including impairment of dendritic cell (DC) function and suppression of T cells caused by tumor-derived factors or myeloid-derived suppressor cells infiltrating the tumors (Gabrilovich, 2004; Melief, 2008; Sica and Bronte, 2007). This immunosuppressive environment induced by tumors limits the effectiveness of anticancer chemotherapy and γ-irradiation. Therefore, great efforts have been made to enhance innate and acquired antitumor immunity.

The generation of tumor antigen-specific cytotoxic T cells (CTLs) is considered crucial for the induction of antitumor immunity. To generate and activate T cell immunity against tumor antigens, antigen-presenting cells (APCs) must initially acquire tumor cell-associated antigens, and one of the major sources of tumor antigens is dead tumor cells (Melief, 2008). Massive tumor cell death induced by anticancer therapy and particulate matter from the dead tumor cells provide large amounts of tumor-associated antigens. However, previous studies have been unable to clarify the mechanism by which APCs in the lymph node (LN) internalize and crosspresent these types of antigens to CD8+ T cells.

Phagocytes, such as macrophages and DCs, swiftly phagocyte apoptotic cells by recognizing molecules exposed only on the surface of these corpses (Henson et al., 2001; Lauber et al., 2004; Ravichandran and Lorenz, 2007; Savill and Fadok, 2000). Failure of apoptotic cell clearance results in autoimmune disorders, indicating that apoptotic cell clearance by phagocytes is essential for the maintenance of self-tolerance under physiological conditions (Asano et al., 2004; Hanayama et al., 2004; Miyanishi et al., 2007). Consistent with these findings, the intravenous injection of apoptotic cells can induce cell-associated antigen-specific immunosuppression or tolerance (Green et al., 2009). This mechanism of tolerance induction requires the contributions of two populations of cells in the splenic marginal zone (MZ): macrophages that rapidly clear corpses in the blood flow, and CD8α−CD103+ DCs that selectively phagocytose dead cell and subsequently present dead cell-associated antigen (Miyake et al., 2007; Qiu et al., 2009).

In contrast to the outcome after intravenous injection, subcutaneously injected apoptotic cells are often immunogenic and many investigators have taken advantage of immunogenic tumor corpses for tumor vaccination (Apetoh et al., 2007; Green et al., 2009; Obeid et al., 2007; Tesniere et al., 2008). These findings suggest that apoptotic cells in the periphery are cleared and processed in a very different way from blood-borne apoptotic cells in spleen.

Antigen presentation in peripheral LNs is thought to be a process that is coordinately performed by migratory DCs from peripheral tissues and LN-resident APCs (Allan et al., 2006; Allenspach et al., 2008; Lee et al., 2009). However, little is known about the role of different APCs in the clearance of...
dead cells and the presentation of dead cell-associated antigens in peripheral tissue or LNs.

Here we document how tumor cell-associated antigens derived from dead tumor cells are crosspresented in the draining LNs. We concluded that CD169+ macrophages that reside in the LN sinus take up dead tumor cells and directly crosspresent tumor antigens to CTLs. Mice lacking CD169+ macrophages at the time of dead tumor cell vaccination or chemotherapy-induced tumor degradation fail to induce antitumor immunity. Surprisingly, neither migratory DCs nor LN-resident conventional CD8α+ DCs, cells well known for their crosspresentation ability, are involved in the crosspresentation of dead cell-associated antigens. These findings should improve our understanding of how to generate and activate tumor-specific T cell immunity in response to tumor cell death.

RESULTS

Subcutaneous Immunization with Dead Tumor Cells Activates Tumor-Specific CTL

The generation of tumor antigen-specific CTLs is an essential component of tumor immunity. As has been previously reported, subcutaneous injection of dead cells induced the proliferation of tumor antigen-specific CTLs in the draining LNs (Figure 1A). After the subcutaneous immunization with dead tumor cells, dead cell-associated antigens are transported to the draining LN in some manner, where they are crosspresented by APCs to activate tumor-specific CTLs. This immunization with dead tumor cells serves as an effective tumor “vaccination,” as shown by the fact that mice injected with X-ray-irradiated EG7 cells, ovalbumin (OVA)-transfected T cell thymoma, reject subsequently inoculated syngeneic tumor cells (Figure 1B).

