

# C-X-C Motif Chemokine Receptor 4 Blockade Promotes Tissue Repair After Myocardial Infarction by Enhancing Regulatory T Cell Mobilization and Immune-Regulatory Function

**BACKGROUND:** Acute myocardial infarction (MI) elicits an inflammatory response that drives tissue repair and adverse cardiac remodeling. Inflammatory cell trafficking after MI is controlled by C-X-C motif chemokine ligand 12 (CXCL12) and its receptor, C-X-C motif chemokine receptor 4 (CXCR4). CXCR4 antagonists mobilize inflammatory cells and promote infarct repair, but the cellular mechanisms are unclear.

**METHODS:** We investigated the therapeutic potential and mode of action of the peptidic macrocycle CXCR4 antagonist POL5551 in mice with reperfused MI. We applied cell depletion and adoptive transfer strategies using lymphocyte-deficient *Rag1* knockout mice; DEREg mice, which express a diphtheria toxin receptor–enhanced green fluorescent protein fusion protein under the control of the promoter/enhancer region of the regulatory T ( $T_{reg}$ ) cell–restricted Foxp3 transcription factor; and dendritic cell-depleted CD11c-Cre iDTR mice. Translational potential was explored in a porcine model of reperfused MI using serial contrast-enhanced magnetic resonance imaging.

**RESULTS:** Intraperitoneal POL5551 injections in wild-type mice (8 mg/kg at 2, 4, 6, and 8 days) enhanced angiogenesis in the infarct border zone, reduced scar size, and attenuated left ventricular remodeling and contractile dysfunction at 28 days. Treatment effects were absent in splenectomized wild-type mice, *Rag1* knockout mice, and  $T_{reg}$  cell–depleted DEREg mice. Conversely, treatment effects could be transferred into infarcted splenectomized wild-type mice by transplanting splenic  $T_{reg}$  cells from POL5551-treated infarcted DEREg mice. Instructive cues provided by infarct-primed dendritic cells were required for POL5551 treatment effects. POL5551 injections mobilized  $T_{reg}$  cells into the peripheral blood, followed by enhanced  $T_{reg}$  cell accumulation in the infarcted region. Neutrophils, monocytes, and lymphocytes displayed similar mobilization kinetics, but their cardiac recruitment was not affected. POL5551, however, attenuated inflammatory gene expression in monocytes and macrophages in the infarcted region via  $T_{reg}$  cells. Intravenous infusion of the clinical-stage POL5551 analogue POL6326 (3 mg/kg at 4, 6, 8, and 10 days) decreased infarct volume and improved left ventricular ejection fraction in pigs.

**CONCLUSIONS:** These data confirm CXCR4 blockade as a promising treatment strategy after MI. We identify dendritic cell–primed splenic  $T_{reg}$  cells as the central arbiters of these therapeutic effects and thereby delineate a pharmacological strategy to promote infarct repair by augmenting  $T_{reg}$  cell function in vivo.

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## Clinical Perspective

### What Is New?

- Inflammatory cell trafficking between hematopoietic organs and sites of tissue injury is controlled by C-X-C motif chemokine ligand 12 (CXCL12) and its receptor, CXCR4. We show that bolus injections of a highly selective peptidic macrocycle CXCR4 antagonist enhance tissue repair and functional recovery after reperfused acute myocardial infarction in mice.
- The therapeutic effects require dendritic cell priming and are specifically mediated by regulatory T cells. Intermittent CXCR4 blockade mobilizes regulatory T cells from their splenic reservoir, leading to their enhanced recruitment to the infarcted region and regulatory T cell–dependent attenuation of inflammatory gene expression in monocytes and macrophages.
- Highlighting the translational potential of this strategy, CXCR4 blockade also reduces infarct volume and improves systolic function in a porcine closed-chest model of reperfused acute myocardial infarction.

### What Are the Clinical Implications?

- This study outlines a new pharmacological approach to augment regulatory T cell function and tissue repair after acute myocardial infarction.
- These findings should stimulate further research into the therapeutic potential of CXCR4 blockade after myocardial infarction and in other acute conditions wherein excessive innate or adaptive immune responses cause immunopathology.

**A**cute myocardial infarction (MI) is caused by thrombotic occlusion of a coronary artery, which leads to progressive cell death in the nonperfused territory. Improved reperfusion strategies have enhanced myocardial salvage in most patients with acute MI,<sup>1</sup> but patients with extensive myocardial injury remain at risk of developing chronic heart failure.<sup>2</sup> MI triggers an inflammatory response that leads to the necrotic area being replaced with highly vascularized granulation tissue and eventually a collagen-rich scar. The heart can undergo deleterious changes in left ventricular (LV) geometry and function during this vulnerable period before scar formation has stabilized the infarct area. The inflammatory response must therefore be tightly regulated to promote healing and prevent postinfarction heart failure.<sup>3,4</sup>

Inflammatory cell trafficking from hematopoietic organs to sites of tissue injury is coordinated by chemokine-chemokine receptor networks.<sup>5</sup> Several chemokines produced in the infarcted myocardium provide exit cues for circulating leukocytes expressing their cognate receptors.<sup>6–8</sup> Therapeutically modulating chemokine-chemokine receptor interactions may promote infarct healing by limiting excessive inflammation-induced tissue damage or by enhancing the recruitment of angiogenic cell populations to the infarct wound.<sup>9–11</sup> Pharmacological blockade of the C-X-C motif chemokine receptor 4 (CXCR4) with the small-molecule antagonists plerixafor (AMD3100) and burixafor (TG-0054) has been shown to improve heart function after MI in mice and pigs, respectively.<sup>10–12</sup> Plerixafor is clinically used to mobilize CXCR4-expressing hematopoietic stem and progenitor cells from the bone marrow to the peripheral blood for collection and subsequent transplantation in patients with hematologic malignancies.<sup>13</sup> Under steady-state conditions, hematopoietic stem and progenitor cells are retained in the bone marrow by interacting with the CXCR4 ligand, C-X-C motif chemokine ligand 12 (CXCL12), which is constitutively produced by bone marrow stromal cells.<sup>14</sup> After MI, CXCL12 expression increases in the infarct area and serves as a homing signal for circulating stem and progenitor cells and proangiogenic cell populations referred to as endothelial progenitor cells.<sup>15–18</sup> Along these lines, the beneficial effects of plerixafor after MI have been associated with enhanced mobilization and cardiac recruitment of endothelial progenitor cells and increased infarct vascularization.<sup>10,11</sup> CXCR4, however, is widely expressed in the hematopoietic system,<sup>19</sup> and mobilization of hematopoietic stem and progenitor cells by CXCR4 antagonists is accompanied by marked leukocytosis that affects all hematopoietic lineages in patients and mice.<sup>20</sup> Notably, neither hematopoietic stem and progenitor cells, endothelial progenitor cells, nor any specific myeloid or lymphoid cell population has been causally linked to the beneficial effects of CXCR4 blockade after MI.

POL5551 and its close analogue and clinical-stage compound POL6326 are potent, selective, and reversible CXCR4 antagonists that have been developed by protein epitope mimetics technology.<sup>21–23</sup> Both compounds are peptidic macrocycles that mimic the  $\beta$ -hairpin structure of the CXCR4-binding motif of CXCL12. Here, we investigated the therapeutic potential and mode of action of POL5551 in a mouse model of reperfused acute MI. We show that POL5551 promotes tissue repair and functional adaptation after MI via Foxp3+ regulatory T (T<sub>reg</sub>) cells. Supporting the translational potential of this strategy, we find that POL6326 enhances systolic function in a porcine closed-chest model of reperfused acute MI. Our observations define a specific mode of action of CXCR4 blockade after MI and outline a new pharmacological approach to enhance T<sub>reg</sub> cell function and tissue repair after acute MI.

**METHODS**

On reasonable request, the data, analytic methods, and study materials will be made available to other researchers for the purposes of reproducing the results. Extended

methods are provided in the Methods section in the [online-only Data Supplement](#).

## Reagents

The CXCR4 antagonists POL5551 and POL6326 were provided by Polyphor.<sup>21,22</sup> Mice were treated with POL5551 (8 mg/kg per dose) or POL6326 (8 mg/kg per dose) and pigs with POL6326 (3 mg/kg per dose). For continuous dosing in mice, an osmotic minipump (Alzet, model 1007D) was implanted in a subcutaneous interscapular pocket. Pumps were filled with vehicle only or POL5551 to deliver the compound at a dose of 8 mg/kg/d for up to 7 days.

## Mice

C57BL/6NCrl wild-type (WT) mice were purchased from Charles River. DEREg (depletion of regulatory T cell) mice express a simian diphtheria toxin (DT) receptor–enhanced green fluorescent protein (eGFP) fusion protein under the control of the endogenous forkhead box P3 (Foxp3) promoter/enhancer region on a bacterial artificial chromosome transgene. The transcription factor Foxp3 is highly restricted to T<sub>reg</sub> cells and confers immunosuppressive functions to naïve T cells.<sup>24,25</sup> In DEREg mice, DT receptor–eGFP expression is observed in fully functional CD4<sup>+</sup> Foxp3<sup>+</sup> T<sub>reg</sub> cells, thereby allowing their DT-induced ablation or fluorescent detection. To deplete Foxp3<sup>+</sup> T<sub>reg</sub> cells, mice were intraperitoneally injected with DT (Merck, catalog number #322326, 25 ng/g body weight) immediately before and 24 hours after MI.<sup>26</sup> DT-treated WT littermates served as controls. Recombination activating gene 1 (*Rag1*) deficient (knockout) mice (which do not produce mature T and B cells), CD11c-Cre mice (which express Cre recombinase under the control of the CD11c promoter/enhancer region), iDTR mice (in which Cre-mediated excision of a STOP cassette renders cells sensitive to DT), and Tie2-GFP mice (which express green fluorescent protein under the control of the murine endothelium-specific Tie2 promoter) were purchased from the Jackson Laboratory. To deplete CD11c<sup>+</sup> dendritic cells (DCs), CD11c-Cre iDTR mice were intraperitoneally injected with DT (50 ng/g body weight) 24 hours before and immediately before MI.<sup>27</sup>

## Mouse Model of Reperfused Acute MI

All procedures in mice were approved by the authorities in Hannover, Germany (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit). MI was induced in 8- to 10-week-old male mice by transient left anterior descending coronary artery ligation. In sham-operated control mice, the ligature around the left anterior descending coronary artery was not tied. LV end-diastolic area (LVEDA) and LV end-systolic area (LVESA) were recorded by high-resolution 2-dimensional transthoracic echocardiography. Fractional area change (%) was calculated as [(LVEDA–LVESA)/LVEDA]×100. LV pressure-volume loops were recorded with a micromanometer-tipped conductance catheter.

