Circulating cMet-expressing memory T-cells define cardiac autoimmunity.

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Short title: cMet$^+$ T-cells detect heart inflammation

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Abstract:

Background: Autoimmunity is increasingly recognized as a key contributing factor in heart muscle diseases. The functional features of cardiac autoimmunity in humans remain undefined, due to the challenge of studying immune responses in-situ. We have previously described a subset of cMet-expressing ($cMet^+$) memory T-lymphocytes, which preferentially migrate to cardiac tissue in mice and humans.

Methods: In-depth phenotyping of peripheral blood T-cells, including $cMet^+$ T-cells, was undertaken in groups of patients with inflammatory and non-inflammatory cardiomyopathies, patients with non-cardiac autoimmunity and healthy controls. Validation studies were carried out using human cardiac tissue and in an experimental model of cardiac inflammation.

Results: We show that $cMet^+$ T-cells are selectively increased in the circulation and in the myocardium of patients with inflammatory cardiomyopathies. The phenotype and function of $cMet^+$ T-cells are distinct from cMet-negative ($cMet^-$) T-cells, including preferential proliferation to cardiac myosin and co-production of multiple cytokines (IL-4, IL-17 and IL-22). Further, circulating $cMet^+$ T-cell subpopulations in different heart muscle diseases identify distinct and overlapping mechanisms of heart inflammation.

In experimental autoimmune myocarditis, elevations in autoantigen-specific $cMet^+$ T-cells in peripheral blood mark the loss of immune tolerance to the heart. Importantly, disease development can be halted by pharmacological cMet inhibition, indicating a causative role for $cMet^+$ T-cells.

Conclusions: Our study demonstrates that the detection of circulating $cMet^+$ T-cells may have utility in the diagnosis and monitoring of adaptive cardiac inflammation, and

additionally define new targets for therapeutic intervention when cardiac autoimmunity causes or contributes to progressive cardiac injury.

Non-standard Abbreviations and Acronyms.

AHR	Aryl hydrocarbon receptor
AM	Acute myocarditis
c-Met	c-mesenchymal epithelial transition factor
CCR	Chemokine receptor
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CMR	Cardiac Magnetic Resonance imaging
CS	Patients undergoing elective cardiac surgery
DCM	Dilated cardiomyopathy
EAM	Experimental autoimmune myocarditis
ECG	Electrocardiogram
Echo	Echocardiogram
ELISA	Enzyme linked immunosorbent assay
EMB	Endomyocardial biopsy
fHMD	Familial heart muscle disease
GARP	Glycoprotein A repetitions predominant
GATA3	GATA family of conserved zinc-finger transcription factors 3
HC	Healthy controls
HGF	Hepatocyte growth factor
IFN	Interferon
iDCM	Idiopathic dilated cardiomyopathy
IHF	Ischemic heart failure

Ig	Immunoglobulin
IL	Interleukin
MHCa	Myosin Heavy Chain alpha
NOD	Non-obese diabetic
PCR	Polymerase chain reaction
RORγt	RAR-related orphan receptor gamma isoform t
STEMI	ST-elevation myocardial infarction
SS	Sjögren's syndrome
T-bet	T-box transcription factor TBX21
TCR	T-cell receptor
TEMRA	T effector memory RA^+ cells
Th	T-helper cell subset
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cells

Clinical Perspective.

What is new?

- We can detect evidence of active autoimmune myocardial inflammation through assays of peripheral blood that identify and quantify circulating heart-homing cMet^{+} memory T-cells
- Phenotyping these T-cells provides insights into the mechanisms through which the adaptive immunological system contributes to myocardial injury in heart muscle diseases where there is an inflammatory component

What are the clinical implications?

- Assays for circulating cMet^{+} T cells are readily obtained and may have utility as a non-invasive diagnostic marker for inflammatory heart muscle disease.
- The persistence, phenotype and magnitude of a circulating cMet^{+} T cell response may also indicate prognostic risk and identify individuals at risk of developing persistent myocardial inflammation and chronic myocarditis.
- The cMet^{+} T cell response provides new therapeutic targets for the treatment of myocardial inflammation. We demonstrate that blockade of cMet signalling will abrogate myocardial inflammation in an experimental model of auto-immune myocarditis.

Introduction

T-cell-mediated immunity has been linked to a variety of heart diseases, from classical inflammatory cardiac conditions such as myocarditis to diseases without a readily evident pathogenic inflammatory component such as hypertensive cardiomyopathy. [1] T-cell activation in cardiac inflammation often results from the interaction of an external environmental trigger (viral infection) or an endogenous stimulus (mechanical or oxidative stress) with the host's immune system. [2] Persistence of pro-inflammatory stimuli or development of autoimmunity lead to chronic myocardial inflammation and ultimately cardiac dysfunction.

Acute myocarditis (AM) is mediated by T-cell myocardial inflammation following infectious and non-infectious triggers. [3] The acute myocardial injury associated with AM is also the commonest cause of acute presentations with features of acute myocardial infarction but with angiographically-normal coronary arteries. [4] AM is a relatively frequent cause of sudden cardiac death in young adults and athletes[5, 6], and also in older populations. [7]

In some of these patients autoimmunity develops, and progressive cardiomyocyte damage leads to systolic impairment and dilated cardiomyopathy (DCM). [8, 9] AM is considered the most common cause of DCM[10] with some reports suggesting that almost 50% of patients with a clinical diagnosis of 'idiopathic' DCM (iDCM) have immunohistochemically detectable features of lymphocytic myocarditis[11, 12], suggesting that chronic myocarditis can be sub-clinical.

Despite the many experimental models available, it has been difficult to define autoimmunity in inflammatory cardiomyopathy in humans, largely due to the technical challenges of studying the immune response in situ. While there is consistency in reports

implicating IL-17-producing T-cells in myocarditis, the contribution of Th1 and Th2 responses in human disease remains controversial.[13,14] In addition, the identification of an immunophenotype linked with clinical disease progression in human myocarditis has remained elusive.

We have previously described a Hepatocyte Growth Factor (HGF)-induced memory T-cell subset that preferentially migrates to the heart. [15] These T-cells are characterized by expression of the Hepatocyte Growth Factor receptor cMet and chemokine receptors CXCR3 and CCR4.

In this study we detect and analyze the presence of cMet-expressing T-cells in the blood of patients with heart muscle disorders where an inflammatory etiology is suspected (AM in particular). We show that circulating $cMet^{+}$ T-cells mark the presence of autoreactive inflammation of the heart in humans and mice and define the features of this heart-selective immune response in acute and chronic disease.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request. Raw data CEL files have been deposited to Gene Expression Omnibus under accession GSE186270

Detailed information on Methods not covered in the main manuscript is provided in the Data Supplement and specific details on all the reagents used in this study are provided in Table I in the Data Supplement.

Study Populations

This study was approved as a component study (sub-study 34) of the Barts BioResource (Research Ethics Committee reference 14/EE/0007 and 17/WS/0172). All patients provided written consent according to the principles of the Declaration of Helsinki. Study subjects were recruited into the following patient groups: acute myocarditis (AM), idiopathic dilated cardiomyopathy (iDCM), acute ST-elevation myocardial infarction (STEMI), patients undergoing cardiac surgery (CS), ischemic heart failure (IHF) and active Sjögren' s syndrome (SS). A group of individuals with known familial heart muscle disease (fHMD) was also studied. Healthy volunteer controls (HC) were recruited locally (Queen Mary, University of London) with two Ethics Research Committee (ERC) approvals (QMERC 2014/61 and QMERC 2014/61).

