The transcription factor Gata6 links tissue macrophage phenotype and proliferative renewal§

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Abstract

Tissue-resident macrophages are heterogeneous as a consequence of anatomical niche-specific functions. Many populations self-renew independently of bone marrow in the adult, but the molecular mechanisms of this are poorly understood. We determined a transcriptional profile for the major self-renewing population of peritoneal macrophages in mice. These cells specifically expressed the transcription factor Gata6. Selective deficiency of Gata6 in myeloid cells caused substantial alterations in the transcriptome of peritoneal macrophages. Gata6-deficiency also resulted in dysregulated peritoneal macrophage proliferative renewal during homeostasis and in response to inflammation, which was associated with delays in the resolution of inflammation. Our investigations reveal that tissue macrophage phenotype is under discrete tissue-selective transcriptional control and that this is fundamentally linked to the regulation of their proliferation renewal.

Tissue-resident macrophages play fundamental roles specific to their micro-anatomical niche, ranging from dedicated homeostatic functions to immune surveillance (1). Such heterogeneity predicts that discrete transcriptional controls likely exist in specific macrophage populations that determine both their particular phenotypes and tissue-specific functions.

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Many resident macrophages self-renew by local-proliferation ((1) and citations within). This is initiated after seeding of macrophages into tissues during development, and their expansion during the neonatal period (1). Under specific conditions, these tissue-resident macrophages may also be derived from blood monocytes (1). Classic F4/80$^{\text{high}}$CD11b$^{\text{high}}$ peritoneal-resident macrophages fit this model (2-7) and they proliferate above homeostatic levels in response to inflammation (6). Proliferation of human macrophages has also been observed in several contexts (reviewed in (1)). However, the factors controlling these processes remain ill-defined. We hypothesized that discrete transcriptional controls would govern both the specific phenotype of tissue macrophages and their proliferative renewal in a select tissue microenvironment.

We performed a transcriptional analysis of murine monocyte-like cells during acute peritonitis (Fig. S1-3 and Tables S1-3). Our approach analyzed populations specifically enriched in tissue-resident macrophages (6) allowing definition of a tissue macrophage-restricted transcriptional profile, which was associated with homeostatic and metabolic processes (‘Cluster 15’, Fig. S2, Table S3A-C). Gata6 was selectively expressed in peritoneal macrophages when compared to both in vitro-generated bone marrow-derived macrophages and, when isolated during inflammation, contemporary monocyte-derived (8) macrophages (Fig. S1D, F, G). Perhaps best known for its essential requirement in the development of heart, gut and liver (9-11), the role of Gata6 in macrophages is unknown.

We crossed conditional knockout (KO) Gata6$^{\text{tm2.1Sad/J}}$ mice (12) with Lysozyme M (Lyz2) Cre-recombinase ‘knock-in’ mice (‘Lyz2$^{\text{Cre}}$, B6.129P2-Lyz2$^{\text{tm1(cre)Ifo/J}}$) (13) to generate mice with a myeloid deficiency of Gata6 (‘Gata6-KO$^{\text{mye}}$’) (14). Lyz2$^{\text{Cre}}$ mediates recombination in approximately 95% of peritoneal macrophages (13). Flow-cytometric analysis of peritoneal cells from Gata6-KO$^{\text{mye}}$ mice compared to their wild type (WT) littermates indicated a gross change in the characteristic F4/80$^{\text{high}}$CD11b$^{\text{high}}$ phenotype, with the majority (~95%) of classic peritoneal macrophages exhibiting reduced F4/80 and CD11b expression (Fig. 1A). Further analysis of peritoneal myeloid cells (CD11b$^{+}$CD19$^{-}$), indicated that whilst the F4/80$^{\text{low}}$ macrophages exhibited relatively normal expression of Tim4 (a marker expressed by the majority of peritoneal resident macrophages and found in this study to be selectively expressed by these cells during acute peritonitis; Fig. S1 and (6)), there was a reduction in their numbers, and an increase in eosinophils and MHCII$^{\text{high}}$F4/80$^{\text{low}}$ macrophages/dendritic cells (Fig. 1B, C). However, there were no substantive alterations in the numbers of peritoneal lymphocytes (Fig. S4A) or peripheral blood cells (Fig. S4B, C).

We established a panel of lentiviral vectors (Fig. S5A, Table S4) with which we achieved selective high expression of transgenes in peritoneal-resident macrophages in vivo (Fig. S5A, B). Lentiviral delivery of Cre to the peritoneal-resident macrophages of adult Gata6$^{\text{tm2.1Sad/J}}$ mice resulted in alteration of phenotype, including lower F4/80 expression (Fig. S5C). This confirmed that Gata6 was important for phenotype maintenance in the adult, and we also excluded a role for Cre toxicity (15) (Fig. S5C-E).

