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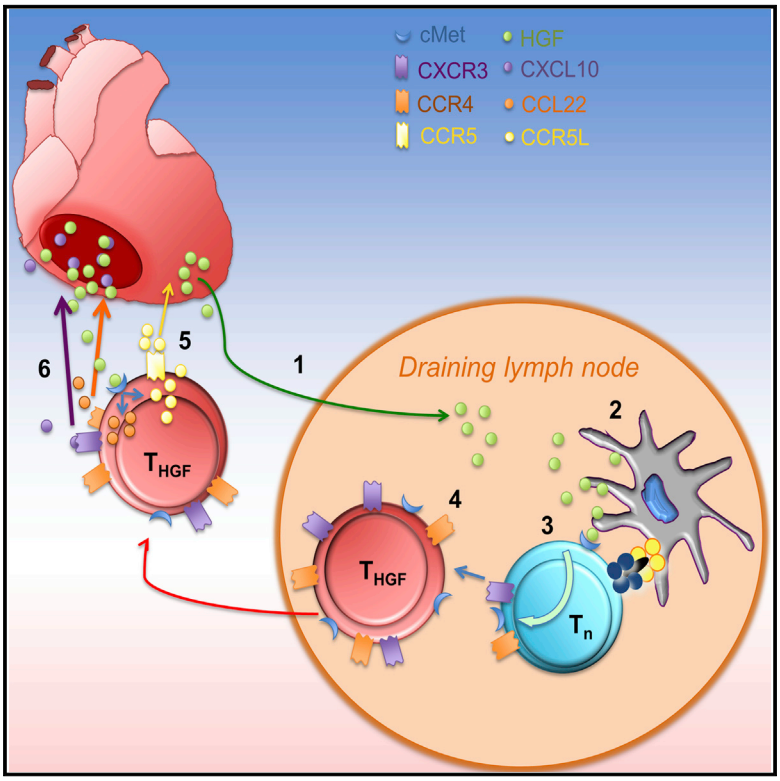
Graphical Abstract



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In Brief

Understanding the signals that imprint tissue-specific immune cell homing might result in novel approaches to target inflammatory disease. Marelli-Berg and colleagues report that hepatocyte growth factor binding to its receptor c-Met instructs T cell homing to the heart, and pharmacological blockade of c-Met prevents T-cell-mediated inflammation in the heart but not the skin.

Highlights

- HGF-induced c-Met signals during activation induce cardiotropic memory T cells
- Cardiotropic T cells express a specific molecular signature (c-Met$^+$CCR4$^+$CXCR3$^+$)
- By inducing an autocrine chemokine loop, c-Met also promotes T cell recruitment
- Blockade of the HGF-c-Met axis prevents heart, but not skin, allograft rejection

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Hepatocyte Growth Factor Receptor c-Met Instructs T Cell Cardiotropism and Promotes T Cell Migration to the Heart via Autocrine Chemokine Release

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SUMMARY

Effector-T-cell-mediated immunity depends on the efficient localization of antigen-primed lymphocytes to antigen-rich non-lymphoid tissue, which is facilitated by the expression of a unique set of "homing" receptors acquired by memory T cells. We report that engagement of the hepatocyte growth factor (HGF) receptor c-Met by heart-produced HGF during priming in the lymph nodes instructs T cell cardiotropism, which was associated with a specialized homing "signature" (c-Met$^+$CCR4$^+$CXCR3$^+$). c-Met signals facilitated T cell recruitment to the heart via the chemokine receptor CCR5 by inducing autocrine CCR5 ligand release. c-Met triggering was sufficient to support cardiotropic T cell recirculation, while CCR4 and CXCR3 sustained recruitment during heart inflammation. Transient pharmacological blockade of c-Met during T cell priming led to enhanced survival of heart, but not skin, allografts associated with impaired localization of alloreactive T cells to heart grafts. These findings suggest c-Met as a target for development of organ-selective immunosuppressive therapies.

INTRODUCTION

The migratory patterns of naive and memory T lymphocytes are well defined and vary depending on their activation, differentiation, and function. Through expression of a unique set of adhesion molecules and chemokine receptors, so-called "homing" receptors, distinct memory T cell populations are able to interact with organ-specific endothelial cells (ECs) and are recruited to distinct target tissues. For example, lymphocyte trafficking to the intestinal lamina propria is mediated by the interaction between intestinal mucosal addressin cell adhesion molecule-1 expressed by gut endothelium, and lymphocyte $\alpha_4\beta_7$ integrin. T cell migration to the skin is promoted by cutaneous lymphocyte-associated antigen (CLA) interaction with vascular E-selectin with the involvement of chemokine-receptor pairs CCR4-CCL17, CCR10-CCL27, and CCR8-CCL1 (McCully et al., 2012; Mora and von Andrian, 2006).

During antigen activation of naive T cells, the microenvironment of the draining lymphoid tissue provides vital cues for the acquisition of peripheral homing preference. For example, dendritic cells (DCs) derived from gut-associated lymphoid tissue (GALT) have been shown to instruct gut tropism, via the production of retinoic acid from vitamin A (Mora and von Andrian, 2006). Similarly, skin DCs produce the vitamin D3 metabolite $1,25(OH)_2D_3$, which favors the induction of skin-homing lymphocytes (Mora and von Andrian, 2006). With the exception of gut and skin, the molecular signature and imprinting mechanisms that define preferential homing to other organs are largely elusive. Most studies have focused on the role of adhesion and chemokine receptors, but the extensive overlap in the expression of these molecules by lymphocytes retrieved from different tissues has prevented the identification of tissue-selective area codes.

It has been recently proposed that soluble factors produced by the tissue itself might contribute to T cell homing imprinting. For example, skin-derived soluble factors have been shown to induce the skin homing receptor CCR8 in T cells (McCully et al., 2012). It is known that tissue-derived small molecules can be directly delivered to draining lymph nodes by anatomically defined conduits (Gretz et al., 2000). Some of these molecules are produced in a tissue-specific manner and can therefore define the topographic identity of the tissue where they are generated in the draining lymphoid tissue and possibly contribute to T cell homing imprinting (Campbell et al., 2003).

HGF is a pleiotropic cytokine that plays important functions in organ development, regeneration, and cancer by activating its tyrosine kinase receptor c-Met (Zhang and Vande Woude, 2003). A key feature of HGF is its ability to promote cell migration

(Zhang and Vande Woude, 2003). In immune processes, HGF can induce chemotactic responses by liver-derived human T lymphocytes (Adams et al., 1994) and maintains the differentiation of human hepatic sinusoidal endothelial cells, which specialize in lymphocyte recruitment to the liver (Lalor et al., 2006). The HGF-c-Met axis has also been implicated in the mobilization of cardiac progenitor cells (Leri et al., 2005). Based on these reports, we have investigated the effects of T cell exposure to HGF during activation on their migration and homing patterns. Our findings reveal that engagement of HGF-c-Met axis during priming induces ''heart-homing'' signature T cells and also indirectly mediates their recirculation in cardiac tissue.

RESULTS

Engagement of the HGF-c-Met Axis during Activation Induces Distinctive Migratory Features in T Cells

To investigate the effects of HGF on T cell migration, we first assessed the expression of the HGF receptor c-Met and the effects of its stimulation on T cell functions. Naive T (Tn) cells were found to express low amounts of c-Met (Figure 1A) and displayed modest but reproducible migratory responses to HGF (Figure 1B), which were prevented by pre-treatment with the c-Met-specific inhibitor PHA-665752 (Rodig and Shapiro, 2010) or by selective inhibition of c-Met in T cells with lentiviral-delivered, specific shRNAs (Figures S1A–S1C), which confirmed the specificity of the c-Met small molecule inhibitor. In contrast, c-Met inhibition did not affect T cell migration induced by the chemokines CCL19 and CCL21 (Figure 1C), which stimulate naive T cell migration via CCR7 (Okada et al., 1998). Naive T cells were subsequently activated for 7 days by plastic-bound anti-CD3 and anti-CD28 antibodies either in the presence (Thgf) or absence (Tnt) of HGF, which was added at a concentration of 15 ng/ml, a dose determined by preliminary experiments of HGF-induced chemokinesis by naive T cells. Exposure to HGF during activation did not affect T cell division or cytokine production (Figures S1F–S1I). In contrast, only Thgf cells upregulated c-Met (Figures 1D, 1E, and S1J–S1L) and maintained the ability to migrate to this cytokine, which was prevented by the c-Met inhibitor (Figures 1F and 1G). The inhibitor did not affect either spontaneous migration of Thgf or Tnt cells or migration of Thgf cells in response to the chemokine CCL5 (Figures 1G–1I), which induces migration of activated T cells (Okada et al., 1998). In addition, we observed that the presence of HGF increased adhesion of Thgf cells to VCAM-1 in a flow system (Figures S1M and S1N), suggesting that c-Met signaling might enhance integrin-mediated adhesion, as previously reported (Zhang and Vande Woude, 2003). c-Met-mediated migratory responses to HGF were mediated by an Akt-dependent pathway, which was not activated in c-Met-negative Tnt cells (Figures S2A–S2F). In addition, HGF-induced chemokinesis required signal transducer and activator of transcription 3 (STAT3) activity (Figures S2G and S2H), which has been reported to be dispensable for chemokine receptor signaling (Khabbazi et al., 2013).