## Splenectomy

Mice were injected with butorphanol 2 mg/kg SC and anesthetized with 2% isoflurane via face mask. The abdominal cavity was entered through a small incision under the left rib

cage, splenic vessels were cauterized, and the spleen was removed. In control mice, the abdomen was opened, but the spleen was left in situ (laparotomy only).

## Adoptive Splenic Mononuclear Cell Transfer

Spleens were aseptically removed from donor mice and placed in DMEM (Thermo Fisher Scientific). Splenocyte suspensions were prepared by carefully mincing the spleen with the plunger tip of a 1-mL syringe while passing the tissue through a 100- $\mu$ m cell strainer (BD Biosciences). Erythrocytes were depleted by NH<sub>4</sub>Cl lysis. Cells were suspended in DMEM, layered onto Ficoll-Paque (GE Healthcare), and centrifuged for 20 minutes at 2000g at room temperature. Splenic mononuclear cells (MNCs) were collected from the buffy coat and washed with PBS. Splenic MNCs from mice belonging to the same experimental group were pooled and suspended in PBS. Cells were injected via a jugular vein catheter into splenectomized recipient mice (1.7×10<sup>7</sup> MNCs per mouse, corresponding to the average number of MNCs isolated from 1 donor mouse).

## Adoptive Splenic Monocyte Transfer

Splenic monocytes were prepared from splenic MNCs with a kit from Miltenyi Biotec (#130-100-629). In brief, highly pure, untouched monocytes were isolated by depleting nontarget cells (T cells, B cells, natural killer cells, DCs, erythroid cells, and granulocytes) using magnetic cell labeling and separation. Monocytes from mice belonging to the same experimental group were pooled, suspended in PBS, and infused via a jugular vein catheter into splenectomized recipient mice (1×10<sup>6</sup> monocytes per mouse, corresponding to the average number of monocytes isolated from 1 donor mouse).

## Inflammatory Cell Isolation

Peripheral blood was drawn from the right ventricle. Splenocyte suspensions were prepared with a gentleMACS dissociator (Miltenyi Biotec). Blood and spleen erythrocytes were depleted by NH<sub>4</sub>Cl lysis. Inflammatory cells were isolated from the infarcted region of the LV by enzymatic digestion and mechanical dissociation with a gentleMACS dissociator.

## T<sub>reg</sub> Cell Quantification and Isolation

Inflammatory cells were isolated from DEREg mice, incubated with a CD16/CD32 antibody (clone 2.4G2, mouse BD Fc Block, BD Biosciences; dilution 1:100), and stained with a CD4-APC antibody (clone RM4-5, BioLegend; dilution 1:100). CD4<sup>+</sup> Foxp3<sup>+</sup>/eGFP<sup>+</sup> T<sub>reg</sub> cells were counted by flow cytometry. For cell transfer experiments, CD4<sup>+</sup> Foxp3<sup>+</sup>/eGFP<sup>+</sup> T<sub>reg</sub> cells were isolated by fluorescence-activated cell sorting using a FACSAria IIu instrument (Becton Dickinson) and infused via a jugular vein catheter into splenectomized recipient mice (2×10<sup>5</sup> T<sub>reg</sub> cells per mouse, corresponding to the average number of T<sub>reg</sub> cells isolated from 1 donor mouse).

## Closed-Chest Model of Reperfused MI and Magnetic Resonance Imaging in Pigs

All procedures in pigs were approved by the Animal Ethics Committee of the Hungarian National Food Chain Safety

Office (approval number 23.1./02322/009/2008). Acute MI was induced in domestic (DanBred hybrid) female pigs by percutaneous balloon occlusion of the mid left anterior descending coronary artery as described previously by our group.<sup>28</sup> Three days and 6 weeks after MI, LV end-diastolic and end-systolic volumes (LVEDV and LVESV) and infarct volume were determined by contrast-enhanced magnetic resonance imaging, and LV ejection fraction (LVEF; %) was calculated as  $[(LVEDV-LVESV)/LVEDV] \times 100$ . LV myocardium showing late contrast enhancement was quantified to assess infarct volume.

## Statistical Analyses

We randomly allocated mouse littermates and pigs to the different experimental groups. Whenever possible, the investigators were blinded to group allocation during the experiment and when assessing the outcome. No animals were excluded from the analyses. On the basis of visual inspection, data were normally distributed, with similar variances in the different groups. With small sample sizes, we did not apply statistical tests for normality or equality of variances. Data are presented as mean  $\pm$  SEM unless otherwise stated. The 2-independent-sample *t* test was used to compare 2 groups. For comparisons among >2 groups, 1-way ANOVA was used if there was 1 independent variable and 2-way ANOVA if there were 2 independent variables. The Dunnett post hoc test was used for multiple comparisons with a single control group. The Tukey post hoc test was used to adjust for multiple comparisons. In the pig study, we used ANCOVA to compare LVEF changes within (3 days to 6 weeks) and between treatment groups (placebo versus POL6326), with treatment group as the main factor and LVEF at 3 days as a covariate. Differences in least-squares means and corresponding 95% CIs were calculated based on the ANCOVA model. Other end points were analyzed using the same methods. We considered a 2-tailed *P* value <0.05 to indicate statistical significance. K.C.W. had full access to all the data in the study and takes responsibility for the integrity of the data and the data analysis.

## RESULTS

### POL5551 Promotes Tissue Repair and Improves Heart Function After MI

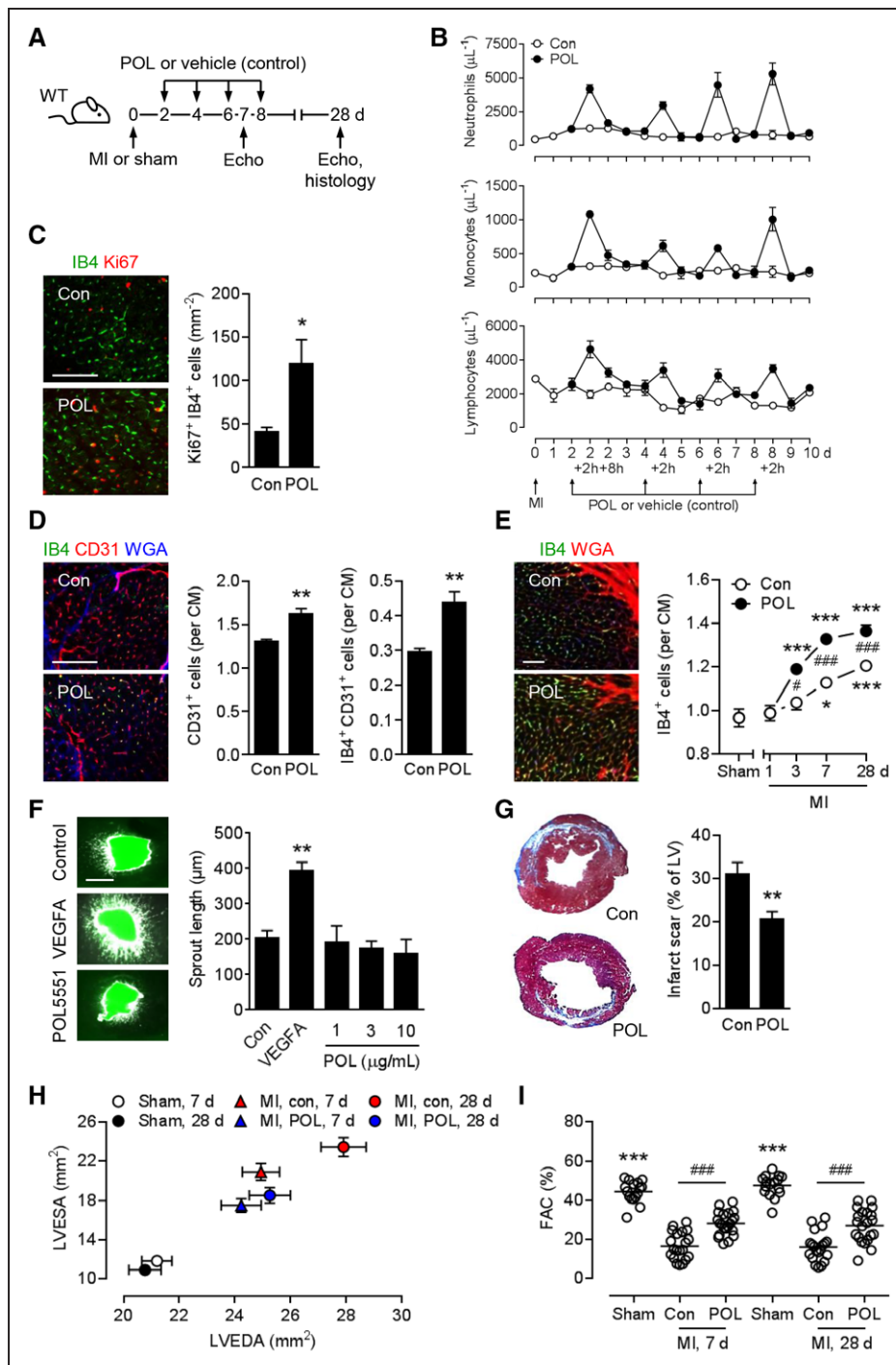
To explore the therapeutic potential of POL5551 after MI, we subjected mice to transient coronary artery ligation. This model simulates the situation in patients with acute MI receiving reperfusion therapy and was used throughout the present study. At 2, 4, 6, and 8 days after MI, mice were injected intraperitoneally with POL5551 or vehicle (0.9% NaCl) only (Figure 1A). Each POL5551 bolus injection was followed by a rapid (within 2 hours) mobilization of neutrophils, monocytes, and lymphocytes into the peripheral blood (Figure 1B). Cell numbers returned to baseline levels within 24 hours after each injection (Figure 1B). Ly6C<sup>high</sup> and Ly6C<sup>low</sup> monocyte populations showed similar mobilization kinetics (Figure I in the online-only Data Supplement). Compared with vehicle-only-treated control

