Spatiotemporal dynamics of macrophage heterogeneity and a potential function of Trem2hi macrophages in infarcted hearts

Heart failure (HF) is a frequent consequence of myocardial infarction (MI). Identification of the precise, time-dependent composition of inflammatory cells may provide clues for the establishment of new biomarkers and therapeutic approaches targeting post-MI HF. Here, we investigate the spatiotemporal dynamics of MI-associated immune cells in a mouse model of MI using spatial transcriptomics and single-cell RNA-sequencing (scRNA-seq). We identify twelve major immune cell populations; their proportions dynamically change after MI. Macrophages are the most abundant population at all-time points (>60%), except for day 1 post-MI. Trajectory inference analysis shows upregulation of Trem2 expression in macrophages during the late phase post-MI. In vivo injection of soluble Trem2 leads to significant functional and structural improvements in infarcted hearts. Our data contribute to a better understanding of MI-driven immune responses and further investigation to determine the regulatory factors of the Trem2 signaling pathway will aid the development of novel therapeutic strategies for post-MI HF.

With the technical advances and widespread adoption of cardiac revascularization strategies and guideline-directed medical therapeutic interventions, the age-standardized death rates and heart failure hospitalization rates after acute myocardial infarction (AMI) have been gradually declining. However, the rate of recurrent events, including heart failure-associated hospitalization and death after myocardial infarction (MI) are still very high. Heart failure is a common condition among individuals who survived an MI attack, mainly due to adverse left ventricular remodeling.

The ischemic injury following AMI induces the mobilization and recruitment of a diverse repertoire of innate and adaptive immune cells to the infarcted heart. Of these, macrophages are critical for the clearance of infarcted tissues as well as for wound repair and remodeling processes. Macrophages are broadly divided into M1 and M2 subtypes according to their in vitro construction; M1 macrophages express high levels of pro-inflammatory cytokines and promote a pro-inflammatory milieu, whereas M2 macrophages release anti-inflammatory cytokines and promote angiogenesis and wound healing. The balance between M1 and M2 macrophages during immune response...
responses was defined as essential for effective healing and remodeling processes\(^6\). However, this dichotomous classification was obsolete, as in vivo environment of macrophages are more complex\(^7\). Therefore, a more precise evaluation is needed for accurate characterization of the dynamics of macrophage heterogeneity during the acute period of MI. Single-cell RNA-sequencing (scRNA-seq) allows the investigation of the transcriptional states of heterogeneous cell populations at a high resolution and has already been applied to non-myocyte cardiac cell populations of healthy mice\(^\text{8}\) and immune cell populations of mice hearts after MI\(^\text{9,12}\).

In this study, we perform spatial and scRNA-seq analyses of cardiac immune cells in a mouse MI model to investigate the spatiotemporal dynamics of MI-associated immune cells. We identify a macrophage subset, Trem2\(^\text{20}\) macrophages, with anti-inflammatory characteristics, specifically dominant in the late-stage infarcted heart. Moreover, in vivo injection of soluble Trem2 leads to significant functional and structural improvements in infarcted hearts. Overall, our data may enable the identification of biomarkers and the development of therapeutic strategies for MI.

**Results**

**Immune cell dynamics after MI**

To obtain a comprehensive landscape of immune cells after ischemic injury of the heart, we performed scRNA-seq on flow cytometry-sorted CD45\(^+\) leukocytes isolated from infarct and peri-infarct areas 1, 3, 5, and 7 days after the induction of MI in mice (Fig. 1a). CD45\(^+\) cells in the steady-state (before MI induction) were also used as a control for the Seurat R package\(^\text{13}\).

Uniform manifold approximation and projection (UMAP) plots using post-MI [day 3]: 4446 cells; day 5 post-MI [day 5]: 7271 cells; day 7 post-MI [day 7]: 8316 cells) were integrated, clustered, and visualized in uniform manifold approximation and projection (UMAP) plots using the Seurat R package\(^\text{14}\).

To annotate cell clusters, the SingleR package\(^\text{14}\) was used to assess the expression of well-known cell-type-specific markers; a total of 12 broad cell clusters were defined within CD45\(^+\) cells (Fig. 1b). The most abundant cell population was macrophages (64.2% of total CD45\(^+\) cells); they highly expressed prototypical macrophage genes such as Cd14, Cdx6, and Adgre1 (Fig. 1c and Supplementary Data 1). Non-macrophage cell populations included three clusters of dendritic cells (DC: CD209\(^+\) DC, CD209a\(^+\) and Flt3 enriched; Xcr1 DC, Xcr1l and Itgae enriched; and migratory DC, Fscn1, and Cacnb3 enriched), monocytes (enriched genes: Ly6c2, Chil3, and Ace), neutrophils (enriched genes: S100a8, S100a9, and Retnlg), B cells (enriched genes: Ms4a1, Cd79a, and Ly6d), T cells (enriched genes: Cd3e, Cd3d, and Lef1), group 2 innate lymphoid cells (ILC2; enriched genes: Rora, Ccrr6, and Gat3a), natural killer (NK) cells (enriched genes: Nkg7, Klrblc, and Gzma), plasma cells (enriched genes: Igk1l1 and Mzb1), and mast cells (enriched genes: Cma1 and Kit) (Fig. 1c and Supplementary Data 1).

