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Macrophages are crucial for epithelial cell death and adipocyte repopulation during mammary gland involution

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SUMMARY

Mammary gland development is dependent on macrophages, as demonstrated by their requirement during the expansion phases of puberty and pregnancy. Equally dramatic tissue restructuring occurs following lactation, when the gland regresses to a state that histologically resembles pre-pregnancy through massive programmed epithelial cell death and stromal repopulation. Postpartum involution is characterized by wound healing-like events, including an influx of macrophages with M2 characteristics. Macrophage levels peak after the initial wave of epithelial cell death, suggesting that initiation and execution of cell death are macrophage independent. To address the role of macrophages during weaning-induced mammary gland involution, conditional systemic deletion of macrophages expressing colony stimulating factor 1 receptor (CSF1R) was initiated just prior to weaning in the Mafia mouse model. Depletion of CSF1R$^+$ macrophages resulted in delayed mammary involution as evidenced by loss of lysosomal-mediated and apoptotic epithelial cell death, lack of alveolar regression and absence of adipocyte repopulation 7 days post-weaning. Failure to execute involution occurred in the presence of milk stasis and STAT3 activation, indicating that neither is sufficient to initiate involution in the absence of CSF1R$^+$ macrophages. Injection of wild-type bone marrow-derived macrophages (BMDMs) or M2-differentiated macrophages into macrophage-depleted mammary glands was sufficient to rescue involution, including apoptosis, alveolar regression and adipocyte repopulation. BMDMs exposed to the postpartum mammary involution environment upregulated the M2 markers arginase 1 and mannose receptor. These data demonstrate the necessity of macrophages, and implicate M2-polarized macrophages, for epithelial cell death during normal postpartum mammary gland involution.

KEY WORDS: TGFβ, Delayed involution, STAT3, Macrophage programming, Perilipin, Mouse

INTRODUCTION

Postnatal mammary gland development research focuses primarily on mammary epithelial cells (MECs), as the epithelium undergoes dramatic expansion in preparation for milk synthesis (Lyons, 1958; Nandi, 1958). The MEC is also highlighted in studies on postpartum involution, a developmental window characterized by massive epithelial cell death that is necessary to return the gland to a non-secretory state (Green and Streuli, 2004; Lund et al., 1996). Although fluctuations in hormones and growth factors are essential for postnatal MEC development (Brisken and O'Malley, 2010; Hynes and Watson, 2010), the epithelial cell interacts with a complex microenvironment and evidence for stromal participation in postnatal mammary development is accumulating (Cunha et al., 1995; Howlett and Bissell, 1993; Lyons et al., 2011). Of note, immune cells, including macrophages, eosinophils and mast cells, are required for pubertal and gestational gland development (Coussens and Pollard, 2011; Lilla and Werb, 2010; Reed and Schwertfeger, 2010). The role of macrophages in mammary development has been investigated using mice that lack the macrophage growth factor colony stimulating factor 1 (CSF1) or its receptor (CSF1R). Macrophage depletion during puberty decreases terminal end bud number, mammary duct length and branching (Dai et al., 2002; Gouon-Evans et al., 2000). Macrophage depletion during pregnancy leads to diminished branching and precocious lobuloalveolar development (Pollard and Hennighausen, 1994). These pioneering studies demonstrate that macrophage function lies at the confluence of proliferation, tissue remodeling and terminal differentiation events required for mammary gland morphogenesis and function. Potential contributions of macrophages to postpartum mammary gland involution have not been elucidated, in large part owing to the inability to investigate normal involution in the background of abnormal pubertal and pregnancy development that occurs with embryonic macrophage depletion. However, recent immunohistochemistry (IHC) and microarray data have identified macrophage influx and associated gene expression profiles following weaning, with maximal influx occurring after the peak window of MEC apoptosis (Clarkson et al., 2004; Lund et al., 1996; O'Brien et al., 2010; Stein et al., 2004).

MEC apoptosis, which is a hallmark of postpartum involution, is described as an intrinsically regulated developmental program (Strange et al., 1992). Proposed initiation mechanisms include mechanical stress from milk stasis, accumulation of pro-apoptotic factors in the milk, and autocrine/paracrine production of pro-apoptotic/anti-survival factors by the MEC (Green and Streuli, 2004). Dramatic changes in gene expression occur at the time of weaning, and several genetic experiments demonstrate the requirement of MEC-intrinsic pro-apoptotic factors for execution of apoptosis (Green and Streuli, 2004; Watson, 2006). In mouse models, MEC death is an early event, with histological evidence observed between 12 and 72 hours of forced weaning, and later

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stages of involution described by, but not limited to, extracellular matrix (ECM) remodeling and adipocyte repopulation (Clarkson et al., 2004; Lund et al., 1996; Schedin et al., 2004; Stein et al., 2007; Strange et al., 1992). Anticipated roles for involution-associated macrophages have focused on later stage activities, including phagocytosis of cell debris, milk clearance and protease production related to ECM restructuring, events that occur 3-4 days post-weaning, after the peak in apoptosis (Atabai et al., 2007; O'Brien and Schedin, 2009; Schwertfeger et al., 2006).

To address potential roles for macrophages in postpartum mammary gland involution, macrophages expressing CSF1R were systemically depleted prior to weaning. Unexpectedly, in the absence of macrophages, postpartum involution processes, including MEC death and adipocyte repopulation, failed to occur. Injection of bone marrow-derived macrophages (BMDMs) directly into mammary glands of macrophage-depleted mice at the time of weaning restored involution, confirming a requirement for macrophages in postpartum involution.