Migratory DCs Are Not Primarily Involved in the Delivery and Crosspresentation of Dead Cell Antigens

APCs in LNs consist of migratory DCs from peripheral tissues and LN-resident cells. Early studies have demonstrated the involvement of migratory DCs in the delivery and/or presentation of various types of antigens in LNs. Waithman et al. (2007) reported that a cellular antigen derived from dead cells in the course of natural tissue turnover is delivered and crosspresented in LNs by skin-derived DCs. Allan et al. (2006) demonstrated that migratory DCs in the skin transport viral antigens to draining LNs, where they transfer antigens to LN-resident DCs. These studies initially led us to assume that immunized dead tumor cell-associated antigens would be taken up and transported to LNs by migratory DCs.

To analyze the contribution of migratory DCs to the delivery of cellular antigens, we first utilized Kaede transgenic (Tg) mice that express a photoconvertible fluorescent protein (Tomura et al., 2008). Kaede mice were immunized with complete Freund’s adjuvant (CFA) or apoptotic tumor cells in the hind leg foot pads. At 12 and 18 hr after immunization, the foot pads were exposed to violet light to induce photoconversion. In control mice, a small but detectable number of photoconverted CD11c+ cells were observed in the draining LNs (Figure 1C), indicating the constant migration of these cells from peripheral tissues to the draining LNs in the steady state. When the mice were injected subcutaneously with CFA, the number of migratory CD11c+ cells was greatly increased. On the other hand, the subcutaneous injection of dead cells did not stimulate the migration of CD11c+ DCs (Figure 1C). This finding indicates that migratory DCs do not primarily participate in the delivery of dead cell-associated antigens.

To further analyze the ability of migratory DCs to present cellular antigens, we next used Langerin-diphtheria toxin receptor (DTR) mice (Kissenpfennig et al., 2005). In those mice, diphtheria toxin (DT) injection causes the selective deletion of Langerhans cells and dermal DCs (Bursch et al., 2007; Poulin et al., 2007). OT-I OVA-specific T cells were transferred into wild-type (WT) or Langerin-DTR mice. 2 days after DT injection into the mice, OVA-expressing apoptotic cells were injected...
Phagocytosis of Dead Tumor Cells by CD169+ Macrophages in LNs

We then explored the possibility that the cellular antigens directly transit to draining LNs. We injected fluorescently labeled apoptotic cells into the foot pads of mice and then examined the draining LNs at various time points for the appearance of the labeled cells. The apoptotic cell corpses reached the draining LNs as early as 3 hr after the injection (Figure 2A). The accumulation of dead cells continued up to 4 days after the injection (Figure S2A). This time course suggests that the arrival of injected dead cells at the draining LNs precedes that of migratory DCs, which take days to reach draining LN (Kissenpfennig et al., 2005). Immunohistochemical examination revealed that the fluorescent cells were mainly located in the subcapsular and paracortical sinus and not in T and B cell areas (Figure 2B). On the basis of confocal microscopic analysis, dead cell antigens accumulated in the sinus were actually phagocytosed by CD169+ macrophages in vivo (Figure S2B). This distribution indicates that the cell corpses traveled to the draining LNs shortly after the injection via lymphatic flow and were trapped in the sinus by CD169+ macrophages.

We next determined which cells phagocytosed the apoptotic cell corpses. Two types of phagocytes are present in LNs: CD11b+CD169+ sinus macrophages and CD11c+ DCs. We isolated total phagocytes from LNs by enriching CD11b- and/or CD11c-positive cells by magnetic beads. Flow cytometric analysis of these cells revealed that CD169+ sinus macrophages could be further divided into CD11c+ and CD11c- subpopulations, whereas CD169+ CD11c- conventional DCs consisted of CD8α+ and CD8α- subpopulations. 24 hr after the injection of fluorescence-labeled dead cells, approximately 25% of both CD11c+ and CD11c- CD169+ macrophages contained fluorescence-labeled apoptotic cells (Figure 2C). In contrast to this extensive phagocytosis by CD169+ macrophages, dead cell phagocytosis by CD8α+ DCs was minimal and only 7% of CD8α- DCs phagocytosed dead cells. These results clearly indicate that subcutaneously injected dead cells reach LNs via...
lymphatic flow and are predominantly taken up by CD169+ macrophages.