In the steady-state, macrophages represented the largest cell population (58.7% of total CD45\(^+\) cells) in the mouse hearts, followed by B cells (18.2%), NK cells (8.6%), T cells (4.3%), monocytes (3.7%), and neutrophils (3.1%) (Fig. 1d and Supplementary Table 1); the other cardiac leukocyte populations occupied less than 3% in the steady state. Interestingly, the proportion of macrophages drastically dropped at day 1 (24.9%) but then gradually recovered from day 3 (66.8%), reaching a peak on day 7 (84.0%) post-MI (Fig. 1d). On the contrary, the proportion of neutrophils increased steeply at day 1 (54.4%) and then decreased rapidly (14.8, 1.7, and 1.6% on days 3, 5, and 7, respectively), which was consistent with previous reports\(^\text{15}\). Monocytes were increased in the early phase of MI (days 1 and 3) and then decreased in the late phase of MI (days 5 and 7), while DCs were predominant at days 3 and 5. Lymphocytes (T cells, B cells, NK cells, and ILC2 cells), occupying 31.9% of total CD45\(^+\) cells in the steady state, decreased at day 1 and did not reach the steady-state values until day 7 post-MI. Collectively, scRNA-seq targeting cardiac leukocytes showed not only major cell populations but also minor populations, such as mast cells and DC subtypes; our data clearly show that the temporal dynamics after MI are different depending on the cell populations.

**Spatiotemporal profiles of immune cells after MI**

To validate the above landscape of immune cells infiltrated into the infarcted area, we performed spatial transcriptome sequencing (ST-seq) using frozen samples from days 1, 3, 5, and 7 post-MI (Fig. 2a). Principal component analysis and unsupervised clustering identified four or five cell clusters per sample based on the differentially expressed genes (Fig. 2b). We then applied SPOTlight\(^\text{16}\), a nonnegative matrix factorization (NMF)-based spatial deconvolution framework, to infer the cell-type composition of each spot (Fig. 2b). Notably, neutrophils highly infiltrated into the infarcted area on day 1 (41.2% on average) but their numbers were then decreased rapidly (18.7, 10.6, and 9.5% on days 3, 5, and 7, respectively), which was consistent with the scRNA-seq data (Figs. 2b and 2c). On the contrary, macrophages were dispersed across the whole heart rather than clustered in the infarcted area on day 1 (Fig. 2b). However, from day 3, macrophages infiltrated into the infarcted area, and their abundance peaked at late MI (days 5 and 7: 18.8% on average) (Fig. 2c). Monocytes and fibroblasts also infiltrated the infarcted area of the heart in a time-dependent manner (Supplementary Figs. 1–5). Other immune cell populations, such as B-cell and T-cell populations, were always dispersed across the entire mouse heart (not clustered in the infarcted area), with a very low abundance (Supplementary Figs. 2–5). Together with the scRNA-seq showing immune cell temporal dynamics after MI, ST-seq analysis with deconvolution algorithm further provided their spatial heterogeneity and spatiotemporal dynamics; our ST-seq data clearly showed that monocytes and neutrophils infiltrated into the infarcted area at early MI while macrophages and fibroblasts acted oppositely.

The heterogeneity of monocytes and macrophages and their temporal dynamics after MI

scRNA-seq showed that macrophages represent the largest population of immune cells in the heart. For further insight regarding the dynamics of macrophage subtypes after MI, we performed a sub-clustering analysis in the context of scRNA-seq data, obtaining 16 sub-clusters (Fig. 3a). Detailed information of marker genes for each sub-cluster is provided in Supplementary Data 2. Three macrophage sub-clusters (clusters 1, 3, and 13) were predominant in the steady state (77.9% of the total monocyte/macrophages), thus we named them steady-state macrophage (SS-M) clusters (Fig. 3b). In the SS-Mp1 cluster (cluster 3), elevated expression of genes such as Lyve1, Flx1a, Cbr2, Cd163, Fol2, and Timd4 was observed (Supplementary Data 2 and Supplementary Figs. 6, 7); importantly, the increased expression of Lyve1 and Flx1a was previously reported in tissue-resident macrophages\(^\text{21}\) and recent single-cell studies also suggested that the upregulation of Fol2, Timd4, and Cbr2 is a marker of resident macrophages\(^\text{22}\). The SS-Mp2 cluster (cluster 1) showed relatively higher expression of antigen-presentation-related genes, including H2-Eb1, H2-Aa, H2-Ab1, and Cd74, resembling the tissue-resident Mhc-II macrophage cluster suggested by Dick et al\(^\text{12}\). Additionally, the marker genes of both SS-Mp1 and SS-Mp2 were detected in the SS-Mp3 cluster (cluster 13), indicating that these cells represent an intermediate state between those in the SS-Mp1 and SS-Mp2 clusters. Expectedly, Ccr2, a marker of monocyte-derived macrophages, was not expressed in the SS-Mp1, whereas it was partially expressed in the SS-Mp2 (Supplementary Fig. 7). Interestingly, the proportions of the SS-Mp clusters dropped drastically after the induction of MI and were then gradually restored from day 3 (SS-Mp2 and SS-Mp3) or day 5 (SS-Mp1) after MI (Fig. 3b). However, their proportion did not reach that in the steady-state until day 7 post-MI.
Interestingly, in the early phase of MI (days 1 and 3), monocytes and two macrophage clusters were predominant (clusters 4, 10, and 14), occupying 59.7% of the total monocyte/macrophage cells at day 1 (termed Early-Mφ clusters). Since cluster 14 showed the upregulation of Chit3, Plac8, Ccr2, and Ly6c2, it was defined as classical Ly6c2hi monocytes (Supplementary Data 2 and Supplementary Fig. 6); Ly6c2hi monocytes peaked one day post-MI and then quickly decreased (Fig. 3b). Additionally, Early-Mφ1 (cluster 10) and Early-Mφ2 (cluster 4) expressed not only Cdh8, Fcgr1, and Itgam (canonical macrophage markers) but also Ccr2, suggesting that these populations were composed of monocyte-derived macrophages (Supplementary Fig. 6). Like monocytes, these two Early-Mφ populations peaked at day 1 and then...
expression than cluster 6, indicating that this cell population may be in phase (cluster 11) and G2/M phase (cluster 9) populations (Fig.3a, b and Supplementary Fig. 9). Overall, the sub-clustering analysis clarified that Hmox1 may have central functions in attenuation of the inflammation at day 3, peaking 7 days post-MI (Fig. 3b).

