



Published in final edited form as:

J Mol Cell Cardiol. 2021 August ; 157: 98–103. doi:10.1016/j.yjmcc.2021.05.003.

B cells modulate the expression of MHC-II on cardiac CCR2⁻ macrophages

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Abstract

The uninjured murine heart contains a heterogeneous population of macrophages with disparate ontogenies and functions. These macrophages are often associated with blood vessels and can be subclassified based on the expression of CC chemokine receptor 2 (CCR2) and major histocompatibility complex class II (MHC-II). The biological cues that modulate these macrophage pool subpopulations have not been completely identified. It has been recently shown that a sub-population of circulating naïve B cells adheres to the myocardial microvasculature. We hypothesized that B cells might modulate the phenotype of myocardial macrophages. To test this hypothesis, we analyzed both the relative location of B cells and macrophages in myocardial histological section and the prevalence of myocardial macrophage subsets in hearts from B cell-deficient mice (μ MT) and mice depleted of B cells through administration of an anti-CD20 antibody. We found that B cells pause in the microvasculature in proximity of macrophages and modulate the number of myocardial CCR2⁻MHC-II^{high} cells. Through *in vitro* studies we found that this is likely the result of a paracrine effect of B cells on the expression of MHC-II in CCR2⁻ cells. These results reveal an unexpected relationship between B cells and resident macrophages

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Contribution to the Field Statement

The naïve myocardium hosts a heterogeneous pool of resident macrophages with disparate ontogeny and function. The biological cues that regulate the composition of the myocardial macrophage pool in the naïve heart remain mostly unexplored. Studying both B cell-deficient and B cell-depleted animals, we found that B cells modulate the composition of the myocardial macrophage pool in the naïve murine heart. We performed histological analysis and *in vitro* studies that support the notion that intravascular myocardial B cells directly modulate the phenotype of myocardial resident macrophages.

Our observations highlight a novel level of complexity in cardiac immunology and indicate that the currently held belief that naïve B cells merely recirculate between primary and secondary lymphoid organs awaiting to encounter their cognate antigen might need revision. Moreover, to the best of our knowledge, our work is the first to describe a cross-talk between B cells and macrophages outside of inflammatory responses.

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Disclosures

LA is cofounder of i-Cordis LLC, which is focused on the development of B cell-modulating therapies for the treatment of heart failure, and coinventor on patent WO2019028062, "Pirfenidone derivatives for modulation of B lymphocyte activity and organ protection," owned by Washington University. CRR and FP have no conflict of interest to disclose.

and, highlighting a direct intramyocardial effect of circulating B cells, challenge the currently held belief that naïve recirculating B lymphocytes merely shuttle between lymphoid stations.

Keywords

Tissue-associated B cells; resident macrophages; naïve B cells

Introduction

Current models of B cell biology posit that naïve B cells merely recirculate between primary and secondary lymphoid stations until meeting their cognate antigen [1, 2]. However, recent studies have highlighted that: 1) the naïve myocardium contains a sizeable population of naïve circulating B cells that pause on the myocardial microvascular endothelium; 2) B cell-deficient and B cell-depleted mice have alterations in myocardial mass, myocardial contractility and number of myocardial T cells [3, 4]. These studies pointed to an unexpected biological relationship between B cells and the heart but did not investigate whether B cells exhibit any local effects in the myocardium [3]. The demonstration of a local B cell effect within the myocardium would broaden our understanding of the relationship between circulating immune cells and the myocardium and challenge current paradigms of B cell biology.

The murine myocardium contains four distinct subsets of CD64⁺ macrophages defined by different expression levels of CC chemokine receptor 2 (CCR2) and major histocompatibility complex class II (MHC-II) [5]. The vast majority of macrophages in the naïve heart are CCR2⁻ resident macrophages (MHC-II^{high} or MHC-II^{low}) of embryonic origin. CCR2⁺ cells are bone marrow-derived monocytic cells that acquire higher levels of MHC-II expression as they fully differentiate into tissue macrophages [5–7]. These macrophage subsets have several important roles in the context of myocardial physiology and pathology [6, 8–11]. Recently, a population of intra parenchymal myocardial macrophages has been identified in close association with the vasculature [8]. The factors that modulate the myocardial macrophage pool composition in the naïve heart have not been completely understood.