We examined whether CD169+ macrophages migrated from peripheral tissue into LNs after dead cell injection. First, injection of dead cells did not increase the population of CD169+ cells in the draining LNs (Figure S2C). We next quantitated the percentages of photoconverted CD169+ macrophages in the LNs of Kaede transgenic (Tg) mice. As was shown in Figure 1C, CFA injection induced robust migration of DCs into draining LN. Even under this condition, the percentage of photoconverted CD169+ macrophages in the LNs of CD169-DTR mice was very low among CD169+ cells (Figure S2D). These findings indicate that CD169+ macrophages reside in LNs and are not migratory.

Failure of Antitumor Immunity in CD169-DTR Mice

Given the selective phagocytosis of dead cells by CD169+ macrophages, it became imperative to examine the role of these macrophages in the crosspresentation of dead tumor cell antigens. We previously generated genetically targeted mice expressing the diphtheria toxin receptor (DTR) under the control of the CD169 gene (CD169-DTR mice) (Miyake et al., 2007). In these mice, DT injection induced the transient deletion of macrophages located in the MZ of the spleen. We also found that CD169+ sinus macrophages were completely depleted in LNs of CD169-DTR mice treated with DT. This deletion was maintained for up to 7 days after DT injection (Figures S3A–S3C). On the other hand, DT administration neither depleted B cells or T cells nor changed the proportions of CD8+/C0 DCs in the LNs of CD169-DTR mice with systemic oxaliplatin on day 7 and LN cells draining dead tumor cells were restimulated in vitro with OVA on day 12. Concentrations of IFN-γ in the culture medium were measured by ELISA. Mean concentrations are shown with SEM. n = 10 WT or n = 13 CD169-DTR mice per group. (B) CD169+ macrophages are required for immune activation subsequent to tumor degradation. WT or CD169-DTR mice were each inoculated or not inoculated with EG7 cells in the right flank on day 0. The mice were treated with systemic oxaliplatin on day 7 and LN cells draining dead tumor cells were restimulated in vitro with OVA on day 12. Concentrations of IFN-γ in the culture medium were measured by ELISA. Mean concentrations are shown with SEM. n = 10 WT or n = 13 CD169-DTR mice per group. (C) CD169+ macrophages are required for crosspresentation of dead cell-associated antigens. CFSE-labeled OT-I T cells were injected intravenously into WT or CD169-DTR mice. OVA-expressing dead cells or OVA in CFA were injected subcutaneously into foot pads of DT-treated mice. Proliferation of OT-I T cells in the draining LNs was analyzed. (D) OT-I cells were transferred into WT and CD169-DTR mice. The DT-treated mice were immunized with X-ray-irradiated EG7 cells in the flank and were rechallenged with live EG7 cells in the foot pads. 2 days after this injection, numbers of CD8+ T cells in the draining and nondraining LNs were quantitated. Numbers of CD8+ T cells in draining LNs are divided by those in nondraining LNs. Mean values are shown with SD of three mice/group. Dotted line indicates baseline amounts in naive mice. Results are representative of two independent experiments. *p < 0.05, n.s., not significant.
either restimulated or not restimulated with OVA protein. In parallel with the finding obtained from the tumor vaccination model, tumor degradation-induced CD8+ T cell activation was abolished in CD169+ macrophage-depleted mice (Figure 3B).

These results highlight the critical role of CD169+ macrophages in the induction of antitumor immunity both by vaccination with tumor cells killed ex vivo and by degradation of the established tumors in vivo.

Next, we analyzed the CD8+ T cell response to various forms of antigen in CD169+ macrophage-depleted mice. OT-I T cells were transferred into WT or CD169-DTR mice, which were then immunized subcutaneously with OVA emulsified in CFA or with OVA-expressing apoptotic cells. Immune stimulation with the OVA-expressing apoptotic cells failed to induce OT-I T cell proliferation in CD169+ macrophage-depleted mice, whereas T cell proliferation was unimpaired in CD169+ macrophage-depleted mice immunized with OVA in the form of a soluble protein (OVA in CFA) (Figure 3C). These in vivo data clearly demonstrate that CD169+ sinus macrophages are required for antigen-specific T cell proliferation specifically in response to cell-associated antigens.