We also identified three transient macrophage populations (peaking 3 or 5 days after MI); two macrophage populations enriched in interferon-stimulated genes, and two proliferating populations, albeit their proportions were low (Fig. 3b). Saa3, Fnd1, and Lct4 genes were enriched in transient-Mφ (cluster 8) cells, Fabp5, Spil, and Gpnmb genes were enriched in Transient-Mφ2 (cluster 7) cells, and Hmoxl, Prdx1, and Gclm genes were enriched in Transient-Mφ3 (cluster 12) cells. The significantly enriched genes in Transient-Mφ populations were similar to those in Late-Mφ cells; however, the expression levels of Trem2 were relatively lower in Transient-Mφ cells (Supplementary Figs. 6, 7). Additionally, many genes associated with interferon signatures such as Il1f7, Ilg15, and Ifi32 were enriched in clusters 6 and 15, which were therefore defined as IFN-Mφ (Fig. 3a, b). The proportions of the IFN-Mφ clusters did not significantly change over time after MI (Fig. 3b). Cluster 15 showed a higher level of Ccr2 expression than cluster 6, indicating that this cell population may be in a resting state after MI (Fig. 3c). However, Transient-Mφ1 and IFN-Mφ populations diverged at a relatively early pseudo-time, and Transient-Mφ1 was not linked to Late-Mφ. The selected genes that are specifically expressed in each cell population were then plotted to track changes across pseudo-times (Fig. 3d). The expression levels of Early-Mφ specific genes, such as Ccr2, Chil3, and Clec4e, were upregulated in Ly6c2hi monocytes/Early-Mφ and then gradually decreased in Transient-Mφ, reaching a low level in Late-Mφ cells. On the contrary, Late-Mφ-specific genes, such as Trem2, Rgs10, and Fcrls, were downregulated in Ly6c2hi monocytes/Early-Mφ and then gradually upregulated in Transient-Mφ and Late-Mφ cells (Fig. 3d). Trem2 was specifically upregulated in Late-Mφ cells, while Fdrl2, Ly6c1, and Mgl2 expressions were very low. Importantly, when we checked the expression of candidate genes and the proportion of macrophage subsets in injured heart tissues using scRNA-seq, marker genes and proportions for each subset were enriched in the infarcted area of the heart in a time-dependent manner, while SS-Mφ1 cells were dispersed in the heart at all time points after MI (Fig. 3e and Supplementary Figs. 10, 11).

Neutrophil and DC subpopulations

Neutrophils, previously reported as the first immune cells recruited into the infarcted area, were also the largest cell population one day post-MI (Fig. 1d). Using the 5,135 neutrophils (steady-state: 204 cells; day 1: 4009 cells; day 3: 660 cells; day 5: 126 cells; day 7: 136 cells), we further identified five distinct neutrophil sub-clusters (Fig. 4a). When we calculated the proportion of each cluster in relation to the total neutrophil counts, cluster 1 (enriched genes: Fpr1, S100a9, and Lcn2) and cluster 3 (enriched genes: Ifi32, Pglypr, and Sild) were predominant in the steady state (31.9 and 63.7%, respectively; Fig. 4b). However, the proportion of cluster 3 notably decreased early after MI (day 1: 11.9%) and then gradually increased to 36.0% at day 7, while cluster 1 did not show any evident trend. Conversely, the proportion of cluster 2 (enriched genes: Icam1, Tnf, and Siglec5) was rarely present in the steady state (0.5%) but notably increased at day 1 (21.9%) and peaked at day 3 (46.8%) post-MI. This is consistent with a previous report suggesting that neutrophils in the infarcted heart are characterized by the acquisition of a Siglec5 signature. Cluster 4 was enriched in genes associated with interferon signatures such as Ifi32, Rd1a2, and Ifg15, and cluster 5 was characterized by the upregulation of endoplasmic reticulum heat-shock proteins such as Hspa5, Dna1b9, Manf, and Hcytl (Fig. 4a). The gene expression levels of typical cluster-specific genes are provided in Fig. 4c and Supplementary Data 3. Notably, proportions of both clusters 4 and 5 peaked one day after MI.