mice, treatment with POL5551 enhanced the number of proliferating Ki67<sup>+</sup> IB4<sup>+</sup> endothelial cells in the infarct border zone at 3 days after MI (Figure 1C). This angiogenic effect was associated with more perfused capillaries at 7 days (Figure 1D) and an increase in total capillary density in the infarct border zone at 3, 7, and 28 days (Figure 1E). POL5551 is a highly selective CXCR4 antagonist with no C-X-C motif chemokine receptor 7 (CXCR7) agonistic activity (Figure II in the online-only Data Supplement). POL5551 did not directly act on endothelial cells, as has been reported for TC14012,<sup>29</sup> a combined CXCR4 antagonist and CXCR7 agonist (Figure 1F and Figure III in the online-only Data Supplement). POL5551-treated mice developed smaller infarct scars (Figure 1G) and less pronounced LV remodeling and systolic and diastolic dysfunction as shown by echocardiography and pressure-volume measurements (Figure 1H and 1I and Table I in the online-only Data Supplement).

### Splenic MNCs Are Required and Sufficient for the Treatment Effects of POL5551

After MI, the spleen continuously supplies neutrophils and monocytes via the bloodstream to satisfy the high demand for these cells at the site of inflammation.<sup>30,31</sup> To delineate the importance of specific cell subsets for the beneficial effects of intermittent CXCR4 blockade, we reassessed the therapeutic potential of POL5551 in splenectomized mice. Mice underwent splenectomy 2 days after MI, before the first injection of either POL5551 or vehicle only (Figure 2A). POL5551 did not enhance capillary density in the infarct border zone (Figure 2B), did not reduce infarct scar size (Figure 2C), and did not attenuate LV remodeling and systolic or diastolic dysfunction in splenectomized mice (Figure 2D and 2E and Table II in the online-only Data Supplement), thereby indicating that the spleen is required for its therapeutic effects. The treatment effects of POL5551 were not affected in infarcted mice that had undergone laparotomy only 2 days after MI (data not shown).

We next explored whether splenic MNCs from POL5551-treated mice can transfer the therapeutic effects into untreated mice. Sham or MI surgeries were performed in donor mice (Figure 3A). After 2 days, donor mice were treated with POL5551 or vehicle only, and splenic MNCs, consisting mainly of lymphocytes and monocytes (Figure IV in the online-only Data Supplement), were prepared 24 hours later. Two days after undergoing MI surgery, recipient mice were splenectomized and then infused with donor MNCs. Capillary density in the infarct border zone was greater in mice that had received MNCs from POL5551-treated infarcted donors than in mice that had received MNCs from vehicle-only-treated infarcted donors (Figure 3B). MNCs from POL5551-treated infarcted donors also re-



**Figure 1. POL5551 mobilizes inflammatory cells and promotes therapeutic effects after MI.**

**A**, Experimental setup for all panels except **F**. Myocardial infarction (MI) was induced in wild-type (WT) mice. Additional mice underwent sham surgery. POL5551 (POL) or vehicle only (control, Con) was intraperitoneally injected 2, 4, 6, and 8 days after MI. Echo indicates echocardiography. **B**, Neutrophil, monocyte, and lymphocyte numbers in the peripheral blood; 3 to 5 mice per time point. **C**, Ki67<sup>+</sup> isolectin B4 (IB4)<sup>+</sup> endothelial cells in the infarct border zone 3 days after MI. Representative fluorescent images (scale bar, 100  $\mu$ m). Summary data from 5 mice per group. \* $P$ <0.05 (2-independent-sample  $t$  test). **D**, CD31<sup>+</sup> capillary density and IB4<sup>+</sup> CD31<sup>+</sup> perfused capillary density in the border zone 7 days after MI. For quantification, cardiomyocyte (CM) borders were visualized by wheat germ agglutinin (WGA) staining. Scale bar, 100  $\mu$ m. Data from 4 control and 5 POL5551-treated mice. \*\* $P$ <0.01 (2-independent-sample  $t$  test). **E**, Fluorescein-labeled IB4<sup>+</sup> capillary density in the border zone 1, 3, 7, and 28 days after MI. Extracellular matrix and CM borders were highlighted by WGA staining. Fluorescent images were taken at 28 days (scale bar, 50  $\mu$ m). Data from 7 sham-operated mice (28 days) and 6 to 14 infarcted mice per group and time point. \* $P$ <0.05, \*\*\* $P$ <0.001 vs sham (for each treatment arm, 1-way ANOVA with Dunnett post hoc test). # $P$ <0.05, ### $P$ <0.001, POL5551 vs control (2-independent-sample  $t$  test). **F**, MI was induced in Tie2-GFP mice. After 24 hours, tissue samples from the infarcted region were removed and cultured for 3 days in the absence (Con) or presence of vascular endothelial growth factor A (VEGFA, 50 ng/mL) or POL5551 (concentration range covering mean and maximum plasma concentrations after bolus injection of 8 mg/kg POL5551 in mice).<sup>21</sup> Images show green fluorescent protein fluorescence (scale bar, 500  $\mu$ m). Bar graph depicts average sprout length. Explants from 3 to 6 mice per group. \*\* $P$ <0.01 vs control (1-way ANOVA with Dunnett post hoc test). **G**, Scar size 28 days after MI. Tissue sections stained with Masson's trichrome. Data from 12 mice per group. LV indicates left ventricle. \*\* $P$ <0.01 (2-independent-sample  $t$  test). (Continued)

**Figure 1 Continued.** **H** and **I**, Sixteen sham-operated mice, 20 infarcted control mice, 21 infarcted POL5551-treated mice. **H**, LV end-diastolic area (LVEDA) and LV end-systolic area (LVESA) as determined by echocardiography 7 and 28 days after sham surgery or MI. LVEDA and LVESA:  $P < 0.001$ , MI vs sham for both treatment groups at both time points (1-way ANOVA with Dunnett post hoc test). LVEDA:  $P < 0.05$ , control vs POL5551-treated mice at 28 days; LVESA:  $P < 0.01$ , control vs POL5551 at 7 days,  $P < 0.001$ , control vs POL5551 at 28 days (2-independent-sample *t* tests). **I**, Fractional area change (FAC). Circles represent individual mice. Horizontal bars are the means. \*\*\* $P < 0.001$  vs both MI groups at each time point (1-way ANOVA with Dunnett post hoc test). ### $P < 0.001$  (2-independent-sample *t* tests).

duced infarct scar size (Figure 3C) and attenuated LV remodeling and systolic and diastolic dysfunction in recipient mice (Figure 3D and 3E and Table III in the online-only Data Supplement). MNCs from sham-operated donors did not exert any therapeutic effects regardless of whether the donors had been treated with POL5551 or vehicle only (Figure 3B through 3E).

Considering the key role of splenic monocytes in orchestrating inflammation and repair after their relocation to the infarcted myocardium,<sup>30,31</sup> we repeated the previous experiment and transplanted only magnetic cell labeling and separation–enriched splenic monocytes from POL5551-treated infarcted donor mice into infarcted and splenectomized recipient mice (Figure 3F and Figure IV in the online-only Data Supplement). To our surprise, adoptively transferring splenic monocytes from POL5551-treated infarcted donors did not attenuate LV remodeling and systolic dysfunction in recipient mice (Figure 3G and 3H).

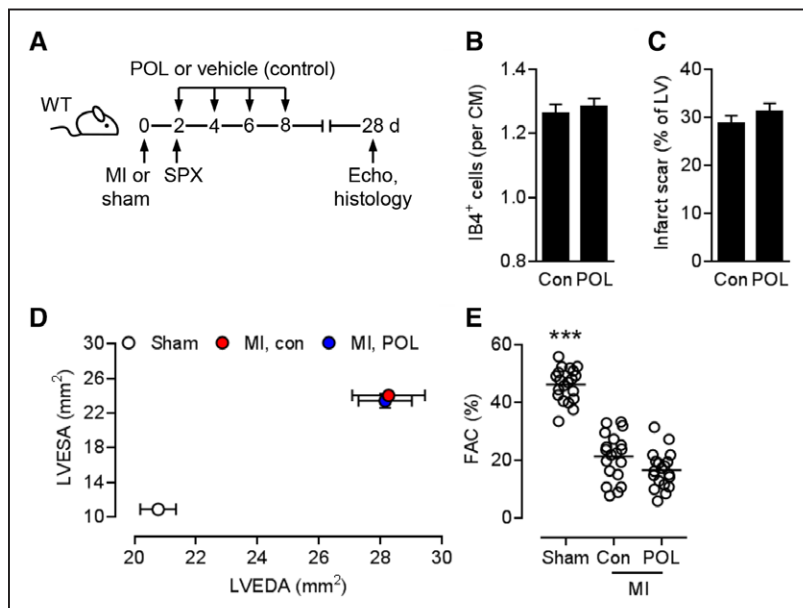
## Splenic $T_{reg}$ Cells Mediate the Treatment Effects of POL5551

The above adoptive cell transfer experiments pointed to splenic lymphocytes as potential mediators of the treatment effects of POL5551. To address this hypothesis, we induced MI in *Rag1* knockout mice and their WT littermates and treated the animals with POL5551 or vehicle only. POL5551 attenuated adverse LV remodeling and systolic dysfunction in *Rag1* WT but not *Rag1* knockout mice, which indicates that T cells or B cells

are required for the treatment effects (Figure V in the online-only Data Supplement).

