ARTICLE

FcγR engagement reprograms neutrophils into antigen cross-presenting cells that elicit acquired anti-tumor immunity

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Classical dendritic cells (cDC) are professional antigen-presenting cells (APC) that regulate immunity and tolerance. Neutrophil-derived cells with properties of DCs (nAPC) are observed in human diseases and after culture of neutrophils with cytokines. Here we show that FcγR-mediated endocytosis of antibody-antigen complexes or an anti-FcγRIIIB-antigen conjugate converts neutrophils into nAPCs that, in contrast to those generated with cytokines alone, activate T cells to levels observed with cDCs and elicit CD8+ T cell-dependent anti-tumor immunity in mice. Single cell transcript analyses and validation studies implicate the transcription factor PU.1 in neutrophil to nAPC conversion. In humans, blood nAPC frequency in lupus patients correlates with disease. Moreover, anti-FcγRIIIB-antigen conjugate treatment induces nAPCs that can activate autologous T cells when using neutrophils from individuals with myeloid neoplasms that harbor neoantigens or those vaccinated against bacterial toxins. Thus, anti-FcγRIIIB-antigen conjugate-induced conversion of neutrophils to immunogenic nAPCs may represent a possible immunotherapy for cancer and infectious diseases.

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Classical DCs (cDCs) are highly active at presenting peptides derived from internalized extracellular antigens on MHC class II molecules (MHCIi) to CD4+ T cells and on MHC class I molecules (MHCII) to CD8+ T cells. The latter process referred to as cross-presentation, is essential for anti-tumor immunity and eradication of pathogens. Among antigen-presenting cells (APC), only cDCs are specialized for cross-presentation and robust activation of immunologically naive CD4+ and CD8+ T cells, which have a higher activation threshold than memory T cells. However, cDC-based cancer therapy is difficult in practice due to the low abundance of DCs that cross-present antigens and the need to administer short-lived, toxic TLR agonists to convert cDC populations into immunogenic APCs.

Neutrophils are widely considered terminally differentiated, short-lived innate immune effector cells. However, neutrophils with DC markers are present in patients with cancer, infections, and autoimmune diseases, and several studies show that neutrophils upregulate the cDC surface molecules CD11c, MHCIi, and T cell co-stimulatory molecules when cultured with cytokines (e.g., GM-CSF) or autologous T cells, while retaining select neutrophil markers and functions. These cells were described as transcriptionally similar to monocytic-derived DCs and their generation required the downregulation of the transcription factor Ikaros. Although in vitro studies demonstrate that cytokine-generated neutrophil-derived APCs (nAPC) can promote CD4+ T cell responses, it is debated whether they efficiently cross-present antigen or if mature, differentiated neutrophils can acquire DC-like features. Furthermore, the molecular drivers of conversion are poorly understood and whether nAPCs are pathogenic or can be harnessed for immunotherapy remain largely unexplored.

IgG antibody–antigen immune complexes (ICs) trigger several neutrophil effector functions by binding to activating Fcg receptors (FcγRs). Mouse neutrophils express activating FcγRs, FcγRI, FcγRII, and FcγRIV, each associated with a Fcγ-chain that is required for their expression and function. Notably, Fcγ-chain-deficient γ−/− mice are protected from IgG-mediated tissue injury. Human neutrophils, by contrast, express FcγRI, FcγRIIA (which promotes cytotoxic functions), and FcγRIIB, a glycosylphosphatidylinositol (GPI)-linked neutrophil-specific receptor of uncertain function. We have explored the in vivo functions of human FcγRs by expressing human FcγRIIA and/or FcγRIIB selectively on neutrophils of mice that lack the Fcγ-chain (FcγRγ−/−) and therefore their endogenous activating FcγRs. Our studies revealed that FcγRIIA and FcγRIIB both promote IgG-mediated neutrophil accumulation in tissues, while only FcγRIIA induces tissue injury.

Here, we investigate whether neutrophils can convert to cells with the antigen-presenting capabilities of cDCs following engagement of their FcγRs and explore the therapeutic potential of this conversion. Our data demonstrate that neutrophil endocytosis of antibody–antigen complexes via FcγRs or FcγRIIB engagement with an anti-FcγRIIB-antigen conjugate rapidly converts them into fully immunogenic nAPCs, and the number of nAPCs in lupus patient blood correlates with disease outcomes. We also define the transcriptome of nAPCs and the transcriptional program driving neutrophil to APC conversion downstream of GM-CSF and/or FcγRs. Of therapeutic relevance, we show that nAPCs induce anti-tumor immunity in mice and when generated from human neutrophils can reactivate autologous antigen-specific memory T cells in vitro. Thus, neutrophil FcγRs provide a direct link between innate and adaptive immunity and may be targeted as a strategy to generate a large number of immunogenic nAPCs for T cell-based immunotherapy.

Results

Immune complexes engage activating FcγRs on mature neutrophils to promote their conversion to nAPCs. Murine peripheral blood mature neutrophils (Supplementary Fig. 1a) were treated with Ovalbumin (Ova)-anti-Ova immune complexes (Ova-IC), anti-Ova, or Ova for 2 h, washed and cultured with GM-CSF, known to preserve viability. At day 3, Ly6G+ neutrophils under all three conditions exhibited >65% survival and acquired DC surface markers (CD11c+MHCIi), as previously reported for neutrophils cultured in GM-CSF alone. Importantly, Ova-IC treatment significantly increased the percentage of neutrophils expressing CD11c, MHCIi, T cell co-stimulatory molecules (CD80 and CD86), and CCR7 (which promotes DC migration to secondary lymphoid organs) as compared to the other treatments (Fig. 1a, b). We obtained similar results with bone marrow neutrophils (BMNs) from G-CSF (Supplementary Fig. 1b–d) and non-G-CSF (Supplementary Fig. 1e) treated mice and with ICs made with antibody to bovine serum albumin (BSA) or hapten-conjugated-Ova (Supplementary Fig. 1f); levels of DC markers on converted BMNs were equivalent to those of splenic and monocye-derived cDCs (Supplementary Fig. 1g) while monocyte and macrophage markers were minimal (Supplementary Fig. 1b). Neutrophils treated with Ova-ICs may release cytokines that contribute to nAPC conversion, independent of FcγR engagement with ICs. To test this, neutrophils isolated from mice expressing CD45.1 or CD45.2 isoforms were differentially pre-treated and co-cultured with GM-CSF. We found that CD45.1+ Ova-IC-pretreated neutrophils were unable to upregulate nAPC markers in CD45.2+ Ova or anti-Ova pre-treated neutrophils (Fig. 1c). Thus, ICs enhance the conversion of neutrophils to nAPCs in a cell-autonomous manner.

Systemic Lupus Erythematosus is a prototypical IC-mediated disease associated with circulating ICs containing autoantibodies against double-stranded DNA and RNA-protein complexes. We observed that sera from SLE patients, as well as ICs formed in vitro with IgG isolated from SLE patients and Ribonucleoprotein, RNP (SLE-IC), promoted conversion of neutrophils to nAPCs. Neutrophils, which lack all TLR receptors, FcγRIIA or FcγRIIIB triggers this conversion. Of therapeutic relevance, we show that sera from SLE patients, as well as ICs formed in vitro with IgG isolated from SLE patients and Ribonucleoprotein, RNP (SLE-IC), promoted conversion of neutrophils to nAPCs. Neutrophils, which lack all TLR receptors, FcγRIIA or FcγRIIIB triggers this conversion. Of therapeutic relevance, we show that sera from SLE patients, as well as ICs formed in vitro with IgG isolated from SLE patients and Ribonucleoprotein, RNP (SLE-IC), promoted conversion of neutrophils to nAPCs. Neutrophils, which lack all TLR receptors, FcγRIIA or FcγRIIIB triggers this conversion. Of therapeutic relevance, we show that sera from SLE patients, as well as ICs formed in vitro with IgG isolated from SLE patients and Ribonucleoprotein, RNP (SLE-IC), promoted conversion of neutrophils to nAPCs. Neutrophils, which lack all TLR receptors, FcγRIIA or FcγRIIIB triggers this conversion.

Immune complex induced conversion of neutrophils to nAPCs is rapid and is accompanied by polymorpho- to mononuclear changes. To assess the kinetics of neutrophil to nAPC conversion after IC treatment and potential changes in nuclear morphology that accompany this change, we imaged mature peripheral blood neutrophils obtained from mice with YFP “knocked-in” to the CD11c locus (CD11c-YFP), labeled with a nuclear dye and treated with SLE-IC (in the absence of GM-CSF). At day 2 of SLE-IC treatment, CD11c-YFP negative neutrophils acquired YFP signal, became mononuclear-like with decondensed nuclear chromatin, as observed by confocal imaging of several fields (Fig. 1f, Supplementary Fig. 2, Movie S1–S2). While in vitro treatment of band neutrophils (immature, immediate precursors of end-stage
neutrophils with GM-CSF for 6–9 days has been shown to result in nuclear morphology changes, we demonstrate the capacity of fully segmented, mature blood neutrophils to undergo changes in nuclear shape. Using live cell imaging, we tracked the conversion of individual neutrophils to nAPCs to examine the kinetics of conversion and thus exclude the possibility that the observed CD11c+ cells are derived from contaminating mononuclear cells. Within 11 h of SLE-IC stimulation, segmented blood neutrophils acquired CD11c-YFP and a mononuclear appearance with decondensed chromatin (Fig. 1g, Supplementary Fig. 3a, b, Movie S3). In summary, SLE-IC treatment rapidly converts fully differentiated blood neutrophils into nAPCs that exhibit marked alterations in nuclear morphology.

