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Ly6C$^+$ monocyte efferocytosis and cross-presentation of cell-associated antigens

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Recently it was shown that circulating Ly6C$^+$ monocytes traffic from tissue to the draining lymph nodes (LNs) with minimal alteration in their overall phenotype. Furthermore, in the steady state, Ly6C$^+$ monocytes are as abundant as classical dendritic cells (DCs) within the draining LNs, and even more abundant during inflammation. However, little is known about the functional roles of constitutively trafficking Ly6C$^+$ monocytes. In this study we investigated whether Ly6C$^+$ monocytes can efferocytose (acquire dying cells) and cross-present cell-associated antigen, a functional property particularly attributed to Batf3$^+$ DCs. We demonstrated that Ly6C$^+$ monocytes intrinsically efferocytose and cross-present cell-associated antigen to CD8$^+$ T cells. In addition, efferocytosis was enhanced upon direct activation of the Ly6C$^+$ monocytes through its corresponding TLRs, TLR4 and TLR7. However, only ligation of TLR7, and not TLR4, enhanced cross-presentation by Ly6C$^+$ monocytes. Overall, this study outlines two functional roles, among others, that Ly6C$^+$ monocytes have during an adaptive immune response.
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The initiation of the adaptive immune response is highly attributed to non-lymphoid dendritic cells (DCs) that traffic antigen to the draining lymph nodes (LNs). In most tissues, like the skin and lung, there are two overarching non-lymphoid DC subsets that are known as Batf3$^+$ DCs (CD103$^+$) and Irf4$^+$ DCs (CD11b$^+$ DCs).[1] However, in addition to DCs, there is another mononuclear phagocyte that constitutively traffics into tissue and drains to the LNs: the Ly6C$^+$ monocytes. During steady state conditions, extravasated Ly6C$^+$ monocytes maintain most of their original transcriptional signature found in blood.[2] Nonetheless, some transcriptional and protein changes that do occur during extravasation include the up-regulation of molecules associated with antigen presentation.[2–4] Due to this finding, we set out to investigate whether Ly6C$^+$ monocytes play a role in antigen presentation. Although Ly6C$^+$ monocytes have been shown to cargo soluble antigen from tissue to the draining LNs and present *in vitro* soluble antigen to CD4$^+$ and CD8$^+$ T cells,[2,5] a question still remains as to whether, Ly6C$^+$ monocytes have the capacity to efferocytose (acquire dying cells) *in vivo* and cross-present cell-associated antigen to CD8$^+$ T cells.

In this study, we examined whether Ly6C$^+$ monocytes have an intrinsic ability to efferocytose, similar to Batf3$^+$ DCs, and cross-present cell-associated antigen to CD8$^+$ T cells, a property characteristic of Batf3$^+$ DCs.[6–8] The main reasons behind this line of investigation were two: first, Ly6C$^+$ monocytes are precursors to non-embryonic derived macrophages, and macrophages, albeit substantially larger and unable to migrate to draining LNs, are known to be highly efferocytic. Second, we have shown that Batf3$^+$ DCs, and not Irf4$^+$ DCs, predominantly efferocytose and cross-present cell-associated antigen to the adaptive immune response but it is unclear whether Ly6C$^+$ monocytes also contribute to this process.

Here we demonstrate that Ly6C$^+$ monocytes do efferocytose and cross-present cell-associated antigen, and both processes are enhanced under discrete TLR stimulation. This study suggests that in tissue, trafficking Ly6C$^+$ monocytes, along with Batf3$^+$ DCs, have the innate ability to acquire and cross-present cell-associated antigen for an adaptive immune response, which are enhanced by selective TLR agonists.

Results

TLR ligated Ly6C$^+$ monocytes display enhanced efferocytosis. Initial experiments addressed the ability of Ly6C$^+$ monocytes to ingest apoptotic cells *in vivo*. CFSE-labeled apoptotic thymocytes were injected intravenously into C57BL/6 mice and 4 h later the spleen was harvested to assess efferocytosis among different potential phagocytes. As previously shown, of the two main classical DCs, Batf3$^+$ DCs (also known as CD8α^+ DCs at this site) predominantly acquired apoptotic cells compared to Ir4$^+$ DCs (also known as CD11b$^+$CD8$^-$ DCs) (Figure 1a).[2] In total, there were five splenic cell populations that exhibited uptake of the apoptotic cells. In addition to the Batf3$^+$ DCs (CD8$^+$), efferocytosis was observed in red pulp macrophages (F480^{hi}), neutrophils (Ly6C^{int}CD11b^{hi}), Ly6C$^-$ monocytes and Ly6C$^+$ monocytes (Figure 1a). Within the spleen, red pulp macrophages and Ly6C$^-$ monocytes are not located within the para-cortex, and along with neutrophils are not thought to be capable of priming naïve T cells. Therefore, in addition to Batf3$^+$ DCs in the spleen, Ly6C$^+$ monocytes may have the potential to