MATERIALS AND METHODS

Mouse breeding and tissue preparation

The University of Colorado IACUC approved all mouse procedures. Mafia and C57BL/6J wild-type mice (Jackson Laboratories, Bar Harbor, ME, USA) were bred in static micro-isolator cages with 12-hour light/dark cycles. Two days postparturition litter sizes were standardized to 5-6 pups. AP20187 drug (Ariad Pharmaceuticals, Cambridge, MA, USA) or vehicle (4% ethanol, 10% PEG 400, 2% Tween 20) intraperitoneal treatment was initiated 72 hours before weaning at 10 mg/kg body weight daily for 4-9 days. Weaning commenced (denoted InvD0) at lactation days 10-14. Mice were anesthetized with Isoflurane (VetOne, Meridian, ID, USA), followed by cardiac puncture to collect plasma. Lymph node-free mammary gland 4-5 tissue was collected, flash frozen and stored at –80°C. Mammary tissue adjacent to the lymph node region and abdominal fat were fixed in 10% neutral buffered formalin for 24 hours and paraffin embedded.

For macrophage add-back experiments, isolated bone marrow from 8- to 12-week-old female C57BL/6J mice was differentiated to BMDMs or M2-activated macrophages as described (Edwards et al., 2006). Two thousand five hundred macrophages were resuspended in 5 μl PBS for mammary orthotopic injections at InvD0. Results are representative of three separate experiments.

Flow cytometry

CD45-APC and/or F4/80-PE antibodies (Invitrogen, Carlsbad, CA, USA) were added to 100 μl whole blood and incubated at room temperature for 20 minutes. Samples were treated with Red Cell Lysis Buffer (eBioscience, San Diego, CA, USA) and then analyzed on a Beckman Coulter CyAn. In vitro cultured macrophages were stained with F4/80-FITC (AbD Serotec, Raleigh, NC, USA), CD45-APC, CD11b (ITGAM – Mouse Genome Informatics) (eBioscience) and GR1-PE (LY6G – Mouse Genome Informatics) (BD Pharmingen, San Diego, CA, USA) antibodies and analyzed on a Beckman Coulter Gallios.

Quantitative immunohistochemistry

Paraffin-embedded 4 μm mammary tissue sections were pre-treated in Dako (Carpinteria, CA, USA) Target Retrieval Solution (TRS) or EDTA Antigen Retrieval Solution at 125°C under pressure for 5 minutes. Primary antibody incubations were for 1 hour at room temperature: F4/80 (TRS, 1:100; AbD Serotec), perilipin (TRS, 1:200; Cell Signaling, Danvers, MA, USA), smooth muscle actin (SMA) (EDTA, 1:200; Dako), p63 (TRP63 – Mouse Genome Informatics) (EDTA, 1:200; Biocare, Concord, CA, USA), cleaved caspase 3 (TRS, 1:200; Cell Signaling), phosphoSTAT3 (EDTA, 1:100; Cell Signaling), GFP (TRS, 1:400; Vector Laboratories, Burlingame, CA, USA) and mannose receptor (MR) (TRS, 1:600; Sigma, St Louis, MO, USA). Immunoreactivity was detected using Envision+ Systems (Dako) for non-conjugated primary antibodies or streptavidin-HRP (Dako) for biotinylated antibodies. Images were extracted from scanned slides using Aperio (Vista, CA, USA) hardware and software. F4/80, GFP, perilipin and MR IHC stains were quantified as percentage positive alveolar area utilizing the Aperio color deconvolution algorithm, with ten fields/gland/mouse, n=2-6 mice/time point. Average lumen area was quantified for eight to ten lumen/400× field, five to six fields/gland/mouse, n=2-6 mice/time point utilizing ImageJ software (NIH). The average number of acini per lobule was quantified from five lobules/mouse and three mice/time point. The average number of cleaved caspase 3-positive cells/200× field was quantified from ten fields/gland/mouse, n=2-6 mice/time point.

Western blots

Mammary tissue samples were prepared (three mice/group) and loaded by equal volume as described (Schedin et al., 2004). Membranes were incubated (overnight at 4°C unless stated otherwise, diluted in 5% BSA in TBS-T) with the following antibodies: STAT5 (1:200; Santa Cruz, Santa Cruz, CA, USA), STAT3 (1:1000; Cell Signaling), phosphoSTAT5 (1:200; Cell Signaling), phosphoSTAT3 (1:1000; Cell Signaling), phosphoERK1/2 (1:1000; Cell Signaling), ERK1/2 (1:1000, 1 hour at room temperature; Millipore, Billerica, MA, USA), cathepsin B (1:1000; Abcam, Cambridge, MA, USA), cathepsin L (1:1000; R&D Systems, Minneapolis, MN, USA), iNOS (1:100, 4 hours at 4°C; Abcam), arginase 1 (1:120; Santa Cruz), MR (1:100; Sigma) and GAPDH (1:1000, 1 hour at room temperature; Sigma).

RNA analyses

RNA was isolated from 15 mg mammary tissue or 1×10^6 $CD11b^+$ macrophages using Trizol reagent (Invitrogen). Concentration and purity were determined by 260 nm and 280 nm fluorescence. cDNA was synthesized from 500 ng RNA using the Verso cDNA Synthesis Kit (ThermoScientific, Waltham, MA, USA) and cDNA was amplified using primers for *Csn2*, *Tgfb3* and *Actb* (supplementary material Table S1). Real-time RT-PCR conditions were: 95°C for 5 minutes, then 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. mRNA was normalized using the geometric average of *Actb*.

Statistics

Unpaired t-tests were performed using InStat (GraphPad) software.