Regarding DCs, six distinct sub-clusters were identified as per the sub-clustering analysis (steady-state: 160 cells; day 1: 309 cells; day 3: 349 cells; day 5: 568 cells; day 7: 535 cells; Fig. 4d). Three of them were predominant before MI (clusters 2, 3, and 4; 93.8% of the total DCs) but their proportions gradually decreased to 44.5% 5 days post-MI (Fig. 4e). In cluster 2, genes such as Tgfb1 and Ccr2 were upregulated, while cluster 3 showed a relatively higher expression of DC-SIGN (Cd209a) and MHC II molecules (H2-DMa and H2-Aa) (Fig. 4d). Additionally, cluster 4 showed a distinct transcriptional state with a high expression of Xcr1 and Clec9a, suggesting that this population is composed of cDC1. On the other hand, macrophage (Ms4a7 and Fcrls) and migratory DC (Fscn1 and Ccr7) signatures were detected in clusters 1 and 5.

Single-cell trajectories in the infarcted heart

To better understand the process of monocyte/macrophage infiltration into the infarcted area, we performed a trajectory analysis using the Monocle R package. Ly6c2hi monocytes share several properties with human CD16 CD14 monocytes, and it is well known that they enter the circulation, contribute to excessive monocytes, preferentially accumulate in lesions, and differentiate into macrophages after MI. When Ly6c2hi monocytes were set as the root of the trajectory, the pseudo-time of cells increased in the order of Early-Mφ, Transient-Mφ, and Late-Mφ, which were ranked in a similar temporal manner (Fig. 3c). However, Transient-Mφ1 and IFN-Mφ populations were inversely correlated between Ly6c2hi monocytes, and Early-Mφ cells were dispersed in the heart at all time points after MI (Fig. 3e). On the other hand, macrophage (Ms4a7 and Fcrls) and migratory DC (Fscn1 and Ccr7) signatures were detected in clusters 1 and 5.
respectively (Fig. 4d); their abundance was relatively higher in the late phase than that in the early phase of MI (Fig. 4e). Finally, cluster 6 was characterized by the upregulation of plasmacytoid DC marker genes such as *Siglech* and *Bst2* (Fig. 4d). The gene expression levels of typical cluster-specific genes are provided in Fig. 4f and Supplementary Data 4.

The expression of Trem2 after MI

Previous studies showed the increased expression of *Trem2* in mouse hearts during atherosclerosis progression and regression. In this study, immunohistochemistry also showed a gradual increase in the expression of Trem2 over time in the infarcted area after the induction of MI, which was almost absent in the steady state (Fig. 5a). Consistent
with scRNA-seq, ST-seq, and immunohistochemistry results, western blotting showed that the expression of full-length Trem2 (~32 kDa) steadily increased, peaking 5 days post-MI and decreasing thereafter (Fig. 5b). Interestingly, the expression of the soluble form of Trem2 (sTrem2, ~18 kDa) increased after day 3 and peaked on day 7. The expression of total Trem2 (full-length Trem2 and sTrem2) peaked on day 5 (Fig. 5b). Therefore, we decided to investigate the main source of Trem2 in the heart; to investigate whether Trem2hi macrophages are the major cell population responsible for the Trem2 expression pattern after MI, we performed a co-localization assay. Importantly, CD68hi macrophages and Trem2hi macrophages, as major subsets in the early and late phases of MI, were then collected from the infarcted heart (Fig. 5e, f). Additionally, the mean size of infarcted hearts in vivo. sTrem2 promotes the functional and structural improvements of remodeled LV with thicker infarcted walls and lower end-systolic volume (ESV) than the mice treated with PBS or GH alone (Fig. 6c, d, Supplementary Table 2 and Movies 1–3). Histologically, the mice treated with sTrem2-GH showed less dilated and well-remodeled LV with thicker infarcted walls and lower fibrosis (Fig. 6b and Supplementary Fig. 16). The infarct size was also significantly smaller in sTrem2-GH-treated MI mice (26.0%) than that in PBS- and GH-treated mice (Fig. 6d). Importantly, based on the mechanical strength of GH, the crosslinking densities of GH alone and sTrem2-GH were fixed at 1.5 kPa. In vitro, the sTrem2 release profile test from sTrem2-GH showed that 70% of sTrem2 was released out of sTrem2-GH at day 1 and approximately 90% was cumulatively released over 7 days at a slow pace (Supplementary Fig. 15). Importantly, 28 days post-MI, echocardiography showed that the mice treated with sTrem2-GH had significantly higher left ventricular ejection fraction (LVEF), fractional shortening (FS), and left ventricular ejection fraction (LVEF), fractional shortening (FS), and lower end-systolic volume (ESV) than the mice treated with PBS or GH alone (Fig. 6c, d, Supplementary Table 2 and Movies 1–3). Histologically, the mice treated with sTrem2-GH showed less dilated and well-remodeled LV with thicker infarcted walls and lower fibrosis (Fig. 6b and Supplementary Fig. 16). The infarct size was also significantly smaller in sTrem2-GH-treated MI mice (26.0%) than that in PBS- (48.4%), and GH-treated (38.0%) mice (Fig. 6e) and an increased survival rate was observed in the mice treated with sTrem2-GH compared with other groups (Fig. 6f). Altogether, these results indicate that sTrem2 promotes the functional and structural improvements of infarcted hearts in vivo.