We assessed the importance of Gata6 as a regulator of the characteristic peritoneal macrophage phenotype by microarray analysis of macrophages from WT and Gata6-KO$^{\text{mye}}$
mice (14) (Fig. 2A, Tables S5A, B). Analysis of peritoneal macrophage-specific transcripts indicated that there was a significant over-representation of probesets that were downregulated in the absence of Gata6 (Fig. 2B). The array data were validated by examination of surface receptors whose mRNA was altered (Fig. 2C-E). An additional study (16) identified genes specific to peritoneal macrophages when compared to other tissues and there was a similar over-representation of genes from this list that were downregulated in the absence of Gata6 (Fig. S6, Table S6). Using both datasets, in addition to Gata6, we identified a gene-list that could be considered peritoneal macrophage-specific by both criteria (within and between tissues), of which 60% of genes were down-regulated in the absence of Gata6 (Fig. S6). This confirmed Gata6 as a major regulator of the peritoneal macrophage phenotype. Peritoneal macrophage-selective transcripts were not the only transcripts that were altered in the absence of Gata6, however, indicating a more broad impact on phenotype (Fig. 2A, B). Consistent with a role in peritoneal phenotype specialization, enforced Gata6 expression in bone marrow-derived macrophages promoted their peritoneal retention and altered their phenotype towards that of peritoneal resident macrophages (Fig. S7). Although we have not addressed this, we would anticipate that Gata6 would also be upregulated in bone marrow-derived cells recruited to the peritoneum when replacing the tissue resident pool. For example, as can occur in irradiation chimeras (17).

Peritoneal macrophages in WT mice are capable of renewal without monocytic input (6, 7), particularly under homeostatic conditions. In accordance with this, genes associated with the regulation of cell proliferation (GO:0042127) were also altered by Gata6-deficiency (Table S5A), including Cdkn2b, Csf1, Igf1, Tgfb2, Tgfbr2, and Bmpr1a. Moreover, Gata6-deficient peritoneal macrophages exhibited increased basal proliferation compared to macrophages in WT mice (Fig. 3A-B). As with WT cells (8), proliferation of Gata6-deficient peritoneal macrophages in vivo is dependent on the cytokine macrophage-colony stimulating factor (M-CSF). We observed marked polyploidy in Gata6-deficient cells (Fig. 3A, C, S8). Polyploid Gata6-deficient cells were multinucleate, and this could reflect failed cytokinesis (18) or the creation of a fusogenic phenotype by the marked alteration in membrane associated molecules (enrichment of GOTERM_CC_FAT GO:0005886, Benjamini P=0.000007). We took advantage of the existence of F4/80high peritoneal macrophages in the mice (Fig. 1A), which we confirmed had escaped Cre-mediated Gata6-deletion, and were phenotypically normal (Fig. S9). Within the Gata6-KOmyc mice, we observed a significantly lower level of proliferation of F4/80high WT cells compared to F4/80low KO macrophages (Fig. 3D), which was comparable to that observed in WT mice. Similar results were obtained by lentiviral mediated Cre-delivery into peritoneal macrophages of adult conditional-KO mice (Fig. 3E). These studies demonstrated a cell-intrinsic role for Gata6 in limiting basal proliferation. Although the mechanisms controlling this response are unclear, it is likely that Gata6 influences proliferation through both direct and indirect impacts on the cellular phenotype of peritoneal macrophages. Given the similarities between tissue-resident peritoneal and pleural macrophages, we examined Gata6 expression and found it comparable at both sites (Fig. 3F). Similar to peritoneal macrophages, the pleural macrophages of Gata6-KOmyc mice were predominantly F4/80low and exhibited a cell-intrinsic increase in proliferation and polyploidy, when compared to the contemporary F4/80high pleural macrophages from the same microenvironment (Fig. 3G, S8D).
During acute inflammation, peritoneal macrophage numbers first decrease. This is followed by M-CSF-dependent (Il4ra-independent) proliferation of surviving macrophages (6, 8). We induced acute peritonitis with 2×10^6 zymosan particles and observed increases in the numbers of neutrophils and eosinophils in both WT and Gata6-KO^{mye} mice (Fig. S10). The initial decrease in the number of Tim4^+ macrophages was seen in both genotypes; however, whereas the Tim4^+ macrophages were mostly restored to pre-inflammation levels in WT mice, this did not occur in the Gata6-KO^{mye} mice 48 hours post-challenge (Fig. 4A). In contrast to the inflammation enhanced proliferative response in WT mice, proliferation of Gata6-KO^{mye} macrophages remained unaltered (Fig. 4B). Since alterations in inflammation or basal conditions between WT and Gata6-KO^{mye} mice could impact on proliferative recovery of the tissue macrophages, we compared the proliferative state of the Tim4^+F4/80^{high} WT macrophages to the Tim4^+F4/80^{low} KO cells within the same Gata6-KO^{mye} mice (Fig. 4C, D). Unlike the KO macrophages, the F4/80^{high} WT cells in the same environment responded to inflammation with elevated proliferation confirming that the role of Gata6 was cell-intrinsic and not a consequence of secondary/environmental factors (Fig. 4C, D). The mechanism underlying these phenotypic alterations is likely to be complex. A bioinformatic analysis indicated a high probability that multiple transcriptional networks were influenced by Gata6-deficiency (Table S7). Thus, the altered phenotype imposed by the loss of Gata6 activity may arise from both direct Gata6 signaling and indirect responses mediated downstream of Gata6. Gata6 can therefore alter both cell proliferation and the phenotypic specialization of macrophages within the resident tissue. As validation of our approach we selected Map3k8, which exhibits significantly-altered expression in the absence of Gata6 (Fig. S11A, B). We anticipated that Map3k8 may be involved in the proliferation of peritoneal macrophages because we had found that this process has an absolute requirement for M-CSF (8). Lentiviral shRNA-mediated Map3k8 knockdown resulted in significantly reduced proliferation during inflammatory resolution (Fig. S11C-E).