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Abbreviations: APC, antigen-presenting cells; DCs, dendritic cells; LN, lymph node; TLR, toll-like receptor; CFSE, carboxyfluorescein succinimidyl ester
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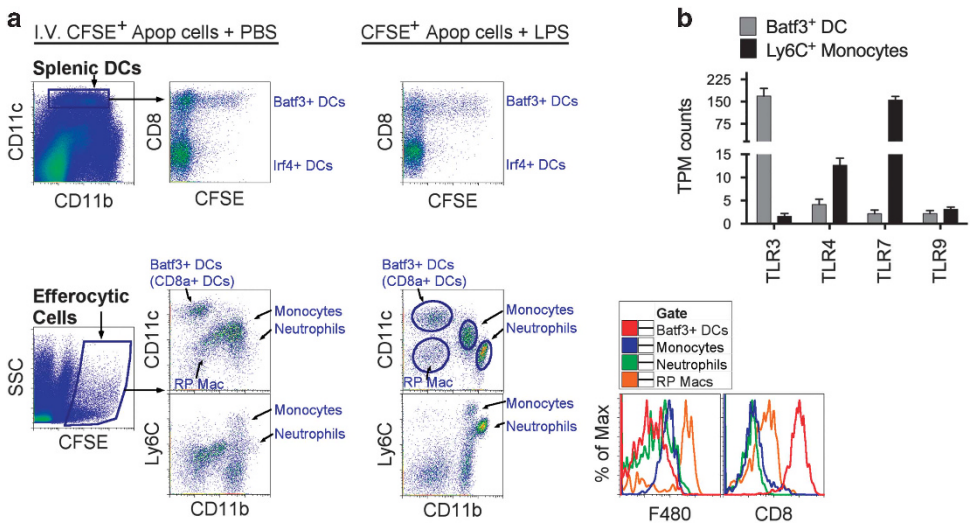

Figure 1 Splenic Ly6C$^+$ monocytes acquire apoptotic cells *in vivo*. (**a**) Spleens from C57BL/6 mice were harvested 4 h after i.v. injection of CFSE-labeled apoptotic thymocytes with or without LPS. Efferocytic cells were identified by flow cytometry as CFSE$^+$. Data are representative of 3 independent experiments. Red pulp macrophages (RP Mac), Apoptotic cells (Apop cells). (**b**) Splenic Batf3$^+$ DCs and Ly6C$^+$ monocytes from naïve mice were enriched and FACS sorted. RNA was isolated for RNA sequencing and quantitation analysis. Triplicate samples were averaged and plotted as transcript per million values for indicated genes.

cross-present cell-associated antigen to CD8$^+$ T cells. Furthermore, when we initiated an inflammatory environment using a standard inflammatory agent, LPS, we also observed efferocytosis by the same five splenic populations, as seen under steady-state conditions. However, during inflammation there was an increase in the total number of Ly6C$^+$ monocytes that were CFSE$^+$, as indicated by flow cytometry dot plots (Figure 1a).

Due to these results, we next examined whether direct TLR stimulation on monocytes could enhance Ly6C$^+$ monocyte efferocytosis. First, we determined the spectrum of TLR expression on purified Batf3$^+$ DCs and splenic Ly6C$^+$ monocytes. RNA sequencing analysis measuring total RNA transcript per million showed that, as expected, Batf3$^+$ DCs expressed high levels of TLR3 (senses dsRNA), compared with TLR4 (senses LPS), TLR7 (senses ssRNA) and TLR9 (senses CpG).[8,9] By contrast, Ly6C$^+$ monocytes expressed higher levels of TLR4 and TLR7 compared with TLR3 and TLR9 (Figure 1b). This observation is also supported by Immgen Affymetrix microarrays (immgen.org).[2,8] Due to the monocyte TLR repertoire – high levels of TLR4 and TLR7 expression – we hypothesized that direct stimulation of these receptors might enhance Ly6C$^+$ monocyte efferocytosis, compared with direct stimulation of TLR3 and TLR9. To test this, CFSE-labeled, UV irradiated apoptotic thymocytes were adoptively transferred into mice in the presence or absence of Poly I:C (TLR3 ligand), LPS (TLR4 ligand), R848 (TLR7 ligand) or CpG (TLR9 ligand). Uptake of the apoptotic cells was assessed 1 h later. To identify efferocytic cells, Batf3$^+$ DCs were identified as CD11c^{hi}CD8α^+CD11b$^-$CFSE$^+$ and monocytes as Ly6C^{hi}CD11b$^+$CFSE$^+$ (Figure 2a). We observed that the frequency of apoptotic cell acquisition by Batf3$^+$ DCs was similar whether a TLR ligand was present or absent. However, when monocytes were stimulated by their corresponding TLRs, TLR4 and TLR7, compared with their low-expressing TLRs, TLR3 and TLR9, their capacity to acquire apoptotic cell was significantly enhanced compared with those treated with apoptotic cells alone (Figure 2a). Similar acquisition of apoptotic cells was observed whether apoptosis was induced either by UV or dexamethasone treatment. Furthermore, in the absence of TLR7 expression (TLR7 deficient mice), the acquisition of apoptotic cells in the presence of R848 was abrogated (Figure 2b). These data suggest that monocytes *in vivo* increase their efferocytic capacity when directly stimulated by their corresponding TLRs.