**Discussion**

In this study, we attempted to identify the heterogeneity and spatio-temporal dynamics of leukocytes at a single-cell level in the mouse heart after the induction of MI using longitudinal scRNA-seq and ST-seq. Our data support four major conclusions: First, leukocytes are heterogeneous in the heart tissues with respect to not only inter-cell (12 broad cell populations) but also intra-cell (16 monocytes/macrophage subsets, 5 neutrophil subsets, and 6 DC subsets) populations; second, leukocyte populations after the initiation of MI are remarkably dynamic, changing depending on the cell type or cell subpopulations; third, the single-cell trajectory analysis supports a sequential differentiation from Ly6C+ monocytes to Late-Mp rather than the obsolete dichotomous MI-M2 paradigm; fourth, in the late phase of MI, the number of Trem2hi macrophages abundantly expressing anti-inflammatory signature genes is significantly increased in the

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infarcted heart tissues and the soluble form of Trem2, the expression of which increased after the peak of full-length Trem2 expression, significantly improves remodeling and cardiac function in the infarcted heart. Altogether, our results demonstrate that the cellular components of the immune system in the mouse heart are heterogeneous and have plasticity in response to ischemic injury. These findings can provide a rich resource to explore the molecular targets and underlying mechanisms of tissue repair after MI and thus may guide the development of novel therapeutic strategies aimed at enhancing myocardial repair and regeneration.

Macrophages are composed of several heterogeneous subpopulations and recent investigations adopting single-cell
Fig. 3 | Monocyte/macrophage cell subsets. a The UMAP visualization of the 21,533 cardiac monocytes/macrophages identified 16 subsets (left panel). Each point represents a single cell, colored according to the sub-cluster assigned. In the right panel, the heatmap shows the top 10 most differentially expressed genes in each sub-cluster. Blue and red indicate lower and higher expression, respectively. The typical markers strongly and specifically associated with each sub-cluster are shown on the left. b The proportion of each sub-cluster among total monocytes/macrophages according to the time-point after MI. c Pseudo-time trajectory as per the pseudo-time algorithm. Pseudo-time analysis is an approach used to investigate the path and progress of individual cells undergoing differentiation. Ly6c2φ monocytes were set as the root of the trajectory. The scale indicates the temporal status, from dark blue (recruited Ly6c2φ monocytes) to yellow (Late macrophages). d Spline plots showing the expression of typical markers associated with each cluster across the pseudo-time (Ly6c2φ monocytes: 632 cells; Early-Mφ: 2838 cells; Transient-Mφ: 3416 cells; IFN-Mφ: 1856 cells; Late-Mφ: 4380 cells). e Gene expression levels of Clec9a, Hmox1, Trem2, and Ly6c2φ per ST-seq. Blue and red indicate lower and higher expression, respectively. The scale bar is marked on the H&E stained section in Fig. 2b. Results are representative of four different samples. Day 1, day 1 post-MI; Day 3, day 3 post-MI; Day 5, day 5 post-MI; Day 7, day 7 post-MI. Source data for Figs. 3b and 3d are provided in the Source Data file.
Fig. 4 | Neutrophil and dendritic cell subsets. a The UMAP visualization of 5135 neutrophils identified five distinct subsets (left panel). Each point represents a single cell, colored according to the sub-cluster assigned. In the right panel, the heatmap shows the top 10 most differentially expressed genes in each sub-cluster. Blue and red indicate lower and higher expression, respectively. Typical markers strongly and specifically associated with each sub-cluster are shown on the left. b Bar plot showing the proportions of cells in each of the 5 sub-clusters according to the time-point after MI. c Violin plots of the expression of Fpr1, Tnf1, Ifitm2, Ig15, and Manf in neutrophil sub-clusters (Cluster 1: 1864 cells; Cluster 2: 1243 cells; Cluster 3: 844 cells; Cluster 4: 698 cells; Cluster 5: 486 cells). d The UMAP visualization of 1921 dendritic cells identified 6 distinct subsets (left panel). Each point represents a single cell, colored according to the sub-cluster assigned. In the right panel, the heatmap shows the top 10 most differentially expressed genes in each sub-cluster. Blue and red indicate lower and higher expression, respectively. Typical markers strongly and specifically associated with each sub-cluster are shown on the left. e Bar plot showing the proportions of cells in each of the 6 sub-clusters according to the time-point after MI. f Violin plots of the expression of Ms4a7, Ccr2, Cd209a, Xcr1, Fscn1, and Siglech in DC sub-clusters. (Cluster 1: 541 cells; Cluster 2: 507 cells; Cluster 3: 377 cells; Cluster 4: 264 cells; Cluster 5: 184 cells; Cluster 6: 48 cells). Regarding the box plots of c and f, the lower whisker, lower hinge, box center, upper hinge, and upper whisker represent the minimum, lower quartile, median, upper quartile, and maximum calculated without outlier values which are more than 1.5× interquartile range of the lower and upper quartiles. Source data for b, c and e, f are provided in the Source Data file.
in the infarcted area, while SS-Mφ cells were dispersed in the heart at all time points after MI. Considering that tissue-resident macrophages can prevent fibrosis, promote electrical conduction, and facilitate the healing of injured areas\cite{11,12}, SS-Mφ might contribute to modulating the immune responses in a way different from the other macrophage subsets. Although less prominent, fibroblasts and DCs, which are known to be involved not only in tissue homeostasis but also in repair and regeneration of the heart\cite{13}, were relatively abundant in the infarcted area, suggesting that they may also be involved in infarcted tissue repair.