Sample sizes were estimated from pilot data and studies reporting quantitative changes in peripherally circulating T-cells.[16-19]

For immunohistology, post-mortem tissue samples from five AM and five DCM patients were obtained.

Details of each population can be found in Methods and Table II-IV in the Data Supplement.

Experimental Autoimmune Myocarditis (EAM).

The EAM model has been previously described [20], and it is summarized in Methods in the Data Supplement.

Statistical analysis

All data is presented as median and interquartile range (IQR) or mean +/- standard deviation (SD) as indicated.

Power calculations were performed in GPower based on a Wilcoxon-Mann-Whitney test a=0.05, power = 0.8 and a predicted effect size of 0.87. The effect size was defined as the mean standardized difference of the cMet+CD45RO+CD4+% between patients with acute myocarditis and DCM

Standard paired and unpaired t-tests were performed on normally distributed data.

All non-normally distributed data were assessed with Wilcoxon-signed rank and Mann Witney U tests for paired and unpaired data as appropriate and with a Kruskal-Wallis test if a comparison between more than 2 groups was performed; using a standard Dunn's post-hoc analysis for multiple comparisons when the Kruskal-Wallis test was statistically significant. Upon assessment of two independent variables on a dependent variable a two-way ANOVA was used. Repeated measures two -way ANOVA was used as a statistical method for measuring paired dependent variables. If the two-way ANOVA was

statistically significant a Tukey's post-hoc test for multiple comparisons was used. Grouped, ordinal data was compared with Chi-squared tests.

The ROC analysis was performed in GraphPad V8 software, using default settings. The list of thresholds was estimated by sorting all the values in all groups and averaging adjacent values in the sorted list. Each threshold value is midway between two values in the data. Sensitivity is the fraction of values in the patient group that are above the threshold. Specificity is the fraction of values in the control group that are below the threshold. Each confidence intervals are computed from the observed proportion by the Clopper method without any correction for multiple comparisons. Significance is defined at two-tail level of 0.05

Cell proliferation was assessed using the cell 'proliferation modelling' module on FlowJo flow cytometry software according to the manufacturer's instructions. The proportion of cells dividing was determined after automatic identification of the resting, peak 0, T-cells.

Statistical analysis was performed in Prism (Version 8.3.0, GraphPad Software, San Diego, USA), and a two-tailed p-value of <0.05 was considered statistically significant.

Results

Adaptive cardiac inflammation is marked by an increase in circulating c-Met+ memory T-cells.

To test the hypothesis that circulating c-Met+ memory T-cells mark the presence of myocardial inflammation, we first assessed their presence in the peripheral blood of subjects presenting with presumed myocardial infarction (MI) but who are subsequently diagnosed with acute myocarditis (AM) as a cause of their acute myocardial injury. Additional groups included patients with apparently idiopathic DCM (iDCM), often associated with cardiac inflammation [21], first-time ST-elevation MI (STEMI) where acute myocardial injury is a manifestation of coronary atherosclerosis in which inflammation localizes to the arteries but not the heart [22], patients undergoing elective cardiac surgery (CS) but with no evidence of inflammation, patients with ischemic heart failure (IHF) and healthy controls (HC). A cohort of patients with active Sjögren' s Syndrome (SS) a non-cardiac autoimmune condition was also included as a control. [23] Cohort demographics and diagnostic criteria are shown in Table II-IV in Data Supplement. Memory ($CD45RO^+$) $cMet^+$ $CD4^+$ and $cMet^+$ $CD8^+$ T-cells were significantly elevated in the blood of AM and iDCM subjects compared to STEMI, CS, SS, IHF and HC (Figure 1A-B; Data Supplement Figure I for gating and Figure IIA for absolute numbers). Importantly, $CD3^+cMet^+$ T-cells were identified within AM cardiac tissue, where they represent the majority of infiltrating T-cells (Figure 1C-D).

As previously described[24], an increased proportion of $c\text{-}Met^+CD4^+$ memory T-cells co-expressing the CXCR3 and CCR4 chemokine receptors was detectable in AM and iDCM

patients compared to the other groups (Figure 1E). In contrast, no significant difference was detected in memory c-Met$^+$CD8$^+$CXCR3$^+$CCR4$^+$ T-cells However, both CD4$^+$ and CD8+ c-Met$^+$CCR4$^+$ T-cells were significantly increased (Figure 1F).

STEMI and AM can have almost identical clinical presentations, with similar ECG and serum troponin concentrations and differential diagnosis often requires emergency coronary angiography and cardiac magnetic resonance (CMR) imaging. To address the sensitivity and specificity of circulating cMet$^+$ memory T-cells, we performed receiver operating characteristic (ROC) analyses limited to subjects with AM and STEMI (Figure 1G-H). For memory c-Met$^+$CD4$^+$ T-cells the area under the curve (AUC) for AM is 0.99 ($p < 0.0001$); a threshold of <6.1% c-Met expression has a sensitivity and specificity of 93.3% and 94.1% respectively. For memory c-Met$^+$CD8$^+$ T-cells the AUC is 0.90 ($p<0.0001$) and <2.7% threshold had a sensitivity of 86.7% and specificity of 88.2%.

Phenotypic and functional characterization of circulating cMet+ T-cells in AM.

A phenotypic and functional analysis of total circulating memory T-cell populations of subject groups detected differences in T-cell subsets and in markers of T-cell activation, detailed in Data Supplement, Figure II. [25] [26]

To identify selective functional features of circulating cMet$^+$ T-cells we applied pairwise comparisons of c-Met$^+$ and c-Met$^-$T-cells in AM patients. First, despite an overall increase of circulating cMet$^+$ T-cells, we observed a significant reduction in the proportion of cMet$^+$ T-cells with an effector phenotype in both CD4$^+$ and CD8$^+$ T-cell subsets compared to cMet$^-$ T-lymphocytes. In contrast, naïve, central memory and TEMRA phenotypes in

$CD4^+$ and $CD8^+$ T-cell subsets were similarly represented between $cMet^+$ and $cMet^-$ T-cells (Figure 2 A-C).

We then investigated the activation status of $cMet^+$ T-cells and found that they expressed the early activation marker CD69 at significantly higher levels than $c\text{-}Met^-$ T-cells, both in the $CD4^+$ and $CD8^+$ T-cells subsets (Figure 2D-E). In AM patients, circulating $cMet^+$ T-cells included a substantial increase in the regulatory T-cell (Treg) subset, and within this subset, of Tregs expressing the activation marker GARP (Figure 2F-G).

Finally, we profiled cytokine production by $cMet^+$ T-cells. As shown in Figure 2H-I, memory $cMet^+CD4^+$ T-cells were significantly more likely to produce IL-4, IL-17A and IL-22 compared to $cMet^-$ T-cells, while they were significantly less likely to produce IFN-γ. The dominant production of IL-4, IL-17 and IL-22 by $cMet^+CD4^+$ T-cells led us to investigate whether individual cells in this T-cell subset could co-produce combinations of these cytokines. As shown in Figure 2J, we observed a significant increase in single positive $IL\text{-}17A^+$ in $cMet^+$ T-cells compared to $cMet^-$ T-cells as well as single positive $IL\text{-}22^+cMet^+$ T-cells compared to $cMet^-$ T-cells (Figure 2K). We also observed an increase in $IL\text{-}17^+IL\text{-}22^+$ co-expressing $cMet^+$ T-cells compared to $cMet^-$ T-cells (Figure 2L). In addition, the $cMet^+$ T-cell population was significantly enriched in $IL\text{-}4^+IL\text{-}17^+$ but not $IL\text{-}4^+IL\text{-}22^+$ co-producing T-cells compared to their $cMet^-$ counterparts (Figure 2M).