We further investigated the migratory signature induced in T cells by HGF by comparing the phenotype of Thgf and Tnt cells. Expression of a number of surface molecules (Figure S2I) and migratory characteristics also revealed that, compared to Tnt cells, Thgf cells expressed higher amounts of the chemokine receptor CXCR3 (Figures 1J and 1K), which correlated with enhanced chemotactic responses to CXCL10 and increased responsiveness to CCL22, this occurring with various degrees of upregulation of its receptor CCR4 (Figures 1L–1O).

Overall, these data indicate that signals induced by the HGF-c-Met axis during T cell activation favor the emergence of a T cell population with distinctive migratory features, including c-Met expression and the ability to migrate in response to HGF via an Akt- and STAT3-dependent pathway.

c-Met Promotes T Cell Migration by Inducing Autocrine Chemokine Release

Although HGF has been defined as a chemoattractant by a number of studies (Zhang and Vande Woude, 2003), its cognate receptor c-Met does not belong to the G protein-coupled receptor family, the canonical trans-membrane proteins that transduce chemotactic activity. Many of them effect of c-Met signaling, including activation of integrins and intracellular mediators of cytoskeleton rearrangements (Zhang and Vande Woude, 2003), and our own observations that HGF induces chemokinesis and T cell adhesion (Figures 1, S1M, and S1N) closely resemble those of chemokine receptors. We therefore investigated in more depth the mechanism of HGF-induced chemokinesis. First, the effect of Thgf cell pretreatment with the GPCR-G_1 inhibitor pertussis toxin (PTX) on HGF-induced chemokinesis was investigated in vitro and in vivo. As shown in Figure 2A, PTX treatment abrogated Thgf cell migration elicited by HGF in transwell. In vivo, migration of adoptively transferred (i.v.) Thgf cells to the peritoneal cavity of mice, which received HGF (100 ng/mouse) i.p. 30 min earlier, was inhibited by T cell pretreatment with PTX (Figures 2B and 2C). These observations suggest that GPCR-Gαi signals are mediating HGF-induced chemokinesis, implying a crosstalk between c-Met and these receptors.

Although c-Met has been described to be physically associated to—and potentiate signaling of—other membrane receptors, including integrins (Trusolino et al., 2001), the possibility that c-Met might increase GPCR signaling is unlikely based on the observation that Thgf cells do not display enhanced responses to CCL5 (as previously shown in Figure 1I). In addition, HGF stimulation of a variety of cells has been shown to induce chemokine production and secretion (Kaibori et al., 2006; Matsuda-Hashii et al., 2004; Tu et al., 2009). We therefore investigated the alternative possibility that c-Met triggering might indirectly induce chemokinesis by stimulating the release of GPCR ligands by the T cell itself. Migration of Tnt cells—which do not migrate in response to HGF—to supernatants (SNs) from Thgf cells unstimulated or re-exposed to HGF overnight was measured in a transwell assay. As shown in Figure 2D, Tnt cells migrated in response to SNs from Thgf cells stimulated with HGF but not those kept in medium alone. As expected, HGF alone did not induce chemokinesis of Tnt cells, thus ruling out the possibility of chemokinesis induced by HGF contained in the SNs. These data suggest that c-Met might increase chemokinesis of Thgf cells by inducing the release of GPCR ligands.

T lymphocytes have been reported to secrete members of the β-chemokine family, particularly CCL4, which in turn can affect T cells in an autocrine loop (Casazza et al., 2009; Tanaka et al.,

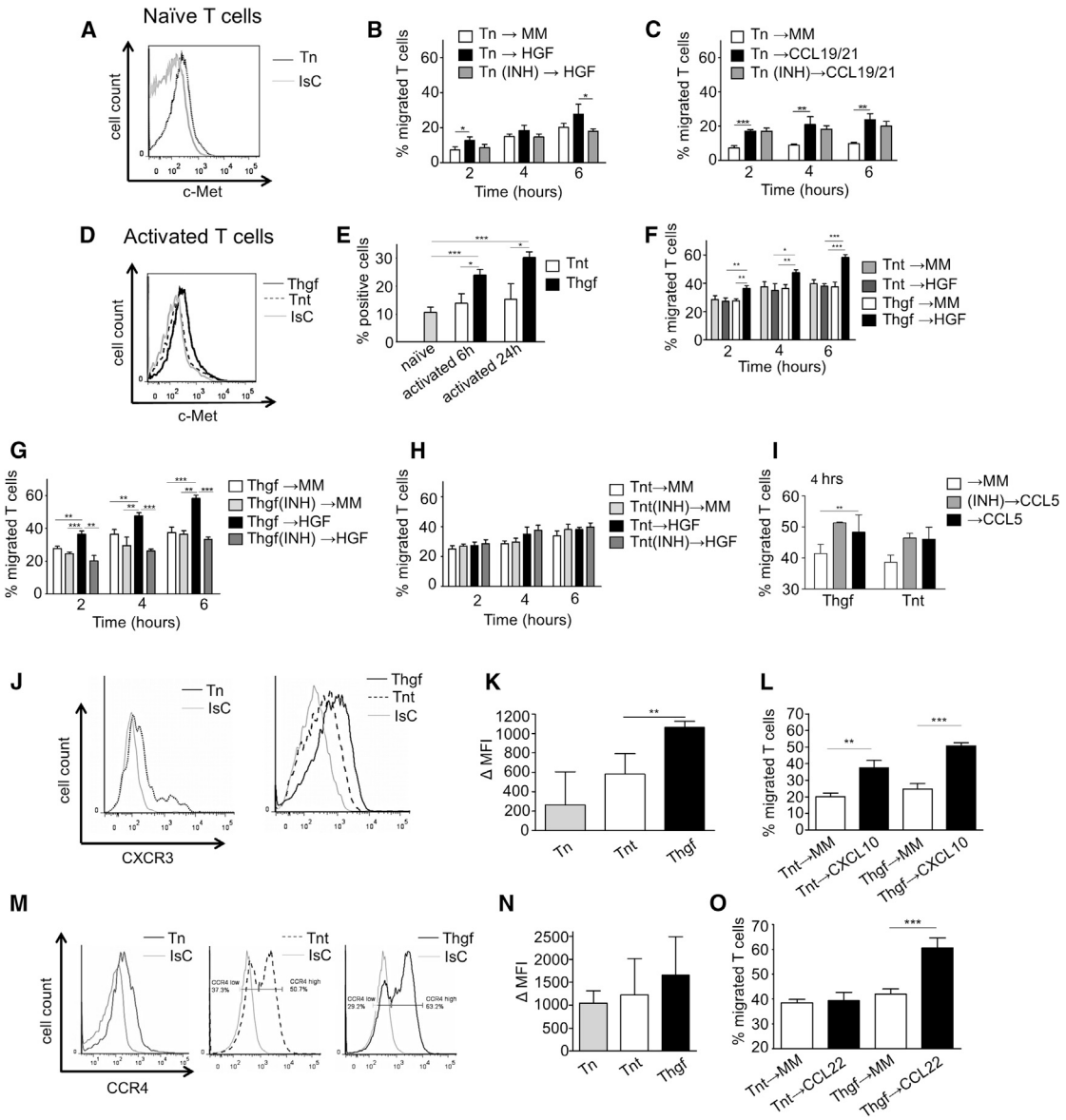

Figure 1. T Cell Activation in the Presence of HGF Induces a Distinct Migratory Phenotype

(A–C) Naive T (Tn) cells were isolated from lymph nodes of C57BL/6 mice. In (A), expression of the HGF receptor c-Met is shown. Naive T cell (10^6 cells/well) migration in response to HGF (15 ng/ml) (B) or to CCL19/21 (200 ng/ml) (C) was measured by transwell assay at the indicated time points. In some instances, T cells were treated with the c-Met inhibitor (INH) PHA-665752 (0.3 ng/ml) overnight at 37°C prior to the migration assay.

(D–I) Naive T cells were subsequently activated by plastic-bound anti-CD3 and anti-CD28 antibodies in the presence of HGF (Thgf) or in medium alone (Tnt). c-Met expression by Thgf and Tnt 24 hr after stimulation is shown in (D), and the mean differential MFI ($MFI_{cMet} - MFI_{IsC}$) in three experiments of identical design is shown in (E). T cells were harvested after a week of culture and their migratory responses to HGF (F) measured by transwell assays. Some T cells were pretreated with the c-Met inhibitor (INH) PHA-665752 (0.3 ng/ml) overnight at 37°C (G and H). Chemokinesis of Thgf and Tnt cells in response to CCL5 (100 ng/ml) is shown in (I).

(J–O) Expression of CXCR3 (J and K) and CCR4 (M and N) and response to their chemokine ligands CXCL10 and CCL22 by naive T, Thgf, and Tnt cells; (A) and (D) show representative histograms. Chemokinesis to CXCL10 and CCL22 (at 4 hr), is shown in (L) and (O), respectively. Data are represented as the differential mean fluorescence intensity from isotype control (ΔMFI) (K and N) or mean percentage T cell migration (L and O) measured in three independent experiments of identical design (±SD).