In conclusion, we present a comprehensive single-cell and spatial transcriptomic landscape of infarcted hearts following MI. We explore...
the spatiotemporal dynamics of macrophage heterogeneity and the potential function of Trem2hi macrophages in cardiac tissue repair. Our data make important contributions to the current understanding of MI-driven immune responses, and further investigations and discovering the regulatory factors of the Trem2 signaling pathway will help establish novel therapeutic strategies for post-MI HF.

Methods

Ethical considerations

All animal experiment procedures were conducted in compliance with the approval of the Institutional Animal Care and Use Committee (IACUC) at the Catholic University School of Medicine (CUMC-2018-0033-07).

Animal models

We used male wild-type 7–8 week-old C57BL/6 mice purchased from Orient Bio (Gyeonggido, Korea). Mice were housed in a specific pathogen-free facility maintained on a 12 h light-dark cycle at 20–26 °C and 50 ± 10% humidity. Mice weighting 20–22 g were anesthetized with an intraperitoneal injection of a mixture of Zoletil (30 mg/kg; Zoletil 50, Virbac, France) and xylazine (10 mg/kg; Rompun, Bayer HealthCare, Leverkusen, Germany). Mechanical ventilation was carried out using a Harvard Apparatus ventilator and supplemental oxygen to maintain general anesthesia. Mice were intubated with BD Angiocath plus 22GA and placed in an operating table. After a left-sided thoracotomy, experimental MI was induced by permanent ligating the proximal portion of left anterior descending artery (LAD). The protein C57BL/6 male

| Day 0 | Day 14 | Day 28 |
|-------|-------|-------|
| LAD ligation | Injection: PBS, GH, sTrem2-GH | Echocardiography Histological examination |

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of soluble Trem2 (sTrem2, mouse trem2 aa 12–171 His N-term) was purchased from LifeSpan BioSciences (LSBio, Seattle, WA, USA). To evaluate the efficacy of sTREM2, mice were injected with PBS, gel, or gel containing 12 μg sTrem2 into the myocardium at the infarct border zone after the LAD ligation. Each treatment was injected at two sites of the peri-infarcted area (10 μL per site), right after MI. Mice were initially anesthetized with 5% isoflurane and then anesthetized with 1% isoflurane during the echocardiography procedure to maintain the heart rate. The ejection fraction (EF) and fractional shortening (FS) were calculated from M-mode echocardiography procedure to maintain the heart rate. The ejection fraction (EF) and fractional shortening (FS) were calculated from M-mode echocardiography procedure to maintain the heart rate.

**Cell staining and flow cytometry**

For flow cytometric analysis, heart tissues were digested with collagenase type II solution (Worthington Biochemical Corporation, USA) at a concentration of 500 unit/ml for 40 min at 37 °C and homogenized in gentleMACS Dissociator (Miltenyi Biotech, USA). After homogenization, the tissues were passed through a 40 μm cell strainer, washed with HBSS Buffer, and resuspended in FACS staining buffer. For FACS analysis, the following antibodies were used: FITC-anti-mouse CD45 antibody (1 μg/mL; BD; #553080), BV421-anti-mouse Gr1 (1 μg/mL; Biologend; #100605), APC-anti-human/mouse Trem2 (1 μg/mL; R&D system; #FAB17291A), and PE-Cy7 anti-human/mouse Arg1 (1 μg/mL; Invitrogen; #25-3697-S2). CD45 positive cells were sorted by flow cytometry sorter (Beckman Coulter, USA). After staining each sample, FACS analysis was performed using the FlowJo software (TreeStar Inc., USA).