RESULTS AND DISCUSSION

Macrophage depletion blocks postpartum mammary gland involution

The abnormal pubertal and gestational mammary gland development resulting from macrophage depletion in CSF1-deficient mice renders this model unsuitable for investigating postpartum involution. To examine involution-specific macrophage function(s), conditional depletion of CSF1R-expressing macrophages following full lactation was performed 3 days prior to weaning, utilizing previously described Mafia (macrophage Fas-induced apoptosis) transgenic mice (Burnett et al., 2004). In these mice, CSF1R-expressing cells express a suicide transgene and green fluorescent protein (GFP). Treatment of Mafia mice with a modified dimer (AP20187) triggers Fas-mediated apoptosis in CSF1R-expressing cells (Burnett et al., 2004). Peripheral blood isolated from vehicle- or AP20187-treated mice at involution day (InvD) 1, 3 and 5 was analyzed for CD45 (PTPRC – Mouse Genome Informatics; a leukocyte-specific antigen), GFP (transgene-expressing cells) and F4/80 (EMR1 – Mouse Genome Informatics; a mouse macrophage antigen), which confirmed an ~85% decrease in $CD45^+$ GFP^+ $F4/80^+$ cells with AP20187 treatment as early as InvD1 (supplementary material Fig. S1A). IHC analyses for F4/80 and GFP in mammary tissue confirmed that systemic delivery of AP20187 resulted in significant macrophage depletion (Fig. 1A,B; data not shown).

Compared with control Mafia mice, mammary glands of AP20187-treated mice exhibited distended alveoli filled with milk at InvD3, a trend that persists at InvD5 and InvD7 (Fig. 1A,C,D,E).

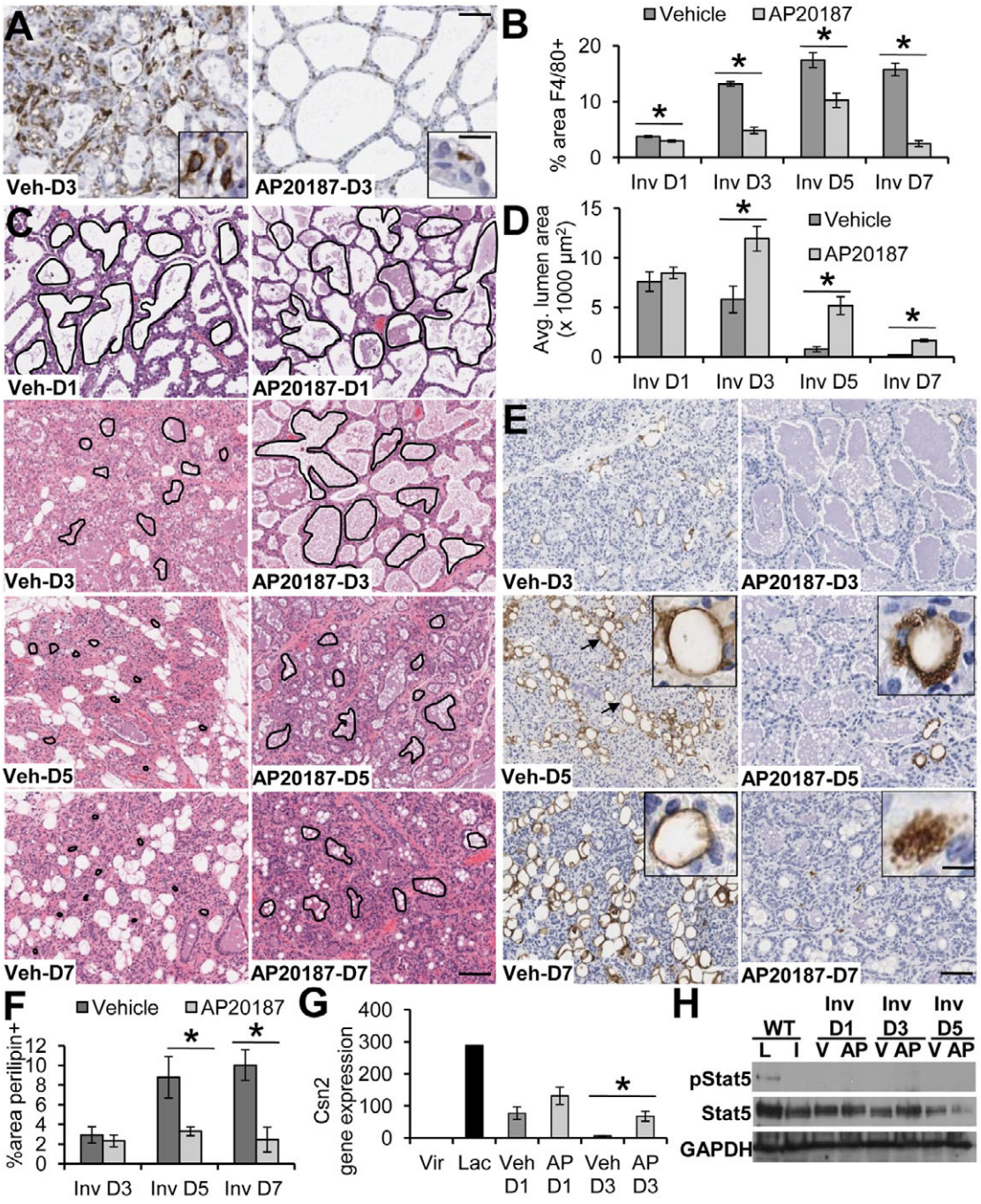

Fig. 1. Systemic macrophage depletion delays postpartum involution. (**A**) F4/80 immunohistochemistry (IHC) at involution day (InvD) 3 in mouse mammary tissue treated with vehicle or AP20187. (**B**) F4/80 IHC quantification. *, $P<0.03$; $n=2$-5. (**C**) Hematoxylin and Eosin (H&E)-stained mammary tissue at InvD1, 3, 5 and 7. The lumen are outlined. (**D**) Lumen area quantification. *, $P<0.01$; $n=2$-5. (**E**) Perilipin IHC in mammary tissue at InvD3, 5 and 7. Arrows indicate perilipin$^+$ adipocytes. (**F**) Perilipin IHC quantification. *, $P<0.04$; $n=2$-5. (**G**) β-casein expression as assessed by RT-PCR (for *Csn2*). *, $P=0.002$; $n=3$. (**H**) PhosphoSTAT5 western blot. Vir, virgin; L, lactation day 10; I, involution; V, Veh, vehicle; AP, AP20187. Error bars indicate s.e.m. Scale bars: 90 µm in A; 20 µm in A inset; 120 µm in C,E; 10 µm in E inset.