A subset of $IL\text{-}4^+IL\text{-}17^+IL\text{-}22^+$ co-producing T-cells was also significantly increased in the $cMet^+$ but not $cMet^-$-T-cell population (Figure 2N), suggesting extreme plasticity in $cMet^+$ T-cell differentiation.

In keeping with these data, $cMet^+CD4^+$-enriched T-cell populations displayed increased levels of the transcription factors RORγT, GATA3 and AHR, respectively associated with

the development of Th17, Th2 and Th22 responses, while the cMet- population showed increased transcription of Th1-inducer T-bet associated with Th1 development (Figure IIIA in Data Supplements) [27]. We also used bulk RNA transcriptomics to compare the transcriptional activity between cMet$^{+/-}$ T-cells (CD4^{+} and CD8^{+}), which detected significantly increased transcripts of the Olfactory Transduction Pathway [28]. This pathway, together with cMet, has been implicated in induction of motility in neuronal cells [29] but never in T-cells, was subsequently confirmed by RT-PCR (Figure IIIB-H and Table V in Data Supplement).

A recent study of single-cell RNA sequencing and single T-cell receptor sequencing in cardiac tissue from DCM patients has described the presence of T-cells expressing STAT3, known to be involved in cMet signaling, as well as the chemokine receptor CXCR3. [30] We performed an in-silico re-analysis of the gene expression in T-cells populations (Figure IIII in Data Supplement) and compared a T-cell cluster resembling the cMet^{+} population (T-cell A) with the rest of the T-cells populations (T-cell B) (Supplementary Methods). The T-cell A cluster showed upregulation of genes positively regulating IL-4 and IL-13 (*MAF*, *PARP1*, *NELL2*, *ID2*) and IL-17 (*TIGIT*, *IL32*, *CREM*) and downregulation of genes positively associated with IFN-γ gene transcription (*AIF1*, *PLAC1*, *S100A4*, *IFIM3*, *ZNF683*), compared to the rest of T-cell populations (T-Cell B) (Supplementary Figure III, panel I).

The cMet^{+} memory T-cell subset contains autoreactive T-cells specific for cardiac myosin.

Irrespective of the initial trigger, the pathogenic immune response in AM can develop autoimmune specificity for self-antigen. [3] We therefore evaluated the antigen-specificity of cMet^{+} T-cells in AM, iDCM, IHF patients and HC by assaying proliferation of Tag-it-violet-labelled PBMCs from AM patients exposed to selected cardiac sarcomeric antigens and the recall antigen tetanus toxoid (TT) after 7 days of culture. As shown in Figure 3A-D, proliferative responses to cardiac myosin and TT were detected in cell cultures from all AM patients tested. Importantly, responses to the autoantigen cardiac myosin segregated with the cMet^{+} T-cell population (Figure 3C-D). Conversely, most of the TT-responding T-cells were in the c-Met^{-} fraction. We did not detect T-cell proliferation in response to troponin antigens in any of the patients (A-B).

Similar antigen-specific responses were detected in PBMC from DCM: T-cells responding to cardiac myosin were detected identified in the cMet^{+} T-cell subset (Figure 3E-F). In contrast, although T-cells proliferated in response to TT, no responses to cardiac antigens by cMet^{-} T-cells were detectable in IHF (Figure 3G-H) and HC (Figure 3I-J) samples. Due to their minute proportion, it was not possible to detect proliferative responses in cMet^{+} T-cells from the latter cohorts.

In summary, autoreactive responses mediated by cMet^{+} T cells are selectively detectable in individuals with cardiac inflammation. In line with these data, autoantibodies to Myosin Heavy Chain have been detected in patients with acute myocarditis, and DCM. [31]

Circulating cMet^{+} T-cell subpopulations in different cardiac diseases identify distinct and overlapping mechanisms of cardiac inflammation.

Myocardial inflammation may contribute to myocardial injury in up to half the cases of iDCM, where unresolved chronic inflammation can lead to progressive myocardial injury and poorer clinical outcomes. [2] However, in clinical practice, the cause(s) of myocardial injury in any individual case is often unclear following extensive diagnostic work-up and, in many cases, is likely to be multifactorial. [32, 33 32, 33 32, 33 37,38] In this imprecise context, we sought to investigate whether phenotypic and/or functional differences in circulating cMet$^+$ T-cells could provide evidence of an evolving chronic autoimmune response in iDCM.

As previously shown (Figure 1A-D), both CD4$^+$ and CD8$^+$ memory cMet$^+$ T-cells were increased in iDCM patients, which shared most features with those described for AM including specificity (Figure 3E-F), cytokine production, gene transcription and phenotype (Data Supplement, Figure IV and V).

cMet$^+$ T-cells were also present in iDCM cardiac tissue (Figure 4A-B). However, despite the decreased number of infiltrating T-lymphocytes compared to AM (Figure 4C), almost all T-cells in tissue from iDCM expressed cMet (Figure 4D). The proportion of infiltrating CD4+ and CD8+ T-cell subsets was similar in AM and iDCM, with cMet+ CD4+ and CD8+ T cells representing the dominant phenotype, with a further significant enrichment in iDCM infiltrates (Data Supplement, Figure VI).

Alterations in Treg status were the most striking difference between circulating cMet$^+$ T-cells in AM and iDCM. Despite a similar increase in cMet$^+$ Treg cells in iDCM, unlike in AM (Figure 2G), cMet$^+$ Treg cells from iDCM patients failed to upregulate the activation

marker GARP (Figure 4E-G). This difference in Treg status may indicate a mechanism for progression to-, and maintenance of chronic inflammation in iDCM.

Other than potential deficiencies of cMet$^+$ T-cell regulation, the absence of other distinctive features between T-cells in AM and iDCM and the further enrichment in cardiac cMet$^+$ T-cell infiltrates point to a spectrum of heart muscle inflammatory diseases where myocardial injury develops as a result of similar or shared autoimmune pathways.

We next sought to investigate the hypothesis that circulating T-cells might provide clues of the pathogenic process underlying cardiac inflammation. To this aim, we compared cMet$^+$ T-cells in the blood of patients with AM, iDCM and with proven familial/genetic Heart Muscle Disease (fHMD, Table II and IV in Data Supplement). Myocarditis-like 'hot-phase' clinical episodes and inflammatory cell infiltrates are well recognized in the natural history of several genetic heart muscle diseases that are conventionally considered distinct from each other and from 'acquired' myocarditis. [34] In some cases, particularly when fHMD is due to mutations in genes encoding desmosome components or filamin, the initial presentation may be clinically indistinguishable from AM. [35, 3635, 3635, 3634, 35] We therefore investigated whether cMet$^+$ T-cells can be detected in PBMCs from 14 patients with genetic mutations known to be causative for fHMD. There were no significant age differences between fHMD and iDCM groups. Table IV in the Data Supplement shows the genetic variants present in the fHMD group.