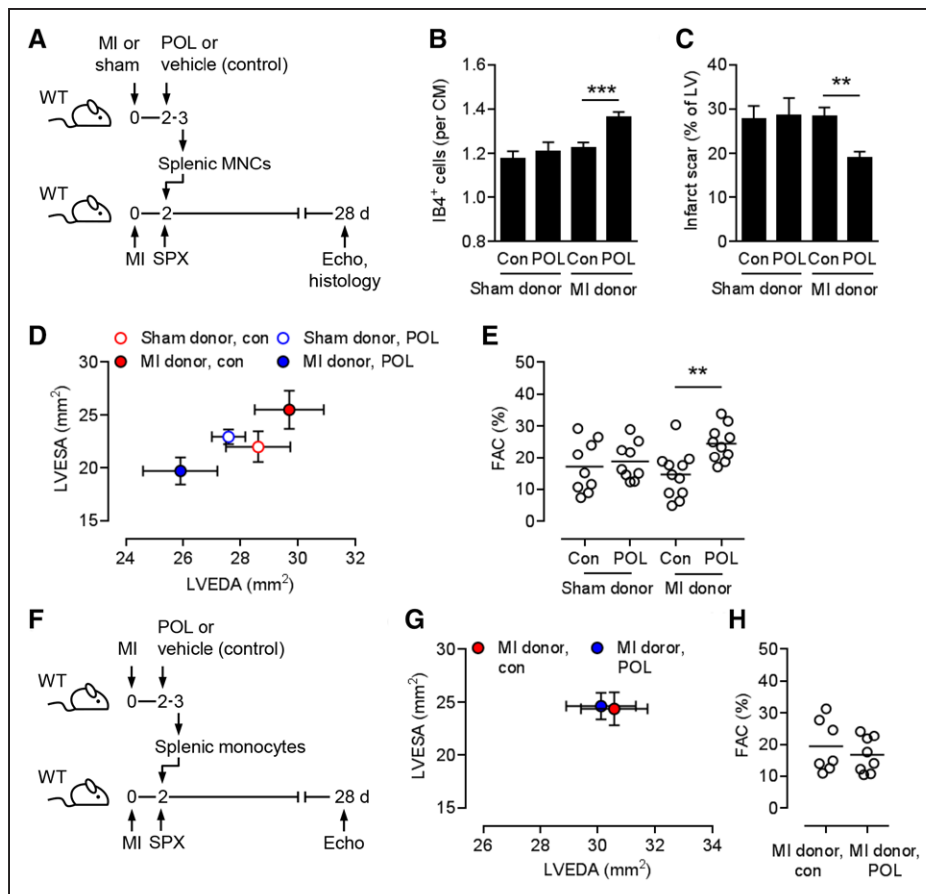
Recent studies have implicated  $T_{reg}$  cells in promoting tissue repair and functional recovery after MI.<sup>32–35</sup> We therefore repeated the experiment in  $T_{reg}$  cell–depleted DREG mice. DREG mice were injected with DT or saline immediately before and 24 hours after MI. Treatment with POL5551 or vehicle only was started 2 days after MI (Figure 4A). DT efficiently depleted  $CD4^+ Fopx3^+/eGFP^+ T_{reg}$  cells in DREG mice (Figure 4B). POL5551 enhanced the capillary density in the infarct border zone (Figure 4C), reduced infarct scar size (Figure 4D), and ameliorated adverse remodeling and systolic dysfunction (Figure 4E and 4F and Table IV in the online-only Data Supplement) in saline-injected, non- $T_{reg}$  cell–depleted DREG mice.  $T_{reg}$  cell depletion, however, completely abolished the treatment effects of POL5551 (Figure 4C through 4F and Table IV in the online-only Data Supplement). The treatment effects of POL5551 were not affected in DT-treated WT mice (Figure VI in the online-only Data Supplement).

To further examine the importance of  $T_{reg}$  cells for the therapeutic effects of CXCR4 blockade, we performed adoptive splenic MNC transfer experiments using  $T_{reg}$  cell–depleted or non- $T_{reg}$  cell–depleted DREG mice as donors and WT mice as recipients (Figure 5A). MI was induced in donor and recipient mice. Donor mice were injected with DT or saline immediately before and 24 hours after MI. Two days after MI, donor mice were treated with POL5551, and splenic MNCs were prepared 24 hours later. Two days after MI, recipi-



**Figure 2. Splenectomy abolishes the therapeutic effects of POL5551.**

**A**, Experimental setup. Myocardial infarction (MI) was induced in wild-type (WT) mice. Additional mice underwent sham surgery. POL5551 (POL) or vehicle only (control) was intraperitoneally injected 2, 4, 6, and 8 days after MI. Infarcted mice underwent splenectomy (SPX) immediately before the first injection. **B**, Fluorescein-labeled isolectin B4 (IB4)<sup>+</sup> capillary density in infarct border zone 28 days after MI; 6 mice per group. CM indicates cardiomyocyte. **C**, Scar size 28 days after MI; 14 mice per group. **D** and **E**, 20 sham-operated mice, 19 control mice, 19 POL5551-treated mice. **D**, LVEDA and LVESA as determined by echocardiography 28 days after sham surgery or MI. LVEDA and LVESA:  $P < 0.001$ , MI vs sham for both treatment groups (2-way ANOVA with Tukey post hoc test). **E**, Fractional area change (FAC). \*\*\* $P < 0.001$  vs both MI groups (2-way ANOVA with Tukey post hoc test). Con indicates control; Echo, echocardiography; LV, left ventricular; LVEDA, left ventricular end-diastolic area; and LVESA, left ventricular end-systolic area.



**Figure 3. Importance of splenic MNCs for the therapeutic effects of POL5551.**

**A**, Experimental setup for **B** through **E**. Sham or myocardial infarction (MI) surgeries were performed in wild-type (WT) donor mice. Two days after MI, donor mice were intraperitoneally injected with POL5551 (POL) or vehicle only (control), and splenic mononuclear cells (MNCs) were isolated 24 hours later. Recipient WT mice underwent MI surgery, were splenectomized after 2 days, and then were infused intravenously with donor MNCs. Echo indicates echocardiography; and SPX, splenectomy. **B** through **E**, 9 to 11 mice per group. **B**, Fluorescein-labeled isolectin B4 (IB4)<sup>+</sup> capillary density in the infarct border zone 28 days after MI. CM indicates cardiomyocyte; and Con, control. **C**, Scar size 28 days after MI. LV indicates left ventricle. **D**, LV end-diastolic area (LVEDA) and LV end-systolic area (LVESA) as determined by echocardiography 28 days after MI. LVESA:  $P < 0.05$ , recipients receiving MNCs from infarcted POL5551-treated vs infarcted vehicle-only-treated donors (2-independent-sample  $t$  test). **E**, Fractional area change (FAC). **F**, Experimental setup for **G** and **H**. MI was induced in WT donor and recipient mice. Two days after MI, donor mice were intraperitoneally injected with POL5551 or vehicle only (con), and splenic monocytes were isolated 24 hours later. Two days after MI, recipient mice were splenectomized and then infused intravenously with donor monocytes. Seven to 8 mice per group. **G**, LVEDA and LVESA 28 days after MI. **H**, FAC. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (2-independent-sample  $t$  tests).

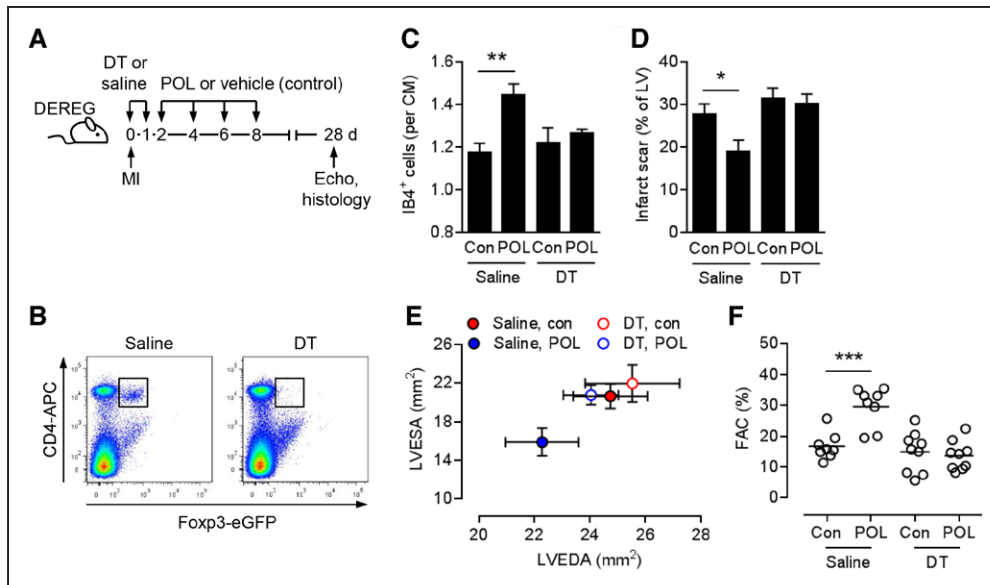
ent mice were splenectomized and then infused with donor MNCs (Figure 5A). Capillary density in the infarct border zone was greater (Figure 5B), infarct scar size was smaller (Figure 5C), and postinfarction LV remodeling (Figure 5D) and systolic dysfunction (Figure 5E) were less severe in recipient mice transplanted with non- $T_{reg}$  cell-depleted MNCs than in mice that were transplanted with  $T_{reg}$  cell-depleted MNCs. These findings further supported our conclusion that splenic  $T_{reg}$  cells are required for the treatment effects of POL5551.