nAPC frequency in blood of patients with systemic lupus erythematosus correlate with clinical scores. Given the ability of SLE-IC to promote neutrophil to nAPC conversion, we looked for...
Fig. 1 Engagement of FcγRs on mouse neutrophils generates nAPCs. a, b Wild-type (WT) blood neutrophils treated with Ova, anti-Ova, or Ova-IC, cultured with GM-CSF and evaluated 3 days later by flow cytometry for survival and acquisition of CD11c and MHCII on Ly6G+ cells (left panel), and CD80, CD86, and CCR7 on Ly6G+CD11c+MHCII+ cells (right panel) (a). Representative gating strategy for Ova-IC generated nAPCs (b). c Neutrophils from CD45.1 (45.1) or CD45.2 (45.2) mice pre-treated with Ova or Ova-IC, or anti-Ova or Ova-IC, co-cultured with GM-CSF and analyzed for percent of Ly6G+ cells expressing CD11c and MHCII. d WT bone marrow neutrophils (BMN) pre-treated with SLE patient or normal human (NH) sera, or SLE-IgG+ RNP (SLE-ICs) or normal human sera-IgG+RNP, cultured without GM-CSF and analyzed as in (a). Representative FACS plots are shown. RNP alone resulted in 0.38 ± 0.11% conversion. e BMNs from WT, γ−/−, FcγRIIB(3B)/γ−/− or FcγRIIA(2A)/γ−/− mice treated with SLE or NH sera and analyzed as in (a). f Blood neutrophils from CD11c-YFP (yellow fluorescent protein) reporter mice treated with SLE-ICs, labeled with a nuclear stain and imaged at day 0 and 2 of culture for nuclear changes (i, iii) and CD11c induction (YFP positive) (ii, iv). Arrows: YFP. Blood neutrophils from CD11c-YFP (yellow fluorescent protein) reporter mice treated with SLE-ICs, labeled with a nuclear stain and imaged at day 0 and 2 of culture for nuclear changes (i, iii) and CD11c induction (YFP positive) (ii, iv). Arrows: YFP. μm; all images are at the same magnification.

Fig. 2 The frequency of nAPCs in lupus blood correlates with disease scores. a, b Blood samples from normal human controls (N) and SLE patients were analyzed for CD11c and HLA-DR markers on lineage negative (Lin−) cells. CD11c-HLA-DR positive cells were examined for neutrophil markers, CD10 and CD15. In SLE samples, CD10 and CD15 positive cells were further evaluated for CD80, CCR7, and Clec9A. Gating strategy for the samples is shown (a). The percent of neutrophils with indicated markers was determined and correlated with SLE disease activity index (SLEDAI) scores (b). Data are mean ± s.e.m. Non-parametric test using Mann–Whitney analysis; correlation analyzed by a Spearman test. *p < 0.05.

nAPCs retain the ability to phagocytose and generate reactive oxygen species. Previous studies showed that GM-CSF treatment of immature neutrophils generates nAPC that capture E. coli, release DNA neutrophil extracellular traps (NETs) and generate reactive oxygen species (ROS) like neutrophils. We found that nAPCs generated from mature bone marrow-derived neutrophils treated with SLE-IC, Ova-IC + GM-CSF, or GM-CSF (i.e., anti-Ova alone) were equivalent to neutrophils in their ability to phagocytose E. coli and IgG coated beads (Fig. 3a) and generate ROS in response to E. coli and Zymosan (Fig. 3b). Therefore, nAPCs retain some functions of canonical neutrophils.

nAPCs generated with antibody–antigen complexes present antigen to naïve CD4+ T cells, cross-present to CD8+ T cells and generate immunogenic cytokines. To address whether IC-generated nAPCs can activate naïve T cells and cross-present to CD8+ T cells, we assessed their ability to stimulate the proliferation of Ova-peptide specific naïve CD4+ and CD8+ T cells isolated from OT-II and OT-I TCR transgenic mice, respectively. nAPCs were generated by pretreating neutrophils with Ova-IC, soluble Ova or vehicle (−) and culturing them with GM-CSF. After 3 days, adherent cells, which were primarily nAPCs (Supplementary Fig. 4a) were harvested and co-cultured with T cells. Ova-IC generated nAPCs stimulated proliferation of greater than 80% of naïve CD4+ (Fig. 3c) and CD8+ (Fig. 3d) T cells. Ova-IC-β2-microglobulin deficient nAPCs...
Fig. 3 Ova-IC generated nAPCs retain neutrophil functions, promote naive CD4\(^+\) and CD8\(^+\) T cell proliferation and generate immunogenic cytokines. a Freshly isolated FcgRIIIB(3B)/γ\(^-\)/− BMNs (Neut) or the same treated with anti-Ova, Ova-IC or SLE-IC and cultured to generate nAPCs were incubated with inactivated FITC-E. coli or IgG-coated, FITC-labeled latex beads and analyzed for FITC-uptake by flow cytometry. b Reactive oxygen species (expressed as relative light units/sec, RLU/s) generated over time by GM-CSF-primed 3B/γ\(^-\)/− Neut and Ova-IC- or SLE-IC- generated nAPCs incubated with serum opsonized E. coli or zymosan. c, d Proliferation of CellTrace Violet-labeled CD4\(^+\) (OT-II) (c) and CD8\(^+\) (OT-I) (d) T cells after co-culture with Ova, Ova-IC or vehicle (−), GM-CSF alone) generated nAPCs of indicated genotypes assessed by CellTrace Violet dilution. In d, vehicle generated nAPCs pulsed with Ova SIINFEKL-peptide (pSIINF) is a positive control. Representative profiles for CD8\(^+\) T cells (d) are shown. e, f CellTrace Violet-labeled CD4\(^+\) (e) or CD8\(^+\) (f) T cells co-cultured with Ova- or Ova-IC-generated nAPC, and Ova or Ova-IC treated splenic monocyte-derived (mDC) or Flt3L-induced splenic DCs, and analyzed as in (c, d). g, h Cytokine concentrations in supernatant of Ova- and Ova-IC generated nAPCs (g) and splenic cDCs (h). Data are mean ± s.e.m. For c-f one-way analysis of variance and Dunnett’s multiple comparison test; g, h Multiple t test between pairs of samples. *p < 0.05, **p < 0.005.
lacking MHCI failed to stimulate CD8+ T cells, confirming the
dependence of this response on MHCI-restricted cross-pre-
sentation of Ova, while presentation to CD4+ T cells was normal
as expected (Supplementary Fig. 4b). TLR agonists, known to
enhance antigen presentation by DCs,44,45 were not required, as T
cell proliferation was unimpaired in MyD88/TRIF-deficient Ova-
IC-nAPCs (Supplementary Fig. 4c). By contrast to Ova-IC-
nAPCs, Ova-IC-nAPCs stimulated the proliferation of less than 20%
of T cells (Fig. 3c, d), none of which were CD8+ (Fig. 3d). As
a positive control, nAPCs generated in all conditions promoted
CD8+ T cell proliferation when pulsed with high concentrations
of Ova-SIINFEKL peptide (Fig. 3d), which directly binds MHC I
and bypasses the need for antigen processing and co-stimulatory
molecules.46 Consistent with a requirement for IC engagement of
FcγRs to generate immunogenic nAPCs, Ova-IC-nAPC derived
from γ−/− neutrophils were impaired in their ability to activate T
cells; levels of activation were similar to nAPCs generated with
soluble Ova. On the other hand, Ova-IC-nAPCs generated from
neutrophils expressing FcγRIIA or FcγRIIB and WT neutrophils
robustly47,48,49,50 activated T cells (Fig. 3c, d). Differences in antigen
internalization cannot account for the significantly greater T cell
stimulation by Ova-IC-nAPCs versus Ova-nAPCs as neutrophil
uptake of Ova-IC was only twice that of soluble Ova (Supple-
mentary Fig. 4d). The degree of CD4+ and CD8+ T cell pro-
liferation supported by Ova-IC nAPCs was comparable to that
observed with Ova-IC stimulated Flt3-induced splenic DCs47 and
monocyte-derived cDCs18 (Fig. 3e, f). Thus, nAPCs generated by
engaging FcγRs with Ova-IC are far superior to nAPCs made
with soluble Ova in stimulating CD4+ T cells, and only Ova-IC-
nAPCs with functional FcγR cross-present antigen to CD8+ T cells.
Moreover, levels of T cell proliferation were comparable in
Ova-IC-nAPCs and Ova-IC treated cDCs.

Cytokines secreted by cDCs promote T cell priming,
differentiation and polarization.49,50 Notably, levels of the key T
cell immunomodulatory cytokines IL-1β, TNFa, IL-15, and IL-23,
were 5- to 3500-fold higher in supernatants from cultures of Ova-
IC versus Ova generated nAPCs (Fig. 3g). In comparison to Ova-
IC-nAPCs, cultures of Ova-IC treated splenic cDCs also had
significantly less IL-1β, and TNFa (Fig. 3h) and undetectable
levels of IL-15 and IL-23. Other cytokines and chemokines
examined were present at similar levels in culture supernatants
of Ova- and Ova-IC- generated nAPCs and, for the most part,
cDCs (Supplementary Fig. 4e, f).

Adaptively transferred nAPCs generated with
antibody–antigen complexes migrate to draining lymph nodes,
support a delayed-type hypersensitivity response and are anti-
tumorigenic. Tissue-resident DCs traffic to draining lymph
nodes to activate T cells.54 We observed by intravital microscopy
that Ova-IC-nAPCs injected into the mouse footpad accumulated
in the draining popliteal lymph node, where they displayed
dendritic extensions and made prolonged contacts with Ova-
peptide specific CD8+ T cells. Only a few T cells were present in
areas without nAPCs (Fig. 4a, Movies S4 and S5). We also
observed that adaptively transferred Ova-IC-nAPCs, but not
Ova-nAPCs, elicited a delayed-type hypersensitivity (DTH)
response, which requires an effector CD4+ Tth T cell-driven
recall response to antigen.51 (Fig. 4b). cDCs can acquire pre-
formed peptide–MHC complexes from the surface of antigen-
loaded APCs, referred to as cross-dressing.52 However, cross-
dressing is unlikely to explain the stimulatory in vivo effects of
Ova-IC-nAPCs, as splenic cDCs from CD45.2+ mice that
received adaptively transferred CD45.1+ Ova-IC-nAPCs failed to
promote CD4+ and CD8+ T cell proliferation ex vivo, while
CD45.1+ Ova-IC-nAPCs retrieved from the same organ did
(Fig. 4c). In parallel, as a control, we show that in vitro Ova-IC-
loaded cDCs and Ova-IC-generated nAPCs comparatively pro-
tected T cell proliferation (Fig. 4c).