1998). We therefore investigated the production of CCL4 by Thgf cells either stimulated with HGF overnight or unstimulated, and we observed an increase in intracellular CCL4 contained in large vesicles in Thgf cells after stimulation with HGF (Figures 2E–2G). Antibody neutralization of CCL4 significantly inhibited migration of Tnt cells in response to SNs obtained from Thgf cells re-stimulated with HGF (Figure 2H). Further, CCL4 neutralization inhibited Thgf cell adhesion to VCAM-1 in a flow chamber system (Figure 2I), suggesting that c-Met signals not only promote CCL4 production, but also induce its release from the T cell. This,

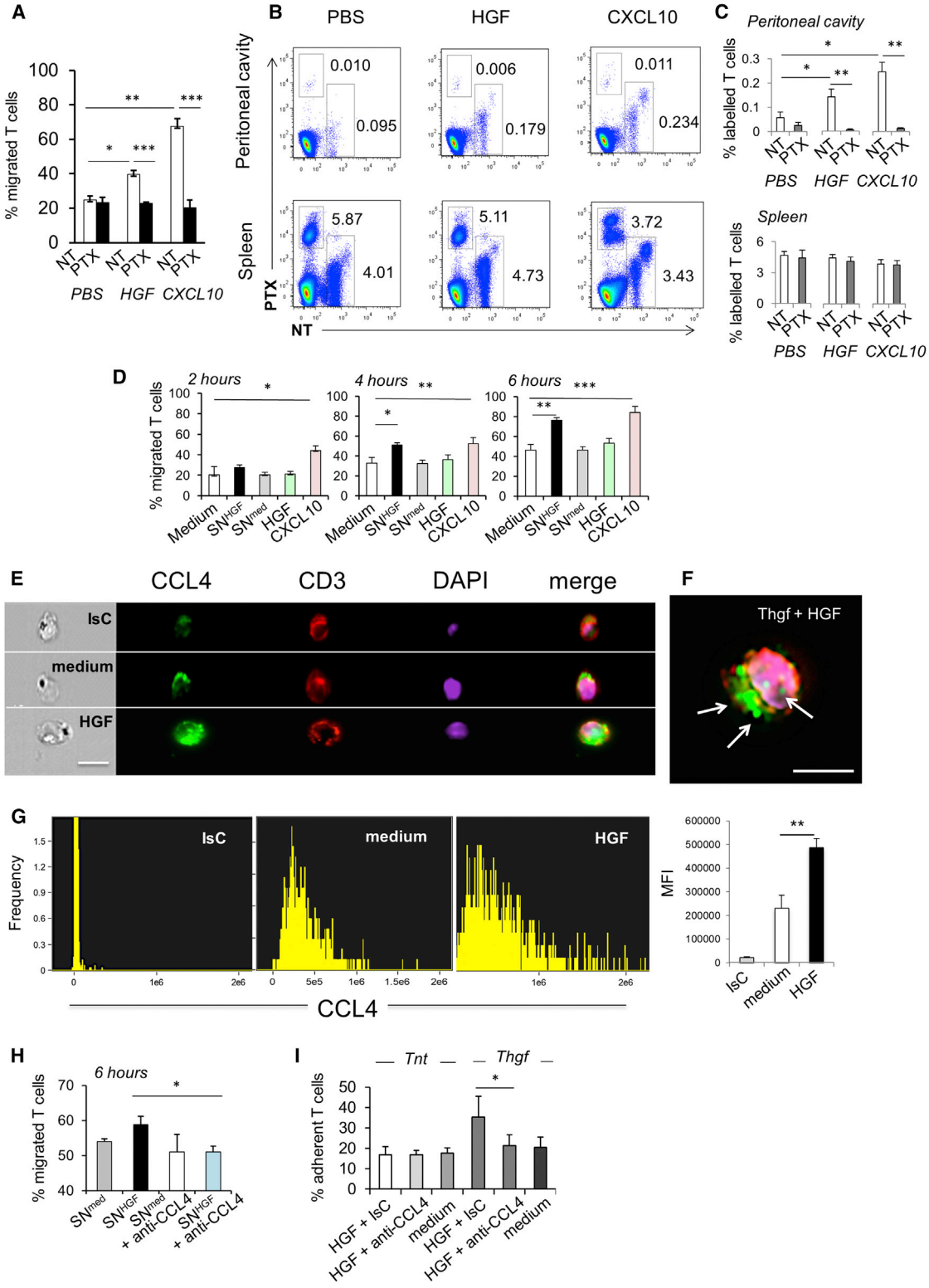

Figure 2. Mechanisms of c-Met-Induced Migration

(A–C) Thgf cell migration in response to HGF (15 ng/ml) (B) or to CXCL10 (200 ng/ml) (C) was measured by transwell assays in vitro (A) and by assessing their migration to i.p.-administered HGF (100 ng/mouse) and CXCL10 (1,200 ng/mouse) upon adoptive transfer into syngeneic recipients in vivo (B and C). In some instances, T cells were treated with the GPCR (G1) inhibitor pertussis toxin (PTX, 0.1 ng/ml) for 1 hr at 37°C prior to use.

(legend continued on next page)

in turn, could enhance adhesion and migration via GPCR stimulation.

Similarly, a number of other chemokines that have been implicated in heart transplant rejection (Schnickel et al., 2008; Hüser et al., 2005) were detected in Thgf cells including other CCR5 ligands (CCL3, CCL5) and the CCR4 ligands CCL22 and CCL17, which are increased by c-Met signals (except CCL17) (Figure S3).

The molecular mechanisms of c-Met-induced CXCR3 and CCR4 expression and responsiveness were further investigated by a series of in vitro experiments, which revealed that STAT-3 activation by c-Met is instrumental for the increased expression and responsiveness of these chemokine receptors by Thgf cells (Figure S4).

HGF Is Produced in the Cardiac Parenchyma and Instructs T Cell Cardiotropism

In addition to its canonical source, the liver, HGF has been previously invoked to be present in heart tissue where it is required for the mobilization of cardiac stem cells (Leri et al., 2005). In animal models, HGF is transiently produced in the heart upon myocardial ischemic injury (Ono et al., 1997), which in humans can be followed by autoimmune responses against cardiac tissue.

To identify physiologic sites of T cell priming in the presence of HGF, we analyzed and compared the production of HGF mRNA by a number of murine tissues, including liver and heart, both in steady-state conditions and 24 hr after the injection of 100 μg LPS. As shown in Figures 3A–3C, in addition to the liver, heart parenchyma was found to express HGF mRNA, particularly in inflammatory settings provoked by endotoxin. Immunohistochemical analysis confirmed that this cytokine is constitutively present in low amounts in cardiac tissue, and it is upregulated by inflammatory stimuli induced by LPS (Figure 3C). In addition, mRNA transcripts for the chemokine CXCL10, but not CCL4 or CCR4 ligands, were found induced in inflamed cardiac tissue (Figure 3D).

Given the ability of HGF to affect the reprogramming of migratory receptors in T cells, we subsequently sought to investigate its distribution within the cardiac parenchyma and heart-draining LNs. Analysis of murine cardiac tissue and draining LNs confirmed the presence of endogenous HGF in both tissues where it co-localized with $CD11c^+$ and $CD31^+$ cells (Figures S5A and S5B), suggesting that locally produced HGF is displayed by DCs and the vasculature—probably via binding to glycosaminoglycans (GAGS), which are used by both cell types to immobilize chemokines and growth factors (Bao et al., 2010; Hartmann et al., 1998; Santiago et al., 2012). Thus, it is likely that HGF produced in the heart parenchyma reaches the draining LNs both by direct transfer (to HEV, possibly via conduits) and bound to migrating DCs.

Based on the observations above and the finding that HGF imprints a unique migratory phenotype in T cells, we next sought to establish whether this phenotype correlates with a specific trafficking pattern of T cells activated in the presence of HGF. First, in vitro activated Thgf or Tnt cells were labeled and injected intravenously into syngeneic recipients and their localization to a variety of tissues was assessed by wide-field fluorescence microscopy. As shown in Figure 3E, unlike control Tnt cells, Thgf cells preferentially localized to the heart and the liver compared to other organs, suggesting that HGF contributes to imprinting these homing patterns in T cells. In addition, pre-treatment of Thgf cells with the c-Met inhibitor prevented their localization to the heart, indicating that HGF not only instructs, but also mediates, T cell cardiotropism. In contrast, c-Met inhibition did not affect Thgf cell localization to the liver, suggesting that additional molecular interactions support T cell migration to this organ (Lalor et al., 2002; Shetty et al., 2011). Similar results were obtained upon selective inhibition of c-Met in T cells with lentiviral-delivered, specific shRNAs (Figures S1D and S1E).