First, we found that cMet$^+$CD4$^+$CD45RO$^+$ T-cells were present in similar proportions in PBMCs from iDCM and fHMD patients and significantly higher than in HCs (Figure 4H-

I). In striking contrast, cMet$^+$CD8$^+$CD45RO$^+$ T-cells were much higher in iDCM than in fHMD compared to iDCM, and proportions of cMet$^+$CD8$^+$CD45RO$^+$ T-cells in fHMD and HC were similar.

This is consistent with the hypothesis that although memory CD4$^+$cMet$^+$ T-cells are similarly elevated in fHMD and iDCM, the mechanisms of cardiac injury (i.e., cell- and/or antibody-mediated) may be different in these two clinical states and/or at different disease stages. Notably, CD4$^+$ T-cell-dependent auto-antibody responses are thought to play a pathogenic role in several forms of fHMD. [37, 3837, 3837, 3843]

This hypothesis was further investigated by assessing the presence of CD45RA$^+$CCR7$^+$CD95$^+$ stem-memory T-cells (TSMCs). This long-lived, self-renewing T-cell subset has been detected in CD4$^+$ and CD8$^+$ T-cell populations of mice and humans [39] and has been suggested to provide a reservoir of self-reactive T-cells in autoimmune diseases. [39,40] We observed increased proportions of both CD4$^+$cMet$^+$ TSMCs in iDCM compared to fHMD (Figure 4J-K). These data imply that while in iDCM autoimmunity has the potential to support itself, additional triggers (exercise and other environmental factors, for example) may be needed to precipitate autoimmunity episodes (hot phases) in fHMD.

cMet$^+$ T-cells play a key role in experimental autoimmune myocarditis and their rise in peripheral blood marks the loss of immune tolerance to the heart.

Defining the causative role of cMet$^+$ T-cells in the development of the autoimmune phase of inflammatory cardiomyopathies is logistically difficult to address in human studies. We therefore modeled the role of cMet$^+$ T-cells in experimental autoimmune myocarditis (EAM), a murine model of progressive cardiac autoimmune inflammation leading from AM to DCM. [20] EAM is a T-cell-dependent model sharing key features with human AM and DCM (T-cell-mediated, autoantigen, disease course, male-sex bias and more).

Male Balb/cAnN mice were immunized subcutaneously with the MHCα peptide (RSLKLMATLFSTYASADR). Control mice received adjuvant alone with the same schedule (Figure 5A). We have previously shown that pharmacologic cMet inhibition during T-cell priming prevents the upregulation of this receptor and cardiac allograft rejection in mice. [15] Accordingly, a third group of mice received the cMet-selective small molecule inhibitor PHA-665752 for 10 days following the initial immunization, a time frame when topographical memory is thought to occur. [15]

Immunization of mice with MHCα led to acute myocarditis assessed as EAM incidence (Figure 5B), area of mononuclear cell infiltrate (Figure 5C), and collagen deposition (Figure 5D) measured by histology on day 28 after the first immunization. Echocardiography revealed functional alterations consistent with AM (Figure VIIA in the Data Supplement). Importantly, pharmacological inhibition of cMet (EAM+INH) significantly reduced EAM incidence, mononuclear cell infiltrates and collagen deposition (Figure 5B-D) as well as echocardiographic sign of AM mentioned above.

The proportion of circulating memory $CD44^+cMet^+$ T-cells was monitored by flow cytometry for the duration of the experiment (Figure VIII in Data Supplement for gating strategy). Development of disease was marked by a progressive increase of circulating $CD44^+$ $cMet^+$ T-cells in immunized mice (Figure 5E, and Figure VIIB in Data Supplement for absolute numbers). Conversely, in mice treated with the cMet inhibitor, reduction of disease severity was mirrored by a reduction in circulating $cMet^+$ T-cells.

We further analyzed the distribution and functional characteristics of $cMet^+$ T-cells ex-vivo at day 28 of EAM. Increased proportions of both $CD4^+$ and $CD8^+$ $cMet^+$ memory T-cells were found in heart tissue, heart-draining lymph nodes (dLN) and spleen (Data Supplement Figure VIIC-E) but not in non-draining LN (ndLN, Figure VIIF in Data Supplement). $cMet^+$ T-cells displayed signs of recent activation in the heart tissue and dLN, but less in the spleen. In addition, we detected a significant increase of $cMet^+CD4^+CD25^{high}FoxP3^+$ Treg cells in heart, dLN, and spleen, but not in ndLN. Treatment with PHA-665752 blunted the expansion of $cMet^+$ T-cells in the heart, dLN, and spleen, as well as reducing the proportion of Tregs.

As a control, we also investigated the presence of circulating $cMet^+$ T cell subsets in pre-diabetic and diabetic NOD mice, a model of autoimmune type I diabetes but we were unable to detect an expansion of this T cell subset in this model of autoimmunity, suggesting that cMet+ T-cells selectively associate with heart autoimmunity (Figure VIIG-H in Data Supplement).

The specificity of cMet$^+$ T-cells was determined by assaying proliferation of CFSE-labelled splenocytes from diseased and control mice exposed to cardiac myosin after 5 days of culture.

As shown in Figure 6A-B, proliferation to autoantigen was detected only in the cMet$^+$ T-cell population of immunized mice, confirming their auto-reactive nature. As expected, no proliferative responses against the MHCα peptide were detected in either cMet$^+$ or cMet$^-$ CD8$^+$ T-cells (Figure IXA in Data Supplement). Interestingly, unlike in human AM, cMet$^+$CD4$^+$ T-cells also produced IFN-γ (Figure 6C), with a proportion of these T-cells also co-producing IL-17 (Figure 6D) and TNFα (Figure 6E). Surprisingly, IFN-γ was also produced by cMet$^+$ T-cells from mice that received adjuvant alone (Figure 6C), possibly because of non-specific systemic immune activation by the adjuvant. Like what was observed in human PBMCs from AM and iDCM patients, IL-17- and IL-13-producing CD4$^+$ T-cells were significantly increased in the cMet$^+$ T cell populations (Figure 6F-G). IL-13 was used as surrogate for Th2 responses as IL-4 could not be measured by intracellular antibody staining. These cytokines were not produced by cMet$^-$ T-cells. IL-22-producing T-cells were also enriched in the cMet$^+$ T-cell population (Figure 6H). Importantly, when multiple cytokine producers were analyzed, like in human AM, IL-17$^+$IL-13$^+$ double positive and IL-17$^+$IL-13$^+$IL-22$^+$ triple positive cMet$^+$ T-cells were significantly increased in the cMet$^+$ T-cell population (Figure 6I-J) in the heart-draining LNs, but not the spleen of immunized mice (Data Supplement Figure IX, depicting cytokine production by cMet$^+$ and cMet$^-$ T cells in the spleen).