**Single-cell RNA sequencing library construction**

The single-cell library preparation relied on a commercially available droplet method using the 10x Genomic Chromium System (10x Genomic Inc., San Francisco, CA) and Single Cell 3' v3 Reagent Kit (10x Genomics Inc.) according to the manufacturer’s protocol. In brief, dissociated cells were counted by hemocytometer (ThermoFisher) and 16,000 cells per sample were added to each channel. The cells were then partitioned into gel beads in emulsion (GEMs) in the Chromium instrument, where cell lysis and barcoded reverse transcription of RNA occurred. cDNA was synthesized and amplified for 14 cycles. cDNA clean-up was performed using a SpeSelect Reagent Kit (Beckman Coulter, Brea, CA). 50 ng of the amplified cDNA were used for each sample to construct indexed sequencing libraries. Sequencing libraries were sequenced on an Illumina HiSeq2500 platform. This resulted in an average read depth of 93,445 reads/cell (steady-state: 77,078 reads/cell; day 1 post-MI: 87,068 reads/cell; day 3 post-MI: 143,667 reads/cell; day 5 post-MI: 84,228 reads/cell; day 7 post-MI: 75,185 reads/cell).

**Single-cell RNA sequencing data analysis**

The sequenced data were processed into expression matrices with the Cell Ranger Single Cell software suite v3.0.1 by 10x Genomics. Raw base-call files from HiSeq2500 sequencer were demultiplexed into library-specific FASTQ files using Cell Ranger mkfastq option. Sequencing reads were mapped to the mm10 version 3.0.0 reference, downloaded from the 10x Genomics (https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/latest). Subsequently, cell barcodes and unique molecular identifiers underwent filtering and correction. Reads associated with the retained barcodes were quantified using Cell Ranger count option and used to build a transcript count table.

Bioinformatics processing of the scRNA-sequencing data was performed with the R package Seurat (version 3.2.0). To exclude low-quality cells in scRNA-sequencing, we filtered cells with an expressed gene count fewer than 2% or greater than 98%. Additionally, cells in which more than 10% of reads corresponded to mitochondrial genes were removed. Data was log-normalized and highly variable features were identified based on a variance stabilizing transformation (VST) method. All datasets (steady-state, day 1, 3, 5, and 7) were then integrated using the canonical correlation analysis (CCA) method, “FindIntegrationAnchors” and “IntegrateData” functions in Seurat. Principal components analysis (PCA) was performed on the integrated datasets. Based on the top 40 principal components (PCs), graph-based clustering was performed using the shared nearest-neighbor (SNN) modularity optimization with resolution set to 1.4, and all cells were classified into 34 clusters. Clustering data was then applied followed by uniform manifold approximation and projection (UMAP) allowing the visualization of identified clusters in UMAP plots. Each cell cluster was annotated for their cell type using the SingleR R package (version 1.2.4) and/or assessment of well-known cell-type specific markers. Isolated clusters of cells that expressed the endothelial markers (Pecam1 and Kdr) or fibroblast markers (Col1a1 and Col1a2) were removed from the further analysis as it was likely due to cellular contamination during FACS. Cell cycle analysis was performed by using the “CellCycleScoring” function in Seurat. Differentially expressed gene (DEG) analysis was used to identify significantly differentially expressed genes within each cluster using the logistic regression test for significance and an average log fold-change. Only genes with a positive average log fold-change value (greater than 0.25) and an adjusted P value lower than 0.05 were kept in the analysis. Raw sequencing data generated for scRNA-sequencing have been deposited in Gene Expression Omnibus (GEO) under accession number GSE163129.

Pseudotime trajectories of differentiation were generated using the Monocle3 R package (version 0.2.3). Pseudotime analysis proceeds on the basis that cells undergo biological processes in an asynchronous manner, and thus that cells can be ordered along a calculated trajectory to infer the transcriptional changes throughout the process. Ly6c2+ monocyte was set as a root of the trajectory, and genes that were most differentially expressed in identified clusters were used to assign pseudotime values to individual cells.

**Spatial transcriptome sequencing (ST-seq)**

Frozen samples from day 1, 3, 5, and 7 post-MI were embedded in OCT (TissueTek), and cryosectioned into 10 μm slices at −10°C. Sections were then placed on chilled Visium Spatial Gene Expression slide (10x Genomics), and adhered by warming the back of the slide. Sections were then fixed in chilled methanol and stained according to the manufactures guideline. Tissues were permeabilized for 30 (Day 1), 6 (day 3), 18 (Day 5), and 12 min (Day 7), which was selected as the optimal time based on Visium Tissue Optimization experiments. For tissue optimization experiments, fluorescent images were taken with a TRITC filter using a confocal microscopy LSM800 (Carl Zeiss, Oberkochen, Germany). Brightfield histology images were taken using a 10x objective on an Olympus BXS1 microscope with Olyvia software (Olympus, Tokyo, Japan). CDNA libraries were generated using Visium Spatial Gene Expression slide & Reagent Kit according to the manufacturer’s instruction (10X Genomics), and sequenced on a NovaSeq 6000 system (Illumina). This resulted in an average read depth of 90,805 reads/spot (day 1 post-MI: 88,083 reads/ spot; day 3 post-MI: 94,479 reads/spot; day 5 post-MI: 105,089 reads/ spot; day 7 post-MI: 75,568 reads/ spot).