To support the concept that enhanced efferocytosis by TLR stimulated Ly6C$^+$ monocytes is cell intrinsic and not due to surrounding inflammatory mediators, we examined efferocytosis by Batf3$^+$ DCs and Ly6C$^+$ monocytes *ex vivo*. CD11c and CD11b expressing splenic cells were positively enriched using Miltenyi magnetic bead separation. Enriched cells were placed in flat bottom wells for 3 h prior to adding CFSE-labeled apoptotic thymocytes in the presence or absence of Poly I:C, LPS, R848 and CpG. Similar to our findings *in vivo*, *ex vivo* Batf3$^+$ DCs efferocytosed apoptotic cells with a similar frequency whether or not TLR ligands were present (Figure 2c), whereas Ly6C$^+$ monocytes displayed enhanced efferocytosis when TLR4 and TLR7 were directly stimulated (Figure 2c). Hence, these data support that the enhanced efferocytosis by Ly6C$^+$ monocytes in the presence of TLR4 and TLR7 ligands is cell intrinsic.

To describe the physical interactions of monocytes with apoptotic cells, an image was obtained using an *ex vivo* platform. Enriched monocytes were co-cultured with apoptotic cells, then fixed and stained with fluorescently labeled anti-Ly6C for imaging. Here, the image shows a Ly6C$^+$ monocyte with a fully ingested apoptotic cell (CFSE^{dim}) as well as a tethered apoptotic cell (CFSE^{hi}) (Figure 2d).

Transcriptome analysis of activated monocytes. Next we investigated the transcriptional change that occurs when monocytes acquire apoptotic cells with LPS or R848 (RNA