We next assessed whether Ova-IC-nAPCs can promote CD8+
T cell-dependent immunity to B16F10 melanoma cells expressing
Ova (B16F10-Ova)53. In vitro generated nAPCs were injected
intravenously into WT mice prior to the subcutaneous implanta-
tion of B16F10-Ova cells. Mice given Ova-IC-nAPCs showed no
in vivo, whereas tumor growth in Ova-IC-nAPC
immunized mice was comparable to that of mice given nAPCs without
loaded antigen (Fig. 4d). Tumor immunity in Ova-IC-nAPC
mice associated with higher accumulation of Ova-
specific endogenous CD8+ T cells in draining lymph node
and spleen compared to Ova-IC-nAPC immunized mice (Fig. 4d). Ova-
IC-generated nAPC-anti-tumor immunity was CD8+ T cell
dependent (Fig. 4e). Antitumor immunity was intact in mice
given Ova-IC-generated MyD88−/− TRIF−/− nAPCs (Fig. 4d),
which excludes a role for TLR ligands, potentially present in
experimental reagents and known to elicit antitumor responses,
in the observed results. To examine nAPC trafficking,
labeled Ova-IC nAPCs were adaptively transferred into mice
implanted with B16F10-Ova tumor cells 6 days prior; 24 h after
transfer, Ova-IC-nAPCs were found mostly within the tumor
bed, whereas by 96 h these cells were present in draining lymph
node and spleen (Fig. 4f). Thus, it is possible that, like cDCs,55
nAPCs facilitate T cell recruitment to tumors and migrate to
draining lymph nodes to cross-present endocytosed tumor antigens to CD8+ T cells.

An anti-FcγRIII-antigen conjugate promotes neutrophil to
nAPC conversion. FcγRIIB is sufficient for IC-induced conver-
sion of neutrophils to nAPCs (Fig. 1e) and for antigen
presentation (Fig. 3c, d) in vitro. We therefore next asked if binding of a
FcγRIIB antibody conjugated to Ova antigen reproduced the
effects of ICs as this could have therapeutic potential. A murine
IgG1 antibody specific for FcγRIII, 3G8,56 was conjugated to FITC-
labeled Ovalbumin (fOva) and is hereafter referred to as 3G8-fOva.
We observed that 3G8-fOva treated humanized neutrophils
expressing both FcγRIIA and FcγRIIB and cultured with GM-CSF
acquired DC markers while similar treatment of γ−/− neutrophils
failed to do so (Fig. 5a). The effect of 38-fOva was seen consistently
with several independent conjugate preparations. 3G8 isotype
control had no effect (Fig. 5a). Conversion also occurred in
humanized neutrophils expressing only FcγRIIC (FcγRIIB/b−/+),
while it did not occur in γ−/− or FcγRIIB/γ−/− neutrophils
(Fig. 5b), indicating that 3G8-fOva specifically binds FcγRIIB
to promote neutrophil to nAPC conversion.

To explore how the GPI-linked FcγRIIB triggers neutrophil
conversion, we first evaluated whether monomeric 3G8 alone is
sufficient or whether 3G8-fOva, a heterogeneous species (gen-
erated by conjugation of antibody to fOva) is required. 3G8-fOva
expression of FcγRIIB/γ−/− neutrophils was accompanied by
receptor internalization (Fig. 5c) and acquisition of CD11c and
MHCII (Fig. 5d), which were not observed with monomeric 3G8
or isotype control (Fig. 5c, d). The endocytic machinery is
known to promote receptor signaling and gene transcription57–60
and our prior work demonstrated that FcγRIIB and FcγRII
internalize ICs via a lipid-raft, actin, and cdc42 regulated
pathway.61 Cytochalasin D and MβCD, which disrupt the actin
cytoskeleton and lipid rafts, respectively, both abrogated nAPC
conversion following 3G8-fOva treatment (Fig. 5d) without
affecting binding of 3G8 (Supplementary Fig. 5a). Interestingly,
these treatments also disrupted FcγRIIB internalization (Fig. 5e)
and nAPC generation (Fig. 5f) induced by Ova-IC or SLE-IC,
while generation of nAPCs by GM-CSF alone (in presence of
soluble Ova control) was unaffected (Fig. 5f). Thus, with the caveat that we cannot exclude endocytosis independent effects of chemical inhibitors, these findings suggest that FcγRIIB endocytosis is a common proximal mechanism in 3G8-fOva- and IC-induced neutrophil to nAPC conversion.

Anti-FcγRIII-antigen conjugate induces FcyR internalization and human neutrophil conversion to nAPCs. We next evaluated the ability of the 3G8-fOva conjugate to convert human peripheral blood neutrophils from normal volunteers to nAPCs. Ova, a model protein antigen conjugated to 3G8 for mouse models, is irrelevant.
Fig. 4 nAPCs migrate to draining lymph nodes, elicit a delayed-type hypersensitivity (DTH) response, promote anti-tumor immunity, and accumulate in the tumor and draining lymph nodes. A. Ova-IC generated RFP+nAPCs injected in the footpad and GFP-CD8+ T cells injected intravenously detected in the draining lymph node at day 3. Dwell time and number of CD8+ cells in field of view (FOV) and interacting with RFP+nAPCs are given. Data are average of three FOV per mouse. Representative images of OT-I cells (green) distribution in areas without (left) and clustering around (red) (right) nAPCs. Scale bar: 15 μm. B. DTH response in mice given Ova or Ova-IC generated nAPC in footpad and re-challenged with Ova or vehicle. Footpad swelling and inflammation score are given. Representative images of Ova-IC-nAPCs infiltrated with indicated nAPCs. C. Experimental scheme for analysis of “cross-dressing.” Endogenous cDC and Ova-IC loaded nAPCs (injected 3 days prior) were retrieved from spleen and co-cultured with Cell Trace Violet labeled CD4+ (OT-II) and CD8+ (OT-I) T cells ex vivo. In parallel, CD4+ and CD8+ T cell proliferation on in vitro generated Ova-IC-nAPCs and Ova-IC loaded cDCs was assessed. Direct loading of nAPCs and cDCs with Ova-peptide for CD4+ (p233-339) or CD8+ (pSILNFKEK) T cells were controls. Proliferation of CD4+ or CD8+ T cells for ex-vivo (left panels) and in-vitro (right panels) samples are shown. D. Tumor volumes in wild-type (WT) mice immunized with no Ova− (−), Ova− or Ova-IC− generated WT nAPCs or Ova-IC−MyDb8/−TRIF−/− nAPCs 7 days prior to s.c. B16F10-Ova tumor cells (left panel). Number of mice per group is in parenthesis. Flow cytometric plots for MHCI-tetramer-PE labeled CD4+ cells in the spleen of mice given 3G8-fOva (Supplementary Fig. 6a), leading to the appearance of FITC+Ly6G+CD11c+MHCII+ cells expressing CD80 and CCR7 (Fig. 6a) by 24 h. These changes were not observed in similarly treated γ−/− mice. FcyRIIB and FcγRIIA are expressed on a subpopulation of CD11b+CD115+Ly6chi monocytes in our humanized mice (Supplementary Fig. 6b, but >97% of CD8-fova-positive CD11c+MHCII+ cells in the spleen expressed the neutrophil marker Ly6G and were Ly6ch (Supplementary Fig. 6c), consistent with an origin from converted neutrophils. Moreover, FcγRIIA and FcγRIIB were undetectable in macrophages and CD11c+ splenic DCs (Supplementary Fig. 6d). Next, we examined the accumulation of nAPCs in tissues and secondary lymphoid organs in mice given 3G8-fOva conjugate or monomeric 3G8 control in the absence or presence of GM-CSF. By 72 h, nAPCs generated in mice given 3G8-fova accumulated in lymph nodes, spleen, lung and liver (Fig. 6b), similar to the reported tropisms of blood DCs67, whereas 3G8 treatment did not result in significant nAPC accumulation in any organs. Although GM-CSF treatment produced a small increase in the frequency of nAPCs in some tissues of mice given 3G8 alone (Fig. 6b), it did not in mice treated with 3G8-fova.

We next examined the ability of 3G8-fova-induced nAPCs generated in vivo to promote CD4+ T cell proliferation, using MHCII deficient mice to rule out effects of endogenous APCs on outcomes. These mice were infused with FcγR humanized neutrophils, treated with vehicle, 3G8 antibody, or 3G8-fova, and then administered naïve CellTrace Violet-labeled Ova-specific CD4+ T cells. Robust CD4+ T cell proliferation was observed in the spleen and lymph nodes of MHCII deficient recipient mice treated with 3G8-fova, whereas no proliferation was observed in mice given 3G8 or vehicle control (Fig. 6c). In addition, 3G8-fova treatment of humanized FcγRIIB/γ−/− mice promoted the proliferation of adoptively transferred Ova-specific CD8+ T cells (Fig. 6d). Next, the function of endogenous CD8+ T cells activated by nAPCs in vivo was assessed in a target cell killing assay. For this, 3G8-fova immunized humanized mice expressing FcγRIIB and/or FcγRIIA, or mice lacking FcγRs (γ−/−) were injected with Ova-SIINFEKL peptide pulsed and unpulsed target splenocytes, each loaded with a different amount of fluorescent label to distinguish the populations by FACS analysis. We observed killing specifically of splenocytes pulsed with the Ova peptide only in FcγRIIB expressing humanized mice (Fig. 6e), an effect that was abrogated by neutrophil depletion (Fig. 6f). Thus, 3G8-fova creates nAPCs that promote the proliferation of CD4+ and CD8+ T cells and the generation of endogenous, cytotoxic CD8+ T cells.