Further experiments confirmed that in vivo priming of T cells in the presence of HGF, although not affecting their proliferation and cytokine production (Figures S5C–S5H), induced upregulation of c-Met, CCR4, and CXCR3 expression and function (Figures S5I–S5O), leading to the acquisition of cardiotropism (Figures S6A and S6B).

c-Met, CXCR3, and CCR4 Differentially Contribute to T Cell Migration to the Heart

Both CXCR3 and CCR4 have been shown to promote the development of T cell infiltration of cardiac tissue in heart allograft rejection (Hancock et al., 2001; Hüser et al., 2005). To establish the relative role of c-Met, CXCR3, and CCR4 in mediating T cell homing to the heart, we performed a series of experiment in which WT Thgf cells were co-injected with differentially labeled Thgf cells generated from CCR4- or CXCR3-deficient mice in saline- or LPS-treated mice. Some CCR4KO or CXCR3KO T cells were also treated with a blocking anti-CXCR3 antibody or CCR4 inhibitor, respectively, prior to adoptive transfer. As shown in Figures 4A and 4C, Thgf cell migration to the heart of saline-treated mice was not affected by lack of either CCR4 or CXCR3. In contrast, migration of CCR4- or CXCR3-deficient Thgf cell to the heart of LPS-treated mice was similarly impaired compared to that of WT Thgf cells (Figures 4B and 4D). Additional blockade of either receptor did not further inhibit T cell localization. Co-injected T cells were retrieved in similar numbers in the spleen of recipients, independently of the experimental conditions. These data indicate that both CXCR3- and

(D) Supernatant was harvested from Thgf cells stimulated for 6 hr with HGF (SN^{HGF}) or left in serum-free medium (SN^{med}) and placed in the bottom chamber of 5 μm pore transwells. Migration of Tnt cells to the SNs and indicated controls was measured over the next 6 hr. In parallel, expression of the chemokine CCL4 by Thgf cells stimulated with HGF or unstimulated was measured by ImageStream analysis.

(E–G) Representative images and histograms. In (F), a representative image of Thgf cells stimulated with HGF highlighting the intracellular compartmentalization of CCL4. Scale bar represents 10 μm.

(H) Migration of Tnt cells to SN^{HGF} or SN^{med} in the presence or absence of a neutralizing anti-CCL4 antibody.

(I) Tnt and Thgf cells were perfused over recombinant VCAM-1-Fc-coated flow chambers. Where indicated, a neutralizing anti-CCL4 antibody or isotype control was added to the perfusion medium. T cell adhesion was quantified as described in Supplemental Experimental Procedures.

Data are represented as the mean MFI (A, D, E, H) or mean percentage migration (B, F) or adhesion (I) measured in three independent experiments of identical design ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

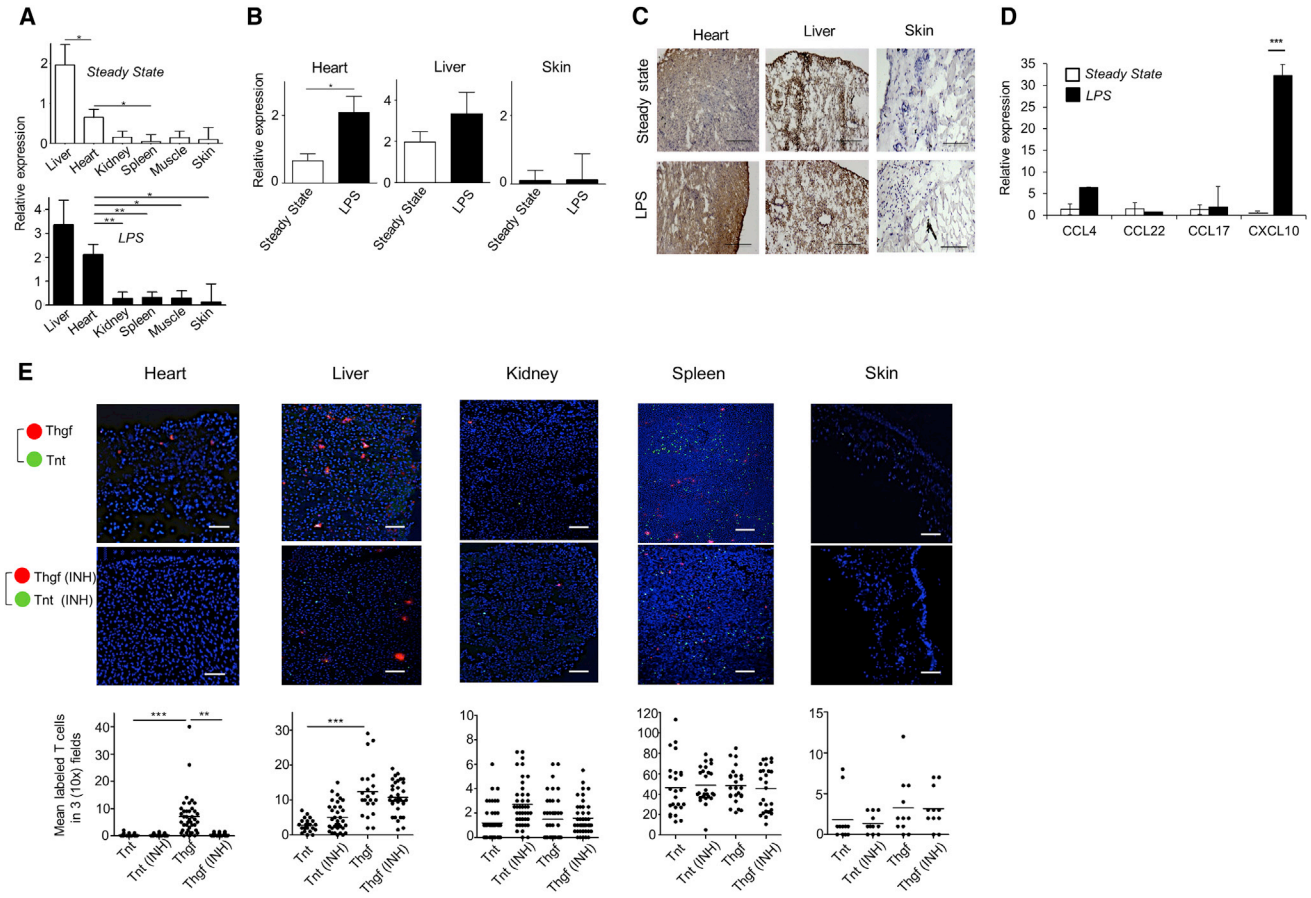

Figure 3. HGF Is Produced in the Heart and Induces T Cell Cardiotropism

(A) Relative HGF mRNA after intraperitoneal injection of saline solution (steady-state) or LPS (100 μg/mouse) as assessed by qPCR in the indicated tissues and normalized to a housekeeper gene (Gapdh).

(B) Comparison of mRNA expression in the heart, liver, and skin tissues from untreated and LPS-treated mice. Data show the mean of three independent experiments (±SEM).

(C) 5 μm-thick frozen sections of either steady-state (SS) or inflamed tissue (LPS) were incubated overnight with an anti-HGF antibody at 15 μg/ml in 4°C. Subsequently, a biotinylated secondary antibody was applied, and HSS-HRP/DAB system used for color development. Sections were counterstained with hematoxylin to visualize nuclei (magnification 10×, scale bars represent 100 μm).

(D) Relative mRNA expression of the indicated chemokines and after intraperitoneal injection of saline (steady-state) or LPS. Data show the mean of three independent experiments (±SEM).

(E) In vitro activated Thgf and Tnt cells were labeled with PKH26 (5 μM) (red) and CFSE (4 μM) (green), respectively, and co-injected (10^7/mouse) i.v. into syngeneic C57BL/6 recipients. Some Thgf and Tnt cells were pre-treated with the c-Met inhibitor (INH) PHA-665752 overnight at 0.3 ng/ml/10^6 cells, in 37°C. Localization of labeled T cells in the various tissues was analyzed 24 hr later by wide-field fluorescence microscopy. Representative images are shown (upper images show co-injected Thgf and Tnt cells and lower images show co-injected Thgf and inhibitor-treated Thgf cells). The mean number of cells detected in ten randomly selected fields (10×-magnified) from at least three sections of tissue samples from each recipient (±SD) is shown below each set of images. Scale bars represent 50 μm. **$p < 0.01$, ***$p < 0.005$.

CCR4-mediated signals are required for T cell infiltration of the heart in inflammatory but not steady-state conditions. Experiments conducted with WT Thgf cells treated with either the CCR4 inhibitor or the CXCR3 blocking antibody prior to adoptive transfer confirmed that both receptors are required for entry of the T cells in the heart of LPS-treated animals (Figures S6C–S6F). In vitro, inhibition of either or both CXCR3 and CCR4 elicited a similar reduction in Thgf cell migration to a combination of CXCL10 and CCL22, further suggesting a crosstalk between the two receptors (Figure S6G).

As expected, PTX pre-treatment inhibited Thgf cell migration to the heart of non-inflamed mice (Figure 4E) consistent with our observation that c-Met signals support their recirculation to cardiac tissue via its effects on GPCRs. Untreated recipients were used in these experiments to minimize Thgf cell recruitment via CCL22 and CXCL10 as observed in LPS-treated recipient mice.