Here, we used histology, flow cytometric analysis of myocardial macrophages from B cell-deficient mice and B cell-depleted mice, and primary cardiac macrophage cultures to investigate the hypothesis that B cells modulate the phenotype of myocardial macrophages through a paracrine effect.

Materials and Methods

Mice

To investigate the effects of B cell deficiency, we used 4-5 week-old male and female wild-type (WT) (background C57BL/6J; Stock No. 000664) and μ MT mice (B cell deficient) (background C57BL/6J; B6.129S2-*Ighm*^{tm1Cgn}/J; Stock No. 002288) age and sex matched. These strains were purchased from The Jackson Laboratory and bred in house. The CD19 reporter model was generated as previously reported [3]. Mice were bred and maintained at

the Washington University School of Medicine Animal Care Facility. Experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Washington University School of Medicine.

Antibody-mediated B cell depletion

Wild-type (WT) mice were injected once intraperitoneally with anti-CD20 antibody (BioLegend; Clone: SA271G2; Cat.#152104; 1 mg/mL). Mice were injected between 21 and 28 days of age (100 μ L/mouse). Littermate control mice were injected with isotype control antibody (BioLegend; Clone: RTK4530; Cat# 400644). All mice were analyzed 7 days post-injection. B cell depletion was assessed by flow cytometry (Sup. Figure 1).

Flow cytometry

Cardiac tissue was digested and stained as described previously [5, 12]. Murine samples were stained with the following fluorescently conjugated antibodies (BioLegend; dilution 0.5 μ L/100 μ L): CD45 (30-F11, PerCP-Cy5); CD19 (1D3/CD19, APC); Ly6G (1A8, FITC); CD11b (M1/70, BV605); CCR2 (SA203G11, BV421); I-A/I-E (MHC-II) (M5/144.15.2) and CD64 (X54-5/7.1, PE). Flow cytometric analysis was performed on a BD LSR Fortessa X-20. Macrophages were gated as CD45⁺CD11b⁺Ly6G⁻CD64⁺CCR2^{+/-}MHC-II^{high/low} (Sup. Figure 2).

Cell isolation

Heart cell suspensions and splenocytes were prepared as previously described [12, 13]. Cardiac macrophages were gated as CD45⁺Aqua⁻Ly6G⁻CD64⁺, splenic neutrophils were gated as CD45⁺Aqua⁻Ly6G⁺ and splenic B cells were gated as CD45⁺Aqua⁻CD19⁺. Antibody dilutions and specifications are described in “Flow cytometry” section. Cell viability was assessed by Zombie Aqua staining (BioLegend; Cat#423102; dilution 1:100). Cells were isolated through FACS sorting on a BD FACS Aria-III (nozzle 100 μ m).

For culture of primary macrophages in B cell conditioned media experiment, discrimination of live vs dead cells was achieved using Vibrant DyeCycle Violet Ready Flow dye (ThermoFisher) to mark viable cells and Propidium Iodide to stain dead cells. CD45⁺Aqua⁻Ly6G⁻CD64⁺ cells and CD45⁺CD19⁺ cells were isolated with a MoFlow sorter.

Macrophage/ B cell culture or co-culture

Cardiac CD45⁺Aqua⁻Ly6G⁻CD64⁺ cells from μ MT mice and splenic CD45⁺Aqua⁻CD19⁺ cells from WT mice were cultured in 96-well plates (5000 cells/100 μ L) for 40 hours at 37°C as described previously [12]. Splenic neutrophils CD45⁺Aqua⁻Ly6G⁺ were used as a negative control. Cultured cells were stained for CD45, CD64, CCR2, MHC-II and CD19 or Ly6G (antibody specifications are shown in “Flow cytometry” section; dilution 0.2 μ L/100 μ L). Flow cytometric analysis was performed on a BD LSR Fortessa X-20 (gating strategy is shown in Sup. Figure 3).