Anti-FcγRIIB-antigen conjugate generates functional nAPCs that elicit CD8+ T cell proliferation and target cell killing in vivo. We next sought to determine whether infusion of 3G8-fova converts nAPCs into functional nAPCs in vivo. Blood neutrophils in FcγRIIB/γ−/− mice internalized intravenously injected 3G8-fova (Supplementary Fig. 6a), leading to the appearance of FITC+Ly6G+CD11c+MHCII+ cells expressing CD80 and CCR7 (Fig. 6a) by 24 h. These changes were not observed in similarly treated γ−/− mice. FcγRIIB and FcγRIIA are expressed on a subpopulation of CD11b+CD115+Ly6chi monocytes in our humanized mice (Supplementary Fig. 6b, but >97% of CD8-fova-positive CD11c+MHCII+ cells in the spleen expressed the neutrophil marker Ly6G and were Ly6ch (Supplementary Fig. 6c), consistent with an origin from converted neutrophils. Moreover, FcγRIIA and FcγRIIB were undetectable in macrophages and CD11c+ splenic DCs (Supplementary Fig. 6d). Next, we examined the accumulation of nAPCs in tissues and secondary lymphoid organs in mice given 3G8-fOva conjugate or monomeric 3G8 control in the absence or presence of GM-CSF. By 72 h, nAPCs generated in mice given 3G8-fova accumulated in lymph nodes, spleen, lung and liver (Fig. 6b), similar to the reported tropisms of blood DCs67, whereas 3G8 treatment did not result in significant nAPC accumulation in any organs. Although GM-CSF treatment produced a small increase in the frequency of nAPCs in some tissues of mice given 3G8 alone (Fig. 6b), it did not in mice treated with 3G8-fova.

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Anti-FcγRIIB-Ova antigen conjugate is anti-tumorigenic in mice. Given the anti-tumorigenic activity of Ova-IC-nAPCs generated in vitro in the presence of GM-CSF (see Fig. 4d) and the success of anti-tumor strategies involving GM-CSF68-72, we combined a single dose of 3G8-fova with sequential doses of GM-CSF. Tumor growth in 3G8-fova-immunized mice expressing FcγRIIB and FcγRIIA was significantly reduced compared to unimmunized, GM-CSF treated humanized mice (Fig. 7a, Supplementary Fig. 7a) and two additional sets of GM-CSF treated controls: Wild-type mice given fOva, which will lead to an anti-tumor immune response driven by endogenous APCs,
such as cDCs, and γ−/− mice given 3G8-fOva that do not make nAPCs as they lack the FcγRIIIB but nonetheless, like wild-type plus fOva, will mount an immune response to fOva (contained in the 3G8-fOva conjugate) (Fig. 7a). The reduction in tumor growth in 3G8-fOva immunized mice expressing FcγRIIIB and FcγRIIA correlated with the generation of Ova-specific CD8+ effector T cells and higher CD8+ effector to Treg cell ratios in the spleens of these mice versus other groups (Fig. 7b, Supplementary Fig. 7b). In summary, a single immunization of GM-CSF treated mice with 3G8-fOva attenuates melanoma growth and results in the generation of antigen-specific CD8+ effector T cells.
Fig. 5 Anti-FcγRIIIB-antigen conjugate converts murine and human neutrophils to nAPCs that is dependent on FcγRIIIB endocytosis. a Blood neutrophils from 2A3B/γ−/− and γ−/− mice incubated with isotype (Iso) or 3G8-fOva, cultured with GM-CSF and analyzed after 3 days for CD11c, MHCII on Ly6G positive cells. CD11c+MHCII+Ly6G− cells in 2A3B/γ−/− + 3G8-fOva sample further analyzed for CD80, CD86, and CCR7. b CD11c and MHCII acquisition after 3G8-fOva or isotype treatment of murine neutrophils from indicated mouse strains and culture with GM-CSF for 3 days. c, d 3B/γ−/− bone marrow neutrophils pretreated with vehicle, methyl-beta cyclodextrin (MβCD) or Cytochalasin D (Cyto D) and treated with isotype, 3G8-fOva or 3G8 antibody. FcyRIIIB on the cell surface evaluated using anti-FcγRIIIB, clone REAS89 (c). Cells from c analyzed for the percent of cells acquiring CD11c and MHCIi after 3 days in culture with GM-CSF (d). e, f Cells treated with Ova, Ova-Ic, or SLE-Ic and analyzed for surface FcγRIIIB (e) and acquisition of CD11c and MHCIi on Ly6G+ cells (f) as in (c, d). g, h Human blood neutrophils treated with 3G8-fOva or isotype control. Evaluation of surface FcγRIIIB (assessed with REAS89 antibody), FcyRIIA (assessed with IV.3 antibody) and FITC (fOva) positivity over time (g). The percent of CD11c and HLA-DR positive cells with neutrophil markers (CD15, CD10) after 2 days in culture with GM-CSF. CD11c and HLA-DR double-positive cells further examined for CD80, CD86, and CCR7 (h). I Flow plots assessing the purity of human neutrophils at day 0 (upper panels) using markers for neutrophils (CD15, CD66b, DCs (CD11c), monocytes (CD14), B cells (B220), NK cells (NK1.1), and T cells (CD3) (Lin−). Gating strategy for nAPC markers at day 2 after 3G8-fOva treatment (lower panels). J Images of purified human neutrophils, treated as in (g, h), FACS sorted on day 1 and stained with Wright-Giemsa. FACS profiles of day 0 human blood neutrophils stained for markers of neutrophils (left), monocytes (middle) and NK, T and B cells (right). Data are mean ± s.e.m. c-g One-way analysis of variance and Dunnett’s multiple comparison test; a, b, h Multiple t-tests between pairs of samples. *p < 0.005.

FcyRIIIB engagement of human neutrophils from patients with myeloid neoplasia or vaccinated normal human volunteers generate nAPCs that activate autologous memory T cells. To explore the therapeutic relevance of our results, we tested if 3G8-fOva treatment of human neutrophils expressing endogenous tumor antigens or loaded with exogenous pathogen associated antigens could generate nAPCs that stimulate autologous T cells. As already noted, the Ova in the 3G8-fOva conjugate is irrelevant in the human samples. For tumor antigen responses, we used neutrophils isolated from patients with myeloid neoplasms, in which neutrophils are components of the malignant clone and hence express tumor neoantigens. We selected two patients that harbored several driver mutations; based on high variant allele frequencies and differential cell counts (Supplementary Table 2), these mutations can be inferred to be present in the neutrophil fractions of these patients. 3G8-conjugate treatment converted neutrophils from both patients into nAPCs, which generated autologous CD3+ T cells as assessed by IFNγ secretion. No IFNγ production was observed with nAPCs generated from three normal human volunteers incubated with autologous T cells indicating that the response seen in patient samples is due to neutrophils carrying tumor neoantigens. For responses to exogenous pathogen antigens, neutrophils from normal volunteers previously immunized with the Tdap (tetanus, diphtheria, and pertussis) vaccine were treated with 3G8-fOva or isotype control antibody, pulsed with the vaccine antigens, and incubated with autologous T cells. IFNγ generation was observed in cultures of 3G8-fOva- but not isotype-treated neutrophils loaded with vaccine antigens. Together, these studies indicate that human nAPCs can stimulate the response of autologous memory T cells to neutrophils carrying tumor neoantigens and exogenously provided vaccine antigens.

Transcriptional profiling reveals the uniqueness of nAPCs and transcription factor PU.1 as a potential driver of neutrophil to nAPC conversion. We next sought to define the transitional cell states and uncover potential molecular mechanisms underlying neutrophil to nAPC conversion by conducting single-cell RNA-seq (scRNA-seq) on (i) splenic Ly6G+CD11c+MHCII+ and Ly6G+CD11c−MHCII− cells (presumptive nAPCs and neutrophils, respectively) isolated from FcγR humanized mice 3 days after i.v. injection of 3G8-fOva treatment and (ii) isolated neutrophils harvested at day 0 and cultured in vitro with 3G8-fOva for 1 to 3 days. We also analyzed splenic cells from untreated wild-type mice and FcγR humanized mice as reference cell types (Supplementary Fig. 8). We identified five neutrophil subpopulations, Nt.1-NT.5, and two CD11c+MHCII+ clusters, referred to as nAPCs, which could be further divided into six subclusters (Fig. 8a). Importantly, similar neutrophil and nAPC cell states were identified in vitro and in vivo (Fig. 8a). These states were robust with respect to potential batch effects (Supplementary Fig. 9) despite the challenges of profiling neutrophils, which are fragile and have low RNA content. These factors explain the observed cluster of monocytes on day 0 in vitro, as these cells (which were >99% neutrophils as assessed by flow cytometry, Supplementary Fig. 1a) are likely to be disproportionally represented in scRNA-seq analyses relative to neutrophils. Importantly, these contaminating monocytes are transcriptionally distinct from Nt and nAPC populations (Fig. 8a). We also observed DC, monocyte, macrophage, and NK clusters in splenic samples from untreated wild-type and humanized FcγR mice (Fig. 6a, Supplementary Data 1).