Given our previous observation that Thgf cells produce a number of CCR5 ligands, we sought to confirm the role of these chemokines in mediating T cell cardiotropism by generating CCR5-deficient Thgf cells and monitoring their recruitment to the heart both in non-inflamed and LPS-treated mice. As shown in Figures 4F and 4G, lack of CCR5 expression inhibited Thgf cell migration to the heart both in steady-state and

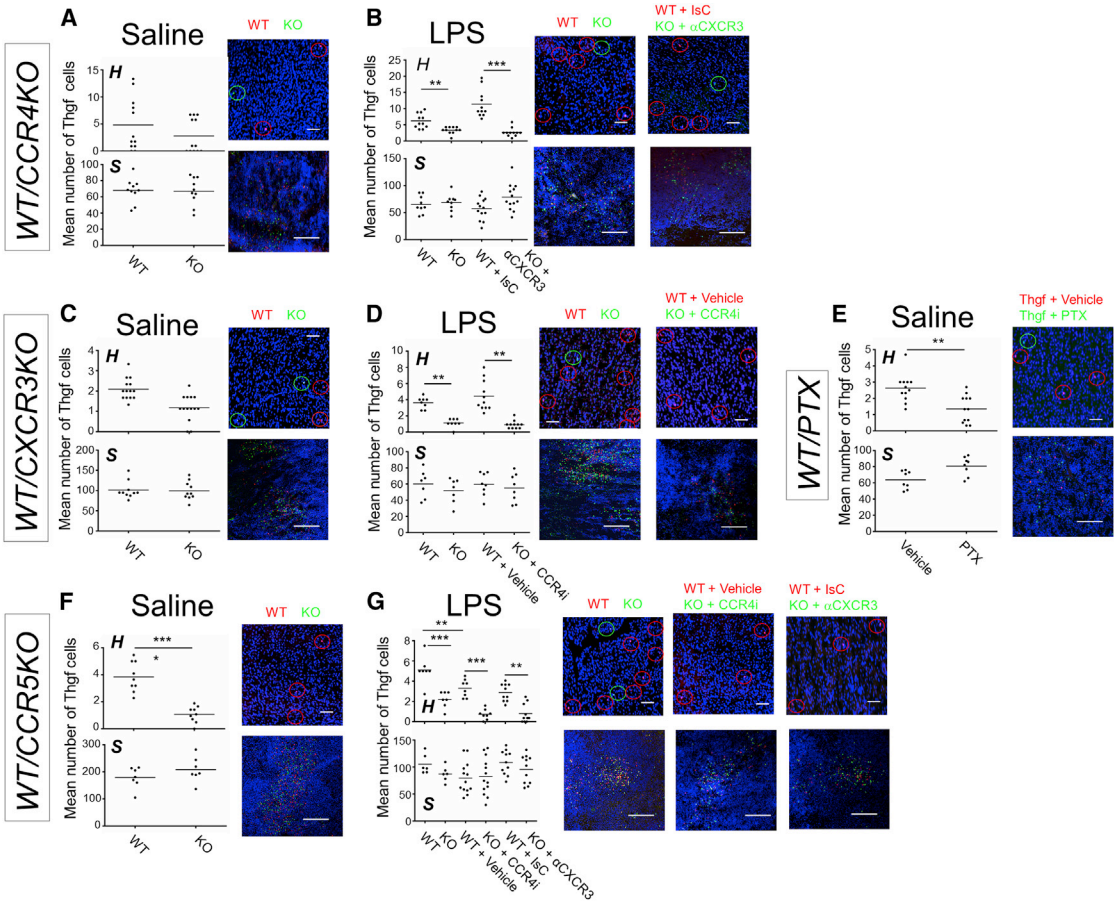

Figure 4. c-Met, CXCR3, or CCR4 Differentially Contribute to T Cell Cardiotopism in Steady-State Conditions and during Inflammation

(A–D) In vitro activated Thgf cells from WT and CCR4 (A and B) or CXCR3 (C and D) mice were differentially labeled and co-injected (10^7/mouse) i.v. into syngeneic C57BL/6 recipients that had received either saline solution or LPS (as indicated in the panels) i.p. 48 hr earlier. Some CCR4- or CXCR3-deficient Thgf cells were treated with an anti-CXCR3 antibody or a CCR4 inhibitor (B and D), respectively, prior to co-injection with untreated cells in LPS-treated recipients.

(E) WT Thgf cells were also treated with PTX (0.1 ng/ml) for 1 hr at 37°C, washed in PBS, labeled with CFSE, and co-injected with untreated PKH26-labeled Thgf cells.

(F and G) Thgf cells from WT and CCR5 mice were co-injected (10^7/mouse) i.v. into syngeneic C57BL/6 recipients that had received either saline solution (F) or LPS (G) i.p. 48 hr earlier. Some CCR5-deficient Thgf cells were treated with an anti-CXCR3 antibody or a CCR4 inhibitor (G) prior to co-injection with untreated cells in LPS-treated recipients.

Localization of labeled T cells in the heart was analyzed 24 hr later by wide-field fluorescence microscopy. T cell localization in the spleen was also assessed to ensure that similar cell numbers had been injected. Representative images are shown in the right side of each panel, with label coding indicated on top. The mean number of cells detected in ten randomly selected fields (10×-magnified) from at least three sections of tissue samples from each recipient (±SD) is shown in the left side of each panel. n = 3, N = 2. Scale bars represent 100 μm. **p < 0.01, ***p < 0.005, ****p < 0.001.

inflammatory conditions. However, CCR5-deficient Thgf cells access to heart tissue of LPS-treated mice was further inhibited by CCR4 or CXCR3 blockade, suggesting that c-Met-mediated production and release of CCR5 ligands provide additional stimuli for the recruitment of cardiotropic T cells during inflammation.

c-Met$^+$CCR4$^+$ Memory T Cells Preferentially Recirculate to the Heart

Having established that HGF-induced cardiotropism is associated with the expression of c-Met and CCR4 by memory T cells, we sought to investigate the physiological relevance of this molecular area code by assessing the phenotype of T cells retrieved from cardiac tissue in steady-state conditions and during heart-specific immune responses. To this aim, T cells were retrieved from B6Kd-derived heart allograft and the native heart of C57BL/6 recipients and analyzed for the expression of c-Met and CCR4. This strain combination gives rise to CD8$^+$ T cell-mediated direct alloresponses against intact K^d molecules and CD4$^+$ T-cell-mediated indirect alloresponses against a single K^d-derived peptide in the context of H2^b molecules (Honjo et al., 2004). As a control, CD44^{hi} T cells retrieved from the spleen were also examined. As shown in Figure 5, T cells from native and transplanted heart expressed high amounts of c-Met (Figures 5A and 5B) and CCR4 (Figures 5C and 5D) compared to memory T cells in the spleen. In addition, a high proportion of heart-resident T cells co-expressed both receptors, including in the lymph nodes draining native hearts (Figures 5E and 5F).