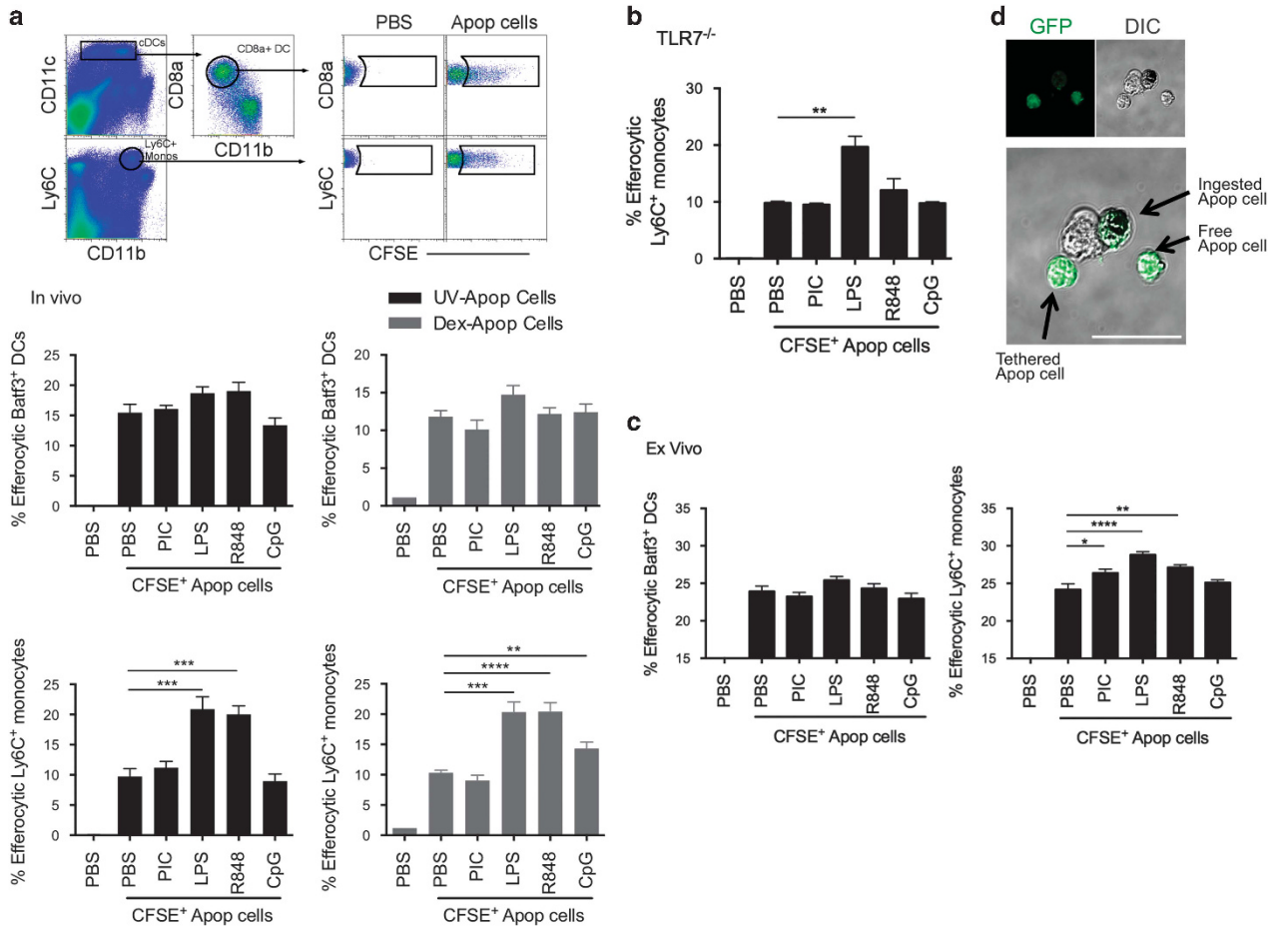

Figure 2 TLR4 and TLR7 stimulated Ly6C$^+$ monocytes exhibit enhanced efferocytosis. (**a**) CFSE-labeled apoptotic thymocytes induced by either UV radiation or dexamethasone treatment were adoptively transferred i.v. in conjunction with TLR agonizts: Poly I:C (PIC), LPS, R848 and CpG. One-hour post transfer of apoptotic cells +/− TLR agonizts, spleens were harvested and the cells analyzed by flow cytometry. Batf3$^+$ DCs were identified as CD11c^{hi}CD8a$^+$ and monocytes were identified as Ly6C^{hi}CD11b$^+$ (left plots). Percent efferocytosis was measured by gating CFSE$^+$ cells (right plots). Graphs display the % efferocytic Batf3$^+$ DCs (top) and Ly6C$^+$ monocytes (bottom). Data pooled from three independent experiments with three mice per group. (**b**) TLR7$^{-/-}$ mice analyzed for *in vivo* uptake of CFSE-labeled apoptotic cells in the presence or absence of TLR agonizts. Data pooled from two independent experiments with three mice per group. (**c**) Splenic Batf3$^+$ DCs (left) and Ly6C$^+$ monocytes (right) were enriched by CD11b and CD11c magnetic microbead selection and co-cultured with CFSE-labeled apoptotic cells in the presence or absence of TLR agonizts for 1-h before analysis for uptake by flow cytometry. Data pooled from three independent experiments with triplicate wells. (**d**) Co-cultured monocytes and CFSE-labeled apoptotic thymocytes (green) were fixed and stained for Ly6C after a 1-h incubation for imaging. Panels include CFSE$^+$ apoptotic cells (top left), differential interference contrast (DIC) (top right) and a merged image of a Ly6C$^+$ monocyte with an ingested apoptotic cell and a tethered apoptotic cell. Bar scale represents 20 μm. ****$P<0.0005$, ***$P<0.005$, **$P<0.005$, *$P<0.05$.

reads and *q* values in Supplementary Table I and Figure 3). To determine the transcriptional regulation of TLR activation upon apoptotic cell ingestion, we performed RNA sequencing on purified monocytes that have efferocytosed (sorted CFSE$^+$Ly6C$^+$ monocytes). Since we observed that monocytes enhance efferocytosis in the presence of LPS and R848, we first examined genes known to participate in efferocytosis (Figure 3a), such as scavenger receptors (top panel), negative regulators (middle panel) and adapter proteins (bottom panel). Of the genes outlined, over 50% were increased when monocytes were stimulated with either LPS or R848; albeit, the relevance of these gene changes are currently unclear and will require future investigations.

Since we observed enhanced efferocytosis by Ly6C$^+$ monocytes, we next examined whether direct TLR stimulation altered the expression of molecules associated with antigen presentation. For efficient antigen presentation we examined co-stimulatory molecules and MHC I and MHC II expression to present antigen-peptides. Indeed, RNA expression of co-stimulatory molecules was increased in Ly6C$^+$ monocytes stimulated with either LPS or R848 compared with unstimulated cells (Figure 3b). In addition, Ly6C$^+$ monocytes that acquired apoptotic cells with LPS or R848 increased their RNA expression for MHC I related proteins (Figure 3c). Notably we also observed differences in the responses to LPS versus R848. R848 stimulated monocytes, unlike LPS stimulated monocytes or monocytes with apoptotic cells only, substantially enhanced the gene expression of essential MHC I pathway molecules: *B2m*, *Tap1* and *Tapbp* (Beta-2 microglobulin, transporter associated with antigen processing 1 and tapasin). In summary, in the presence of LPS and R848, Ly6C$^+$