Discussion

Based on our previous description of cardiac-tropic cMet$^+$ memory T-cells [15], we investigated the possibility that blood-borne cMet-expressing memory T-cells might mark and define adaptive autoimmune inflammation of the heart. Of note, cMet expression by highly cytotoxic CD8$^+$ [41], but not CD4$^+$ T-cells has been reported in encephalitogenic T-cells [42], and has been transduced in T cells to target them to the liver [43], in line with our previous observations. [15]

cMet$^+$ memory T-cells display features that implicate them in the pathogenesis of cardiac autoimmunity. cMet$^+$ T-cells are detected in large numbers in inflammatory infiltrates of AM and iDCM hearts and respond to the autoantigen cardiac myosin.

cMet$^+$ T-cells in display certain unique functional characteristics, including patterns of cytokine secretion dominated by IL-4, IL-17 and IL-22. Production of IL-17A has been previously associated with AM in which it is upregulated in response to cardiac myosin stimulation[14]. Although limited data describe a role of IL-4 in human myocarditis, work in mice has shown the importance of IL-4 in driving AM. [20] Relevant to this study, in a model of autoimmune myocarditis, the c-Met ligand hepatocyte growth factor (HGF) was shown to enhance Th2 responses [44, 45], providing a mechanistic link for IL-4 production by c-Met$^+$ T-cells.

IL-22 plays a central role in wound healing and anti-microbial immunity in the skin [46]. However under pathologic conditions, IL-22 can play a pro-inflammatory role in psoriasis

[47] and murine viral myocarditis [48], particularly when associated with IL-17 production. [47] Further, a recombinant IL-22-Ig molecule has been shown to ameliorate experimental autoimmune myocarditis in mice. [49] Like IL-4, the role of IL-22 in cardiac inflammation has not been described in humans, but IL-22 production in EAM was also reduced by cMet inhibition, suggesting a common induction pathway.

A unique feature of cMet$^+$ Th subsets is the ability to simultaneously produce IL-4/IL-13, IL-17 and IL-22 in both AM and EAM. Precursors of Th17 cells can differentiate into Th17/Th1 cells in response to IL-12, whereas an IL-4-rich microenvironment can induce memory Th17/Th2 cells[50, 51]. Similar observations have been made in the Th22 subset. [52, 53] The protective or pathogenic role of these subsets in human health and disease is still unclear. [54] A similar IL-4$^+$IL-17$^+$IL-22$^+$ Th-cell subset has been reported to play a protective role in pregnancy outcomes [55], with transcripts of relevant transcription factors, including GATA-3, RORc and AHR [54] detected in the sites infiltrated by this subset. Further studies are needed to establish the role of cMet-signals in defining cytokine production by T-cells, particularly as our data in mice suggest that only IL-13 and IL-22, but not IL-17 production is promoted by this pathway.

We also observed an increased production of IFN-γ by cMet$^-$ T-cells in iDCM. The role of this cytokine in inflammatory cardiomyopathy remains contentious. A number of murine studies suggest that IFN-γ plays a protective role in myocarditis due to its role in maintaining the suppressive function of Treg cells. [56,57, 58] Other works in mouse models of persistent adenoviral myocardial infection and EAM, have suggested that IFN-γ is pro-inflammatory although it is not clear if the IFN-γ was produced by T-cells in these studies

as it can also be produced by Natural Killer cells. [59-61] Genetic testing of children with DCM has shown higher frequencies of an IFN-γ genotype (TT) that is associated with higher IFN-γ expression compared to healthy controls. [62] However, another study involving 16 patients with undifferentiated but severe DCM (average LVEF=22) has shown that, after in vitro stimulation, there was a reduction in the proportion of $CD4^+$ T-cells producing IFN-γ compared to healthy controls – suggesting IFN-γ producing $CD4^+$ T-cells do not make a substantial contribution to severe DCM. [63] Increased IFN-γ production might be part of the immune system's attempt to control inflammation. Our finding that c-Met^+ $GARP^+$ Tregs are increased in AM but not in iDCM suggests a failure to develop a protective response in the latter, as it has been reported elsewhere. [64] Increased IFN-γ^+ T-cells in these patients might therefore reflect a compensatory mechanism to increase Treg suppressive and anti-inflammatory activity.

Our observations also indicate that differences in circulating $cMet^+$ memory T cell subpopulations may reflect distinct pathogenic mechanisms of inflammatory cardiomyopathies. While similar proportions of c-Met^+CD4^+ memory T-cells are detectable in both iDCM and fHMD, c-Met^+CD8^+ memory T-cells are nearly undetectable in fHMD patients. $CD8^+$ T-cell responses are essential to viral containment and the presence of $CD8^+$ T-cells has been described as a diagnostic feature for AM by EMB immunohistochemistry analysis [65-67], consistent with commonly described viral triggers of this condition. Other work has shown that $CD4^+$ T-cells are the predominant T-cell infiltrate in EMB from inherited arrhythmogenic cardiomyopathies. [68] Relevant to this observation, the role of $CD8^+$ T cells in autoimmune myocarditis has not been clarified.

Presumably, like $CD4^+$ T-cells they are induced to express cMet during priming. In human AM, they could be remnants of an anti-viral immune response. In EAM, while we find $CD8^+cMet^+$ T-cells, we cannot detect proliferation of these T cells in response to cardiac myosin (as expected, because the immunogen is MHC class II restricted). While epitope spreading during autoimmunity might account to this effect, the precise role of $CD8^+cMet^+$ T cells in autoimmune heart muscle inflammation remains to be fully clarified.

In addition, we show that c-Met positive stem memory T-cells (TSMCs) are detectable in AM and iDCM, but not in fHMD patients. TMSCs play a role in the maintenance of T-cell mediated autoimmune diseases, where they provide a reservoir of self-reactive T-cells that sustain chronic adaptive inflammation [39,40], suggesting persistence of autoimmunity in AM and in iDCM. Their absence in fHMD patients indicates that in these conditions, autoimmunity might arise occasionally or intermittently, possibly in response to transient triggers, such as following exercise or during systemic inflammatory responses following otherwise unrelated disorders.

Finally, we provide evidence that preventing the development of $cMet^+$ T-cells by selective pharmacological inhibition of cMet is effective in significantly blunting disease severity in EAM, in which the functional phenotype of $cMet^+$ cells is remarkably similar to that of their human counterpart. Further, their presence in the peripheral blood can predict EAM development, suggesting a causative role for this T-cell subsets in cardiac autoimmunity.

Study limitations: A limitation of these experiments is that we cannot exclude an effect of the cMet inhibitor on other immune cells, such as antigen-presenting cells. Accordingly,

an alternative approach would use inducible, T-cell-selective cMet-deficient mice, but these are not currently available on the Balb/cAnN genetic background.

Single-cell transcriptomic analysis of both AM and DCM cMet$^+$ T-cells would greatly help the full characterization of cMet$^+$ T-cells in cardiac autoimmunity.

An additional limitation is that AM diagnoses were made based on clinical presentations and cardiac imaging (angiography and CMR) and were not confirmed by biopsy. However, endomyocardial biopsy is not recommended in the diagnostic work-up for most AM patients. [69] Although human cohorts were small, sample sizes exceeded those indicated by power analyses.

Conclusions: In a novel study with a limited number of patients, the potential translational implications of this work are evident, namely the diagnostic and prognostic potential of monitoring cMet$^+$ T-cells in the venous blood of patients, and additionally provide a new target pathway for therapeutic intervention. Ultimately, multi-center studies will be required for the translation of these findings in the diagnosis, prognosis and treatment of inflammatory cardiomyopathies.