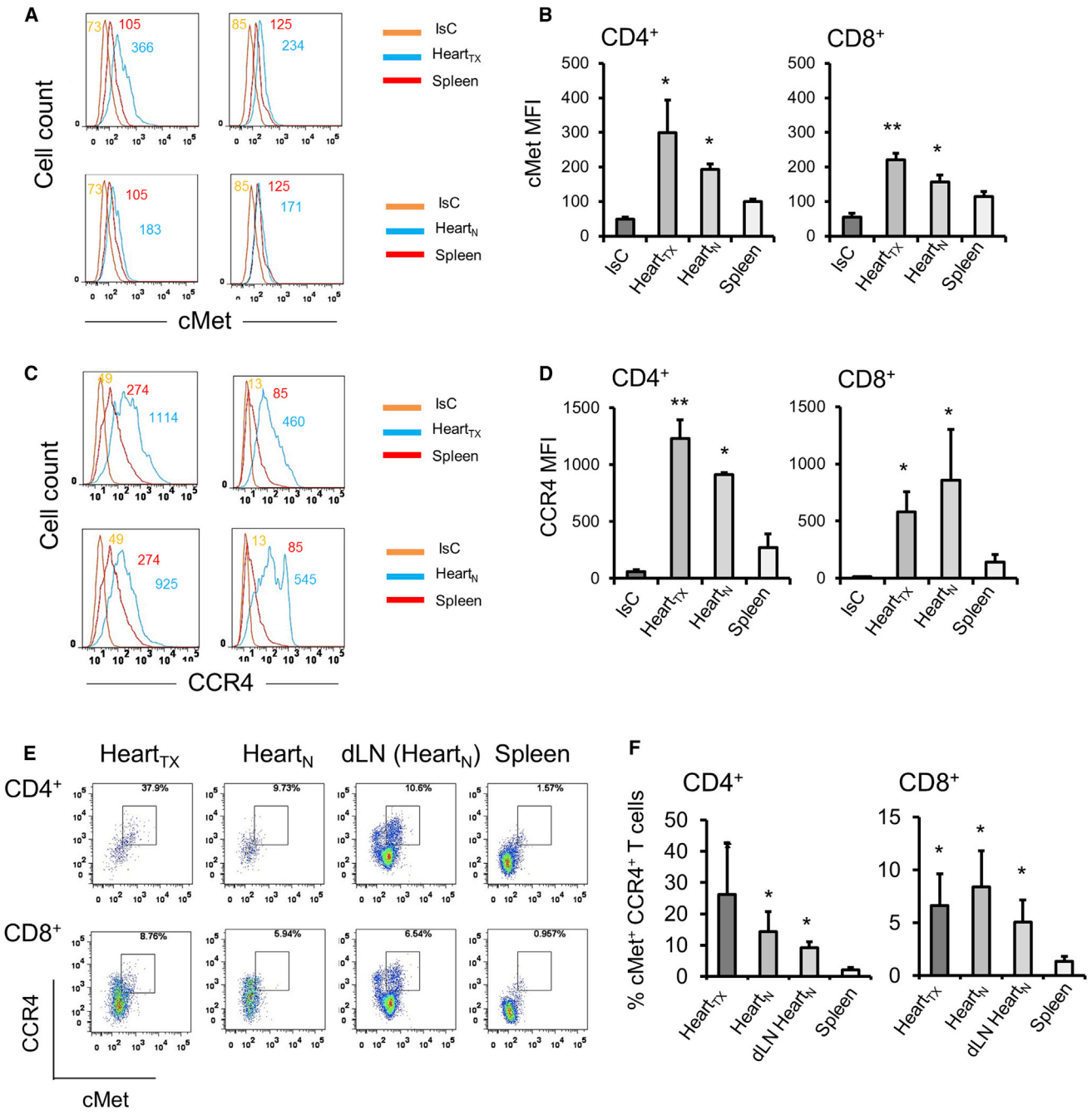

Figure 5. T Cells that Localize to Cardiac Tissue Express c-Met and CCR4

(A–D) T cells were obtained by enzymatic digestion of B6Kd heart allograft 2 weeks after transplantation into C57BL/6 mice (Heart$_{TX}$), and from C57BL/6 native hearts (Heart$_N$). As a control, splenic T cells from naive C57BL/6 mice were used. Memory T cells were identified by gating on the CD44^{hi} T lymphocyte population. Expression of the HGF receptor c-Met by CD44^{hi} CD4$^+$ and CD8$^+$ T lymphocytes from all populations is shown in (A) (representative histograms). Expression of CCR4 by the same T cell populations is shown in (C). The mean MFI of c-Met and CCR4 from three samples are shown in (B) and (D), respectively.

(E) Dot plots depict co-expression of c-Met and CCR4 by T cells populations isolated from B6Kd heart allograft 2 weeks after transplantation into C57BL/6 mice (Heart$_{TX}$) and from native recipients' hearts (Heart$_N$) and their draining lymph nodes (dLN Heart$_N$), and from control spleens.

(F) Mean percentage of double-positive CD44^{hi} CD4$^+$ and CD8$^+$ T cells (±SD, n = 3, N = 2).

*p < 0.05, **p < 0.01.

CXCR3 expression was similar in all the T cell populations analyzed (not shown), possibly due to the relatively late time point of T cell harvesting.

These observations indicate that c-Met- and CCR4-expressing T cells are enriched in heart tissue both in steady-state and inflammatory conditions, suggesting that expression of these

receptors is instrumental for physiological T cell recirculation to the heart and associated lymphoid tissue.

c-Met Blockade Prevents Rejection of Heart Allografts by Promoting Selective Topographic Ignorance

To establish the selectivity of T cell homing imprinting by HGF, we then compared the effect of pharmacological blockade of c-Met on the survival of B6Kd-derived heart or skin grafts transplanted in C57BL/6 recipients. All recipients received a depleting anti-CD8 antibody prior to transplantation (125 μg/mouse i.p. on day −1) (Tsang et al., 2008) to remove potential pre-existing cross-reactive memory alloresponses and ensure that the ensuing response against K^d alloantigens is derived from naive T cells (i.e., still susceptible to HGF effects). $CD8^+$ T cell numbers recovered by 72 hr after depletion, as previously described (Tsang et al., 2008). Some recipients were treated daily with the c-Met inhibitor PHA-665752 i.p. (500 μg/mouse) for 2 weeks after transplantation. This schedule was designed in order to target the priming phase of immunity, when the acquisition of homing properties is known to occur, and to avoid affecting the beneficial effects of HGF on heart tissue, including tissue repair and vascularization (Madonna et al., 2012). Control groups received saline solution with vehicle alone.

As shown in Figures 6A and 6B, pharmacological c-Met blockade led to long-term heart allograft survival in 75% of the recipients compared to controls. In contrast, skin allograft survival was not affected, indicating that c-Met inhibition selectively targets T cell responses against heart tissue irrespective of antigen specificity.

Histological analysis of HE-stained grafts and native heart revealed severe inflammatory lesions in the grafts of recipients treated with vehicle alone, which were attenuated in PHA-665752-treated recipients (Figures 6C and 6D).

Co-staining of elastine and smooth muscle cells (SMCs) alpha actin revealed signs of vasculopathy (narrowing of the lumen and perivascular proliferation of SMCs) in vehicle-treated recipients, which were abrogated by the administration of the c-Met inhibitor (Figures 6C and 6E). Syngeneic graft and native hearts were free of disease.

Graft infiltration by $CD4^+$ and $CD8^+$ T cells was assessed by immunostaining with anti-CD4 or anti-CD8 primary antibodies followed by AlexaFluor488-conjugated secondary antibody. As shown in Figures 6F and 6G, T cell infiltration of the hearts of PHA-665752-treated mice was reduced compared with that observed in vehicle-treated recipients, particularly in the $CD8^+$ T cell subset, which is in fact responsible for direct allorecognition of K^d MHC class I molecules in this combination (Honjo et al., 2004).

T cells separately isolated from heart graft draining or non-draining lymph nodes and the spleen of heart allograft recipients at the time of rejection or 100 days after transplantation displayed strong proliferative responses to B6Kd splenocytes (direct alloresponses; Figure 6H) and to the K^d peptide presented by syngeneic splenocytes ex vivo (indirect alloresponses; Figure 6I) irrespective of the treatment with the c-Met inhibitor and the occurrence of rejection, suggesting that c-Met blockade does not affect T cell priming nor leads to the induction of tolerance. Rather, as confirmed by substantial inhibition of T cell infiltration of heart grafts from inhibitor-treated recipients, c-Met blockade impaired the ability of primed T cells to localize to the heart.

Liver-derived human T cells have been shown to migrate in response to HGF in vitro (Adams et al., 1994). We therefore sought to investigate whether the HGF-mediated induction of cardiotropism in T cells is operational in humans. To this aim, we developed a model of human heart xenograft transplanted subcutaneously in SCID/Beige mice. This model has been previously used to identify molecular patterns of migration to human synovial tissue, lymph nodes, and skin (Garrood et al., 2009). Within 2–3 weeks, human heart xenografts become vascularized by mouse vessels, which formed direct anastomosis with the intact human vasculature (Figures S7A–S7C). Human naive T cells ($CD45RO^-$), isolated by immunomagnetic negative selection, were activated with plastic-bound anti-CD3 and anti-CD28 antibodies in the presence or absence of HGF. Because commercially available anti-human c-Met antibodies are not suitable for flow cytometric analysis (Adams et al., 1994), binding of HGF to human naive, Thgf, and Tnt cells was assessed by incubation with DyLight-488-labeled HGF, which revealed low binding of HGF on naive T cells, which selectively increased in Thgf cells, where it appeared to co-localize on the cell surface with the integrin LFA-1 (Figures S7D–S7F). Thgf and Tnt cells were then labeled with distinct fluorochromes and injected i.v. into SCID/Beige recipients of a double xenograft of human heart and skin tissue transplanted 4 weeks earlier. Grafts and spleen were retrieved 48 hr later and cell localization analyzed by wide-field fluorescence microscopy. As shown in Figures 7A–7F, Thgf but not Tnt cells were enriched in heart but not skin xenografts. Thgf cell migration to heart tissue was inhibited by pretreatment with the c-Met inhibitor. Although not significant, increased numbers of Thgf cells were retrieved in the recipients' spleen, but the c-Met inhibitor did not alter this effect (Figures 7G–7I). Overall, these findings suggest that the induction of T cell cardiotropism via the HGF-c-Met axis is also operational in the human system.

DISCUSSION

The present study describes a novel molecular signature of T cell cardiotropism, which is imprinted in T cells primed in the presence of the cytokine HGF and is characterized by c-Met expression and enhanced CCL22- and CXCL10-induced chemokinesis. In addition to imprinting T cells with cardiotropism, HGF also indirectly promotes migration of T cells to the heart.

Based on our observations, HGF produced by the myocardium is transported to the heart-draining LN either passively or bound to DCs. HGF has high affinity for heparan sulfate proteoglycans that sequester the ligand and probably localize the action of HGF-SF only to the surrounding c-Met-expressing cells. We noticed that ectopically administered HGF localizes only on CD11c-expressing cells in skin-draining LNs, suggesting a specialization of HEV in heart-associated lymphoid tissue. Thus, DC-bound HGF is probably responsible for the imprinting of cardiotropism during T cell priming, whereas HGF associated to the vasculature probably contributes to cardiotropic memory T cell recruitment during inflammation.