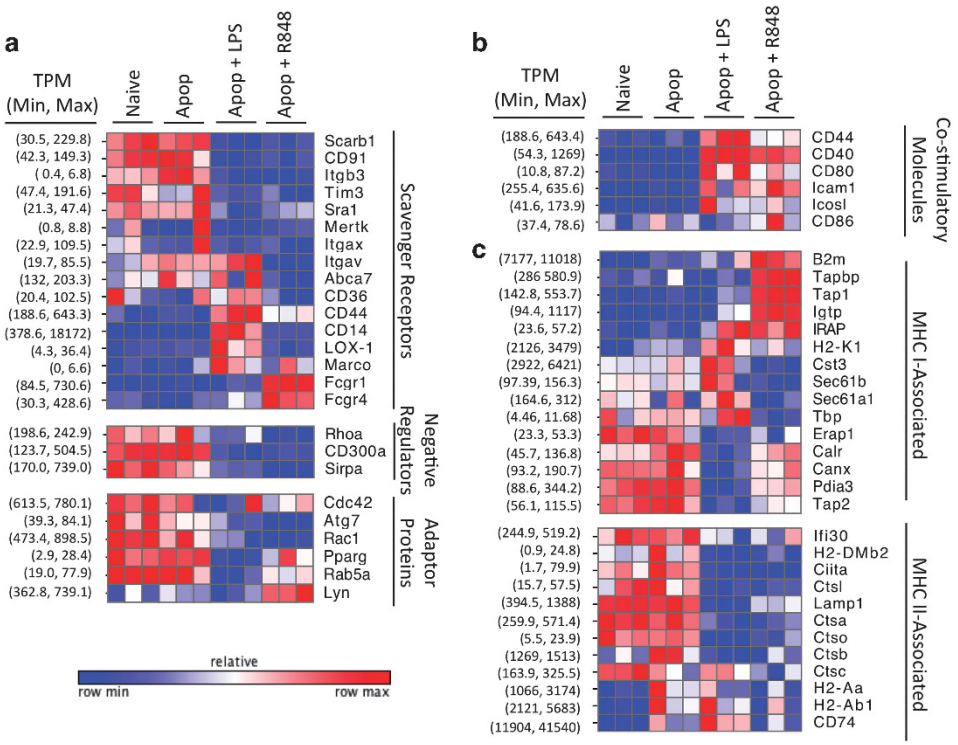

Figure 3 TLR4 and TLR7 stimulated Ly6C$^+$ monocytes display enhanced gene expression for phagocytosis, co-stimulatory molecules and MHC-associated genes. Efferocytic Ly6C$^+$ monocytes were obtained by adoptively transferring CFSE$^+$ apoptotic thymocytes into C57BL/6 mice with or without LPS or R848. Naive control included PBS treated mice without apoptotic cells. One hour post adoptive transfer, spleens were harvested to FACS sort CFSE$^+$ Ly6C$^+$ monocytes. RNA was extracted from the efferocytic Ly6C$^+$ monocytes to perform RNA sequencing. RNA reads for each gene were *z*-scored (mean-subtracted divided by standard deviation). Heat maps were generated using GENE-E software and displayed as row scaled data where red indicates relative gene up-regulation and blue indicates relative gene down-regulation. Minimum and maximum transcript per million values for each gene are listed along the left. Genes shown are (**a**) Scavenger receptors, negative regulators of phagocytosis and adapter proteins (**b**) Co-stimulatory molecules, and (**c**) MHC-I and MHC-II-associated molecules. Heat maps display triplicate samples for each treatment group. *q*-values in Supplementary Table I.

monocytes showed increases in gene expression of efferocytic and antigen presenting pathways.

TLR7 stimulated Ly6C$^+$ monocytes demonstrate enhanced cross-presentation of efferocytosed cell-associated antigen. Recently, we demonstrated that direct (and not indirect) TLR7 stimulation on soluble antigen-bearing Irf4$^+$ DCs promotes cross-presentation.[4] Therefore, we examined whether direct stimulation of the corresponding TLRs, TLR4 and TLR7, on Ly6C$^+$ monocytes would enhance cross-presentation of cell-associated antigen (i.e. the loading of OVA-SIINFEKL peptide on MHC I). To examine cross-presentation, we adoptively transferred intravenously OVA-expressing thymocytes with or without TLR agonists. After 16 h, the spleen was harvested to measure SIINFEKL/H-2 k^b expression on Ly6C$^+$ monocytes. R848 stimulated Ly6C$^+$ monocytes significantly cross-presented the SIINFEKL peptide in comparison with PBS, Poly I:C, LPS, or CpG (Figure 4a).

As an alternative method for measuring cross-presentation, the proliferative response of antigen specific OT-I CD8$^+$ T cells was determined. First, Ly6C$^+$ monocytes were enriched by positively selecting for B220, CD3 and Ly6G expressing cells to remove B cells, plasmacytoid DCs, T cells, eosinophils and neutrophils. Then, the negatively selected cells were positively enriched for Ly6C, to enrich for the Ly6C$^+$ monocytes. This second enrichment step excluded conventional DCs, Ly6C$^-$ monocytes and splenic macrophages that do not express Ly6C. After 3 h, OVA-expressing apoptotic thymocytes were co-cultured in the presence or absence of Poly I:C, LPS, R848 and CpG. Then, CFSE-labeled antigen-specific CD8$^+$ T cells (OT-I cells) were added and co-cultured for four days to assess the cross-presentation capacity of TLR stimulated Ly6C$^+$ monocytes (Supplementary Figure 2 and Figure 4b). Interestingly, TLR7 stimulated, but not TLR4 stimulated, Ly6C$^+$ monocytes demonstrated significantly enhanced cross-presentation of cell-associated antigen compared with no TLR stimulation or TLR3 and TLR9 stimulated monocytes (Figure 4b). To confirm that these observations are substantially attributed to monocytes rather than contaminating cells within the enrichment process, we performed the same assay with FACS sorted Ly6C$^+$ monocytes in comparison with FACS sorted Batf3$^+$ DCs. Purified monocytes showed comparable results, in that only R848 stimulated monocytes significantly enhanced cross-presentation compared with PBS or other TLR ligands (Figure 4c). Furthermore, the intrinsic ability of monocytes to cross-present antigen was comparable with Batf3$^+$ DCs.