Alterations in macrophage phenotype and restoration could impact on inflammatory resolution, for example leading to delayed neutrophil clearance, so we initiated inflammation with a higher dose of zymosan (2×10^7 particles), where the initiation of inflammation is less macrophage-dependent (3, 19-21) (Fig. 4E). Compared to WT animals, Gata6-KO^{mye} mice had substantially lower numbers of recoverable Tim4^+ macrophages, slightly increased levels of Tim4^- macrophages and increased neutrophil numbers during the resolution of inflammation (Fig. 4E).

In summary, we have identified Gata6 as a master controller of peritoneal macrophage-specific phenotype. This phenotype is intrinsically linked to the regulation of proliferation. Our observations demonstrate transcriptional control of tissue-resident macrophage proliferative renewal and have implications for the study of tissue macrophages and tissue physiology in general. They indicate that not only do resident macrophages acquire a specialized phenotype adapted for a specific microenvironment, but that this is integral to the systems that preserve regulated self-renewal. Ex vivo, peritoneal macrophages alter their phenotype (22, 23), including an apparent absence of proliferation (24). We also observed a down regulation of Gata6 in ex vivo cultures, which in itself alludes to the presence of local Gata6 induction within the tissue. Further afield, these observations dictate that to understand the master controllers and interaction of any resident macrophage population...

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within its tissue, a full context-specific characterization of these cells will be required. It can be anticipated that many of the individual downstream pathways used by tissue-resident macrophages to interact with their environment may be common between different sites. The development of viable Gata6-deficient peritoneal macrophages provides an opportunity to dissect the functional interaction between tissue-resident macrophages and their tissue in a highly tractable system to aid in the identification of approaches to promote tissue homeostasis, the resolution of inflammation and host defense.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**References and Notes**

14. Information on materials and methods is available on Science Online.

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Fig. 1. Selective myeloid cell alterations in the peritoneum of mice with myeloid Gata6-deficiency

A. Representative Flow-cytometric and immunofluorescent assessment of peritoneal-resident macrophages from WT and Gata6-KO<sub>mye</sub> mice. F4/80<sup>high</sup> (arrowhead) and F4/80<sup>low</sup> (arrows) macrophages are indicated. Fluorescent images were captured with a 40x objective lens, the scale bar is indicated and the images are representative of 4 mice per group (Fig. S9).

B. Representative flow-cytometric analysis of peritoneal myeloid cell (CD11b<sup>+</sup>CD19<sup>−</sup>) composition of the Gata6-WT and Gata6-KO<sub>mye</sub> mice. Percentages indicate typical proportions of the cell types of all peritoneal cells.

C. Quantification of peritoneal myeloid cells in the Gata6-WT (black bars, n=9♂/7♀) and Gata6-KO<sub>mye</sub> mice (white bars, n=5♂/5♀) analyzed by flow-cytometry in (A) and (B) above. Data represents the mean±SEM of mice pooled from two independent experiments and was analyzed by two-way ANOVA (Int, Interaction statistic; Gata6, Gata6 effects; Sex, sex effects).