Next, we explored whether POL5551-primed splenic  $T_{reg}$  cells are also sufficient to promote tissue repair and functional recovery after MI. To this end, MI was induced in DREG donor and WT recipient mice. Two days after MI, donor mice were treated with POL5551 or vehicle only. Splenic CD4<sup>+</sup> Foxp3<sup>+</sup>/eGFP<sup>+</sup>  $T_{reg}$  cells were prepared 24 hours later (Figure 5F and 5G). Two days after MI, recipient mice were splenectomized and

then infused with donor  $T_{reg}$  cells (Figure 5F). Capillary density in the infarct border zone was greater (Figure 5H), infarct scar size was smaller (Figure 5I), and postinfarction LV remodeling (Figure 5J) and systolic dysfunction (Figure 5K) were less severe in recipient mice transplanted with  $T_{reg}$  cells from POL5551-treated donors than in mice that were transplanted with  $T_{reg}$  cells from vehicle-only-treated donors.

### DCs Are Required for POL5551 Treatment Effects

The MNC transfer experiments shown in Figure 3A through 3E indicated that instructive cues provided by the infarct are required for POL5551 treatment effects. MI has recently been shown to prime DCs, which in turn promote  $T_{reg}$  cell activation.<sup>36</sup> We therefore performed adoptive splenic MNC transfer experiments using DC-



**Figure 4. Importance of regulatory T cells for the therapeutic effects of POL5551.**

**A**, Experimental setup. Myocardial infarction (MI) was induced in DERE mice. Diphtheria toxin (DT) or saline was intraperitoneally injected immediately before and 24 hours after MI. POL5551 (POL) or vehicle only (control) was intraperitoneally injected 2, 4, 6, and 8 days after MI. Echo indicates echocardiography. **B**, Representative flow cytometry panels confirming CD4<sup>+</sup> Fcγ3<sup>+</sup>/eGFP<sup>+</sup> regulatory T cell depletion in splenocytes 24 hours after the second DT injection. **C** through **F**, Eight to 9 mice per group. **C**, Fluorescein-labeled isolectin B4 (IB4)<sup>+</sup> capillary density in the infarct border zone 28 days after MI. CM indicates cardiomyocyte; and Con, control. **D**, Scar size 28 days after MI. LV indicates left ventricle. **E**, LV end-diastolic area (LVEDA) and LV end-systolic area (LVESA) as determined by echocardiography 28 days after MI. LVESA:  $P < 0.05$ , POL5551 vs control in saline-injected mice (2-independent-sample *t* test). **F**, Fractional area change (FAC). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (2-independent-sample *t* tests).

depleted or non-DC-depleted CD11c-Cre iDTR infarcted mice as donors and infarcted WT mice as recipients (Figure 5L). Donor mice were injected with DT or saline 24 hours before and immediately before MI. Two days after MI, donor mice were treated with POL5551, and splenic MNCs were prepared 24 hours later (Figure 5M). Two days after MI, recipient mice were splenectomized and then infused with donor MNCs (Figure 5L). Capillary density in the infarct border zone was greater (Figure 5N), infarct scar size was smaller (Figure 5O), and postinfarction LV remodeling (Figure 5P) and systolic dysfunction (Figure 5Q) were less severe in recipient mice transplanted with MNCs from non-DC-depleted donors than in mice that were transplanted with MNCs from DC-depleted donors.

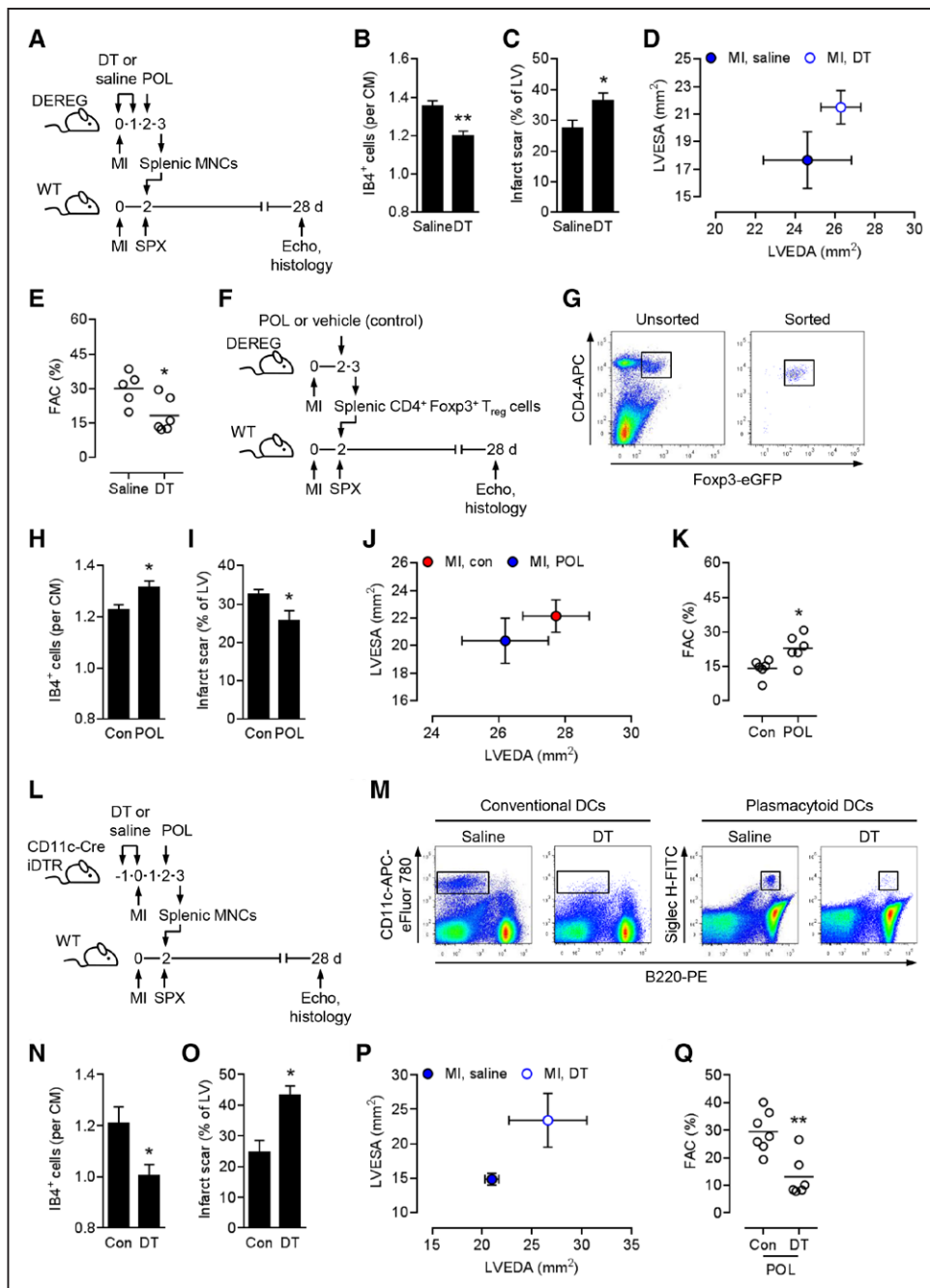
### POL5551 Enhances T<sub>reg</sub> Cell Mobilization, Cardiac Recruitment, and Immune-Regulatory Function

We treated infarcted DERE mice with POL5551 or vehicle only and quantified CD4<sup>+</sup> Fcγ3<sup>+</sup>/eGFP<sup>+</sup> T<sub>reg</sub> cells in the circulation and the infarcted LV by flow cytometry. Each POL5551 injection was followed by rapid T<sub>reg</sub> cell mobilization into the peripheral blood (Figure 6A). T<sub>reg</sub> cell mobilization displayed similar kinetics to other inflammatory cell populations (Figure 1B) and was paralleled by enhanced T<sub>reg</sub> cell accumulation in the infarcted LV (Figure 6B). Enhanced T<sub>reg</sub> cell recruitment was confined to the infarcted region (Figure 6C). In both ve-

hicle-only- and POL5551-treated mice, between 87% and 96% of CD4<sup>+</sup> Fcγ3<sup>+</sup>/eGFP<sup>+</sup> T<sub>reg</sub> cells in the infarcted LV (both in the infarcted and noninfarcted regions) expressed the activation marker CD69 antigen (5 mice per group,  $P = NS$ ). POL5551 did not increase neutrophil, monocyte, or macrophage (Ly6C<sup>high</sup> and Ly6C<sup>low</sup>) or total lymphocyte accumulation in the infarcted region (Figure VII in the online-only Data Supplement). Circulating T<sub>reg</sub> cell numbers were much lower in infarcted mice that had undergone splenectomy before POL5551 injection (Figure 6D), which points to the spleen as the main T<sub>reg</sub> cell reservoir. Indeed, POL5551 did not enhance T<sub>reg</sub> cell accumulation in the infarcted region in splenectomized mice (Figure 6E).