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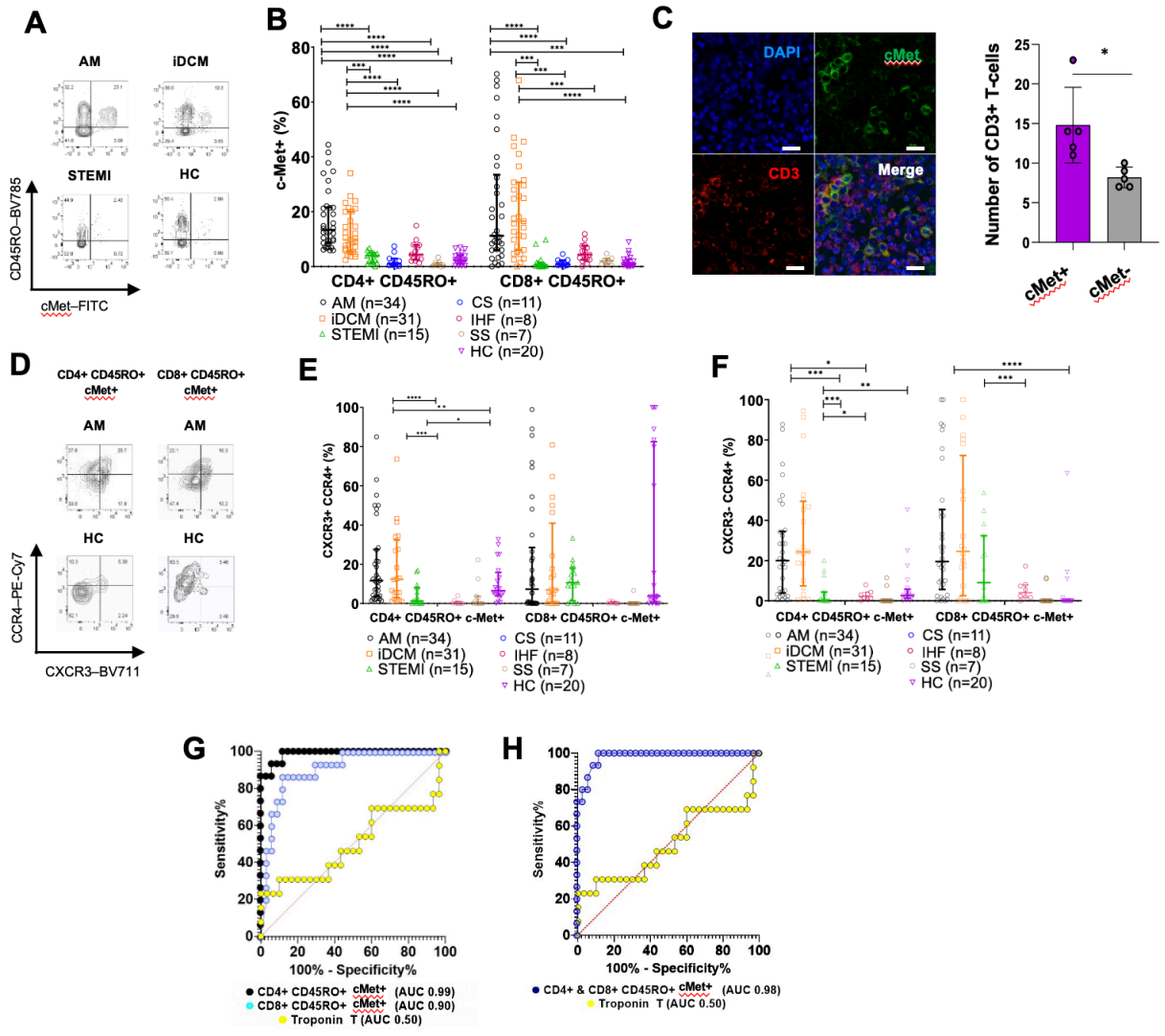
Supplemental Data

Supplemental Methods
Tables I-V
Figures I-VIII

Author contributions: SF, ES designed and performed experiments, analyzed data and wrote the paper; ER-V, AP, ES, VSV, CD, CB-B, GW and SK designed and performed experiments and analyzed data; DC and MPL designed experiments; AP, MDG, SR, CB, MB, PE, DH, SH, MS and KS provided patient samples and clinical data; SAM and FM-B conceived the study, designed experiments and wrote the paper.

Figure Legends

Figure 1: Circulating c-Met-expressing T-cells are increased in inflammatory cardiomyopathies.



Peripheral blood $CD4^+$ and $CD8^+$ memory T-cells were analyzed by flow cytometry for the expression of c-Met. **A**, Representative dot plots from the patient groups. **B**, Grouped data for patient and control groups with significant Kruskal-Wallis for $CD4^+$ and $CD8^+$ T-cells with post-hoc Dunn's multiple comparisons test.

C, Confocal analysis of $CD3^+$ (red) $cMet^+$ (green) cells in paraffin-embedded post-mortem AM samples. Scale bar: 20 μm. The column graph shows the mean number of $cMet^+$ and cMet- $CD3^+$ T-cells in four 20x field from each of 5 patient samples (± SEM, Paired Student's t-Test).

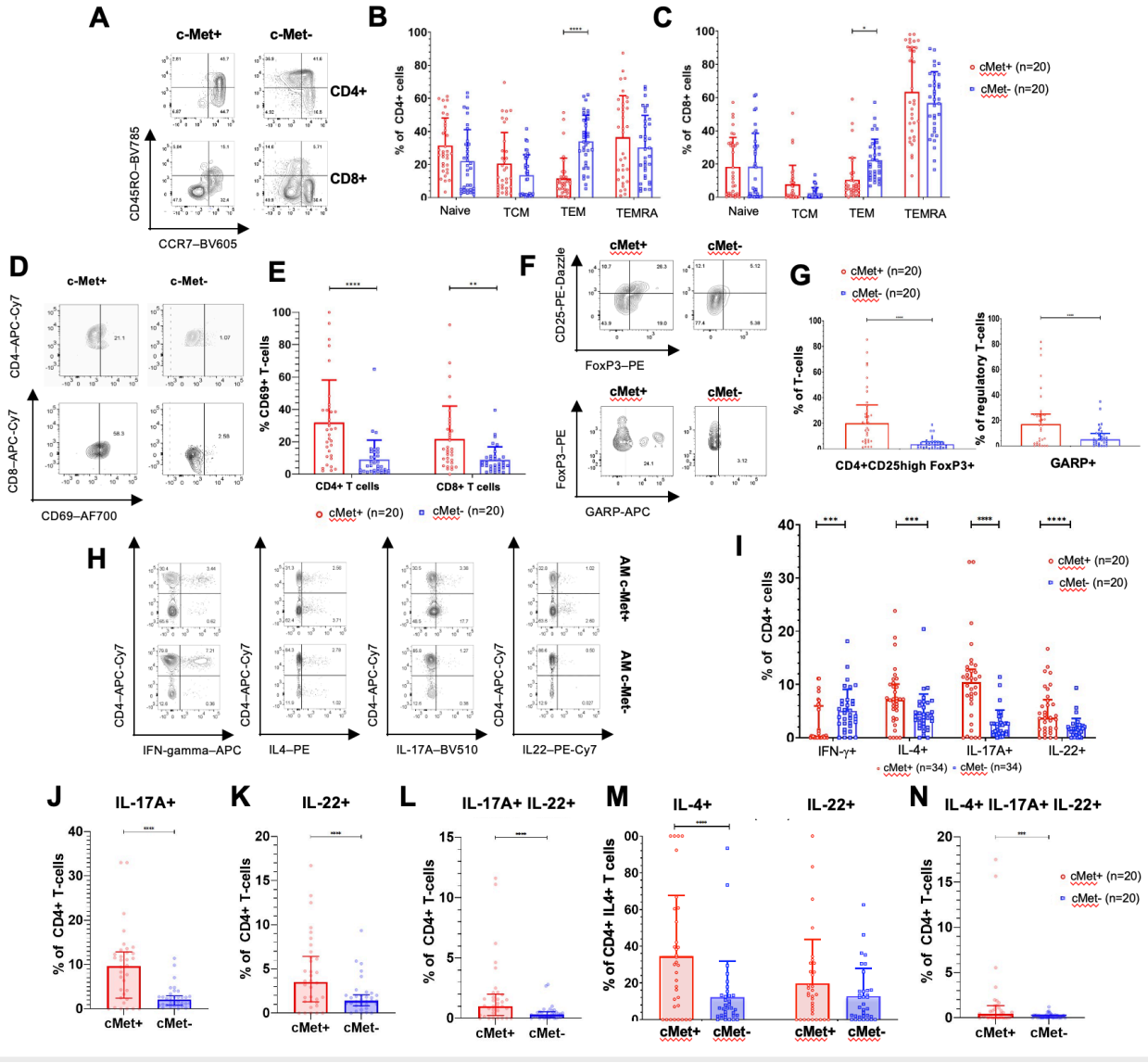
P values are highlighted as: ****p<0.0001, ***p<0.0005, **p<0.005, *p<0.05 and data are represented as median ± interquartile range.