Abbreviations used in this figure: MØ, macrophage; Res, tissue-resident; Eos, eosinophil; DC, dendritic cell.
Fig. 2. Gata6 is fundamental to the peritoneal-resident macrophage phenotype

A. Volcano plot showing the differential gene expression between peritoneal macrophages from Gata6-KO<sup>mye</sup> mice and WT. Significantly 2-fold down-regulated (green) and up-regulated (magenta) probesets are indicated. B. Same volcano plot as (A), overlaid (orange) with the 215 peritoneal macrophage-selective ‘cluster 15’ (Cl. 15) probesets see (Fig. S1-2 (14)), which were significantly (below) disproportionately down-regulated in the absence of Gata6. C-E. Representation (C, E) and quantification (D, E) of flow-cytometric validation of the array data from (A). Data (analyzed by t-test) represents the difference in median fluorescent intensity (ΔMFI) between receptor-specific and isotype-control antibodies (mean

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±SEM) of individual mice (n=4) from one of two experiments (solid bars denote WT, and hatched denote Gata6-KO^mye mice).
Fig. 3. Dysregulated peritoneal macrophage proliferation in the absence of Gata6

A-C. Representative density plots (A) gated on resident-peritoneal macrophages (Fig. 1A) showing proliferation (SG2M) and polyploidy, which were quantified (B) and visualized (arrowheads, C), respectively. Data in (A) and (B) is derived from one of two independent experiments (Gata6-KO*mye, n=5; ‘Het’, n=4; WT, n=3), represented as mean±SEM and analyzed by one-way ANOVA (P value as indicated) with Bonferroni post tests. Immune fluorescence is representative of 5 mice.

D. Examination of proliferative differences between the majority F4/80\textsuperscript{low} (○) KO and the WT F4/80\textsuperscript{high} (●) macrophages (see Fig. S9) within the same Gata6-KO*mye mice. Lines denote paired samples from the same mice (n=9), which were pooled from two similar experiments and analyzed by paired t-test. E. The impact of Gata6 deletion on proliferation was examined 7 days after delivery of Cre-expressing lentiviruses to Gata6\textsuperscript{tm2.1Sad/J} mice intraperitoneally. The proportion of cells in the SG2M phases of cell-cycle were compared between F4/80\textsuperscript{low}Cre\textsuperscript{+} (○) and F4/80\textsuperscript{high}Cre\textsuperscript{−} (●) macrophages. Data is represented as 3 independent experiments (Exp) with lines denoting paired samples from the same mice and analyzed as indicated. F. Gata6 mRNA expression compared by qPCR between peritoneal and pleural leukocytes. Data shows mean ±SEM from one of two independent experiments (129S6 mice, n=≥ 3/group) is normalized to reflect the number of resident macrophages. G. Similar analysis to (D) except using pleural macrophages. Data from two similar experiments were pooled and analyzed by a paired t-test. Abbreviations used in this figure: Res, resident; MØ, macrophage; * = P<0.05, ** = P<0.01, *** = P<0.001. 

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Fig.4. Impaired proliferative recovery of peritoneal macrophages during inflammation in the absence of Gata6

A-B. Quantification of the numbers (A) and proliferation (B) of Tim4+ macrophages at the indicated times after intraperitoneal zymosan injection (2x10^6 particles). Data were pooled from two independent experiments with Gata6-WT (black bars) and Gata6-KO mye (white bars) mice (n=5-11/group). C. Comparison of the SG2M and G1 stages of cell cycle in the Gata6-KO (Tim4+ F4/80low, white bars) and WT (Tim4+ F4/80high, black bars) macrophages within the Gata6-KO mye mice from (A-B). D. Verification of the proliferative alterations shown in (C) by in vivo incorporation of 5-ethynyl-2'-deoxyuridine (EdU) (n=8 mice, pooled from 2 independent experiments). E. Analysis of inflammatory parameters in the resolution phase of a higher dose zymosan (2x10^7 zymosan particles) peritonitis model comparing cell counts from WT (black bars) and Gata6-KO mye (white bars) mice (Data pooled from 3 independent experiments (n=11-13/group). All data in this figure represents mean±SEM of individual mice and were examined by ANOVA (as indicated) with pairing as appropriate and Bonferroni post-tests, except (D), which was analyzed by paired t-test. Abbreviations used in this figure: MØ, macrophage; * = P<0.05, ** = P<0.01, *** = P<0.001.