We further compared the efficacy of dead tumor cell vaccination between WT and CD169-DTR mice in terms of CD8+ T cell proliferation in response to challenge with live tumor cells. OT-I cells were transferred into WT and CD169-DTR mice. Then, the mice were immunized in the right flank with dead EG7 cells. 10 to 14 days after the immunization, mice were injected subcutaneously with live EG7 cells in the left foot pads. 2 days later, numbers of CD8+ T cells in the draining (left) and non-draining (right) LNs were quantitated. In the immunized WT mice, CD8+ T cells vigorously responded to rechallenge with live tumor cells, indicating effective priming with dead tumor cells. On the other hand, CD8+ T cells in DT-treated CD169-DTR mice did not respond to challenge with live tumor cells (Figure 3D). These results clearly demonstrate the requirement of CD169+ sinus macrophages for the efficient priming of CD8+ T cells against dead cell-associated antigens.

Direct Crosspresentation of Cellular Antigens by the CD169+ Macrophage CD11c+ Subset

We next analyzed the in vitro cell-associated antigen-presentation activity of APCs in CD169-DTR mice. Total LN phagocytes from mice injected with OVA-expressing dead cells were enriched by magnetic beads and then cocultured in vitro with OT-I cells. APCs from DT-treated CD169-DTR mice were unable to crosspresent dead cell-associated OVA to CD8+ T cells, as assessed by [3H]-thymidine incorporation (Figure 4A) and by IFN-γ production (Figure 4B). In contrast, when the total APCs were cocultured in vitro with dead cell OVA, CD169-DTR APCs were comparable presenters to WT APCs (Figure S4A). These observations suggest that conventional CD11c+ DCs are intact in their ability to crosspresent dead tumor cell-associated antigens in CD169+ macrophage-depleted mice, but they do not have access to these antigens accumulated in the LN sinus.

To directly address whether CD169+ macrophages can cross-present dead cell-associated antigens, we purified these cells by flow cytometry on the basis of CD169 and CD11c expression (Figure 4C, top; Figure S4B). As shown in Figure 4D, CD11c+ CD169+ macrophages exhibited the strongest presentation ability while the CD11c- subset had no presentation ability. We also examined the CD169+ CD11c+ DC subpopulation (Figure 4C, bottom) and found that their ability to present cellular antigens was significantly lower than that of the CD11c+ CD169+ macrophages (Figure 4D). In the crosspresentation of dead cell antigens transported via lymphatic flow, CD11c+ CD169+ macrophages autonomously accomplished the task without coordination with DCs because the addition of CD8α+ DCs did not enhance the antigen-presentation ability of CD11c+CD169+ macrophages.
macrophages (Figure S4C). These results indicate that CD11c+ CD169+ macrophages phagocytose dead tumor cells transported to the LN via lymphatic flow and crosspresent cell-associated antigens in LNs to stimulate CD8+ T cell proliferation. However, these findings do not exclude the possibility that CD11c-CD169+ macrophages play some role in the priming of T cells against cellular antigens, for example by participating in the retention of cellular antigens in the LN sinus.

It was a mystery how macrophages that capture lymph-borne antigens in the sinus meet T cells in the parenchyma until two different groups demonstrated the migration of T cells from T cell zone to the sinus to receive antigen presentation. In vivo real-time imaging analysis showed T cells probe the surface of cortical sinus and are retained at CD169+ macrophage-rich regions (Grigorova et al., 2009). It has recently been demonstrated that memory OT-I T cells accumulate in the sinus to form stable conjugate with CD169+ macrophages as early as 5 hr after the subcutaneous administration of OVA-expressing Toxoplasma gondii (Chtanova et al., 2009).

In order to examine the interaction between CD169+ macrophages and antigen-specific CD8+ T cells, the kinetics of CD8+ T cells in LN were analyzed sequentially after the subcutaneous injection of dead cells. We transferred carboxyfluorescein succinimidyl ester (CFSE)-labeled CD8+ T cells from OVA-immunized OT-I mice into wild-type mice. The mice were subcutaneously injected with OVA-expressing dead cells and draining LNs were analyzed by immunohistochemistry 4 to 24 hr after the injection. This analysis showed that the number of OT-I T cells that conjugate with CD169+ macrophages peaked at 4 hr after the injection of dead cells and rapidly decreased thereafter (Figure S5A). Fluorescence and confocal microscopic observations indicate that OT-I T cells are in close contact with CD169+ macrophages at 4 hr (Figures S5B and S5C). These results strongly suggest that CD8+ T cells relocalize in the sinus rapidly after the challenge with dead cells to receive antigen presentation from CD169+ macrophages and then return to the T zone for proliferation.