T cell recruitment to the heart by locally produced HGF appears to be a dominant mechanism during constitutive T cell

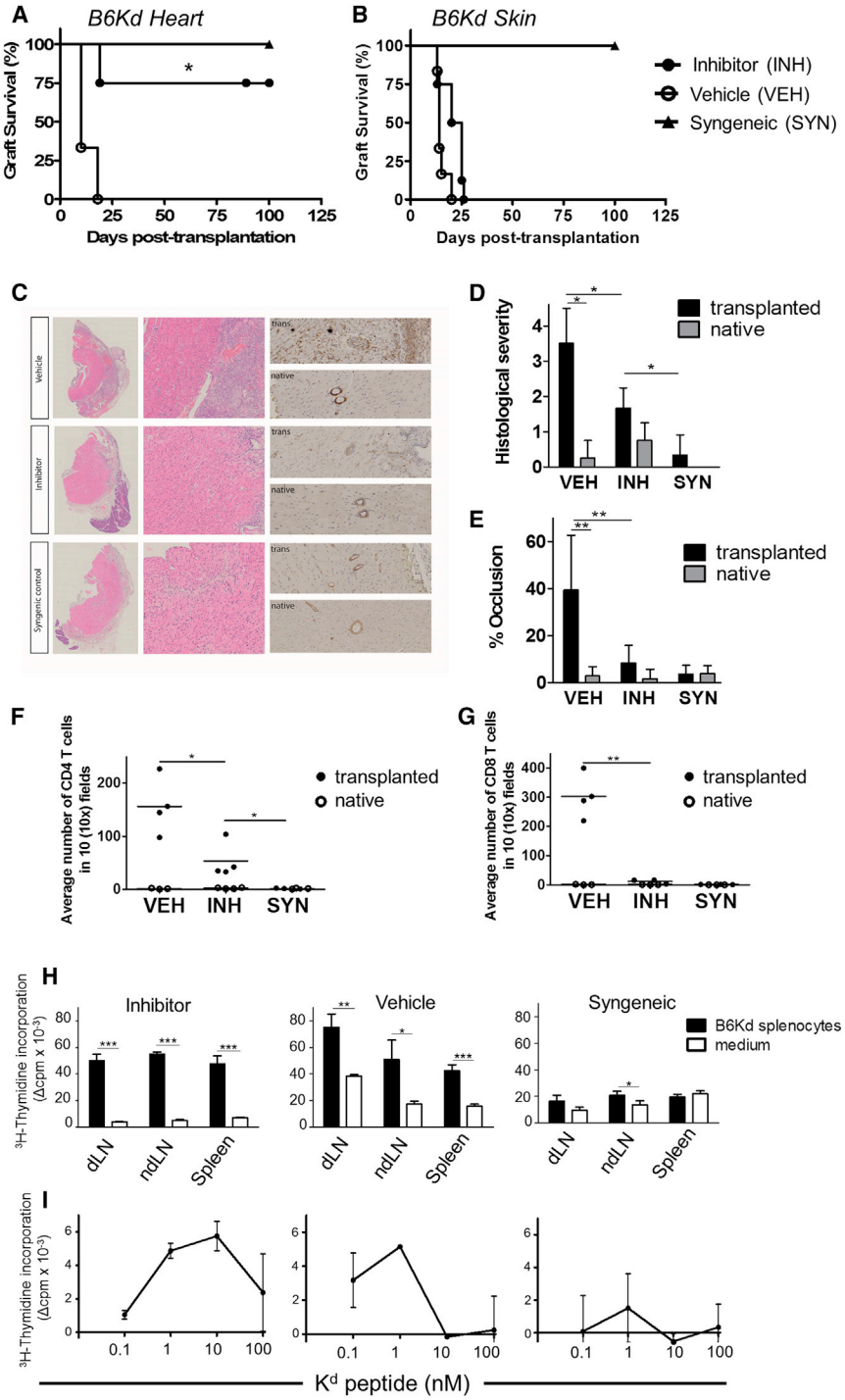

Figure 6. Inhibition of the HGF-c-Met Axis Prevents T-Cell-Mediated Heart but Not Skin Allograft Rejection

(A and B) C57BL/6 mice received either a B6Kd heart or skin graft 24 hr after i.v. administration of a depleting anti-CD8 antibody (125 μg/mouse). Some recipients were treated with the c-Met inhibitor PHA-665752 i.p. (25 mg/kg daily) for 2 weeks after transplantation. Graft survival in heart (A) and skin (B) is shown. N = 2, n = 4.

(C) Both transplanted and native hearts were harvested after rejection or 100 days after transplantation. At least five sections from each heart was processed and stained with hematoxilin/eosin. Left: representative tissue images were scanned with an Olympus in a brightfield mode. Right: Tissue sections underwent immunoperoxidase staining for smooth muscle cells using rabbit monoclonal antibody to mouse alpha-actin, followed by Miller's elastin stain, and counterstained with hematoxylin. Luminal occlusion was evaluated by tracing the cross-section of each vessel's internal elastic lamina and lumen using software in two transverse sections per graft. Each panel shows a representative tissue image. Magnification: 10×.

(D) Each sample was subjected to single blinded pathologic assessment, as described in Experimental Procedures. The mean histopathological scores ± SD of transplanted hearts stained with HE were observed in at least three samples obtained from each recipient (at least three animals/group).

(E) The mean percentage luminal occlusion ± SD was observed in three samples obtained from each recipient (at least three animals/group).

(F and G) Tissue sections were stained with primary purified anti-CD4 or anti-CD8 antibodies and secondary AlexaFluor488 antibody. Shown are the mean $CD4^+$ (F) and $CD8^+$ (G) T cell infiltration observed in three samples obtained from each recipient (at least three animals/group).

(H and I) T cells from draining and non-draining lymph nodes and the spleen of recipient animal were harvested at the time of rejection or 100 days after transplantation. T cells were incubated with B6Kd splenocytes for 48 hr, followed by pulsing with $[^3H]$ thymidine to assess T cell proliferation. Shown are representative proliferation assays (mean of triplicate cultures ± SD) of T cells from C57BL/6 recipients of B6Kd hearts treated with the c-Met inhibitor or vehicle alone (H) or recipient of syngeneic hearts to B6Kd splenocytes or to C57BL/6 splenocytes loaded with the Kd peptide epitope (I) (±SD).

*p < 0.05, **p < 0.005, ***p < 0.001.

recirculation and immunosurveillance, as suggested by the observation that c-Met inhibition abrogated T cell localization to cardiac tissue in steady-state conditions. HGF-mediated recruitment occurred indirectly via the induction of chemokine secretion by c-Met-expressing T cells. A similar mechanism has been described in T cell migration induced by IL-6 (McLoughlin et al., 2005). It is possible that this mechanism also applies to a number of cytokines and growth factors, which have been reported to elicit chemotactic responses by T cells.

During inflammation, the chemokine receptors CCR4 and CXCR3 enhanced T cell recruitment to the heart, as has been previously shown in heart transplantation settings (Hancock et al., 2001; Hüser et al., 2005).

Co-expression of the CCR4 and CXCR3 chemokine receptors and high responsiveness to their cognate ligands, which are

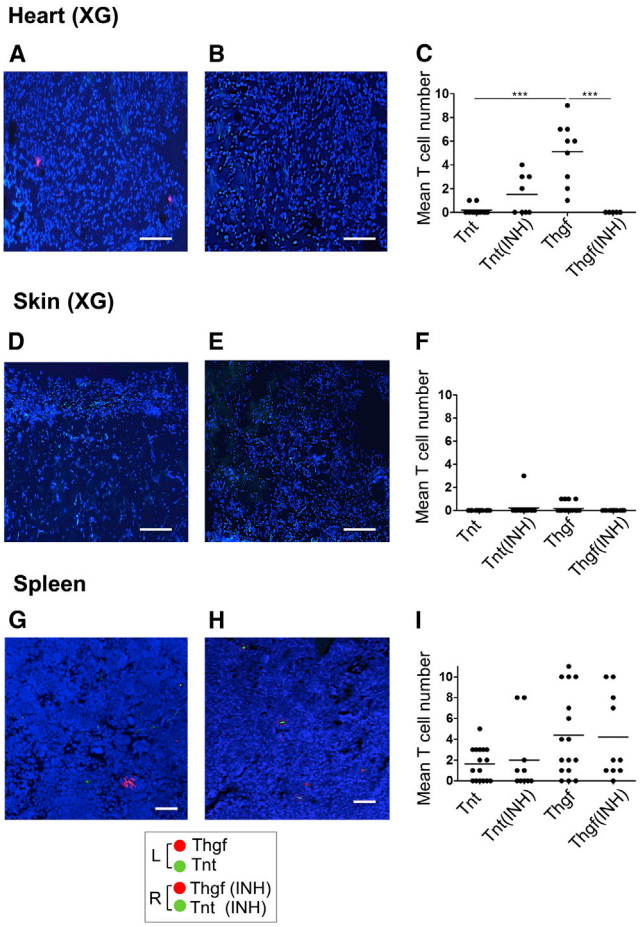

Figure 7. The HGF-c-Met Axis of T Cell Homing to the Heart Is Operational in the Human System