Next, we wanted to understand the transcriptomic features that are associated with two identified nAPC clusters that were heterogeneous and separated from each other in UMAP space, in both trajectory and correlational analyses (Fig. 8a, c, Supplementary Fig. 10a, Supplementary Data 2). A heatmap of differentially expressed genes revealed greater similarity of neutrophils and the nAPC.1 subset than of the nAPC.1 and nAPC.2 subsets (Supplementary Fig. 10b). The close relationship between neutrophils and the nAPC.1 subset was further supported by pseudo-bulk gene correlation analysis (Supplementary Fig. 10a) and the expression of neutrophil-specific chemokines by nAPC.1 cells (e.g., Cxcr2) (Supplementary Fig. 11). nAPC.1 cells differed from Nt.1 cells by showing upregulation of immune stimulatory pathways (e.g., NfkB, JAK/STAT, cytokine, and chemokine signaling) (Supplementary Fig. 12). In contrast to Nt.1 and nAPC.1 cells, nAPC.2 cells shared many differentially expressed genes with DCs, including MHCII and genes associated with antigen processing and presentation (Supplementary Figs. 11 and 12). Most nAPC.2 cells (particularly subset d) also expressed high levels of Ccl17, Ccl22, and Cxcl16 (Supplementary Fig. 11), known to stimulate effector T cell function and/or migration, and included a larger fraction of cells expressing Ccr2 and Ccr5, which promote DC activation and maturation. Comparison of the nAPC.1 and nAPC.2 subsets revealed that nAPC.2 cells showed upregulation of genes within the antigen processing and presentation pathway and pathways related to protein processing and endocytosis (Supplementary Fig. 13a). Within the neutrophil compartment, Nt.1/Nt.2 cells showed high expression of canonical neutrophil markers and genes associated with cytotoxicity (e.g., Lcn2, Cybb, Camp) (Supplementary Fig. 11), while Nt.5 cells corresponded to a more mature neutrophil subset (Supplementary Fig. 13b, Supplementary Data 3) previously described in peripheral blood and mouse spleen.
Characterization of neutrophil to nAPC transition based on in vitro time-course data suggested two lineage trajectories: Nt.1 to nAPC.1 cells; and Nt.1 through Nt.2-Nt.4 cells to nAPC.2 cells (Fig. 8b, Supplementary Fig. 13b). We further explored these trajectories by using Monocle to order cells along a pseudo-time gradient, with Nt.1 cells defined as the starting node (Fig. 8c).

Monocytes exhibited large transcriptomic differences from Nt and nAPC cells (Fig. 8c), indicating that they are unlikely nAPC progenitors. Consistent with neutrophil transition to a cDC-like state, the pseudo-time gradient of Nt.1 to nAPC.2 transition correlated with reduced Ly6G expression and increased expression of CD11c and genes related to antigen presentation (Fig. 8c, Supplementary Fig. 13b).
**Inhibition of PU.1 prevents neutrophil to nAPC conversion.**

To identify transcription factors involved in reprogramming neutrophils to nAPCs, we performed transcription factor enrichment analysis (using MARGE-cistrome) on genes whose expression significantly correlated with neutrophils in the single cell data set, capturing the transition to nAPC. STRING database analysis revealed the activation of two transcriptional factor networks in nAPCs, PU.1 and NFκB. Moreover, inhibition of PU.1 blocked neutrophil to nAPC conversion in response to Ova, Ova-IC and SLE-IC treated in vitro and splenocytes from 3G8-fOva conjugate treated mice revealed similar alterations in gene expression that are consistent with differentiation of neutrophils into two distinct CD11c+ cells with monocellular-like appearance. More-
Fig. 7 Anti-FcγRIIB-antigen conjugate is anti-tumorigenic in FcγR humanized mice and converts human neutrophils into nAPCs that activate autologous T cells. a, b Schematic for the timeline of indicated treatments for examining the effect of 3G8-fOva on B16F10-Ova tumor growth (top panel). Tumor volume was assessed at indicated times after s.c. injection of B16F10-Ova melanoma cells. The number of mice per group is in parenthesis (lower panel). Representative images of harvested tumors are shown, scale bar = 5 mm (a). Spleens of indicated mice from (a) at tumor harvest were analyzed for frequency of Ova-peptide specific effector CD8+ T cells (CD62Llo CD44hi) using MHCI-tetramers (tet+), and CD4+ and Treg (Foxp3+, CD4+) cells (b). c Neutrophils from a patient with primary myelofibrosis (a myeloproliferative neoplasm, MPN) or acute myeloid leukemia (AML) (AML-2.1, AML-2.2 representing two independent blow draws) were treated with 3G8-conjugate (3G8-fOva) or isotype, cultured for 2 days and then co-cultured with autologous T cells and analyzed for IFN-γ secretion on a human IFN-γ ELISpot plate. The percent nAPC generation is shown in the table (avg ± s.e.m.). T cell responses quantified as the number of spots per 1 × 10⁶ cells on a human IFN-γ ELISpot plate is reported and representative images of the same are shown. d Autologous T cell responses against diphtheria toxin (Dipt) or tetanus toxoid (Tet) loaded isotype or 3G8-fOva (3G8-conj) treated neutrophils from normal human volunteers were evaluated using an IFN-γ ELISpot plate as in (e). Representative images are shown. Data are mean ± s.e.m. One-way analysis of variance and Dunnett’s multiple comparison test was used for comparison of multiple groups except for a), for which statistical analysis is described in Supplementary Fig. 7a. *p < 0.05, **p < 0.005.
acquisition of immunogenic functions, a fruitful area for future studies.

Endocytosis of the GPI-linked FcγRIIIB following 3G8-fOva engagement preceded and was essential for nAPC conversion, consistent with evidence that endosomes are signaling hubs required for diverse processes from migration to cell fate. Endocytosis was also required for IC-induced nAPC generation suggesting that the 3G8-fOva conjugate and ICs engage a similar proximal signaling pathway for nAPC generation. Antigen endocytosis that occurs in parallel with FcγRIIIB internalization
is likely targeted to an endocytic pathway with low degradative potential for antigen processing, as in cDCs. Indeed, the exposure of neutrophils to complexed antigen (Ova-anti-Ova ICs) or the OVA-expressing 2A3B cell line conjugate for only 2 h was sufficient for subsequent Ova cross-presentation by newly generated nAPCs, which suggests the existence of a pathway for antigen processing in neutrophils that is distinct from degradative routes. This possibility was not appreciated in previous work studying acquisition of DC-like functions by neutrophils because antigen was present, when noted, throughout the time course of the experiments.

Key features of dendritic cells include their ability to generate immunomodulatory cytokines, migrate to lymph nodes and capture blood borne antigens in the spleen. We show that FcyR generated nAPCs released several-fold higher levels of immunogenic cytokines such as IL-1β, IL-15, and IL-23 than cytokine induced nAPCs or IC-treated cDCs. IL-1β enhances the function and memory response of T cells, IL-15 promotes the survival and function of CD8+ T cells and lowers the threshold for TCR activation and IL-23 promotes memory T cell proliferation and the differentiation of Th17 cells. We show that FcyR-induced nAPCs accumulate in the spleen and can migrate from peripheral tissue (foot pad) to draining lymph nodes, where they interact with T cells. The latter demonstrates that like cDC, nAPCs are migratory and can carry antigen taken up in tissues to secondary lymphoid organs.

Despite significant advances in cancer immunotherapy, clinical benefits remain limited to a minority of patients and there still is an unmet need to achieve durable T cell immunity to many tumors. Targeted DC-based cancer vaccines have been studied extensively but challenges remain, including the low abundance of cross-presenting cDCs and the need for TLR agonists to induce immunogenicity. Conversion of the abundant neutrophils to immunogenic nAPCs with functions previously ascribed exclusively to classical DCs. We propose that converting the abundantly present neutrophils into potent, cross-presenting, immunostimulatory nAPCs by in vivo targeting of FcyRIIB, which is a receptor selectively expressed on neutrophils, could serve as an immunotherapeutic approach that overcomes the obstacles encountered with cDC therapy.

Methods

**Mice.** All mice were on a C57Bl/6 background: Wild-type mice, FcyRγ−/− mice expressing human FcγR (FcγR/Igg2b/IgM, B6.SJL-Pipp/Igg2b/IgM, The Jackson Laboratory #002014), CD11c-YFP (B6.SJL-Tg (Ifg7-Venus)1Mnz/J, The Jackson Laboratory #008829), Granulocyte-specific Pu.1 conditional knock-out mice (MRP8-Cre;Ires;RFP.Spi1#012516). OT-I expressing the transgenic T cell receptor recognizing Ovalbumin residues 257–264 (SINFEKL) in the context of H2Kb (The Jackson Laboratory #003851), OT-I RFP/Fas-FITC (obtained by crossing OT-1 mice with β actin-GFP mice), OT II, transgenic mice expressing T cell receptor recognizing Ovalbumin 323–339 peptide in the context of H2k and are β-actin RFP positive (The Jackson Laboratory, # 005884). Animals were maintained in a specific pathogen-free facility. All in vivo experiments were conducted with age and sex matched animals. The Brigham and Women’s Hospital Animal Care and Use Committee approved all procedures in this study.

**Human volunteers, SLE patients and sera and myeloid neoplasia patients.** For studies with SLE patients, peripheral blood samples were obtained from healthy controls and patients with SLE who fulfilled the 1997 ACR classification criteria. The study was approved by the IRB of the Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran (IRE-2297) and all participants signed informed consent forms. Healthy volunteers without family history of autoimmune diseases were recruited as controls. The clinical and demographic characteristics of the study participants are summarized in Supplementary Table 1. Human SLE serum samples were obtained from the Lupus Clinic at the Beth Israel Deaconess Medical Center and Instituto Nacional de Ciencias Medicas y de la Nutricion Salvador Zubiran. For studies on normal human neutrophils, and collection of normal sera, blood samples were obtained from healthy volunteers consented to Brigham and Women’s Hospital IRB protocol P001694/PHS. Blood samples from patients with myeloid neoplasia were obtained from patients consented to Dana-Farber Cancer Institute IRB protocol 01-206. The clinical and demographic characteristics of the study participants are summarized in Supplementary Table 2. All volunteer subjects gave written informed consent.