Low FASTQ files and Hematoxylin and Eosin (H&E) stained images were processed using Space Ranger software v1.0.0 (10X Genomic). For mapping of the sequencing data, the mm10 version 3.0.0 mus musculus reference genome was used. Raw sequencing data generated for ST-seq data have been deposited in Gene Expression Omnibus (GEO) under accession number GSE165857. Bioinformatics processing of the ST-seq data was performed with the R package Seurat (version 3.2.0). In brief, normalization (“SCTransform” function), dimensionality reduction (“RunPCA” function), graph-based clustering (“FindNeighbors”) and
“FindClusters” functions with 15 PCs, UMAP visualization and DEGs analysis was conducted with default parameters. To infer the cell-type composition of each spot, we applied SPOTlight (version 0.1.7) with default parameters. In brief, clusters of the scRNA-seq data were used to train the SPOTlight. Gene set was the union between the marker genes of the cell types along with the top 3,000 variable genes. All markers were used to initialize the model basis, and unit variance normalization was carried out. Cell types contributing <3% to the spot’s composition were considered fitting noise and were set as 0.

Signature scores were calculated by taking the mean of the scaled and centered expression value across multiple signature genes using the “AddModuleScore” function in Seurat. Cell type signature scores were generated using the following marker genes for each cell type: cardiomyocytes (Myh6, Myh7, Acn2, Nkx2-5, Tnni3, Tnt2), endothelial (Cdh5, Ly6c1, and Kdr) and fibroblast (Col1a1, Pdgfra, and Laml1).

Histological and immunohistochemistry analysis

The excised heart tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue blocks were cut into 4 μm serial sections for histological analysis. The sections were stained with hematoxylin and eosin (H&E) and Masson’s trichrome (MT) according to the manufacturer’s protocol, and myocardial fibrosis and the infarct size were determined by using a digital pathology scanner (Aperio AT Turbo, Leica Biosystems, Germany). For immune-histochemical analysis, after blocking in 10% normal horse serum (Vector Laboratories, USA), the sections were incubated overnight at 4 °C with anti-TREM2 (1:200; Abcam, UK; #ab175525) diluted in PBS. The DAB was used to visualize according to the manufacturer’s specifications (DAB chromogen, GBI Labs, USA).

Co-localization assay

To confirm Trem2 and macrophage Co-localization, the sections performed heat-induced antigen retrieval with Tris/ EDTA buffer pH 9.0 (Abcam). After blocking in 10% normal horse serum (Vector Laboratories, USA), the sections were incubated overnight at 4 °C with anti-CDC68 (Abcam; #ab53444) and anti-TREM2 antibody diluted in PBS (1:200). On the second day, secondary antibodies, Alexa 488 anti-rat antibody (1:500; Invitrogen; #A11006) or Alexa 594 anti-Goat antibody (1:200). On the second day, secondary antibodies, Alexa 488 anti-rat antibody (1:500; Invitrogen; #A11006) or Alexa 594 anti-Goat antibody (1:500; Invitrogen; #A32758), were treated for 1 h at room temperature. Then, DAPI staining was performed. Mounting solution used the Aqua-mount solution (DAKO). The stained sections were examined under a confocal microscope (LSM700, ZEISS, Germany).

Western blotting assay

Total protein was extracted from mouse infarct tissue in lysis buffer (RIPA buffer containing a proteinase inhibitor cocktail). Fifty micrograms of total protein were electrophoretically separated on a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane using a Trans-Blot Turbo transfer system (Bio-Rad). The membrane was blocked in TBST buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.02% Tween 20) containing 5% skim milk and then incubated with anti-TREM2 and anti-alpha tubulin (Abcam; #ab125267) antibodies, diluted 1:1000 in 5% skim milk, respectively. The membrane was then incubated with horseradish peroxidase-conjugated anti-mouse (1:5000, Cell Signaling Technology; #7076S) and anti-rabbit secondary antibodies (1:5000; Cell Signaling Technology; #7074S). Specific protein bands were visualized with an enhanced chemiluminescence system (Amersham, Heights, IL, USA) and quantified using ImageJ software.

Expression of mRNA

Total RNA from Ccr2+ or Trem2+ sorted cells was extracted using RNeasy mini kit (Qiagen, Valencia, USA) according to the manufacturer’s protocol. Total RNA was reverse transcribed using reverse transcriptase (Roche, USA). cDNA samples were subjected to real-time quantitative RT-PCR (qRT-PCR) analyses with specific primers for Il1b, Il10, Alox15, Cx3cr1, Cxcr3, Cxcr7, Ccl2, Tgfβ1, and Spp1 (Supplementary Table 3) using a Bio-Rad CFX96 Real-Time PCR detection system (BIO-RAD Laboratories, Inc., Hercules, CA, USA). The Gapdh was amplified as an internal control. Expression level was calculated by ΔΔCt method, and fold changes were obtained using the formula 2−ΔΔCt. All samples were run in triplicate.

Reporting summary

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

Data availability

The scRNA-seq and ST-seq data sets generated in this study have been deposited in the Gene Expression Omnibus under accession numbers GSE163129 and GSE165857, respectively. Sequencing reads were mapped to the mm10 version 3.0.0 reference, downloaded from the 10x Genomics (https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/latest). Source data are provided with this paper.

Code availability

All codes used in this manuscript are based on 10X Genomics and public library packages that are listed in the “Methods” section. Relevant codes used for data analysis are available from https://github.com/Junglab-CMC/Macrophage-heterogeneity-after-MI.

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