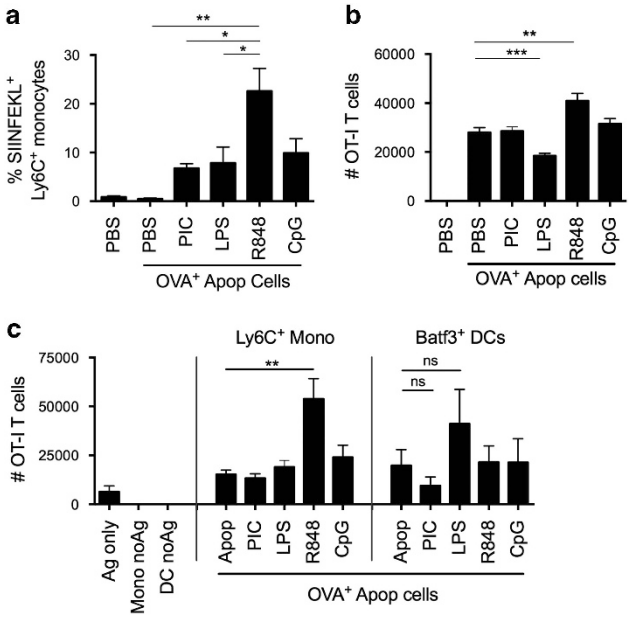

Figure 4 TLR7, and not TLR4, stimulated Ly6C$^+$ monocytes display enhanced cross-presentation. (**a**) OVA-expressing apoptotic cells were adoptively transferred into C57BL/6 mice with or without TLR ligands. After 16 h spleens were harvested and SIINFEKL/H-2 k^b expression on Ly6C$^+$ monocytes was determined by flow cytometry. Graph displays percentage of Ly6C$^+$ monocytes that are SIINFEKL/H-2 k^b positive. Control mice included PBS treated mice without OVA-expressing apoptotic cells. Data pooled from three independent experiments, containing three mice per group. (**b**) Enriched Ly6C$^+$ monocytes were co-cultured in the presence or absence of OVA-expressing apoptotic cells and TLR agonizts. Then, CFSE-labeled OT-I T cells are added to the wells. After four days, cells were analyzed by FACS. Graph displays total number of proliferated OT-I T cells obtained from each well. Data are representative of two independent experiments with 6–7 replicates per group. (**c**) Purified Batf3$^+$ DCs or Ly6C$^+$ monocytes were co-cultured with OVA-expressing apoptotic cells in the presence or absence of TLR agonizts. Then, CFSE-labeled OT-I T cells are added to the wells. Controls included OT-I CD8$^+$ T cells co-cultured with antigen expressing apoptotic cells (antigen only, no APC), monocytes (no antigen) or Batf3$^+$ DCs (no antigen). Graph displays total number of proliferated OT-I T cells from each well. Data is representative of three experiments with three replicates per group.

Discussion

This study corroborates our previous findings that identify the Ly6C$^+$ monocyte as a mononuclear phagocyte with its own immunologic effector function, beyond terminal differentiation into macrophages or DCs. Here we show that Ly6C$^+$ monocytes can acquire and cross-present cell-associated antigen, a function thought to be predominantly by Batf3$^+$ DCs.[8,10,11] Furthermore, we show that direct, and not indirect TLR ligation on Ly6C$^+$ monocytes enhances efferocytosis. This is in contrast to Batf3$^+$ DCs, where the presence of TLR ligands did not increase their overall efferocytic capacity, suggesting that Batf3$^+$ DCs maintain an intrinsic ability to phagocytose independent of TLR ligation, whereas monocytes have inducible effector functions, including the acquisition and antigen presentation of exogenous antigens.

Although other APCs such as splenic CD169$^+$ macrophages, Irf4$^+$CD11b$^+$ DCs or adoptively transferred *ex vivo* monocyte-derived DCs have been shown to cross-present; the cross-presentation for these APCs was demonstrated using either non-apoptotic particulates or soluble antigen.[5,9,12,13] Here we demonstrate cross-presentation in the context of apoptotic cells (i.e. cross-presentation of cell-associated antigen), which are not acquired by Irf4$^+$CD11b$^+$ DCs.

While TLR ligands, LPS and R848, initiated comparable enhancement of Ly6C$^+$ monocyte efferocytosis compared with TLR3 and TLR9 ligands, the downstream signaling responses were shown to be distinct, at the gene level. The differences in the transcriptional profile of TLR4 and TLR7 stimulated monocytes are most likely due to the fact that first, the cellular location of TLR4 and TLR7 are different. TLR4 is on the cell surface, while TLR7 is in the endosomal compartment. Second, downstream signaling differs between TLR4 (MyD88 or TRIF) and TLR7 (MyD88-dependent pathway). Interestingly, R848 compared to LPS stimulated Ly6C$^+$ monocytes displayed enhanced gene expression of essential MHC I pathway molecules: *B2m*, *Tap1* and *Tapbp* (Beta-2 microglobulin, transporter associated with antigen processing 1 and tapasin). Therefore, it is not surprising that in the presence of R848 compared with LPS, Ly6C$^+$ monocytes demonstrated enhanced cross-presentation of the cell-associated antigen by direct antigen loading on MHC I and indirect measurement of antigen-specific CD8$^+$ T cell proliferation.