**Reagents.** Murine GM-CSF (#315-03), human GM-CSF (#300-03), murine G-CSF (#AF-250-05), were from Peprotech. BSA (#A-7970), Ova (#A5053) anti-BSA rabbit antibody (#B2726) and anti-Ova rabbit antibody (#C6534) were from Sigma Aldrich. FITC-Ova (#O23020) was from Thermofisher. NIP-Ova (11 NIPs per Ova) was from Biosearch Technologies and anti-NIP human IgG1 (chimeric antibody with lambda light chain and heavy chain) which were a gift from Richard Blumberg (Brigham and Women’s Hosp, Boston). H-2Kb Ova Tetramer (Ova257–264) were from the NIH Tetramer Core Facility. Anti-FcyRIIB (3G8) (Biolegend) was conjugated to FITC-Ova (#O23020, Thermofisher) as a custom order (Biolegend). Accutase cell detachment solution (#97920) was from STEMCELL Technologies. FACS antibodies used, including catalog numbers, clones, and dilutions, are in Supplementary Tables 3–6. For depletion experiments, anti-mouse Ly6G (1A8, #BP0075-1), anti-mouse CD4 (GK1.5, #BE0003), anti-mouse CD8 (2.43, #BE0061) and rat IgG2a isotype control (2A3, #BP0089) and rat IgG2b Isotype control (LF-2, #BE0090) were obtained from BioXCell. Ova peptide (322–330) (OT1 peptide) and Ova peptide (322–339) (OT2 peptide) were provided by the Partners peptide/protein core facility (Boston, MA), Diphtheria toxoid (Cat #D0556) and tetanus toxoid (#582331) were from Calbiochem.
Generation of model immune complexes. To generate immune complexes, solutions containing 2 mg/ml of anti-Ova, anti-NIP-Ova, or anti-BSA antibodies were added to 0.2 mg/ml of Ova, FITC-Ova, NIP-Ova, or BSA (mixture 1) or 0.1 mg/ml of Ova, FITC-Ova, NIP-Ova, or BSA in PBS (mixture 2) at a 1:1 ratio and incubated overnight at 37 °C to generate IC. The excess antibody in mixtures 1 and 2 results in the appearance of a precipitate (insoluble immune complexes). Equal volumes of mixtures 1 and 2 are combined to generate a solution of ICs. 10 μl of this solution is added per 1 ml of neutrophil suspensions.

Sera, IgG isolation, and generation of immune complexes with RNP. Peripheral blood was drawn into BD Vacutainer® Venous Blood Collection Tubes SST. Tubes were centrifugated at 2500 x g for 30 min. Serum was aliquoted and frozen at −80 °C.

**Isolation and in vitro culture of murine splenic classical dendritic cells and monocyte-derived cDCs.**

For splenocyte DCs, wild-type mice were implanted s.c. with a 1x10^6 expressing B16F10 melanoma. At day 12, spleens were harvested and cDCs were isolated using CD11c microbeads (Miltenyi Biotech) according to manufacturer's instructions. For bone marrow, monocyte-derived cDCs, monocytes were isolated using the Monocyte Isolation kit (Stem Cell Technologies) and cultured for 6 days in media (RPMI1640 plus10%FBS, 20 mM Penicillin/Streptomycin) supplemented with GM-CSF (20 ng/ml) and IL-4 (20 ng/ml). Isolated cells were stained for CD11c and MHCII (Supplementary Tables 3 and 6) and analyzed by flow cytometry. Dead cells were excluded from the analysis based on fixable viability dye fluorescence.

**Analysis of phagocytosis and reactive oxygen species (ROS) generation.**

Phagocytosis of 10^6 freshly isolated neutrophils and adherent nAPCs derived from anti-Ova, Ova-IC, and SLE-IC were assessed using a 1:20 dilution of FITC labeled, inactivated E. coli or IgG complex. Cells were then gently washed with assay buffer twice and FITC uptake was measured by flow cytometry. For analysis of ROS generation, 10^6 freshly isolated neutrophils and adherent nAPCs derived from Ova-IC and SLE-IC suspended in PBS without Ca^2+ and Mg^2+ were incubated with serum opsonized E. coli (MOE200) or 100 μg/ml zymosan followed by luminol (50 μM) in PBS with Ca^2+ and Mg^2+. ROS was measured as production of light over time (expressed in relative light units, RLU), which was continuously monitored at indicated time points using a 6-channel bioluminat LB-953 luminometer (Berthold).

**Analysis of FcRIIIB surface expression and 3G8 binding.**

To assess surface expression of FcγRIIIB (CD16), clone REA589 was used. The cells were incubated with 10 μg/ml FITC-IgG isotype control or FcγRIIIB (3G8)-FITC-Ova or unconjugated CD16 (Clone: 3G8) or (2) Ova, Ova-IC or SLE-IC. The cells were washed thrice, blocked with TruStain FcX antibody (Biolegend) for 10 min at 4 °C and stained with 1:50 dilution of E. coli or IgG complex. Cells were then gently washed with assay buffer twice and FITC uptake was measured by flow cytometry. To detect the integrity of binding of 3G8 to FcγRIIIB after inhibitor treatment, neutrophils were treated with Cytochalasin D or MβCD or vehicle for 30 min at 37 °C and washed twice with PBS. Cells were then incubated with unconjugated anti-human CD16 antibody Clone 3G8 (Stem Cell Technologies) at 1:50 or Isotype IgG1x for 30 min at 4 °C. Cells were washed twice and incubated with Alexa Fluor 647 (Thermo Fisher Scientific) labeled goat anti-Mouse IgG (H + L) cross-adsorbed secondary antibody at a dilution of 1:10,000 for 30 min at 4 °C. Cells were washed and analyzed by flow cytometry.

**Isolation of peripheral blood human neutrophils.**

To isolate human neutrophils, peripheral blood was drawn into tubes containing trisodium citrate, citric acid, and dextrose (Vacutainer ACD Solution A, BD). Autologous serum, used for culture of cells was obtained by drawing blood into BD Vacutainer™ Venous Blood Collection Tubes SST, followed by centrifugation at 2500 × g for 30 min and serum collection. All blood donors provided written informed consent. For normal human blood samples, 10ml human blood was supplemented with GM-CSF (10 ng/ml) for 30 min at 37 °C followed by addition of 30 μg FITC-IgG isotype control or FcγRIIIB (3G8)-FITC-Ova conjugate for 2 h at the indicated concentrations. Blood was then incubated with Hetsap (STEMCELL Technologies) according to manufacturer protocols to deplete red blood cells and enrich leukocytes. Neutrophils were isolated from the leukocyte-rich plasma layer using a EasySep Neutrophil enrichment kit (StemCell Technologies). Neutrophil purity was evaluated using CD15, CD11b, CD66b, and lineage markers (CD3, CD19, and CD56) Supplementary Tables 4 and 6.
Treatment and culture of peripheral blood human neutrophils. Treatment of blood from human volunteers was as follows. 10 ml blood samples were incubated with Gimlet for 3 min and then 30 μg 3G8-FcOva isotype control (FITC-IgG) for 2 h. Neutrophils were isolated and placed in culture with media plus GM-CSF. PBMCs were isolated as described below and frozen in freezing medium (40% RPMI1640 + 50% FBS + 10% DMSO) and preserved for 2 days at ~80 °C. After 2 days, the cells were harvested from isotype and 3G8-conjugate treated samples and co-cultured with autologous CD3 T cells isolated from frozen PBMCs (see method below) on IFNγ and co-cultured with autologous CD3 T cells isolated from frozen PBMCs (see Supplementary Tables 4 and 6). To evaluate cell markers in circulating leukocytes in whole blood, PBMCs patients and controls, were co-cultivated with antibodies for the following surface markers: CD10, CD15, CD11c, MHCII, CD80, CD86, and CD142 (see Supplementary Tables 5 and 6). Within the viable population of lineage negative (CD3, CD19, and CD56) + MHC-II + Ly6G − population. Red blood cells were lysed using lysis buffer (BD Pharm Lyse) and the samples were analyzed by flow cytometry in an LSR Fortessa cytometer (BD bioscience).

Flow cytometry. Flow cytometry was performed on a FACS® Canto II or LSRFortessa-12-color analyzers or FACS®Symphony. FCS (flow cytometry standard format) 3.0 data file was used to export data that was analyzed using FlowJo (Mac version 10.5). Compensation controls were created for each fluorochrome. BD multicolor compensation beads and cells were used to set up compensation for the individual fluorochromes. For all experiments, cells were stained with the Fixable Viability Dye eFlour 780 (ThermoFisher) to gate out dead cells. Forward and side scatter gates were used to discriminate doublets and debris (FSC-A, FSC-H, SSC-A × SSC-H). Matched isotypes were used as controls and negative gating was based on FMO (fluorescence minus one control). Only viable cells were included for the study. For two-color staining, single cell suspensions in FACS buffer (PBS supplemented with 2% FCS and 2 mM EDTA) were incubated with mouse BD Fc block or human TrueStain FcX for 20 min at 4 °C. Samples were incubated with the indicated fluorescence-conjugated antibodies for 30 min at 4 °C, washed with PBS and fixed with 1% paraformaldehyde.