Distinct Localization and Function of CD11c-CD169+ Sinus Macrophages

The differences in cell surface marker profile and antigen-presentation activity suggest that the two CD169+ macrophage subpopulations have distinct localization and functions.

We first examined the localization of the CD11c+CD169+ subpopulation. Immunohistochemical analysis by costaining with CD11c and CD169 antibodies revealed that CD169+ cells were exclusively localized in the sinus, whereas CD11c+ cells were mainly localized in the T cell zone (Figure 5A). However, a small number of CD169+ cells were found to coexpress CD11c. These double-positive cells were mainly localized in the cortical and para cortical sinus, especially at the boundary between the sinus and the T cell zone or B cell follicle (Figures 5B and 5C).

Next we compared the expression of various surface molecules between two fractions of CD169+ macrophages (Figure S6) to find that they uniformly expressed CD11b and F4/80, indicating that they were indeed macrophages. They were positive also for major histocompatibility complex (MHC) and costimulatory molecules that are required for efficient T cell activation.

To further explore the functional differences between the two CD169+ macrophage subpopulations, we analyzed their cytokine production profiles. Both populations were enriched by magnetic beads, further purified by a cell sorter, and then stimulated with toll-like receptor (TLR) ligands such as LPS or CpG in vitro. We measured the concentrations of a panel of cytokines with a cytokine array. LPS (Figure 6A) or CpG (Figure 6B) stimulation induced the production of IL-1β, IL-12 p40, IL-12 p70, MIP-1α, and RANTES by the CD11c+CD169+ subset (Figure 6, filled bars). On the other hand, the concentrations of these cytokines in the culture medium of CD11c- subset were generally lower than those of the CD11c+ subset (Figure 6, open bars) or undetectable. These results clearly indicate that the two CD169+ macrophage subsets have distinct cytokine production profiles on stimulation with TLR ligands.
Mechanisms of Phagocytosis of Apoptotic Cells by CD11c+CD169+ Macrophages

In general, macrophages recognize phosphatidylserine (PS) exposed on the surface of apoptotic cells in order to engulf apoptotic cells. We have previously shown that a milk fat globule-EGF-factor 8 (MFG-E8) protein with a point mutation in an Arg-Gly-Asp (RGD) motif (D89E) inhibited apoptotic cell engulfment by masking PS (Asano et al., 2004; Hanayama et al., 2002). To define the molecular mechanisms of apoptotic cell phagocytosis by CD11c+CD169+ macrophages, we examined the effects of D89E on this process. Apoptotic cells were incubated with D89E and then injected subcutaneously into WT mice. As shown in Figure 7A, D89E treatment significantly inhibited the phagocytosis of apoptotic cells by both CD11c− and CD11c+CD169+ macrophages, whereas treatment with E1E2PT, another MFG-E8 mutant protein that does not bind to PS, had no effect on phagocytosis. As another assay for the impact of masking PS on dead cells, we analyzed antigen-specific T cell proliferation and found that it was greatly reduced by D89E treatment (Figure 7B). These results clearly indicate that CD169+ macrophages phagocytose dead cells in a PS-dependent manner, and this phagocytosis is required for the crosspresentation of cell-associated antigens by CD169+ macrophages in LNs.