The increase in lumen area in macrophage-depleted glands at InvD3 compared with InvD1 suggests stretching due to milk stasis (Fig. 1D). Lumen size is reduced in macrophage-depleted glands at InvD5; however, the number of acini per lobule does not decrease, confirming that acini do not regress but rather decrease in area, probably owing to milk clearance (Fig. 1D, supplementary material Fig. S1B). Additional evidence that mammary involution does not progress in the absence of macrophages is provided by a lack of adipocyte repopulation, as measured by expression of perilipin, an adipocyte-specific lipid droplet protein (Fig. 1E,F). Further, the rare perilipin-positive adipocytes were lipid depleted in AP20187-treated mice (Fig. 1E, insets). AP20187 treatment in wild-type (WT) mice did not affect normal involution, ruling out non-specific drug actions (supplementary material Fig. S1C).

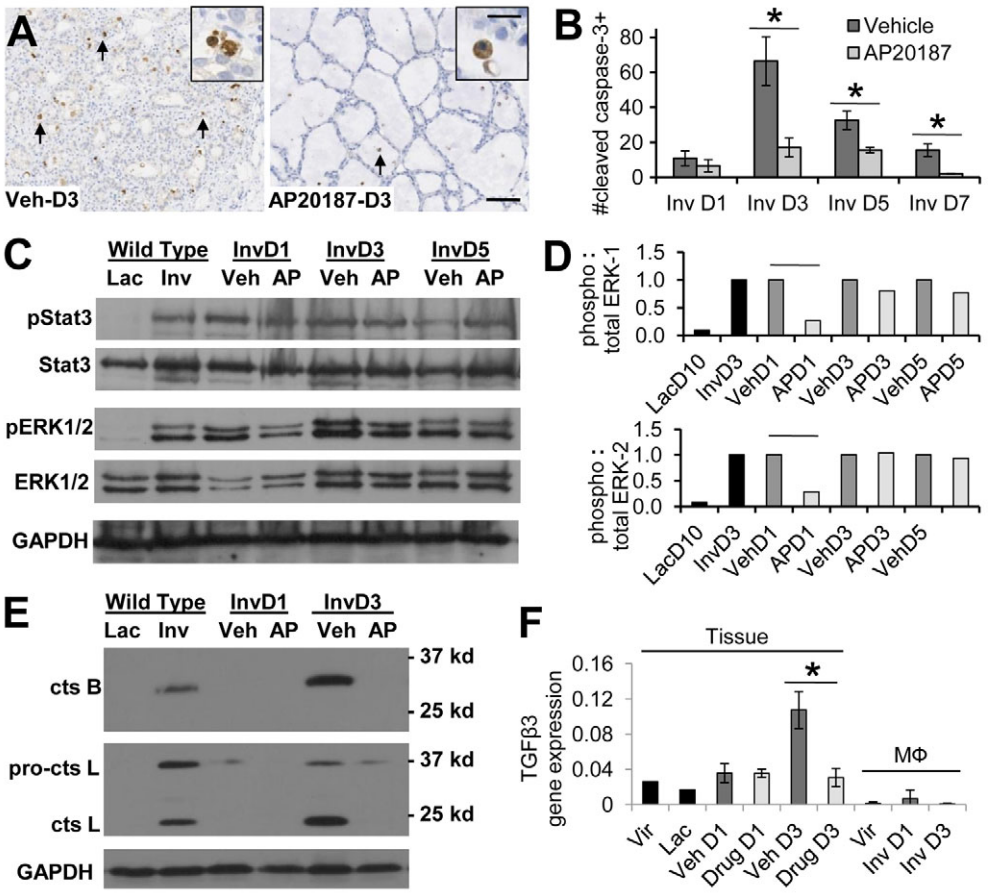

Fig. 2. In the absence of macrophages, milk stasis and STAT3 activation are not sufficient to induce mammary epithelial cell death. (**A**) Cleaved caspase 3 IHC in mouse mammary tissue at InvD3. Arrows indicate cleaved caspase 3^+ cells. (**B**) Cleaved caspase 3 IHC quantification. *, $P \leq 0.03$; $n=2$-5. (**C**) PhosphoSTAT3 and phosphoERK1/2 western blots. (**D**) Quantified ratio of phosphoERK:total ERK. (**E**) Cathepsin B (cts B) and cathepsin L (cts L) western blots. (**F**) Expression of *Tgfb3* as assessed by RT-PCR. *, $P=0.005$; $n=3$. MΦ, macrophages; InvD, involution day; Vir, virgin; Lac, lactation; Veh, vehicle; AP, AP20187. Error bars indicate s.e.m. Scale bars: 120 μm; 10 μm in insets.

AP20187 treatment also did not induce apoptosis in adipocytes directly, as adipocytes are present in the mammary tissue of AP20187-treated nulliparous mice and in the abdominal fat pad of AP20187-treated involution mice (supplementary material Fig. S1D). Myoepithelial cells are also not depleted by AP20187 (supplementary material Fig. S1E). Transcript levels of the milk protein β-casein were assessed. Surprisingly, β-casein expression persisted, although at lower levels, in the AP20187-treated glands through InvD3 (Fig. 1G), even in the absence of the phosphoSTAT5 transcription factor necessary for lactation (Fig. 1H) (Liu et al., 1997).