The implication for enhanced cross-presentation by Ly6C$^+$ monocytes may be to expand a protective immune response in a similar fashion as Batf3$^+$ DCs, which is to induce the differentiation of antigen-specific CD8$^+$ T cells into cytotoxic T cells in the presence of a TLR3 ligand, dsRNA. Thus, our data would suggest that Ly6C$^+$ monocytes could likewise promote cytotoxic T cell development in the presence of a TLR7 ligand, ssRNA. Both of these TLR ligands stimulate cytokine production, known as signal 3, for the induction of cytotoxic T cells (data not shown).[9] Future studies will need to compare the differential effects induced by TLR4 and TLR7 stimulated Ly6C$^+$ monocytes for cell-associated antigen processing and presentation. In contrast to R848 stimulated monocytes, it is possible that LPS enhances antigen loading on MHC II more than MHC I, as the transcriptional data illustrated that TLR4 ligated Ly6C$^+$ monocytes displayed increased gene expression of essential MHC II molecules, *H2-Aa* and *H2-Ab1*, and the invariant chain, *CD74*.

In conclusion, these data demonstrate that Ly6C$^+$ monocytes intrinsically acquire and cross-present cell-associated antigen. In addition, TLR4 and TLR7 stimulation increased the efferocytosis, while TLR7, but not TLR4, enhanced MHC I antigen presentation. Therefore, functional differences between pathogenic stimuli of monocytes provided by TLR ligands promote divergent immune responses. As TLR ligands are increasingly used as adjuvants in current human vaccine studies, our results become relevant and of special interest to the growing field of immunotherapy, where we recently identified the human monocyte trafficking orthologs in tissue and draining LNs.[14]

Materials and Methods

Mice. CD45.1 and CD45.2 C57BL/6, OT-I, TLR7$^{-/-}$ and C57BL/6-Tg (ACTB-OVA) 916Jen/J mice were purchased from Jackson Research Laboratories. Mice used were 6–12 weeks of age and maintained in a specific pathogen-free

environment at National Jewish Health, an AALAC accredited institution, and used in accordance with protocols approved by the Institutional Animal Care and Utilization Committee.

Apoptotic cell delivery. Thymocytes were extracted from C57BL/6 or ACTB-OVA mice. Single-cell suspensions were obtained using a syringe plunger and mashing cells through a 70 μm nylon filter. For detection of apoptotic cell uptake, live cells were labeled with 10 μM CFSE for 10 min (Invitrogen, Carlsbad, CA, USA) and then washed in PBS+10% BSA. Then, apoptosis was induced by either 60 mJ UV radiation exposure (StratLinker 1800; Agilent Technologies, Santa Clara, CA, USA) or 10 μM dexamethasone for 4 h at 37 °C (Sigma-Aldrich, St Louis, MO, USA). We confirmed the induction of apoptosis by phosphatidylserine exposure, detected as Annexin V positive and propidium iodide negative (Supplementary Figure 1).

Tissue collection. Spleens were minced and digested in 1 ml of 2.5 mg/ml collagenase D (Roche, Indianapolis, IN, USA) for 20 min at 37 °C. A total of 100 μl of 100 mM EDTA was added to stop enzymatic digestion. Single-cell suspensions were obtained by pipetting digested tissue using a glass Pasteur pipette and filtering cells through a 70 μm nylon filter. Samples were vortexed for 15 s in 1X ammonium chloride lysing reagent (BD Biosciences, San Jose, CA, USA) and immediately resuspended, filtered, spun and washed in Hanks' Balanced Salt Solution (HBSS).

Flow cytometry. Cells were stained with conjugated antibodies obtained from Biolegend (San Diego, CA, USA), eBioscience (San Diego, CA, USA) and BD Biosciences, for 30 min. The following purified monoclonal antibodies were used for staining: Pacific blue–conjugated anti-CD8 and CD11b; Pacific orange-conjugated anti-IA/IE; PE-conjugated anti-CD4 or Ly6C; PerCP-Cy5.5–conjugated anti- Ly6C or CD8; PE-Cy7–conjugated mAb to anti-CD11c; allophycocyanin-Cy7-conjugated anti- MHCII, B220 and Ly6G; and allophycocyanin-conjugated anti-CD64 and anti-SIINFEKL/H-2Kb (clone 25-D1.16). Flow cytometry was performed using the LSRFORTESSA (BD Biosciences) and data was analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Efferocytosis assay. For *in vivo* studies, apoptotic cells (10×10^6) were delivered simultaneously with or without 20 μg Poly I:C (Enzo Life Sciences, Farmingdale, NY, USA), 5 μg LPS (Sigma-Aldrich), 50 μg R848 (Enzo Life Sciences) or 20 μg CpG ODN 1668 (Enzo Life Sciences). After 1 or 4 h, spleens were harvested to assess efferocytosis. For *in vitro* studies, spleen cells were harvested, RBC lysed and single-cell suspensions obtained (as described above). Myeloid cells were positively enriched using CD11b and CD11c microbeads through a LS column (Miltenyi Biotec, Auburn, CA, USA). One million cells were plated on a 96-well flat-bottom plate on Bend3 cells (3×10^4).[2] Apoptotic cells were added in conjunction with or without the TLR agonizts Poly I:C (500 ng), LPS (125 ng), R848 (250 ng) and CpG (500 ng). After a 1 h of incubation at 5% CO_2 and 37 °C cells were harvested to assess efferocytosis.