Cardiac CD45⁺PI⁻DyeCycle⁺Ly6G⁻CD64⁺ cells from μ MT or WT mice were culture in 96 well plates at about 7000 cells/well. 14 million splenic B cells were sorted and plated in 4 mL of media in 2 C6 wells. After over-night incubation, primary macrophages were rinsed

in HBSS. 700 μ L of media were collected from the B cell cultures (without disturbing B cells on the bottom) and 125 μ L/well of B cell condition media or control media were added to the macrophages. After 10 hours, additional 700 μ L of media was collected from the B cell cultures and additional 125 μ L of B cell conditioned media or culture media were added to the macrophages. After additional 12 hours, flow cytometric analysis was performed on a BD LSR Fortessa X-20 (gating strategy is shown in Sup. Figure 3).

BrdU incorporation

Cells were plated as described in “Cell Culture” section and then allowed to rest overnight. Cell replication was determined by the rate of BrdU incorporation (APC BrdU Flow Kit, Cat#552598, BD Pharmingen). BrdU was added to each well at 10 μ M the morning after plating. BrdU incorporation was assessed by flow cytometry 30-36 hours after BrdU administration.

Immunofluorescence

CD19-reporter mouse hearts were fixed and processed as previously described [3, 13]. Heart sections were stained with the primary antibodies anti-CD68 (Bio-Rad; Cat#MCA1957T; 1:400) and anti-CD31 (R&D Systems; Cat#AF3628; 1:15), overnight at 4°C, followed by incubation with donkey anti-rat Alexa Fluor 488 (ThermoFisher; Cat#A-21208; 1:200) and NL637 (R&D Systems; Cat#NL002; NorthernLights anti-goat IgG-NL637; 1:200) for 1–4 h at room temperature. DAPI was used to stain the nucleus. Fluorescence was visualized using a Zeiss LSM 880 Confocal microscope at the Washington University Center for Cellular Imaging (WUCCI).

Statistical analyses

All results are presented as mean \pm SEM. Statistical comparisons between two groups were performed using two-tailed Student's *t*-test, correcting for multiple comparisons with the Holm-Sidak method. Outliers defined as experimental points two standard deviations away from the mean were excluded. All data were analyzed with Graph-Pad Prism Version 8.

Results and Discussion

We began investigating the relationship between B cells and myocardial macrophages by immunostaining cryosections of naïve murine hearts and analyzing the relative positions of B cells and CD68⁺ macrophages. We found that myocardial-associated B cells were almost exclusively intravascular and myocardial macrophages were frequently perivascular (Figure 1A and Sup. Movies 1–2). Intravascular myocardial associated B cells and perivascular myocardial macrophages were typically in close proximity (Figure 1A and Sup. Movies 1–2). We did not observe any contact between B cells and macrophages. The intravascular location of myocardial B cells and the perivascular location of resident macrophages are consistent with existing literature [3, 8, 14]. However, the proximity between intravascular B cells and perivascular resident macrophages was a novel and unexpected observation that supported the possibility of a cross-talk between macrophages and B cells in the naïve heart.

Next, we analyzed CCR2 and MHC-II expression on cardiac CD64⁺ macrophages in B cell-deficient and B cell-depleted mice (see full gating strategy in Sup. Figure 2). We compared wild-type mice (WT, C57BL/6J) to syngeneic, age- and sex-matched B cell-deficient mice (μ MT, C57BL/6J). The hearts of B cell-deficient mice displayed a significant decrease in CCR2⁻MHC-II^{high} macrophages compared to WT hearts (Figure 1B). CCR2⁻MHC-II^{high} macrophages are resident cardiac macrophages [5, 7]. To confirm these results, we depleted B cells in WT mice by administering an anti-CD20 antibody (Sup. Figure 1). Consistent with the findings using the μ MT mouse model, antibody-mediated B cell depletion reduced the number of myocardial CCR2⁻MHC-II^{high} macrophages (Figure 1C). These combined results indicated that B cells modulate the myocardial macrophage pool in the naïve murine heart by modulating the number of resident myocardial CCR2⁻MHC-II^{high} cells.