Suppressed alveolar regression suggests that MEC death is inhibited without macrophages. MEC apoptosis was essentially absent in macrophage-depleted glands through InvD7, as measured by cleaved caspase 3 (Fig. 2A,B). Of note, this maintenance of MEC survival post-weaning is longer in duration than that reported for most delayed involution phenotypes, including those resulting from knockout of *Il6*, type II TGFβ receptor (*Tgfbr2*) or *Stat3*, where compensatory mechanisms initiate apoptosis by InvD7 (Bierie et al., 2009; Chapman et al., 1999; Radisky and Hartmann, 2009; Zhao et al., 2002). In contrast to these gene studies, here a distinct macrophage population is depleted, suggesting that compensation for a cell type is less likely.

A primary mediator of MEC apoptosis is the transcription factor STAT3, which is phosphorylated (activated) within 12-24 hours post-weaning (Pensa et al., 2009). Western blot analyses for phosphoSTAT3 demonstrated that macrophage-depleted glands activate STAT3 at the same rate and intensity as observed in vehicle-treated glands (Fig. 2C). Although unanticipated, this observation is consistent with previous reports showing that STAT3 phosphorylation is not sufficient to induce apoptosis at the onset of involution in the absence of IL6 or TGFβ (Bierie et al., 2009; Zhao et al., 2002). In *Il6* knockout mice, decreased ERK1/2 (MAPK3/1 – Mouse Genome Informatics) activation was suggested to contribute to delayed apoptosis (Zhao et al., 2002). With AP20187 treatment, phosphoERK1/2 is also decreased in mammary tissue from recently weaned InvD1 dams (Fig. 2C,D), providing evidence for altered MEC signaling in the absence of macrophages. However, ERK1/2 phosphorylation is observed in AP20187-treated glands at InvD3 without induction of MEC apoptosis. Further, mammary tissue *Il6* transcript levels did not differ with macrophage depletion (data not shown).

Recently, lysosomal-mediated epithelial cell death has been observed as a very early event in mammary gland involution, preceding the peak in caspase-mediated apoptosis (Kreuzaler et al., 2011). However, in the absence of macrophages, markers of lysosomal-mediated cell death, cathepsins B and L, were not induced at weaning (Fig. 2E).

In summary, these data show that milk stasis, STAT3 phosphorylation and ERK1/2 phosphorylation, which are all demonstrated inducers/mediators of postpartum involution (Li et al., 1997; Pensa et al., 2009; Zhao et al., 2002), are insufficient to trigger lysosomal- or caspase-mediated MEC death in the absence of macrophages.

Transforming growth factor β3 (TGFβ3) activity is sufficient for induction of MEC apoptosis and its loss suppresses apoptosis during involution (Nguyen and Pollard, 2000). Further, knockout

of *Tgfbr2* during involution delays involution and rescues milk protein gene expression, indicating that TGFβ is necessary to suppress lactation (Bierie et al., 2009). RT-PCR analyses confirm that *Tgfb3* transcripts are significantly increased in normal mammary tissue at InvD3, but not in postpartum mammary tissue from macrophage-depleted mice (Fig. 2F). As *Tgfb3* transcript levels in macrophages isolated from the involuting mammary gland do not account for the elevated levels found in whole tissue (Fig. 2F), TGFβ3 is likely to be indirectly involved in macrophage contributions to postpartum involution. Additional cytokines implicated in macrophage-induced cell death in other models include tumor necrosis factor α, FASL and nitric oxide (Boyle et al., 2001; Boyle et al., 2003; Cui et al., 1994; Reister et al., 2001), which are of interest in follow-up studies.

Reconstitution experiments confirm a crucial role for macrophages in postpartum involution

To confirm that macrophages are sufficient to induce involution in AP20187-treated Mafia mice, macrophage add-back experiments were performed. Macrophages function across a spectrum of programming states, from classical M1 antigen presentation to alternative M2 tissue remodeling roles (Gordon and Taylor, 2005; Martinez et al., 2008). The involuting gland is characterized by the presence of M2 cytokines (IL4 and IL13) and, consistent with these data, involution macrophages express M2 markers [arginase 1 and mannose receptor (MR)] (O'Brien et al., 2010). For add-back studies, WT bone marrow cells were cultured in vitro to promote immature BMDMs and further differentiated in the presence of IL4 to promote M2 maturation, resulting in macrophage populations of greater than 99% purity (supplementary material Fig. S2A). M2 differentiated macrophages expressed arginase 1 and MR and lacked the M1 marker iNOS (NOS2 – Mouse Genome Informatics) (Fig. 3A). BMDMs, M2 macrophages or phosphate-buffered saline (PBS) were orthotopically injected at weaning into mice depleted of endogenous macrophages (Fig. 3B). Successful macrophage reconstitution at 2 days post-injection (InvD2) was confirmed by an increase in F4/80 IHC signal in BMDM-injected or M2-injected glands, which occurred without an increase in endogenous macrophage levels (GFP^+) (Fig. 3C, supplementary material Fig. S2B,C). Histological analyses of BMDM- and M2-injected glands revealed that average lumen size was smaller, adipocyte repopulation higher, and MEC apoptosis elevated over PBS