Detection of SIINFEKL peptide loading. OVA-expressing apoptotic cells ($10x10^6$) were delivered i.v. with or without 20 μg Poly I:C, 5 μg LPS, 50 μg R848 or 20 μg CpG. Sixteen hours post treatment spleens were harvested for flow cytometric analysis to assess the expression of SIINFEKL (OVA peptide) loaded on MHC I (Ab clone: eBio25-D1.16).

T cell proliferation assay. Ly6C$^+$ monocytes were purified with a two-step selection. First: removal of lymphocytes, pDC and neutrophils with biotinylated anti-Ly6g (IA8 clone) and anti-CD3 and anti-B220. Then, flow-through cells were positively selected for Ly6C expression. Ly6C$^+$ cells contained no CD11c$^+$ DCs or Ly6C$^-$ monocytes as these cells do not express Ly6C. Purity of cell populations was confirmed by flow cytometric analysis. The monocyte preparation contained ~40–50% of Ly6C$^+$ monocytes. Contaminating cells included T cells and neutrophils, but no classical DCs, B cells or pDCs were observed. 3×10^5 cells were plated in a 96-well round bottom plate with ACTB-OVA apoptotic cells (1×10^5) and purified CFSE-labeled OT-I T cells (1×10^5) in the presence or absence of TLR agonizts Poly I:C (500 ng), LPS (125 ng), R848 (250 ng) and CpG (500 ng). In addition, Ly6C$^+$ monocytes and CD8α+ DCs (5×10^4) were stained for fluorescence-activated cell sorting (FACSAria Cell Sorter) and plated as described above. After four days the plates were washed and stained for flow cytometric analysis. Proliferating cells were considered CFSE+CD8+.

Monocyte purification and next-generation sequencing of the transcriptome (RNA sequencing). Spleens were harvested as described above. Monocytes were enriched using CD11b microbeads (Miltenyi Biotec) and stained for fluorescence-activated cell sorting (FACSAria Cell Sorter). RNA was isolated from sorted splenic monocytes using the RNeasy Mini Kit (Qiagen). The isolated total RNA was processed for next-generation sequencing library construction as developed in the NJH Genomics Facility for analysis with a Life Technologies (Carlsbad, CA, USA) ion proton next-generation sequencing platform. A modified Clontech SMARTer Ultra Low Input RNA Kit for Sequencing - v3 (Mountain View, CA, USA) and modified Kapa Biosystems (Wilmington, MA, USA) KAPA Hyper Prep Kit was used to primarily target all polyA RNA. Briefly, library construction started from isolation of total RNA species, followed by mRNA (poly-A) isolation, 1st and 2nd strand cDNA synthesis, adapter ligation, amplification and bead templating. Once validated, the libraries were sequenced as barcoded-pooled samples on a P1 ion proton chip, as routinely performed by the NJH Genomics Facility. An Analysis of Deviance was performed on the raw counts using the package DESeq2 (version 1.8.1)[15] in R (version 3.2.0)[16] by comparing a model with the treatment (Naïve, Apop, Apop+LPS, Apop+R848) as variable to a reduced model with only the intercept using the likelihood test. Heat maps were generated using the GENE-E software (http://www.broadinstitute.org/cancer/software/GENE-E/index.html) from the Broad Institute. Statistical analysis: q values and RNA reads are in Supplementary Table 1.

Confocal imaging. Isolated splenic Ly6C$^+$ monocytes were plated at 0.5×10^6 in a 100 μl bubble of media (DMEM+10% FBS+1% PSG) onto MatTek 35 mm glass bottom dishes for 1 h before additional media was added. After 3 h of monocyte settling, CFSE-labeled apoptotic thymocytes were added. Monocytes and apoptotic cells were co-cultured for 1 h in at 5% CO_2 and 37 °C. Non-adherent thymocytes were removed with PBS and cells were fixed with 4% PFA for 15 min at room temperature. Images were taken using a Zeiss LSM700 Confocal with Zen software. Magnification is $\times$ 630 ($\times$ 63 oil objective, $\times$ 10 eyepiece).

Statistical analysis. Statistical analysis was conducted using InStat and Prism software (GraphPad Software). All results were expressed as the mean $\pm$ S.E.M. Statistical tests were performed using two-tailed Student's *t* test. A value of $P < 0.05$ was considered statistically significant. For RNA sequencing, *P*-values were adjusted using the Benhamini-Hochberg false discover rate control using 10 822 genes with sufficient counts tested by DESeq2.[17]

Conflict of Interest

The authors declare no conflict of interest.

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