Human heart and skin tissue (xenografts, XG) were transplanted into Beige SCID C.B-17 mice. Purified human naive T cells previously activated by plastic-bound anti-CD3 and anti-CD28 antibodies in the presence of HGF (Thgf) or in medium alone (Tnt) for 7 days were injected i.v. on day 28 after grafting. Grafts were removed 48 hr later, embedded in OCT, and snap frozen. The presence of Thgf cells labeled with PKH-26 (red) or Tnt labeled with CFSE (green) in the heart (A) and skin (D) grafts and the recipient's spleen (G) was assessed by wide-field fluorescence microscopy. In parallel experiments, Thgf and Tnt cells were treated with the c-Met inhibitor (INH) and differentially labeled prior to co-injections in xenograft recipients (combinations: Thgf plus Thgf [INH] and Tnt plus Tnt [INH]). Representative tissue images of heart (B) and skin (E) xenografts and the recipient's spleen (H) are shown. The mean (±SD) number of labeled T cells counted in at least three 10× fields from sections isolated from eight grafts and the recipient's spleens are shown in (C), (F), and (I) (N = 2, n = 4). ***$p < 0.0005$, scale bars represent 100 μm.

generally thought to be largely restricted to the T helper type 1 and 2 cell subsets, respectively, in humans (Kitamura et al., 1998), is a key feature of cardiotropic T cells. Engagement of both receptors was required for T cell access to the heart in inflammatory conditions, in line with previous reports. The contribution of CCR4 and CXCR3 to cardiotropic T cell recruitment appeared to be cooperative rather than additive: lack or inhibition of each or both receptors similarly reduced T cell access to the heart parenchyma. It is possible that these two receptors cross-regulate each other, as has been described in many GPCRs (Vischer et al., 2011), including the CXCR3-CCR4 pair in which CXCR3 has been shown to enhance CCR4 responsiveness (Sebastiani et al., 2005). In contrast, CCR5 signals only partially inhibited T cell localization to the heart during inflammation, which is further reduced by inhibition of CCR4 or CXCR3 in an additive manner. In line with this observation, CCR4 signaling is enhanced by the CXCR3 but not by the CCR5 ligands CCL3 and CCL4 (Sebastiani et al., 2005).

It is interesting to note that in our model, CCR4 and CXCR3 ligands were produced either by the heart (CXCL10) or by Thgf cells (CCL22/CCL17), and that CCL22 was released by T cells after c-Met triggering. These observations raise the alternative or additional possibility that CCR4 might be crucial for T cell recruitment by contributing to HGF "presentation" and c-Met triggering by the heart endothelium, while CXCR3 might be important for the retention of recruited T cells in the cardiac parenchyma.

Exposure to HGF during priming also promoted the imprinting of T cell hepatotropism. However, differently from what we observed for cardiotropic T cells, HGF-mediated hepatotropic T cell migration to the liver was only partly reduced by c-Met inhibition. These observations indicate that, although HGF is sufficient for the induction of hepatotropism, molecular interactions other than those mediated by c-Met can mediate T cell recirculation in the liver (Lalor et al., 2002; Shetty et al., 2011).

Our findings are in line with the recent observation that soluble factors produced in selected tissues and drained in the local lymphatic stations contribute to the topographic imprinting of T cells during activation (McCully et al., 2012). These observations suggest that specialized, tissue-derived DCs are dispensable in some mechanisms of homing imprinting. Further, the effectiveness of c-Met inhibition in preventing the induction of T cell cardiotropism in a heterotopic heart allograft suggest that the tissue itself dictates the environmental cues that affect T cell differentiation in draining lymph nodes.

The possibility that tissue-specific soluble factors can induce a defined and unique topographic molecular signature paves the way for new investigations aimed to a more comprehensive mapping of organ-selective T cell immunity with a substantial clinical impact. In our study, transient inhibition of HGF-c-Met axis during T cell priming selectively disrupts T cell homing and immune inflammation in the heart without affecting T cell responses in the skin. These findings have immediate implications for the development of novel therapies aimed at selectively targeting heart immunity while preserving immune protection in other sites (organ-selective immunosuppression).

The temporary blockade of c-Met required to achieve this effect should not impact on the well-established long-term beneficial effects of HGF on heart tissue (Hüser et al., 2005), as it is also indicated by the minimal pathological signs in the grafts from treated animals.

Notably, disruption of the HGF-c-Met axis did not induce T cell tolerance, as indicated by the presence of primed alloresponses in non-rejectors. Rather, we argue that temporary c-Met inhibition prevented the development of topographic memory of the heart by T cells resulting in their failure to access the heart allograft. Further studies will be required to fully develop the clinical potential of this strategy, including determining the duration and plasticity of HGF-induced cardiotropism in primed T cells.

EXPERIMENTAL PROCEDURES

Heart Transplantation

Heterotopic heart transplantation was performed by placing the donor heart into the recipient's (WT and B6Kd) sternomastoid cavity, connecting the aortal branch to the carotid artery and the pulmonary vein to the jugular vein. All grafts and native hearts were evaluated by histopathologic criteria in a single-blinded manner and scored to grade the degree of inflammation from 0 to 4 (0, no inflammation; 1, light focal lymphohistocytic infiltrate; 2, moderate focal lymphohistocytic infiltrate with myocardial involvement; 3, moderate to severe inflammation with focal vasculopathy and myocyte degeneration; 4, severe inflammation, vasculopathy, and myocardial fiber loss). Slides were scanned with an Olympus VS-120 using brightfield mode. Images were analyzed with VS-ASW software.

Luminal occlusion was evaluated by tracing the cross-section of each vessel's internal elastic lamina and lumen using Lucia NIS elements software (Nikon UK) in three transverse sections per graft. All vessels in each section, which demonstrated clear staining of elastin laminar and presence of smooth muscle cell (SMC) alpha-actin, were measured in three sections of each heart.

Isolation of T Cells from Heart Tissue

To isolate T cells from heart tissue, native or transplanted murine hearts were harvested and cut into small pieces. The tissue fragments were digested in 1 ml Hanks medium, containing 608 U/ml Collagenase I (Sigma), 187.5 U/ml Collagenase XI (Sigma), 90 U/ml Hyaluronidase (Sigma), and 90 U/ml DNase (Sigma), for 1 hr at 37°C with agitation. Digested samples were passed through a 70 μm cell strainer to obtain a single-cell suspension and were then washed in FACS buffer before cell surface staining with a 1:200 dilution of conjugated antibodies against CCR4-PE (Biolegend), CXCR3-PerCP (Biolegend), CD4-PECy7 (Biolegend), CD8-APC (Biolegend), and CD44-af450 (eBioscience). Cells were incubated for 1 hr at 4°C before being washed twice in FACS buffer and then fixed with 200 μl Fixation Buffer (eBioscience) for 1 hr at 4°C. Cells were washed twice with Fixation/Permeabilisation buffer before intracellular staining with a 1:200 dilution of FITC-conjugated antibody against c-Met (eBioscience). Cells were incubated for 1 hr at 4°C before being washed twice in Fixation/Permeabilisation buffer and being analyzed on a FACS Fortessa (BD).

HGF Binding to Human T Cells

Permeabilized (cell permeabilization kit, eBioscience) T cells were incubated with Dylight 488-labeled (500 ng/ml, ThermoScientific) human recombinant HGF (Peprotech) and rat-anti mouse CD4 and LFA-1 antibodies for 40 min, followed by washing in PBS. Cells were resuspended in fix buffer and analyzed using ImageStream technology (MK II, Amnis). Data were analyzed with IDEAS 6.0 software (Amnis). For analyses, total cells were gated on in focus and single cells. Single cells were then analyzed for HGF intensity within the total $CD4^+LFA\text{-}1^+$ population (mean intensity values shown).

Human Tissue Transplantation into SCID Mice

Small human heart tissue samples were collected from the right atrium and non-ischemic part of the left ventricle in patients undergoing routine coronary bypass surgery and skin tissue was obtained from patients undergoing cosmetic surgery, according to a protocol approved by the local ethical committee and after written informed consent by the patient.

Human heart and skin tissue samples (4 × 4 mm) were transplanted into Beige SCID C.B-17 mice. Two small incisions were made in the skin behind each ear of one mouse and the heart biopsies were carefully inserted subcutaneously in a dorsal position distal to the shoulder joints (two transplants per animal). The incisions were closed with a soluble suture. Mice were monitored for 4 weeks before grafts were exteriorized and removed for analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.05.014.

AUTHOR CONTRIBUTIONS

I.K. performed most experiments; D. Coe, M.K., R.H., and G.W. performed experiments; D.A.S., C.P., G.A., P.B., and G.L. provided essential reagents; I.K., D. Coe, D. Cooper, M.K., R.H., G.W., S.N., H.F., and R.B. analyzed the data; I.K., C.M., and F.M.M.-B. designed experiments; and I.K. and F.M.M.-B. wrote the manuscript. All authors discussed and revised the manuscript.

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