DISCUSSION

During the course of tumor growth, a substantial fraction of tumor cells continually undergo apoptosis. In addition to this constant cell death, irradiation or administration of anticancer drugs causes apoptosis of a large number of tumor cells. It is well accepted that apoptosis is a “silent” cell death that does not trigger immune responses. Rather, the efficient removal of apoptotic cells by phagocytes frequently results in tolerance toward cell-associated antigens. Thus, apoptosis induced by anticancer drugs is accompanied by immunosuppressive side effects. At the same time, it has long been known that the subcutaneous immunization with irradiated syngeneic tumor cells protects mice from subsequent challenge with the same tumor in viable form. Casares et al. (2005) found that tumor cell death induced by anthracyclins, a class of anticancer drugs, or by X-ray irradiation promoted tumor-specific CTL activation. This conversion from tolerogenic into immunogenic cell death was dependent on the exposure of calreticulin on the tumor cell surface and/or the release of endogenous adjuvants from dead cells (Apetoh et al., 2007; Obeid et al., 2007). It is most likely that these immunogenic cell corpses enhance the activation state of APCs. However, APCs that are responsible for the crosspresentation of dead tumor cell-associated antigens have until now not been identified. Our studies here show that (1) subcutaneously injected dead cells are transported to LNs via lymphatic flow and (2) macrophages lining the LN sinus phagocytose and directly crosspresent dead cell-associated antigens to CD8+ T cells. This presentation is critical for the proliferation and activation of tumor-specific CD8+ T cells because mice lacking CD169+ macrophages at the time of vaccination are unable to reject viable tumor cells. Systemic treatment with anticancer chemotherapy induces massive degradation of solid tumor, and dead tumor cell-associated antigens thus generated in the peripheral tissue prime CD8+ T cells in the tumor-draining LNs (Ghiringhelli et al., 2009).
Our findings demonstrate that immune activation subsequent to tumor degradation in vivo is also controlled by CD169+ macrophages. Based on criteria introduced recently by different groups (Junt et al., 2007; Phan et al., 2009), we defined CD169-positive cells that reside in the lymph node sinus as “CD169-positive macrophages.” Although these cells express generally accepted macrophage markers, it remains to be determined whether CD11c+CD169+ macrophages constitute a small subset of CD8α+ DCs.

Very recently, Phan et al. (2009) focused on the function of CD11c+CD11b+CD169+ subcapsular sinus macrophages and found that these cells captured the immune complexes and relayed them to B cell follicles without digestion. In the present study, we showed that subcutaneously injected dead cells were captured by both CD11c+ and CD11c+CD169+ macrophages, but only the CD11c+ subset crosspresented these antigens. As far as we know, this is the first work to identify crosspresenting APCs that reside exclusively in the LN sinus. As described above, the immunogenicity of dead tumor cells depends on the mode of cell death, and even apoptotic cells can become immunogenic under certain circumstances (Tesniere et al., 2008). To better understand antitumor immune regulation, it is very important to explore how immunogenic dead cells affect the function and activation status of CD169+ macrophages. In addition to dead cells, particulate matter, such as bacteria, viruses, parasites, and large immune complexes, accumulates in the LN sinus (Carrasco and Batista, 2007; Chatanova et al., 2008; Junt et al., 2007; Phan et al., 2007). Therefore, it will be of great interest to examine the ability of CD11c+CD169+ macrophages to present antigens derived from particulate matter.

The LN sinus is a highway that links afferent and efferent lymph and is believed to be a filtering zone for lymph-borne molecules. Large particles, including cellular antigens in the lymphatic fluid, make initial contact with CD169+ macrophages that serve as sentinels located along the length of the LN sinus. It is noteworthy that CD8α+ conventional DCs, which are well known for their potent crosspresentation activity (Schnorrer et al., 2006), are not predominantly involved in the crosspresentation of dead cell-associated antigens transported via lymphatic flow. These conventional DCs in LNs are located in the T cell areas, so they may not have access to dead cells that accumulate in the LN sinus. In contrast to dead cell-associated antigens, the crosspresentation of soluble antigens took place normally even in the absence of CD169+ macrophages. It is very likely that soluble antigens passed the layer of sinus lining cells and directly entered the T cell area where they were endocytosed by CD8α+ conventional DCs and subsequently crosspresented. This speculation is consistent with a previous report that macrophages lining the LN sinus capture particles larger than 70 kDa, whereas smaller molecules rapidly enter the T and B cell areas via a conduit system (Roozendaal et al., 2008; Sixt et al., 2005). Sancho et al. (2009) described CLEC9A as a CD8α+ DC-expressing receptor for necrotic cells that regulates the crosspresentation of dead cell-associated antigens. They demonstrated that CLEC9A-deficient bone marrow (BM) DCs failed to crosspresent dead cell-associated antigens cocultured in vitro. It is worth revisiting the contribution of this receptor in the crosspresentation of subcutaneously injected dead cell antigens in peripheral LNs.

Figure 7. CD169+ Macrophages Phagocytose Dead Cells in a PS-Dependent Fashion

(A) Phagocytosis of dead tumor cells by CD169+ macrophages is PS dependent. PKH26-labeled dead W3-OVA cells were coated either with D89E or E1E2PT. Draining LNs were resected 24 hr after the dead cell injection. Total APCs were stained for CD169 and CD11c. Numbers are the percentages of PKH-positive macrophages. Average values of three mice/group are shown with SD. *p < 0.05.