We hypothesized that B cells might affect the myocardial macrophage pool directly. To test this hypothesis, we established an *in vitro* macrophage/B cell coculture system. We isolated primary cardiac CD64⁺ macrophages from μ MT mice and cultured them in the presence or absence of splenic CD19⁺ B cells from WT mice. Primary CD64⁺ macrophages are almost exclusively CCR2⁻ cells (Sup. Figure 3). 40 hours of co-culture with B cells resulted in a higher prevalence of CCR2⁻MHC-II^{high} macrophages (Figure 2A). As a negative control, we co-cultured cardiac CD64⁺ macrophages with splenic neutrophils, which did not change the prevalence of CCR2⁻MHC-II^{high} macrophages (Sup. Figure 4). These *in vitro* results supported our *in vivo* data, and suggested that B cells directly modulate resident myocardial macrophage subpopulations.

We performed proliferation studies via BrdU incorporation to investigate whether the B cell-mediated increase in CCR2⁻MHC-II^{high} macrophages observed in our *in vitro* system was secondary to proliferation of CCR2⁻MHC-II^{high} macrophages or maturation of CCR2⁻MHC-II^{low} cells. Figure 2B shows expression of MHC-II and BrdU incorporation in myocardial macrophages cultured in the presence or absence of B cells. We did not observe any BrdU incorporation in cultured primary myocardial macrophages (Figure 2C), suggesting that the B cell mediated increase in CCR2⁻MHC-II^{high} macrophages observed is likely the result of B cell mediated upregulation of MHC-II on CCR2⁻MHC-II^{low} cells. This is consistent with the notion that the pool of CCR2⁻MHC-II^{high} macrophages in the heart largely results from the maturation of CCR2⁻MHC-II^{low} cells into CCR2⁻MHC-II^{high} macrophages [7].

Since secreted factors can mediate local communication between cells [15], even across endothelial monolayers [16], we hypothesized that B cells might affect the myocardial macrophage pool through a paracrine effect. To test this hypothesis, we cultured myocardial CD64⁺ macrophages in the presence of B cell conditioned media or regular media (Figure 2D–E). Additionally, to investigate whether there was a difference in the response of WT or μ MT macrophages to B cells, we tested the effect of B cell conditioned media in both WT and μ MT derived heart macs. Figures 2D–E show that exposure to B cell conditioned media was associated with an increase in the percentage of CCR2⁻MHC-II^{high} macrophages. B cell conditioned media induced an increase in the percentage of CCR2⁻MHC-II^{high} macrophages of approximately 23% in WT macrophages and 14% in μ MT macrophages. These data support the notion that B cells modulate the phenotype of myocardial macrophages through

secreted factors. In these in vitro experiments, we observed that the proportion of MHC-II^{high} cultured macrophages was higher in macrophages isolated from uMT hearts than WT hearts. This difference could be explained by batch to batch variations or by macrophage plasticity differences in vivo versus in vitro.

In summary, our in vivo findings indicate that B cells modulate the number of myocardial CCR2⁻MHC-II^{high} resident macrophages. The findings of our histological analysis together with our in vitro studies suggest that this is likely the result of a direct paracrine effect of B cells on MHC-II expression in myocardial CCR2⁻ resident cells. Since the vast majority of B cells in the naïve murine heart are intravascular [3], this effect is most likely mediated by intravascular B cells. However, we cannot exclude a contribution of the few intraparenchymal B cells present in the naïve murine heart or a communication between cardiac macrophages and extracardiac B cells.

Our data unveils a previously unappreciated crosstalk between unstimulated B cells and tissue-resident macrophages. Moreover, since myocardial associated B cells are naïve circulating B cells that pause within the myocardial microvasculature [3], our findings challenge the widely believed assumption that naïve B cells merely recirculate between lymphoid stations until meeting their cognate antigen. Further work will be needed to understand the functional implications of our observations and to explore the mechanistic basis of the effect of B cells on CCR2⁻ macrophages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by NIH grant 1K08HL145108-01A1 and institutional funds from the Washington University in St. Louis Division of Cardiology. The authors acknowledge Dr. Doug Mann for critical discussions of this work. The authors acknowledge Dr. Erica Lantelme, Dr. Hao Zhang, the Washington University Flow Cytometry & Fluorescence Activated Cell Sorting Core and the Bloomberg School of Public Health Cell Sorting Facility for assistance with FACS analysis and cell sorting, as well as Dr. Dennis Oakley and the Washington University Center for Cellular Imaging (WUCCI) for assistance with confocal imaging. The authors acknowledge Servier Medical Art by Servier, licensed under a Creative Commons Attribution 3.0 Unported License.