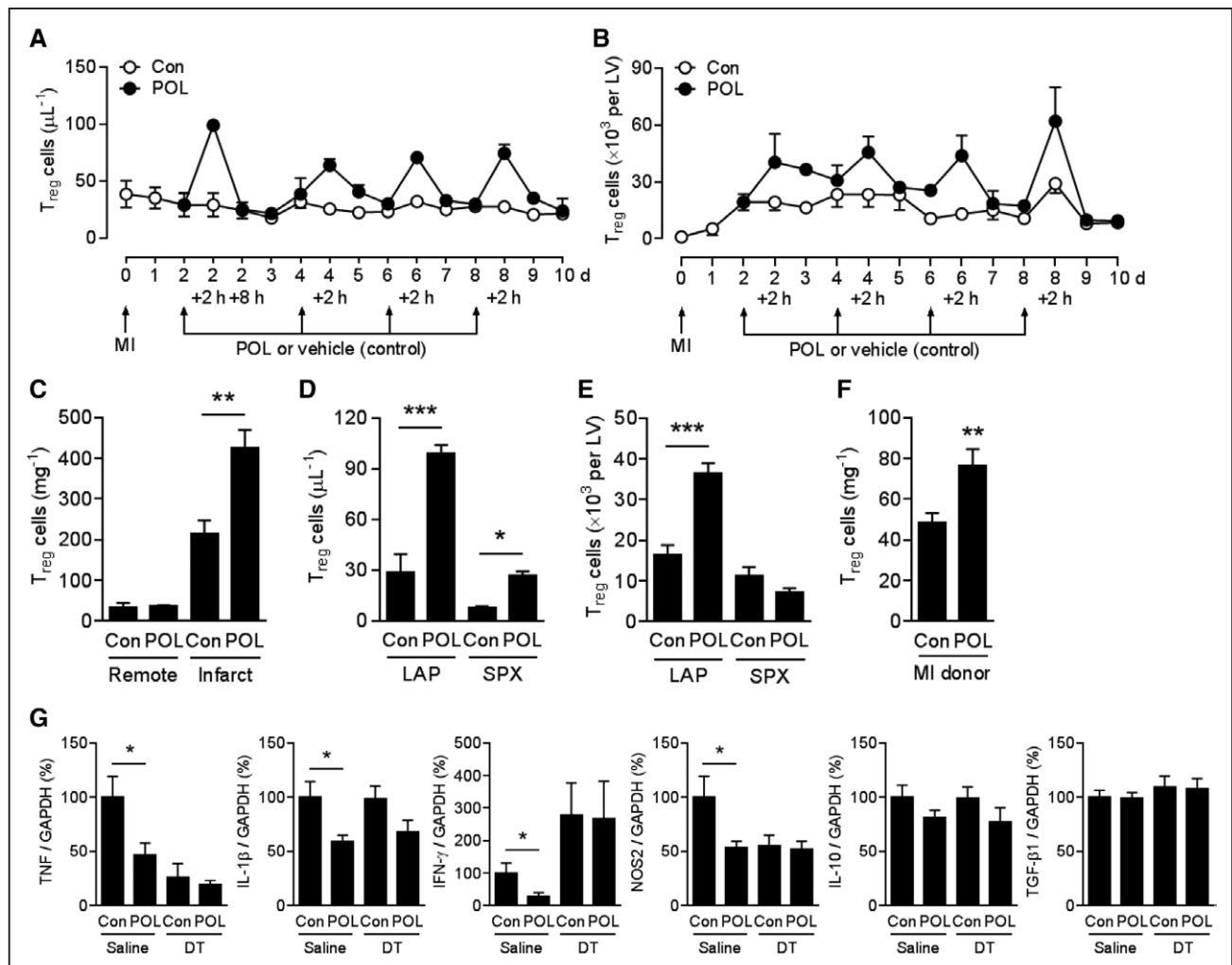
The cell transfer experiments shown in Figure 5F through 5K, where we had transplanted equal numbers of splenic CD4<sup>+</sup> Fcγ3<sup>+</sup>/eGFP<sup>+</sup> T<sub>reg</sub> cells from POL5551- and vehicle-only-treated donors, indicated that POL5551 also enhances T<sub>reg</sub> cell functionality. We therefore explored whether POL5551 enhances the capacity of T<sub>reg</sub> cells to home to the infarct. Indeed, T<sub>reg</sub> cell recruitment to the infarcted region was greater in mice that had been transplanted with T<sub>reg</sub> cells from POL5551-treated donors than in mice that had been transplanted with equal numbers of T<sub>reg</sub> cells from vehicle-only-treated donors (Figure 6F). T<sub>reg</sub> cells from POL5551-treated donors also showed a somewhat greater activity in T<sub>reg</sub> cell suppression assays (Figure VIII in the online-only Data Supplement). Expression of T cell activation markers on T<sub>reg</sub> cells from POL5551- or





**Figure 5. Splenic regulatory T cells are required and sufficient for the therapeutic effects of POL5551.**

**A**, Experimental setup for **B** through **E**. Myocardial infarction (MI) was induced in DEREK donor and wild-type (WT) recipient mice. Donor mice were intraperitoneally injected with diphtheria toxin (DT) or saline immediately before and 24 hours after MI. Two days after MI, donor mice were intraperitoneally injected with POL5551 (POL), and splenic mononuclear cells (MNCs) were isolated 24 hours later. Two days after MI, recipient mice were splenectomized (SPX) and then infused intravenously with donor MNCs. Echo indicates echocardiography. **B** through **E**, Five mice treated with MNCs from saline-injected donors, 6 mice treated with MNCs from DT-injected donors. **B**, Fluorescein-labeled isolectin B4 (IB4) capillary density in the infarct border zone 28 days after MI. CM indicates cardiomyocyte. **C**, Scar size 28 days after MI. LV indicates left ventricle. **D**, LV end-diastolic area (LVEDA) and LV end-systolic area (LVESA) as determined by echocardiography 28 days after MI. **E**, Fractional area change (FAC). **F**, Experimental setup for **H** through **K**. MI was induced in DEREK donor and WT recipient mice. Two days after MI, donor mice were intraperitoneally injected with POL5551 or vehicle only (Con), and splenic CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory T (T<sub>reg</sub>) cells were isolated 24 hours later. Two days after MI, recipient mice were splenectomized and then infused intravenously with donor T<sub>reg</sub> cells. **G**, Representative flow cytometry panels showing unsorted splenic MNCs (left) and sorted splenic T<sub>reg</sub> cells (right) that were used for transplantation. CD4<sup>+</sup> Foxp3<sup>+</sup> T<sub>reg</sub> cells are highlighted in both panels. **H** through **K**, 6 mice that received T<sub>reg</sub> cells from vehicle-only-treated donors, 6 mice that received T<sub>reg</sub> cells from POL5551-treated donors. **H**, Capillary density in the border zone 28 days after MI. **I**, Scar size 28 days after MI. **J**, LVEDA and LVESA 28 days after MI. **K**, FAC. **L**, Experimental setup for **N** through **Q**. MI was induced in CD11c-Cre iDTR donor and WT recipient mice. Donor mice were intraperitoneally injected with DT or saline 24 hours before and immediately before MI. Two days after MI, donor mice were intraperitoneally injected with POL5551, and splenic MNCs were isolated 24 hours later. Two days after MI, recipient mice were splenectomized and then infused intravenously with donor MNCs. **M**, Representative flow cytometry panels showing non-dendritic cell (DC)-depleted (saline) and DC-depleted (DT) splenic MNCs that were used for transplantation. DCs are highlighted in all panels. **N** through **Q**, Seven mice treated with MNCs from saline-injected donors, 6 mice treated with MNCs from DT-injected donors. **N**, Capillary density in the border zone 28 days after MI. **O**, Scar size 28 days after MI. **P**, LVEDA and LVESA 28 days after MI. **Q**, FAC. \**P*<0.05, \*\**P*<0.01 (2-independent-sample *t* tests).



**Figure 6. POL5551 enhances T<sub>reg</sub> cell mobilization, cardiac recruitment, and immune-modulatory function.**

**A and B,** Myocardial infarction (MI) was induced in DEREK mice, and POL5551 (POL) or vehicle only (control [Con]) was intraperitoneally injected after 2, 4, 6, and 8 days. CD4<sup>+</sup> Foxp3<sup>+</sup>eGFP<sup>+</sup> regulatory T (T<sub>reg</sub>) cell numbers (**A**) in the peripheral blood and (**B**) in the infarcted left ventricle (LV); 3 to 9 mice per time point. **C,** CD4<sup>+</sup> Foxp3<sup>+</sup>eGFP<sup>+</sup> T<sub>reg</sub> cell numbers in infarcted and noninfarcted (remote) region 2 hours after first vehicle only (Con) or POL5551 injection; 5 mice per group. **D and E,** Two days after MI, DEREK mice underwent splenectomy (SPX) or sham surgery (laparotomy only [LAP]) and were then intraperitoneally injected with POL5551 or vehicle only (Con). CD4<sup>+</sup> Foxp3<sup>+</sup>eGFP<sup>+</sup> T<sub>reg</sub> cell numbers (**D**) in the peripheral blood 2 hours later and (**E**) in the infarcted LV 24 hours later; 4 to 9 mice per group. **F,** MI was induced in DEREK donor and wild-type recipient mice. Two days after MI, donor mice were injected with POL5551 or vehicle only (Con), and splenic mononuclear cells were isolated 24 hours later. Two days after MI, recipient mice were splenectomized and then infused intravenously with 17×10<sup>6</sup> donor mononuclear cells (as shown in a pilot experiment, mononuclear cells from POL5551- and vehicle-only-treated donors contained comparable numbers of CD4<sup>+</sup> Foxp3<sup>+</sup>eGFP<sup>+</sup> T<sub>reg</sub> cells). Two hours later, T<sub>reg</sub> cells were quantified in the infarcted region in recipient mice; 11 mice per group. **G,** DEREK mice were intraperitoneally injected with diphtheria toxin (DT) or saline immediately before and 24 hours after MI. Two days after MI, mice were intraperitoneally injected with POL5551 or vehicle only (Con). Two days later, Ly6C<sup>low/high</sup> monocytes and macrophages were isolated from the infarcted region, and the expression levels of tumor necrosis factor (TNF), interleukin 1β (IL-1β), interferon-γ (IFN-γ), nitric oxide synthase 2 (NOS2), IL-10, and transforming growth factor β1 (TGF-β1) were determined by reverse-transcription quantitative polymerase chain reaction. Eight to 10 mice per group. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001 (2-independent-sample *t* tests).

vehicle-only-treated donors did not differ significantly (Table V in the online-only Data Supplement).