D-F, $cMet^+$ memory cells were also analyzed for co-expression of CXCR3 and CCR4 co-expression (panel **E**) as well as CCR4 single expression (panel **F**) within the patient groups indicated under the x-axis. A representative dot plot is shown in panel **D**.

Kruskal-Wallis for $CD4^+$ T-cells with post-hoc Dunn's multiple comparisons test.

G, Receiver operating characteristics (ROC) for measurement of c-Met^+ $CD4^+$ (black), $CD8^+$ (blue) memory T-cells and peak Troponin T (yellow) for patients with acute myocarditis vs. patients with STEMI. The ROC analysis was performed in GraphPad, using the default settings. The list of thresholds was estimated by sorting all the values in all groups and averaging adjacent values in that sorted list. Each threshold value is midway between two values in the data. Each sensitivity is the fraction of values in the patient group that are above the threshold. The specificity is the fraction of values in the control group that are below the threshold. Each confidence intervals are computed from the observed proportion by the Clopper method without any correction for multiple comparisons. Significance is defined at two-tail level of 0.05. $CD4^+$ area under the curve (AUC) 0.99, p<0.0001, $CD8^+$ AUC 0.90, p<0.0001, Troponin T AUC 0.50, p=0.98. H: ROC CD4 and CD8 combined: blue (CD4 & CD8 $CD45RO^+$ c-Met^+), yellow (Troponin T). AUC 0.98, p<0.00001

Figure 2: Phenotypic and functional characterization of cMet$^+$ T-cells in AM.



A, Representative flow cytometry plots showing expression of CD45RO and CCR7 by cMet$^+$CD4$^+$ T-cells from a patient with acute myocarditis. Summary flow cytometry data from patients are shown in panels **B** (CD4$^+$) and **C** (CD8$^+$). The dots in the graphs represent single individuals. All the n numbers refer to the number of patients in each respective group in each panel of Figure 2. For panels 2**G**, 2**I**, 2**J**, 2**K**-2**N**, we present results from the Wilcoxon signed rank test as paired patient data is being compared.

For Figure 2 **B**, **C** and **E** we used a 2-way ANOVA as the samples are independent and we are testing whether the cells were c-Met positive or negative and what their expression of CD45RO and CCR7 is.

Panel **D** shows representative dot plots of CD69 expression by $cMet^+$ and $cMet^-$ peripheral blood $CD4^+$ and $CD8^+$ T-cells in a patient with AM. Summary data are shown in panel **E** with a significant two-way ANOVA and post-hoc Sidak's multiple comparisons test.

Panel **F** shows representative dot plots of FoxP3 and CD25 expression (upper dot plots) and expression of GARP by $FoxP3^+CD25^+$ cells (lower dot plots) by $cMet^+$ and cMet- peripheral blood $CD4^+$ T-cells from a patient with AM. Summary data for GARP are shown in panel **G** with a significant Wilcoxon signed rank test.

For both panels E and G the red dots represent c-Met^+ T-cells and the blue dots represent c-Met- T-cells.

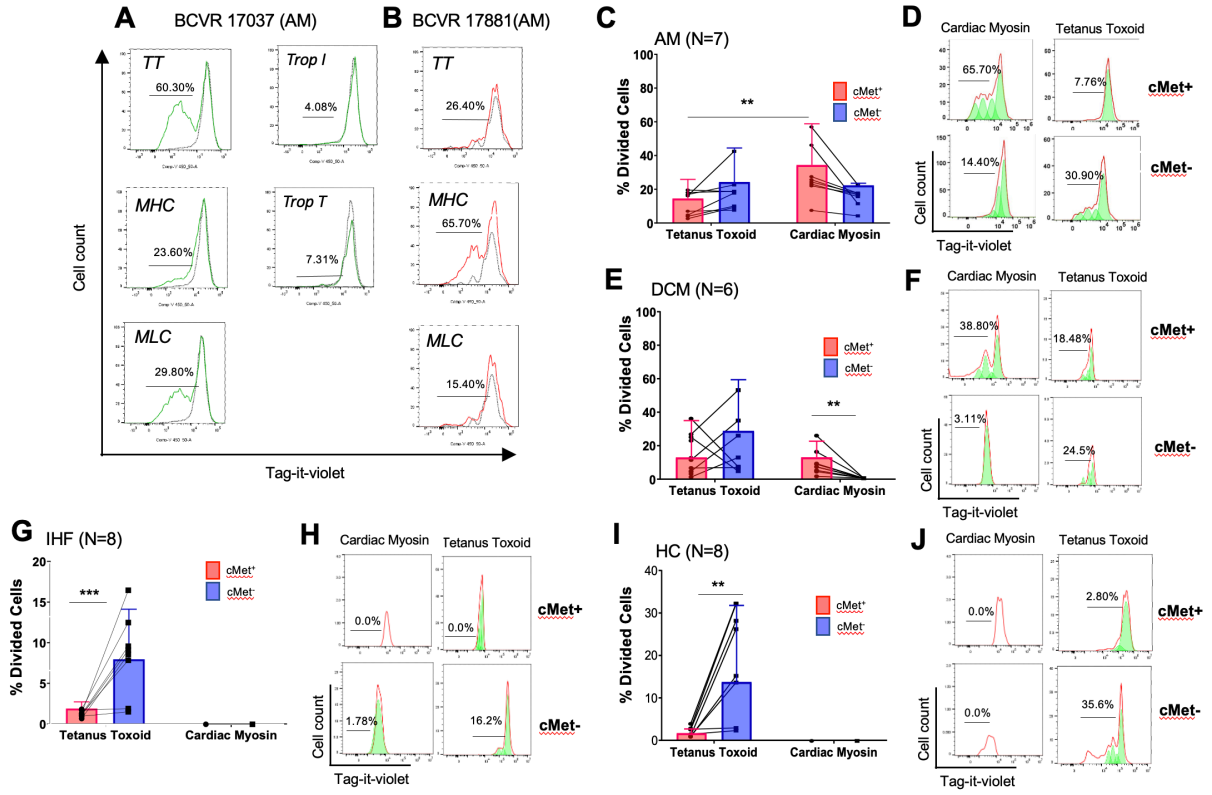
H-I, The production of the indicated cytokines by peripheral blood $cMet^+$ and cMet- $CD4^+$ T-cells from AM patients was assessed by intracellular staining and flow cytometry. Representative dot-plots are shown in panel **H**. In the data summary panel **I** the red dots represent c-Met positive cells and the blue dots represent c-Met negative cells. Statistical analysis was performed by Wilcoxon signed rank tests.