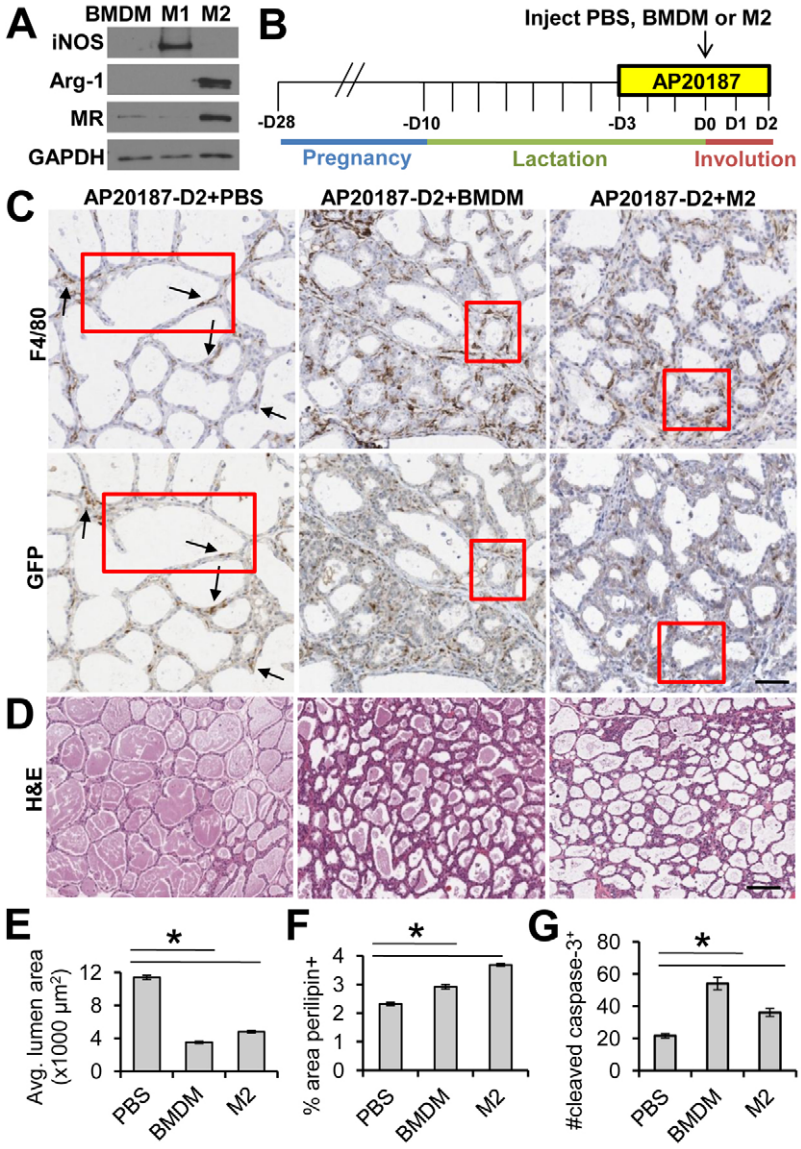

Fig. 3. Macrophage reconstitution experiments in AP20187-treated mice confirm a crucial role for macrophages in postpartum mammary gland involution. (**A**) iNOS, arginase 1 (Arg1) and mannose receptor (MR) western blots of M1, M2 and bone marrow-derived (BMDM) macrophages. (**B**) The add-back experimental design. (**C**) F4/80 and GFP IHC of PBS-, BMDM- or M2-injected glands of AP20187-treated mice 2 days post-weaning. The boxed regions are shown at higher magnification in supplementary material Fig. S2C. Arrows indicate $F4/80^+$ and GFP^+ cells, respectively. (**D**) H&E-stained mammary tissue with macrophage add-back. (**E**) Lumen area quantification. *, $P<0.001$; $n=6$. (**F**) Perilipin IHC quantification. *, $P<0.001$; $n=6$. (**G**) Cleaved caspase 3 IHC quantification. *, $P<0.001$; $n=6$. Error bars indicate s.e.m. Scale bars: 60 μm in C; 150 μm in D.

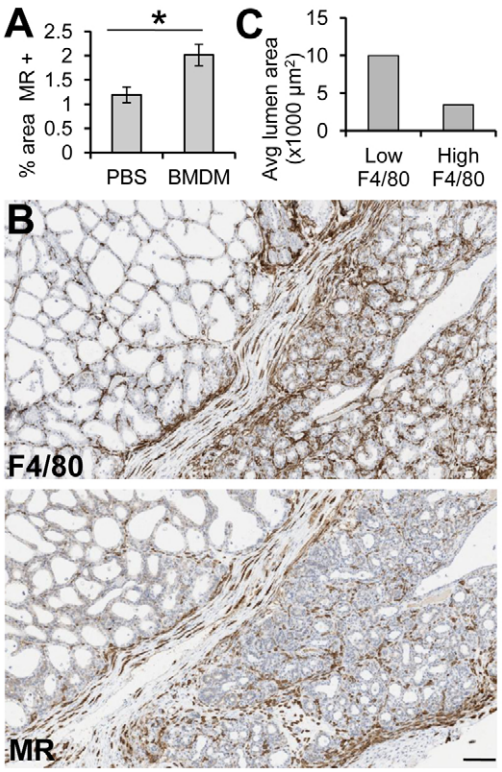

Fig. 4. Bone marrow-derived macrophages upregulate M2 markers when exposed to the postpartum involution mammary microenvironment. (**A**) MR IHC quantification in PBS-injected and BMDM-injected glands of AP20187-treated mice. *, P=0.013; n=6. (**B**) F4/80 (top) and MR (bottom) IHC of BMDM injection site in AP20187-treated mice. (**C**) Lumen area quantification of B. Error bars indicate s.e.m. Scale bar: 120 µm.

controls, demonstrating that WT immature or M2 differentiated macrophages can rescue involution (Fig. 3C-G). Although histological evidence for gland regression was clearly evident in M2-injected glands, induction of MEC apoptosis was lower than in BMDM-injected glands (Fig. 3G). If the time course of apoptosis differs between BMDM- and M2-induced rescue, this could account for the discrepancy in apoptotic indices. Alternatively, immature and mature macrophages might differ in their ability to promote apoptosis in this model. Of note, dendritic cells are also depleted upon AP20187 administration in Mafia mice (Burnett et al., 2004) and therefore might contribute to the delayed phenotype. Nonetheless, these reconstitution experiments confirm that macrophages are sufficient to rescue postpartum involution.