We next explored whether POL5551 modulates gene expression in myeloid cells and whether T<sub>reg</sub> cells may be involved. DEREK mice were injected with either DT or saline immediately before and 1 day after MI. Two days after MI, mice were treated with POL5551 or vehicle only. Ly6C<sup>low/high</sup> monocytes and macrophages were isolated from the infarcted region 2 days later and used for reverse-transcription quantitative polymerase chain reaction analyses. POL5551 attenuated the expression of tumor necrosis factor, interleukin 1β, interferon-γ,

and nitric oxide synthase 2 in Ly6C<sup>low/high</sup> monocytes and macrophages in non-T<sub>reg</sub> cell-depleted but not in T<sub>reg</sub> cell-depleted DEREK mice (Figure 6G). Expression levels of interleukin 10 and transforming growth factor β1 were not altered by POL5551 (Figure 6G).

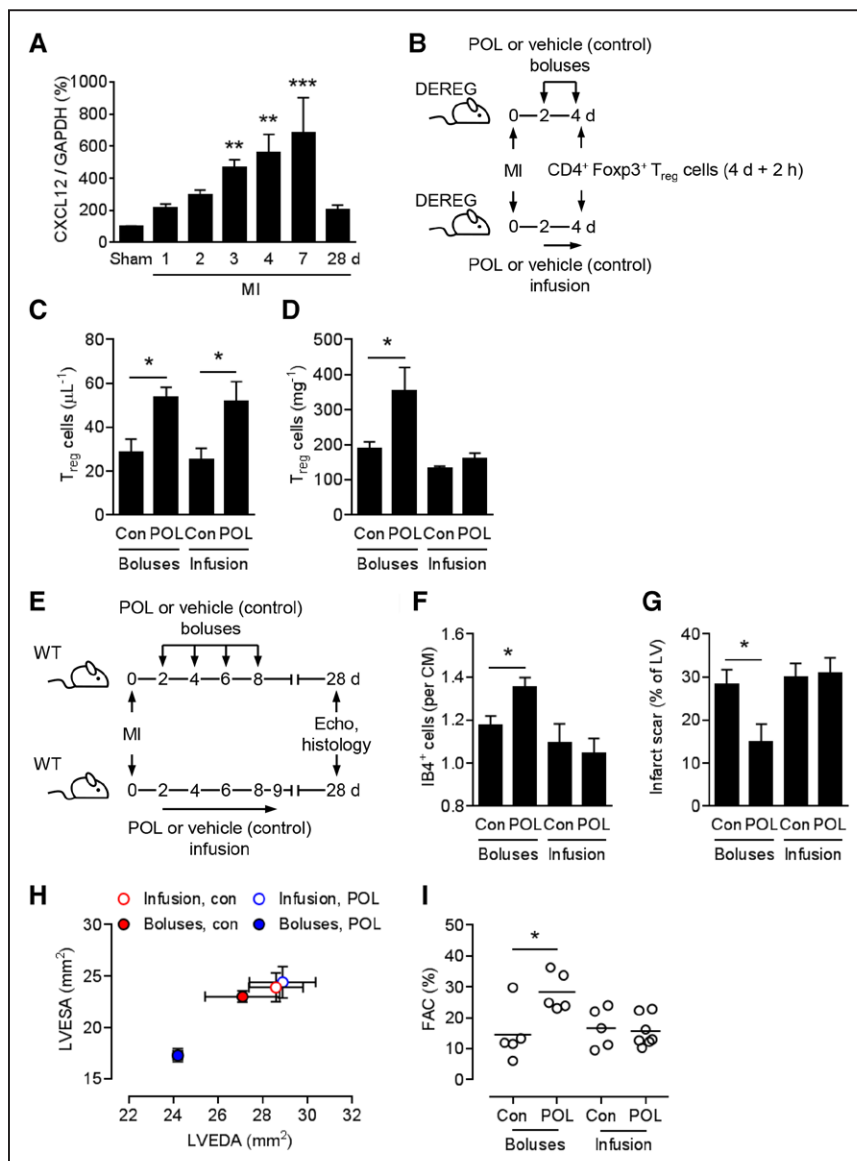
### Continuous POL5551 Infusion Mobilizes T<sub>reg</sub> Cells but Prevents Their Cardiac Recruitment and Therapeutic Effects

Consistent with previous reports,<sup>15,16</sup> CXCL12 expression was significantly increased in the infarcted region,

peaking at 7 days and returning to baseline levels at 28 days (Figure 7A). To assess the importance of CXCL12 for  $T_{reg}$  cell recruitment to the infarcted region, we treated infarcted DERE mice with POL5551 bolus injections as before or with a continuous POL5551 infusion starting 2 days after MI (Figure 7B). Bolus injections and continuous POL5551 infusion both mobilized  $CD4^+ Foxp3^+/eGFP^+$   $T_{reg}$  cells into the circulation (Figure 7C). Continuous POL5551 infusion, however, prevented  $T_{reg}$  cell recruitment to the infarcted region (Figure 7D). In a related experiment, we treated infarcted WT mice with POL5551 bolus injections or with a continuous POL5551 infusion (Figure 7E). Unlike the bolus injections, continuous POL5551 infusion did not enhance infarct border-zone capillarization (Figure 7F), did not reduce infarct scar size (Figure 7G), and did not attenuate LV remodeling and systolic dysfunction (Figure 7H and 7I).

## POL6326 Treatment Effects in Pigs With Reperfused Acute MI

We treated pigs with reperfused acute MI with POL6326 to explore the translational potential of CXCR4 blockade with a peptidic macrocycle antagonist. Like POL5551, POL6326 mobilized  $T_{reg}$  cells, enhanced  $T_{reg}$  cell recruitment to the infarct, attenuated tumor necrosis factor expression in monocytes and macrophages in the infarcted region, enhanced infarct border-zone capillarization, reduced infarct scar size, and attenuated LV remodeling and systolic dysfunction in mice (Figure IX in the online-only Data Supplement). At 4, 6, 8, and 10 days after MI, pigs were infused intravenously with the clinical stage compound POL6326 or vehicle only. Contrast-enhanced cardiac magnetic resonance imaging was performed 3 days and 6 weeks after MI (Table). Infarct volume decreased, on average, by 1.6 mL in the control group ( $P=0.28$ ) and by 6.2 mL in the POL6326



**Figure 7. Continuous POL5551 infusion mobilizes regulatory T cells but prevents their cardiac recruitment and therapeutic effects.** **A**, Expression levels of C-X-C motif chemokine ligand 12 (CXCL12) in the infarcted region at various time points after myocardial infarction (MI) or sham operation (day 7) as determined by reverse-transcription quantitative polymerase chain reaction. Five to 9 mice per group. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs sham (1-way ANOVA with Dunnett post hoc test). **B**, Experimental setup for **C** and **D**. MI was induced in DERE mice. POL5551 (POL) or vehicle only (control [Con]) was injected intraperitoneally 2 and 4 days after MI or infused subcutaneously starting 2 days after MI. **C**, CD4<sup>+</sup> Foxp3<sup>+</sup>/eGFP<sup>+</sup> regulatory T ( $T_{reg}$ ) cell numbers in the peripheral blood and **D** in the infarcted region at 4 days + 2 hours after MI. Four to 5 mice per group. **E**, Experimental setup for **F** through **I**. MI was induced in wild-type (WT) mice. POL5551 or vehicle only (Con) was injected intraperitoneally 2, 4, 6, and 8 days after MI or infused subcutaneously for 7 days starting 2 days after MI. 5 to 7 mice per group. Echo indicates echocardiography. **F**, Fluorescein-labeled isolectin B4 (IB4)<sup>+</sup> capillary density in the infarct border zone 28 days after MI. CM indicates cardiomyocyte. **G**, Scar size 28 days after MI. LV indicates left ventricle. **H**, LV end-diastolic area (LVEDA) and LV end-systolic area (LVESA) as determined by echocardiography 28 days after MI. LVESA:  $P < 0.05$ , POL5551 vs vehicle-only boluses; LVESA:  $P < 0.001$ , POL5551 vs vehicle-only boluses (2-independent-sample  $t$  tests). **I**, Fractional area change (FAC). \* $P < 0.05$  (2-independent-sample  $t$  tests).