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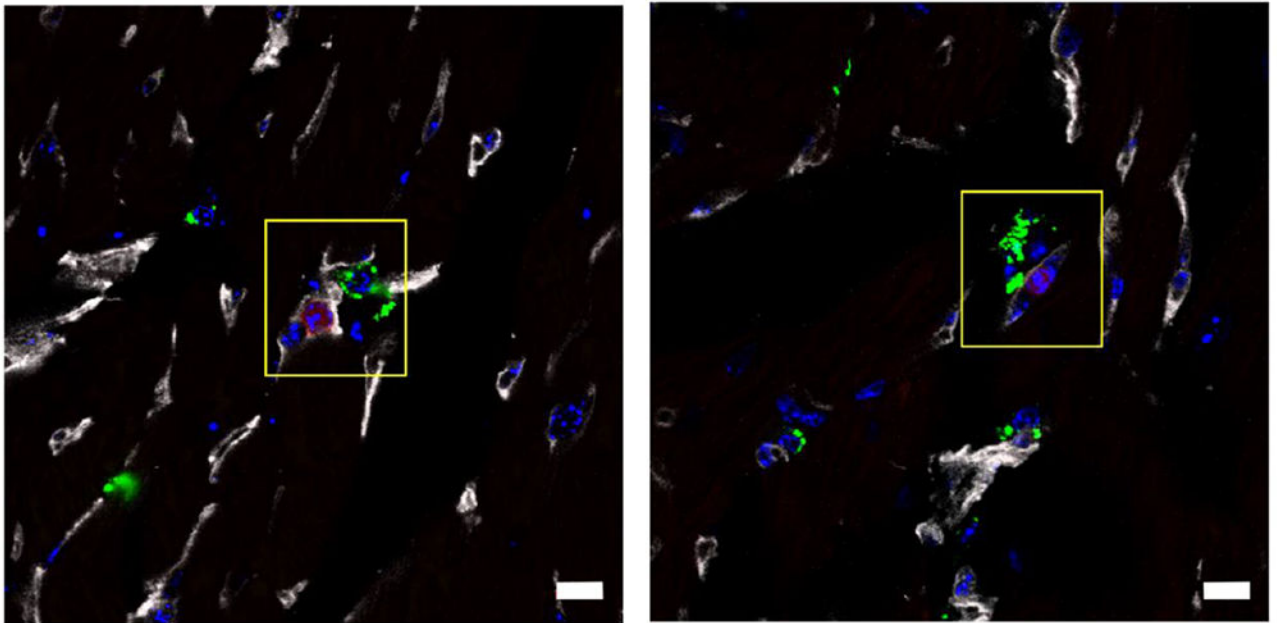
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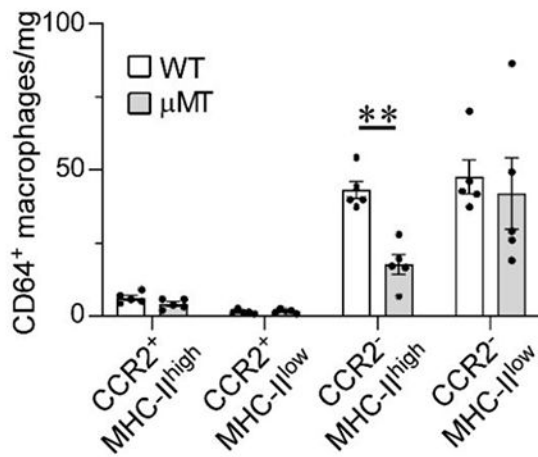
Highlights

- Intravascular myocardial associated B cells are found in proximity to cardiac macrophages.
- B cell depletion leads to a reduction in the number of CCR2⁻MHC-II^{high} resident macrophages in the heart.
- B cell modulate MHC-II expression on cardiac macrophages through a paracrine effect.