Flow cytometric analysis of murine bone marrow-derived neutrophils and nAPCs. For phenotypic and functional analysis of bone marrow-derived neutrophils and nAPCs, the following antibodies for the cell surface markers were used: CD11c, MHC-II, Ly6G, CD80, CD86, and CCR7 (see Supplementary Tables 3 and 6 for details). Within the viable population of lineage negative (CD3, NK1.1, and CD19), CD11c+, and MHC-II+ events were gated and Ly6G expression was analyzed. The CD11c+, MHCII+, and Ly6G− population was further analyzed for expression of co-stimulatory molecules CD80, CD86, and the migratory marker CCR7. The CD11c gates were set based on FMO controls and CD86+, and CCR7− selected based on isotype and FMO controls. Displayed numbers are CD11c+, MHCII+, and Ly6G− events expressed as %. The subset DC markers XCR1, CD103, and DCA were analyzed on the CD11c+, MHC-II+, and Ly6G− population (gating strategy in Supplementary S1). For uptake analysis, FITC and Ly6G gates were set based on isotype control. Anti-Ly6G was coupled with APC fluorochrome. For OT-I and OT-II experiments, anti-CD3, -CD4, and -CD8 antibodies were used. Singlets of CD3+, CD4+, or CD8+ viable cells were gated for further analysis and positive events from one representative sample. Displayed populations of CellTrace Violet+ events (dark gray) were set based on isotype controls. Light gray histograms indicate day 0 staining of CellTrace Violet+ events. For intracellular staining, fixed cells were stained for surface markers and permeabilized with BD Perm/Wash Buffer (BD Biosciences). Cells were then stained with anti- Foxp3, -T-bet, and -Ki-67 antibodies. Cells were washed again with permeabilization buffer and analyzed by flow cytometry.

Flow cytometric analysis of human blood neutrophils and dendritic cells. To evaluate cell markers in freshly isolated human neutrophils, CD15, CD11b, CD66b, and lineage markers (CD3, CD19, and CD56) were used. Dead cells were excluded using fixable viability dye and viable cells were gated for lineage negative markers. Cells were then stained for CD11c and CD66b expression. For analysis of nAPCs after culture of human neutrophils, viable cells were analyzed for expression of CD11c and HLA-DR. Double positive cells were further checked to evaluate co-stimulatory markers CD80, CD86, and migratory marker CCR7 (see Supplementary Tables 4 and 6). To evaluate cell markers in circulating leukocytes in whole blood, PBMC patients and controls, were co-cultivated with antibodies for the following surface markers: CD10, CD15, CD11c, MHCII, CD80, CD86, and CCR7 (see Supplementary Tables 5 and 6). Within the viable population of lineage negative (CD3, CD19, and CD56), CD11c+ and MHCII+ events were gated and Ly6G− population was further analyzed. The CD11c+, and CD15+ population was further analyzed for CD80 and CCR7+ markers. The DC subset markers Clec9a was also evaluated in the CD11c+MHCII−CD10−CD15+ population. Red blood cells were lysed using lysis buffer (BD Pharm Lyse) and the samples were analyzed by flow cytometry in an LSR Fortessa cytometer (BD bioscience).

Live cell imaging of mouse blood neutrophils. Live cell imaging was performed using a CellAsic Omix2 microfluidics cell trap plate (M04T-01- EMD Millipore) (100 um2) traps mounted on a widefield inverted Nikon Ti microscope enclosed in a custom-made environmental chamber heated to 37 °C. Pre-mixed 5% CO2 was perfused into the cells through the glass in the CellAsic Omix2 microfluidics device. The microscope was mapped with a Plan Apochromat 20x/0.75 Nikon linear encoded motorized stage, an Andor Zyla 4.2 plus SMOS monochrome camera and a Lumenera Spectra X light engine. The acquisition software controlling the microscope was Nikon NIS Element AR 4.30. Phototoxicity was minimized by reducing light intensity using an ND8. The signal from YFP-reporter and STO+ deep red-stained nucleus were collected using Chroma ET filter cubes 49003 and 49006, respectively. Neutrophils isolated from the peripheral blood of naive CD11c−YFP mice were stained with SYTO™ Deep Red nucleic acid stain (Invitrogen). SYTO stained neutrophils were introduced into the microfluidics cell trap plate and stimulated with 20 μg/ml SLE-IC for 1.5 h. The cells were washed with RPMI1640 + FCS for 30 min and perfused with RPMI1640 + FCS + SYTO® deep red nucleic acid stain at 3 kPa flow rate for the duration of the experiment. Transmitted light images were collected every 2 min in brightfield to track individual cells whereas fluorescence images in YFP-reporter and STO+ channels were collected every 30 min to minimize phototoxicity. Focus was maintained by the software-built steps in range autofocus routine using transmitted light. A z-stack was collected at each timepoint.

Confocal imaging of mouse blood neutrophils. Isolated peripheral blood neutrophils stained with SYTO™ Deep Red nucleic acid stain (Invitrogen) were stimulated with 20 μg/ml SLE-IC for 1.5 h and placed in culture. Z-stack images were taken at Day 0 and at Day 2 were collected using a DMi8000 (Leica) with a 63X NA1.3 Plan-Apo glycerol immersion objective, CSX1 Yokogawa spinning disc (Andor), Borealis illumination system (Andor), and Zyla Plus camera (Andor), controlled by MetaMorph 7.8 (Molecular Devices).

Cytopsins of human neutrophils. Cytopsins were prepared of purified human neutrophils (day 0) and 24 h after treatment with 3G8-FcOVA or isotype control and cultured in GM-CSF. Cytopsins were stained with Wright-Giemsa.

Analysis of FITC-Ova, FITC-Ova/anti-Ova, and anti-FcγRIII (3G8)-FITC-Ova uptake in vitro. For murine neutrophil uptake experiments, cells from the indicated genotypes were cultured with FITC-Ova or FITC-Ova-anti-Ova model immune complexes generated as described above. At the indicated times, cells were collected, stained with anti-Ly6G, quenched with trypan blue, and subjected to FACS analysis to detect Ly6G/FITC+ cells. For human neutrophil uptake measurement, blood was incubated at 37 °C with FITC-IgG isotype control or anti-FcγRIIIb (3G8)-FITC-OVA antibody conjugate (8.5−12.5 μg/ml) with gentle agitation on a rocking table for the indicated times. Samples were then treated with ammonium chloride solution (STEMCELL Technologies #07800) for 15 min on ice to lyse red blood cells, washed with PBS, and subjected to FACS analysis with the indicated antibodies: anti-CD10, -CD15 -CD32 (IV.3) (Supplementary Table 4).
Adoptive transfer of neutrophils into MHCII deficient mice and immunization with 3G8-Ova. Neutrophils were freshly isolated from bone marrow of naïve FcγRIIA−/− FcγRIIB−/− mice and 1 × 10^6 cells were injected i.v. into MHCII deficient recipient mice at day 3 and day 2. Five hours post injection of neutrophils, 3G8-Ova conjugate or 3G8 (unconjugated) was given i.v. at day 0. Mice were injected i.v. with CellTrace Violet-labeled CD4+ OTI T cells. After 3 days, lymph nodes (brachial, inguinal, renal, mediastinal, popliteal, axillary), spleen, and lung were harvested and percent nAPCs expressing CD11c− MHCII CD80− CD86− CCR7− Ly6G− was evaluated.

Blood and spleen analysis for detection of Ag uptake and neutrophil-DC markers. T cells were freshly isolated from naïve OT-II mice by magnetic cell sorting and labeled with CellTrace Violet (Invitrogen) at 5 μg/ml of Ova257–264 (SIINFEKL Analytical Biotechnology Services, Boulder, C34557) (pulsed) or 1 μg/ml of 3G8 anti-Fcγ with 0.1% bovine serum albumin (Sigma) and incubated with CellTrace Violet (Invitrogen) at 5 μM for 20 min at 37 °C. The reaction was stopped with 10% FCS and the cells were washed twice with cold PBS and resuspended in RPMI supplemented with 10% FCS, L-glutamine, and pen/strep. nAPCs were cultured in vitro in DMEM/high glucose supplemented with 10% FCS and cultured for 3 days to generate nAPCs. At day 3, adherent cells were counted and replated at equal numbers for all samples. As a positive control, nAPCs (with vehicle control, i.e. no Ova) were pulsed with 1 μg/ml of Ova257–264 24 h prior to the addition of T cells. To assess T cell proliferation, OT-I and OT-II T cells were resuspended in PBS (10^5 cells/ml) containing 0.1% bovine serum albumin (Sigma) and incubated with CellTrace Violet (Invitrogen) at 5 μM for 20 min at 37 °C. The reaction was stopped with 10% FCS and the cells were washed twice with cold PBS and resuspended in RPMI supplemented with 10% FCS, L-glutamine, and pen/strep. nAPCs were co-cultured with 2 × 10^5 CellTrace Violet-labeled OT-I or OT-II T cells. After 3 days for OT-I and 2 days for OT-II, cells were washed with PBS and analyzed by flow cytometry for CellTrace Violet dye dilution.

CD8+ (OTI) and CD4+ (OTII) T cell proliferation assays in vivo. T cells from the Ovalbumin specific TCR transgenic OT-I and OT-II mice were isolated from spleen and lymph nodes by positive selection. Cells were enriched with microbeads conjugated to anti-mouse CD8α (Ly-2) (Miltenyi) for enrichment of CD8+ T-cells or anti-mouse CD4 (L4T4) (Miltenyi) for CD4+ T-cell enrichment. Final preparations contained 85–90% CD8+ or 85–90% CD4+ T-cells. 5 × 10^5 bone marrow neutrophils from the indicated genotypes were loaded with Ova, Ova-IC or vehicle control as described earlier, seeded in 96-well microplates in RPMI medium (10% FCS) supplemented with GM-CSF and cultured for 3 days to generate nAPCs. At day 3, adherent cells were counted and replated at equal numbers for all samples. As a positive control, nAPCs (with vehicle control, i.e. no Ova) were pulsed with 1 μg/ml of Ova257–264 24 h prior to the addition of T cells. To assess T cell proliferation, OT-I and OT-II T cells were resuspended in PBS (10^5 cells/ml) containing 0.1% bovine serum albumin (Sigma) and incubated with CellTrace Violet (Invitrogen) at 5 μM for 20 min at 37 °C. The reaction was stopped with 10% FCS and the cells were washed twice with cold PBS and resuspended in RPMI supplemented with 10% FCS, L-glutamine, and pen/strep. nAPCs were co-cultured with 2 × 10^5 CellTrace Violet-labeled OT-I or OT-II T cells. After 3 days for OT-I and 2 days for OT-II, cells were washed with PBS and analyzed by flow cytometry for CellTrace Violet dye dilution.