The question of whether BMDMs injected into the involuting microenvironment respond to differentiation signals and upregulate M2 markers was investigated. BMDMs lack M2 marker expression prior to injection (Fig. 3A); however, IHC quantification demonstrated high MR (Fig. 4A) and arginase 1 (data not shown) expression in BMDM-injected glands as compared with PBS-injected controls. Further evidence that injected BMDMs can acquire an M2 phenotype and promote involution was obtained by evaluating F4/80^{hi} areas, which are likely to demarcate injection sites, versus F4/80^{lo} areas within the same gland. F4/80^{hi} sites clearly express elevated levels of MR and display decreased average lumen size compared with F4/80^{lo} lobules, although many F4/80^{hi} cells lack MR (Fig. 4B,C).

Implications

As macrophages are associated with breast cancer progression (Pollard, 2008) and have been suggested to promote postpartum breast cancer specifically (O'Brien et al., 2010; O'Brien and Schedin, 2009), targeting macrophages for therapeutic benefit has been proposed (Johansson et al., 2008; O'Brien and Schedin, 2009; Sica et al., 2007). Here, we show that conditional depletion of macrophages prevents requisite remodeling of the lactating mammary gland to its non-secretory quiescent state through suppression of MEC death and adipocyte repopulation. Inhibition of MEC death during involution is undesirable as this promotes mammary tumorigenesis in rodents (Radisky and Hartmann, 2009). An alternative strategy involves re-educating tumor-promoting macrophages to become tumor suppressive (Gordon and Taylor, 2005; Hagemann et al., 2008). However, targeting M2-like macrophages for the prevention or treatment of postpartum breast cancer will require further knowledge of their role(s) in mammary involution.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References

Atabai, K., Sheppard, D. and Werb, Z. (2007). Roles of the innate immune system in mammary gland remodeling during involution. *J. Mammary Gland Biol. Neoplasia* **12**, 37-45.

Bierie, B., Gorska, A. E., Stover, D. G. and Moses, H. L. (2009). TGF-beta promotes cell death and suppresses lactation during the second stage of mammary involution. *J. Cell. Physiol.* **219**, 57-68.

Boyle, J. J., Bowyer, D. E., Weissberg, P. L. and Bennett, M. R. (2001). Human blood-derived macrophages induce apoptosis in human plaque-derived vascular smooth muscle cells by Fas-ligand/Fas interactions. *Arterioscler. Thromb. Vasc. Biol.* **21**, 1402-1407.

Boyle, J. J., Weissberg, P. L. and Bennett, M. R. (2003). Tumor necrosis factor-alpha promotes macrophage-induced vascular smooth muscle cell apoptosis by direct and autocrine mechanisms. *Arterioscler. Thromb. Vasc. Biol.* **23**, 1553-1558.

Brisken, C. and O'Malley, B. (2010). Hormone action in the mammary gland. *Cold Spring Harb. Perspect. Biol.* **2**, a003178.

Burnett, S. H., Kershen, E. J., Zhang, J., Zeng, L., Straley, S. C., Kaplan, A. M. and Cohen, D. A. (2004). Conditional macrophage ablation in transgenic mice expressing a Fas-based suicide gene. *J. Leukoc. Biol.* **75**, 612-623.

Chapman, R. S., Lourenco, P. C., Tonner, E., Flint, D. J., Selbert, S., Takeda, K., Akira, S., Clarke, A. R. and Watson, C. J. (1999). Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. *Genes Dev.* **13**, 2604-2616.

Clarkson, R. W., Wayland, M. T., Lee, J., Freeman, T. and Watson, C. J. (2004). Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. *Breast Cancer Res.* **6**, R92-R109.

Coussens, L. M. and Pollard, J. W. (2011). Leukocytes in mammary development and cancer. *Cold Spring Harb. Perspect. Biol.* **3**, a003285.

Cui, S., Reichner, J. S., Mateo, R. B. and Albina, J. E. (1994). Activated murine macrophages induce apoptosis in tumor cells through nitric oxide-dependent or -independent mechanisms. *Cancer Res.* **54**, 2462-2467.

Cunha, G. R., Young, P., Christov, K., Guzman, R., Nandi, S., Talamantes, F. and Thordarson, G. (1995). Mammary phenotypic expression induced in