(B) Blocking of phagocytosis of dead tumor cells impairs antigen-specific CD8+ T cell proliferation. CFSE-labeled OT-I cells were injected intravenously into WT mice. OVA-expressing dead cells incubated either with PBS, D89E, or E1E2PT were injected subcutaneously into the foot pads. OT-I cell proliferation in the draining LNs was analyzed by flow cytometry. These results are representative of four to six different mice in each group.
Waithman et al. (2007) demonstrated that migratory DCs transport and crosspresent dead cell antigens that are generated as a result of tissue turnover. Through the analysis of Kaede Tg mice, we found that DCs constantly migrate from tissue into draining LNs under physiological conditions. These observations implicate that the contribution of migratory DCs, particularly Langerin-negative dermal DCs that are not depleted in Langerin-DTR mice, to the delivery and presentation of dead cell antigens could not be excluded.

In the last two decades, we have witnessed numerous efforts to develop preventive and therapeutic cancer immunotherapy. Two main strategies are vaccine therapy and cell transfer therapy. In the former, tumor antigen-conjugated antibodies are administered to activate APCs in vivo (Bozzacco et al., 2007; Sancho et al., 2008; Trumpfheller et al., 2006). Antitumor immunity achieved by dead tumor cell vaccination may also be included in this category. The latter therapy includes the transfer of DCs loaded ex vivo with tumor-specific antigens (Gilboa and Vieweg, 2004; Kalergis and Ravetch, 2002; Schuurhuis et al., 2006; Su et al., 2005). In either case, the primary goal of the therapies is to enhance tumor-directed T cell responses to effectively destroy tumor cells. However, it is disappointing that the effects of these immunotherapies are often compromised by suppressor leukocytes within a tumor (Marigo et al., 2008; Sica and Bronte, 2007) or by the lack of signals to induce DC maturation (Gabrilovich, 2004; Melief, 2008). It seems likely that one problem with current immunotherapies is that they target DCs that are not primarily responsible for the crosspresentation of dead tumor cell-associated antigens. We have shown here that CD169+ macrophages directly crosspresent tumor antigens to CD8+ T cells. This finding provides new insight into the activation of tumor-directed CTL in LNs, which may help overcome the limitations of current immunotherapeutic approaches.

The structure of the spleen is very similar to that of the LN. In spleen, various types of blood-borne particles, including apoptotic cells, accumulate in the MZ. Those particles are rapidly cleared by two types of macrophages, marginal zone macrophages (MZWMs) and marginal metallophilic macrophages (MMAWs), which are thought to be anatomical and functional counterparts of CD169+ macrophages in the LN. It has not been possible to isolate viable macrophages from the splenic MZ for in vitro functional assays. Therefore, only the in situ phagocytic activity of these macrophages has been demonstrated in previous studies. Because the present study revealed the unique antigen-presentation activity of CD169+ macrophages in LNs, it would be beneficial to examine other functions of these splenic macrophages, such as antigen-presentation activity.

In conclusion, the present study clearly demonstrates the essential role of CD169+ macrophages in tumor immunity induced by tumor cell death and highlights the heterogeneity and the multifunctionality of CD169+ macrophages in LNs.

**EXPERIMENTAL PROCEDURES**

**Mice**

All experiments with mice were approved by the RCAI Animal Use Committee and performed in accordance with applicable guidelines and regulations.
culture was quantitated. IFN-γ production in the culture medium at 72 hr was determined with a mouse IFN-γ ELISA set (BD Bioscience).

**Immunohistochemistry**

OCT-embedded cryosections of LNs were stained and observed under a fluorescence (Keyence) or a confocal microscope (Leica).

**Cytokine Array**

Concentrations of cytokines in the culture medium of CD169+ macrophages were measured with cytokine array (Luminex).

**Statistical Analysis**

All statistical analysis used unpaired two-tailed Student’s t test. p < 0.05 was considered significant.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.immuni.2010.12.011.

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K.A. designed experiments, did research, analyzed and interpreted data, and cowrote the manuscript with M. Tanaka; A.N., Y.M., C.-H.O., and A.K. did experiments; M. Tomura and O.K. did experiments with Kaede mice and analyzed data; S.F. did experiments of tumor vaccination with K.A. and analyzed and interpreted data; and M. Tanaka designed experiments with K.A., analyzed and interpreted data, and wrote the manuscript with K.A.

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