CD8+ (OT-I) and CD4+ (OT-II) T cell proliferation assays in vivo. Enriched OT-I and OT-II T cells were labeled with CellTrace Violet dye as described for in vitro assays. Cells were resuspended in PBS and injected i.v. (3 × 10^6 cells/mouse) into recipient mice (day 0) that had received an i.v. injection of 30 μg of FcγRIIB−/− FITC-Ova conjugate at day 0. On day 4, mice were euthanized and cells from the spleen were harvested and stained for flow cytometry analysis. For MHCII deficient mice, the mice were injected retro-orbitally with 30 μg of 3G8 (anti-FcγRIIB)-FITC-Ova. Blood samples were taken at the indicated times and subjected to red blood cell lysis using ACK lysis buffer, washed with PBS and analyzed by flow cytometry for FITC-FcγRIIB expression. For CD8+ T-cells or anti-mouse CD4 (L4T4) (Miltenyi) for CD4+ T-cell enrichment. For T cell depletion, beginning 1 day prior to injection of 3G8-FcγRIIB injection, mice were treated with intraperitoneal injection of a ketamine/xylazine cocktail (90 mg/kg ketamine, 10 mg/kg xylazine), shaved and injected in the flank subcutaneously with 2 × 10^3 tumor cells in 100 μl HBSS. Tumors were measured as a caliper every 1–2 days once palpable in any one group (long diameter and short diameter) and tumor volume was calculated using an ellipsoid formula (1/2 x long diameter x short diameter). For T cell depletion, beginning 1 day prior to injection of 3G8-FcγRIIB injection, mice were treated with intraperitoneal injection of an initial dose of 200 μg/mouse of anti-CD8 (clone GK1.5, BioXcell) or anti-CD8 (clone 2A3, BioXcell) antibodies in PBS, followed by dosing with 100 μg/mouse every 7 days throughout the course of the experiment.

Organ harvest, T cell depletion, and flow cytometric analysis. Organs and tumors were surgically removed and processed within 30 min of removal. Briefly, on ice, all excess fat was removed. The organs were gently dissociated in FACS buffer (PBS supplemented with 2% FCS and 2 mM EDTA) by shearing the tissue on a 70 μm nylon cell strainer (FisherBrand) using a 3 ml syringe plunger. Tumors were minced/digested in Collagenase type I and dissociated using gentle MACS Dissociator 1X and lymphocytes were purified on a Percoll (GE Healthcare) gradient and resuspended in FACS buffer. Cells from spleen and liver were subjected to red blood cell lysis using ACK lysis buffer solution (Lonza Cat 5-5BB) for 2 min at room temperature, washed once with PBS and resuspended in FACS buffer. Blood was collected from the retroorbital plexus of isoflurane-anesthetized mice using micro-hemocrit capillary tubes into EDTA tubes (final 5 mM). Sample were subjected to red blood cell lysis using ACK lysis buffer for 10 min at room temperature, washed with PBS and resuspended in FACS buffer. For flow cytometric analysis, cells were analyzed for expression of CD4, CD8, C34557, MHCIIγ−RIIIB−/− (H-2Kb Ova Tetramer (Ova257–264)), CD69, CD25, PD-1, Fop3, Ki-67, and T-bet.

Delayed-type hypersensitivity (DTH) responses. Ova-IC or Ova-fed neutrophils were cultured in the presence of GM-CSF. 3 days later, non-adherent cells were discarded, adherent cells were harvested using a 70 μm nylon cell strainer (FisherBrand) using a 3 ml syringe plunger. Tumors were minced/digested in Collagenase type I and dissociated using gentle MACS Dissociator 1X and lymphocytes were purified on a Percoll (GE Healthcare) gradient and resuspended in FACS buffer. Cells from spleen and liver were subjected to red blood cell lysis using ACK lysis buffer solution (Lonza Cat 5-5BB) for 2 min at room temperature, washed once with PBS and resuspended in FACS buffer. Blood was collected from the retroorbital plexus of isoflurane-anesthetized mice using micro-hemocrit capillary tubes into EDTA tubes (final 5 mM). Sample were subjected to red blood cell lysis using ACK lysis buffer for 10 min at room temperature, washed with PBS and resuspended in FACS buffer. For flow cytometric analysis, cells were analyzed for expression of CD4, CD8, C34557, MHCIIγ−RIIIB−/− (H-2Kb Ova Tetramer (Ova257–264)), CD69, CD25, PD-1, Fop3, Ki-67, and T-bet.

Cytokine detection and analysis. Supernatants from nAPCs generated in vitro from Ova-IC or Ova-fed neutrophils were collected and analyzed for cytokine and chemokine levels using the pro-inflammatory focused 32-plex array (Eve Technologies, Calgary, AB). Intravital imaging of popliteal lymph node. Approximately 5 × 10^5 nAPCs generated with Ova-IC as described for the DTH model were resuspended in 25 μl of PBS and injected into the right hind footpad of wild-type C57Bl/6 mice at day 0.
spleens from 3G8-FITC (f)-Ova injected mice and WT control mice were harvested.

Preparation of samples for bulk RNAseq. Bone marrow neutrophils were isolated from wild-type mice by negative selection using the EasySep Mouse Neutrophil Enrichment Kit and incubated with Ova, Ova-IC or SLE-IC for 2 h as described and cultured with GM-CSF for the Ova-IC samples and without GM-CSF for the SLE-IC treated cells. After 3 days, adherent cells were harvested and total RNA was extracted using RNeasy Mini kit (Qiagen, USA) following the manufacturer’s protocol.

Trajectory analysis and trajectory score. To characterize cell differentiation trajectory, we use Monocle 3 software. A principal graph was learned on the UMAP projection of the in vitro cells using the learn graph () function. These cells were then ordered using the order cells () function in order to generate a pseudo-time axis. The following linear model was then fit using poisson regression to counts (X) of gene i in cell j found along the discovered trajectory defined by pseudo-time P with nUMI as an offset term:

The set of genes with p < 0.00005 (1246 genes) defined the set of genes correlated with pseudo-time and were used to calculate trajectory gene scores. These gene scores were calculated by multiplying the fitted beta from the above model for gene i with the expression of that gene in cell j. The sum of these values across all genes within each cell generated a score applied to the in vivo data along with the bulk data. A full list of these genes can be found in Supplementary Data 3.

Maturation score. To characterize maturation levels of Nt.1 and Nt.5 subsets, a maturation score was calculated by adding the expression of a set of 49 maturation-associated genes provided in a recent paper exploring neutrophil heterogeneity. We subsetted the normalized expression matrix on these genes and then scaled the resulting matrix. These scaled expression values were multiplied by an indicator variable (1 or −1) representing positive or negative logFC in the respective cluster and summed across all cells in each cluster. The full list of genes may be found in Supplementary Data 3.

Pseudo-time gene expression correlation. To show changes in gene expression of certain markers and how these changes correlated with pseudo-time, we binned the in vitro cells that fit the trajectory identified above into 25 bins. Within each bin, the mean and standard deviation of the normalized expression of each marker across all cells was determined and plotted vs. pseudo-time. The dots representing the mean normalized expression value within the bin were colored according to the most abundant cell type in that bin.

GO ontology pathway analysis. Pathway analysis was performed using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database. Top 200 genes by auc were used as input into the find enriched pathways function from the KEGG Profile package. Ribosomal genes were excluded from the top 200 genes and disease related pathways were removed from the final list of pathways to consider.

BART TF analysis and STRING database visualization. The genes correlated with pseudo-time were entered into the BART web-browser, which uses the MARGE-cistrome function to predict potential regulators of the input gene list. These regulators were taken and visualized using the STRING database. In the network predicted by the STRING database, the edges were limited to those with high confidence scores (>0.700). The colors of nodes were later changed to highlight the regulatory modules identified in the networks.
Statistics. Statistical analyses were performed using Graphpad prism 8 (La Jolla, CA), STATA 13 (StataCorp. 2013. College Station, TX) and JMP10 software (SAS Institute, Inc, USA). All the data included in the studies are expressed as mean ± SEM. For group analysis, one-way ANOVA with Dunnett’s multiple comparison was used. If only two groups were compared, a two-tailed students t-test with Bonferroni correction was used. The statistical significance of the differences for SLE patient and SLEDAI scores was determined by a nonparametric test using Mann-Whitney analysis. Correlation was analyzed using Spearman test. *P < 0.05 and **P < 0.005 was considered significant. For statistical analysis of tumor growth, the change in volume over time from day 7 to day 14 was estimated in each animal controlling for group and the repeated measures of the same animal through use of a mixed-effects regression model with a random subject effect. The model considered separate intercepts and slopes for animals in each of the four groups. An additional model considered possible non-linear relationships of volume with time in each animal through inclusion of a quadratic effect in time. For estimation, this effect was centered at the mean time of 12.5 days. Models were fitted using maximum likelihood, and likelihood ratio tests were used to test whether slopes differed across groups and whether the inclusion of quadratic terms improved model fit. Models were fitted using the Mixed routine in STATA version 13. A model adding the quadratic term was not significantly better than the model assuming linearity over time with separate slopes per group.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All data generated or analyzed during this study are included in this published article (and its Supplementary information files). Sequence data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) database with primary accession code GSE173569. All reasonable requests for unique materials will be provided under an institutional Materials Transfer Agreement. Source data are provided with this paper.

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**Competing interests**

The authors declare no competing interests.

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