A



B



C

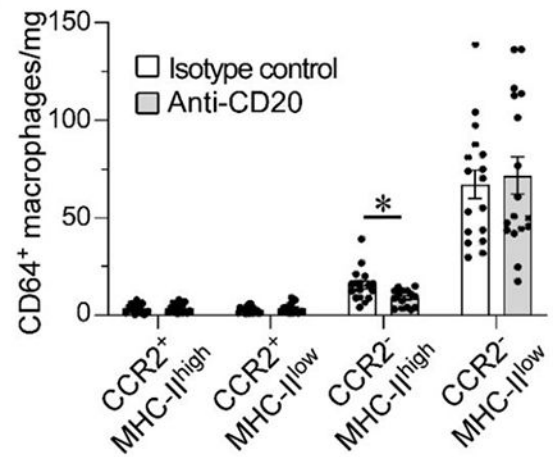


Figure 1. B cells are in proximity of parenchymal macrophages and they modulate the resident myocardial macrophage pool composition in the naïve heart.

(A) Confocal images of immunostained myocardial sections from a CD19-tdTomato reporter mouse. CD19⁺ B cells (red) are intravascular (CD31, gray); CD68⁺ macrophages (green) are intraparenchymal and perivascular in the naïve heart. CD68⁺ macrophages are closely associated to the microvasculature and in proximity to intracapillary B cells (yellow box). Blue stain, DAPI. Scale bar=10 μm. Data collected from 3 hearts (2 slices per heart). (B) Flow cytometric analysis of the cardiac macrophage pool as defined by CCR2 and MHC-II expression in wild-type (WT) and B cell-deficient (μMT) mice. Hearts from B cell-deficient mice display significantly lower numbers of CCR2⁻MHC-II^{high} macrophages than WT

hearts. 5 hearts per condition. (C) Flow cytometric analysis of cardiac macrophage subpopulations in littermate mice treated with anti-CD20 B cell-depleting antibody or isotype control. B cell depletion was associated with reduced numbers of CCR2⁻MHC-II^{high} macrophages. Isotype control: 17 hearts; Anti-CD20: 17 hearts, except for CCR2⁻MHC-II^{hi} (15 hearts) and CCR2⁺MHC-II^{low} (16 hearts). Outliers defined as experimental points two standard deviations away from the mean were excluded. Statistical comparisons between two groups were performed using two-tailed Student's *t*-test, correcting for multiple comparisons with the Holm-Sidak method. Bars represent mean ± SEM. **p*<0.05, ***p*<0.01 (B-C).