**Table. POL6326 Treatment Effects in Pigs With Reperfused Acute Myocardial Infarction**

	3 d (Mean±SEM)	6 wk (Mean±SEM)	Mean Change (95% CI)	P Value	Mean Treatment Effect (95% CI)	P Value
Infarct volume, mL						
Control	18.1±2.4	17.1±1.9	-1.6 (-4.6 to 1.4)	0.28		
POL6326	20.2±1.7	13.4±1.3	-6.2 (-9.2 to -3.2)	<0.001	-4.6 (-8.8 to -0.3)	0.035
LVEDV, mL						
Control	62.2±3.4	69.9±4.7	7.4 (-1.3 to 16.2)	0.09		
POL6326	62.7±2.2	68.0±3.6	5.5 (-3.2 to 14.2)	0.20	-1.9 (-14.2 to 10.4)	0.75
LVESV, mL						
Control	35.1±2.7	41.2±3.9	5.7 (-1.3 to 12.8)	0.11		
POL6326	36.6±1.8	38.4±3.1	2.3 (-4.8 to 9.3)	0.52	-3.5 (-13.4 to 6.5)	0.48
LVEF, %						
Control	44.3±2.2	41.9±2.3	-2.1 (-5.3 to 1.1)	0.19		
POL6326	41.7±2.1	45.0±2.2	3.1 (-0.1 to 6.3)	0.06	5.2 (0.6 to 9.7)	0.027

Four, 6, 8, and 10 days after myocardial infarction, pigs were treated intravenously with POL6326 (n=16) or vehicle only (control, n=16). Three days and 6 weeks after myocardial infarction, infarct volume, LVEDV, and LVESV were determined by contrast-enhanced magnetic resonance imaging. Changes from 3 days to 6 weeks and treatment effects (POL6326 vs control) are expressed as differences in least-squares means with 95% CI (ANCOVA model with adjustment for 3-day values). LVEDV indicates left ventricular end-diastolic area; LVEF, left ventricular ejection fraction; and LVESV, left ventricular end-systolic area.

group ( $P<0.001$ ), which corresponds to a POL6326 treatment effect of  $-4.6$  mL ( $P=0.035$ ). Increases in LV end-diastolic and end-systolic volumes over time tended to be more pronounced in the control group than in the POL6326 group. Mean LVEF decreased by 2.1 percentage points in the control group ( $P=0.19$ ) and increased by 3.1 percentage points in the POL6326 group ( $P=0.06$ ), which corresponds to a POL6326 treatment effect of 5.2 percentage points ( $P=0.027$ ).

## DISCUSSION

Patients who develop heart failure after MI remain at high risk of death and frequent hospitalizations.<sup>37</sup> Available strategies to prevent postinfarction heart failure aim at limiting ischemic injury and chronic LV remodeling.<sup>38</sup> Infarct healing may afford an additional time window of therapeutic opportunity wherein transient interventions can have a lasting impact on cardiac function.<sup>4</sup> In the present study, repeated bolus application of POL5551, a highly selective peptidic macrocycle CXCR4 antagonist, promoted angiogenesis and tissue repair and attenuated LV remodeling and systolic and diastolic dysfunction in a mouse model of reperfused acute MI. As shown by a series of cell depletion and transfer experiments, splenic CD4<sup>+</sup> Foxp3<sup>+</sup> T<sub>reg</sub> cells mediated these therapeutic effects.

T<sub>reg</sub> cells are a subpopulation of CD4<sup>+</sup> T cells that maintain immune homeostasis by controlling adaptive and innate immune responses at multiple levels. Dysregulated T<sub>reg</sub> cell generation or function has been implicated in autoimmunity, cancer, and cardiovascular disease.<sup>39</sup> CXCR4 is expressed on T<sub>reg</sub> cells and other lymphocyte populations.<sup>40</sup> Of note, CXCR4 was original-

ly discovered as a coreceptor for the entry of human immunodeficiency virus into CD4<sup>+</sup> T cells, and the CXCR4 antagonist plerixafor was first developed as an antiviral agent.<sup>41</sup> During the initial pharmacodynamic studies, marked increases in peripheral white blood cell counts were observed after systemic injections of plerixafor, with lymphocytes representing one component of this generalized leukocytosis.<sup>42</sup> In fact, plerixafor mobilizes T cells (including T<sub>reg</sub> cells) and B cells more efficiently than granulocyte-colony stimulating factor.<sup>40</sup> Consistent with this earlier report, POL5551 led to a rapid mobilization of Foxp3<sup>+</sup> T<sub>reg</sub> cells in our study. Within 24 hours after each POL5551 injection, Foxp3<sup>+</sup> T<sub>reg</sub> cell numbers in the circulation returned to baseline levels, consistent with T<sub>reg</sub> cell redistribution into tissues after the interruption of the CXCR4/CXCL12 interaction had ended because of rapid clearance of the drug.<sup>21</sup> POL5551 enhanced cardiac recruitment of Foxp3<sup>+</sup> T<sub>reg</sub> cells, which indicates that some of the mobilized Foxp3<sup>+</sup> T<sub>reg</sub> cells homed to the infarcted myocardium. Neutrophils, Ly6C<sup>high</sup> and Ly6C<sup>low</sup> monocytes, and lymphocytes displayed similar mobilization kinetics after POL5551 injections, but cardiac recruitment of these cells did not increase. Notably, continuous infusion of POL5551 prevented mobilized T<sub>reg</sub> cell homing to the CXCL12-expressing infarcted region and did not promote tissue repair and functional recovery. This indicates that T<sub>reg</sub> cells are recruited to the infarcted region via the CXCR4/CXCL12 axis (not excluding the involvement of additional chemokines)<sup>7</sup> and reconciles our findings with reports from the cancer field that show that continuous CXCR4 blockade inhibits T<sub>reg</sub> cell homing to CXCL12-expressing tumors.<sup>43,44</sup> CXCL12/CXCR4 signals are critical for T<sub>reg</sub> cell trafficking between the bone marrow and the periphery.<sup>45</sup>

Other anatomic niches harboring  $T_{reg}$  cells via CXCR4/CXCL12 interactions remain poorly defined. In our study,  $Foxp3^+ T_{reg}$  cell mobilization was strongly reduced in splenectomized mice, which points to the spleen as the main  $Foxp3^+ T_{reg}$  cell reservoir amenable to POL5551-mediated mobilization. Splenectomy also abolished POL5551 effects on  $T_{reg}$  cell recruitment to the infarct, infarct border-zone angiogenesis, scar size, and LV remodeling. Likewise, depleting mature T and B cells in *Rag1* knockout mice or specifically depleting  $Foxp3^+ T_{reg}$  cells in DREG mice eliminated the therapeutic effects of POL5551. Adoptive cell transfer experiments using splenic MNCs,  $Foxp3^+ T_{reg}$  cell-depleted splenic MNCs, or splenic  $Foxp3^+ T_{reg}$  cells from POL5551-treated infarcted donor mice confirmed splenic  $Foxp3^+ T_{reg}$  cells as the prime mediators of the POL5551 treatment effects.

Splenic MNC transfer experiments comparing infarcted versus noninfarcted and DC-depleted versus non-DC-depleted donor mice revealed that instructive cues provided by the infarct (specifically by DCs) are required for POL5551 treatment effects. Considering that  $Foxp3^+ T_{reg}$  cells are the splenic MNC population that mediates POL5551 treatment effects and that infarcted myocardium-primed DCs promote  $T_{reg}$  cell expansion and activation,<sup>36</sup> we conclude that MI-primed DCs are required to render  $T_{reg}$  cells responsive to POL5551.

Expanding the circulating  $T_{reg}$  cell pool by adoptive  $T_{reg}$  cell transfer or treatment with a superagonistic anti-CD28 antibody enhances  $T_{reg}$  cell recruitment to the infarcted region, improves wound healing, and attenuates LV remodeling in mice and rats with acute MI.<sup>32–34</sup> The therapeutic effects of POL5551 may therefore relate in part to increased  $T_{reg}$  cell availability. Adoptive  $T_{reg}$  cell transfer experiments indicated that POL5551 additionally enhances  $T_{reg}$  cell functionality. Specifically, POL5551 increased the capacity of  $T_{reg}$  cells to home to the infarct and (slightly) promoted  $T_{reg}$  cell immunosuppressive function.

Acute MI activates autoreactive  $CD4^+$  T cells,<sup>46,47</sup> which could trigger autoimmune damage to the myocardium in mice and possibly patients with type 1 diabetes mellitus.<sup>48</sup> Postinfarction autoimmune injury, however, is usually not observed in nondiabetic mice.<sup>47,48</sup> Suppression of autoimmune injury, therefore, might not be the prime mechanism whereby  $T_{reg}$  cell mobilization or  $T_{reg}$  cell expansion<sup>32–34</sup> promotes therapeutic effects after MI. Instead,  $T_{reg}$  cells have been proposed to modulate innate immune responses after MI at multiple levels.<sup>34,35</sup>  $T_{reg}$  cell depletion before the infarct could lead to higher monocyte and macrophage accumulation in the infarcted region.<sup>34,35</sup> Still, treatment with POL5551, starting 2 days after MI, did not affect monocyte and macrophage numbers in the infarcted myocardium. Similarly, treatment with an anti-CD28 antibody 2 days after MI did not affect monocyte and macrophage accumulation,<sup>34</sup> which indicates

that timing could be important or that endogenous  $T_{reg}$  cell effects on innate immune cell recruitment cannot be further enhanced by these interventions.  $T_{reg}$  cells have also been shown to modulate inflammatory gene expression in monocytes and macrophages in the infarcted myocardium.<sup>34</sup> Consistently, we found that POL5551, via  $Foxp3^+ T_{reg}$  cells, attenuated the expression of tumor necrosis factor, interleukin  $1\beta$ , interferon- $\gamma$ , and nitric oxide synthase 2 in monocytes and macrophages in the infarcted region.

In conclusion, our data confirm CXCR4 blockade as a promising treatment strategy to enhance tissue repair and functional recovery after acute MI.<sup>10–12</sup> We identify  $CD4^+$   $Foxp3^+ T_{reg}$  cells as the central arbiters of these therapeutic effects and thereby delineate a new pharmacological strategy to acutely and transiently augment  $T_{reg}$  cell function in vivo.<sup>49</sup> Highlighting the translational potential of this approach, the clinical-stage compound POL6326 reduced infarct volume and promoted LVEF recovery in a porcine closed-chest model of reperfused acute MI.<sup>50</sup> These findings should stimulate further research into the therapeutic potential of CXCR4 antagonists after MI and in other acute conditions wherein excessive innate or adaptive immune responses cause immunopathology.

## ARTICLE INFORMATION

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## Disclosures

Drs Chevalier and Dembowsky were employees of Polyphor during experiments and manuscript preparation. The other authors report no conflicts.

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