epidermal cells by embryonic mammary mesenchyme. *Acta Anat. (Basel)* **152**, 195-204.
Dai, X. M., Ryan, G. R., Hapel, A. J., Dominguez, M. G., Russell, R. G., Kapp, S., Sylvestre, V. and Stanley, E. R. (2002). Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood* **99**, 111-120.
Edwards, J. P., Zhang, X., Frauwirth, K. A. and Mosser, D. M. (2006). Biochemical and functional characterization of three activated macrophage populations. *J. Leukoc. Biol.* **80**, 1298-1307.
Gordon, S. and Taylor, P. R. (2005). Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* **5**, 953-964.
Gouon-Evans, V., Rothenberg, M. E. and Pollard, J. W. (2000). Postnatal mammary gland development requires macrophages and eosinophils. *Development* **127**, 2269-2282.
Green, K. A. and Streuli, C. H. (2004). Apoptosis regulation in the mammary gland. *Cell. Mol. Life Sci.* **61**, 1867-1883.
Hagemann, T., Lawrence, T., McNeish, I., Charles, K. A., Kulbe, H., Thompson, R. G., Robinson, S. C. and Balkwill, F. R. (2008). 'Re-educating' tumor-associated macrophages by targeting NF-kappaB. *J. Exp. Med.* **205**, 1261-1268.
Howlett, A. R. and Bissell, M. J. (1993). The influence of tissue microenvironment (stroma and extracellular matrix) on the development and function of mammary epithelium. *Epithelial Cell Biol.* **2**, 79-89.
Hynes, N. E. and Watson, C. J. (2010). Mammary gland growth factors: roles in normal development and in cancer. *Cold Spring Harb. Perspect. Biol.* **2**, a003186.
Johansson, M., Denardo, D. G. and Coussens, L. M. (2008). Polarized immune responses differentially regulate cancer development. *Immunol. Rev.* **222**, 145-154.
Kreuzaler, P. A., Staniszewska, A. D., Li, W., Omidvar, N., Kedjouar, B., Turkson, J., Poli, V., Flavell, R. A., Clarkson, R. W. and Watson, C. J. (2011). Stat3 controls lysosomal-mediated cell death in vivo. *Nat. Cell Biol.* **13**, 303-309.
Li, M., Liu, X., Robinson, G., Bar-Peled, U., Wagner, K. U., Young, W. S., Hennighausen, L. and Furth, P. A. (1997). Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. *Proc. Natl. Acad. Sci. USA* **94**, 3425-3430.
Lilla, J. N. and Werb, Z. (2010). Mast cells contribute to the stromal microenvironment in mammary gland branching morphogenesis. *Dev. Biol.* **337**, 124-133.
Liu, X., Robinson, G. W., Wagner, K. U., Garrett, L., Wynshaw-Boris, A. and Hennighausen, L. (1997). Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev.* **11**, 179-186.
Lund, L. R., Romer, J., Thomasset, N., Solberg, H., Pyke, C., Bissell, M. J., Dano, K. and Werb, Z. (1996). Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. *Development* **122**, 181-193.
Lyons, T. R., O'Brien, J., Borges, V. F., Conklin, M. W., Keely, P. J., Eliceiri, K. W., Marusyk, A., Tan, A. C. and Schedin, P. (2011). Postpartum mammary gland involution drives progression of ductal carcinoma in situ through collagen and COX-2. *Nat. Med.* **17**, 1109-1115.
Lyons, W. R. (1958). Hormonal synergism in mammary growth. *Proc. R. Soc. Lond. B Biol. Sci.* **149**, 303-325.
Martinez, F. O., Sica, A., Mantovani, A. and Locati, M. (2008). Macrophage activation and polarization. *Front. Biosci.* **13**, 453-461.
Nandi, S. (1958). Endocrine control of mammary gland development and function in the C3H/He Crg1 mouse. *J. Natl. Cancer Inst.* **21**, 1039-1063.
Nguyen, A. V. and Pollard, J. W. (2000). Transforming growth factor beta3 induces cell death during the first stage of mammary gland involution. *Development* **127**, 3107-3118.
O'Brien, J. and Schedin, P. (2009). Macrophages in breast cancer: do involution macrophages account for the poor prognosis of pregnancy-associated breast cancer? *J. Mammary Gland Biol. Neoplasia* **14**, 145-157.
O'Brien, J., Lyons, T., Monks, J., Lucia, M. S., Wilson, R. S., Hines, L., Man, Y. G., Borges, V. and Schedin, P. (2010). Alternatively activated macrophages and collagen remodeling characterize the postpartum involuting mammary gland across species. *Am. J. Pathol.* **176**, 1241-1255.
Pensa, S., Watson, C. J. and Poli, V. (2009). Stat3 and the inflammation/acute phase response in involution and breast cancer. *J. Mammary Gland Biol. Neoplasia* **14**, 121-129.
Pollard, J. W. (2008). Macrophages define the invasive microenvironment in breast cancer. *J. Leukoc. Biol.* **84**, 623-630.
Pollard, J. W. and Hennighausen, L. (1994). Colony stimulating factor 1 is required for mammary gland development during pregnancy. *Proc. Natl. Acad. Sci. USA* **91**, 9312-9316.
Radisky, D. C. and Hartmann, L. C. (2009). Mammary involution and breast cancer risk: transgenic models and clinical studies. *J. Mammary Gland Biol. Neoplasia* **14**, 181-91.
Reed, J. R. and Schwertfeger, K. L. (2010). Immune cell location and function during post-natal mammary gland development. *J. Mammary Gland Biol. Neoplasia* **15**, 329-339.
Reister, F., Frank, H. G., Kingdom, J. C., Heyl, W., Kaufmann, P., Rath, W. and Huppertz, B. (2001). Macrophage-induced apoptosis limits endovascular trophoblast invasion in the uterine wall of preeclamptic women. *Lab. Invest.* **81**, 1143-1152.
Schedin, P., Mitrenga, T., McDaniel, S. and Kaeck, M. (2004). Mammary ECM composition and function are altered by reproductive state. *Mol. Carcinog.* **41**, 207-220.
Schwertfeger, K. L., Rosen, J. M. and Cohen, D. A. (2006). Mammary gland macrophages: pleiotropic functions in mammary development. *J. Mammary Gland Biol. Neoplasia* **11**, 229-238.
Sica, A., Rubino, L., Mancino, A., Larghi, P., Porta, C., Rimoldi, M., Solinas, G., Locati, M., Allavena, P. and Mantovani, A. (2007). Targeting tumour-associated macrophages. *Expert Opin. Ther. Targets* **11**, 1219-1229.
Stein, T., Morris, J. S., Davies, C. R., Weber-Hall, S. J., Duffy, M. A., Heath, V. J., Bell, A. K., Ferrier, R. K., Sandilands, G. P. and Gusterson, B. A. (2004). Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3. *Breast Cancer Res.* **6**, R75-R91.
Stein, T., Salomonis, N. and Gusterson, B. A. (2007). Mammary gland involution as a multi-step process. *J. Mammary Gland Biol. Neoplasia* **12**, 25-35.
Strange, R., Li, F., Saurer, S., Burkhardt, A. and Friis, R. R. (1992). Apoptotic cell death and tissue remodelling during mouse mammary gland involution. *Development* **115**, 49-58.
Watson, C. J. (2006). Involution: apoptosis and tissue remodelling that convert the mammary gland from milk factory to a quiescent organ. *Breast Cancer Res.* **8**, 203.
Zhao, L., Melenhorst, J. J. and Hennighausen, L. (2002). Loss of interleukin 6 results in delayed mammary gland involution: a possible role for mitogen-activated protein kinase and not signal transducer and activator of transcription 3. *Mol. Endocrinol.* **16**, 2902-2912.