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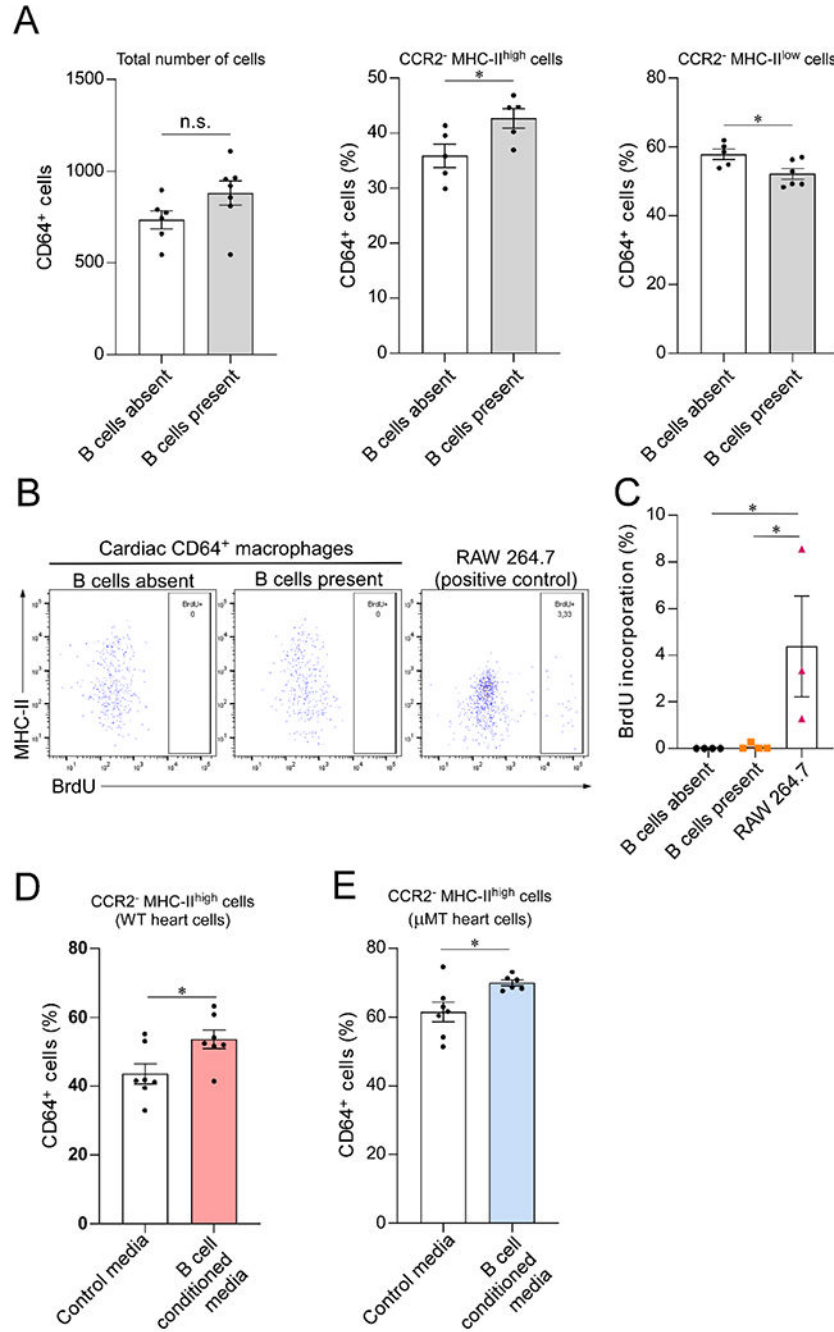


Figure 2. B cells directly modulate the MHC-II expression on CCR2⁻ myocardial macrophages. (A) Cardiac CD64⁺ macrophages isolated from μMT hearts cultured alone or in the presence of WT CD19⁺ splenic B cells. Coculture with B cells increased the prevalence of CCR2⁻MHC-II^{high} macrophages. *Left*, total number of CD64⁺ macrophages analyzed by flow cytometry after 40 hours in culture (n= 6 and 7 samples/condition; p=0.1). *Middle*, CCR2⁻MHC-II^{high} macrophages in relation to the total number of cells (n= 5 samples/condition). *Right*, CCR2⁻ MHC-II^{low} macrophages in relation to the total number of cells (n= 5 and 6 samples/condition). (B) Representative flow cytometry plots representing the

expression of MHC-II and BrdU incorporation by cardiac CD64⁺ macrophages cocultured with B cells (n= 4 samples/condition). RAW 264.7 macrophage-derived cells were used as a positive control for BrdU incorporation (n=3 samples/condition). **(C)** Bar graph showing that BrdU incorporation by cultured primary cardiac CD64⁺ macrophages is close to zero and is not affected by the presence of B cells. **(D-E)** Exposure to B cell conditioned media increased the percentage of CCR2⁻MHC-II^{high} cells in primary cardiac macrophages isolated from both WT (n = 7 samples per condition) and μ MT animals (n = 7 and 6 samples/condition), suggesting that the effect of B cells is, at least in part, mediated by a secreted molecules. Outliers defined as experimental points two standard deviations away from the mean were excluded. Statistical comparisons between two groups were performed using two-tailed Student's *t*-test **(A, D and E)** or One-Way ANOVA followed by Tukey's test for multiple comparison **(C)**. Bars represent mean \pm SEM. n.s. = not significant; **p*<0.05.