J-N, Analysis of multiple cytokine producer $cMet^+$ and c-Met- T-cells. **J**: single positive IL-17A-producing $cMet^+$ and c-Met- T-cells. **K**: single positive IL-22^+ $cMet^+$ and c-Met- cells. **L**: IL-17^+ IL-22^+ co-producing circulating $cMet^+$ and cMet- memory T-cells. **M**: $cMet^+$ and cMet- IL-4^+IL-17^+T-cells and IL-4^+IL-22^+ T-cells. **N**: IL-4^+IL-22^+IL-$17A^+$ triple-

positive cMet^{+} and cMet- memory T-cell populations. Wilcoxon signed rank was used for statistical significance.

P values are highlighted as: ****p<0.0001, ***p<0.0005, **p<0.005, *p<0.05 and data are represented as median ± interquartile range.

Figure 3: cMet$^+$ T-cells proliferate to cardiac myosin.



Peripheral blood T-cells from AM patients were labelled with an intravital fluorescent dye (Tag-it-violet) and co-cultured with the indicated antigens. For controls, no antigen was added to the cultures. **A-B**, Example proliferative responses (tag-it-violet dilution) in 2 AM patients (BCVR 17037 and 17881) depicted histographically by total CD3$^+$ live lymphocytes in response to antigen. Unstimulated T-cells (no-antigen controls) are shown by the black-colored lines. Abbreviations: TT, tetanus toxoid MHC; myosin heavy chain, MHL; myosin light chain; Trop, troponin.

C, Flow cytometry of post-proliferation sample using c-MET antibody to determine cMet$^+$/- status of proliferating cells. Cell replication is indicated by a left-shift on the x-axis. Red line - raw flow cytometry data (cell count). Green peaks - derived exemplar cell replication

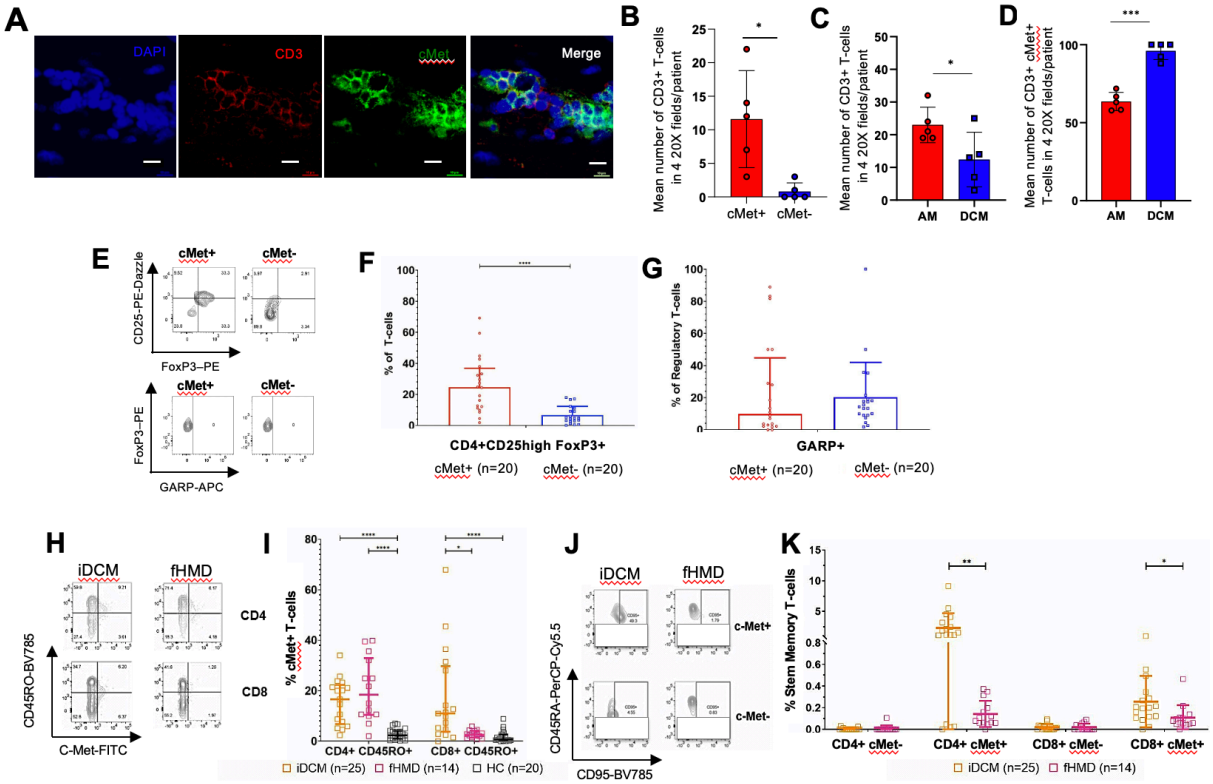
(e.g. cardiac myosin/cMet$^+$ sample: from right-left division (d)0, d1, d2 and d3). Panel **D** shows a summary of the responses to cardiac myosin and TT by cMet$^+$ and cMet$^-$ T-cells from 7 AM patients. Each dot represents cMet$^+$ and cMet- T-cell responses, and the lines linking dots identify the same individual's cMet$^+$ and cMet- T-cell responses to either tetanus toxoid or myosin.

Identical assays were performed using PBMC from 6 iDCM patients (panels **E-F**), 8 IHF patients (panels **G-H**) and 8 HC (panels **I-J**).

Data were analyzed using repeated measures two-way ANOVA followed by a significant Tuckey's multiple comparison test for cMet$^+$ response to TT compared to cMet$^+$ response to cardiac myosin

P values are highlighted as: **p<0.005 and data are represented as median ± interquartile range.

Figure 4: Disease-specific features of cMet⁺ memory T-cells.



A, Confocal analysis of CD3⁺ (red) cMet⁺ (green) cells in post-mortem paraffin-embedded iDCM samples. Scale bar: 20 μm. **B**, mean number of cMet⁺ and cMet- CD3⁺ T-cells in four 20x fields from each of 5 unique iDCM myocardial tissue samples. **C**, mean number of cMet⁺ and cMet- CD3⁺ T-cells in four 20x fields from each of 5 independent samples of AM and 5 samples of iDCM myocardium. **D**, mean percentage of cMet⁺ T-cells in CD3⁺ T cell infiltrates in four 20x fields from each of in 5 independent AM or DCM samples (mean ± SEM, unpaired Student's t-Test).

E-G, FoxP3 and CD25 expression (upper dot plots) and expression of GARP by FoxP3⁺ CD25⁺ cells (lower dot plots) by peripheral blood cMet⁺ and cMet- CD4⁺ T-cells iDCM

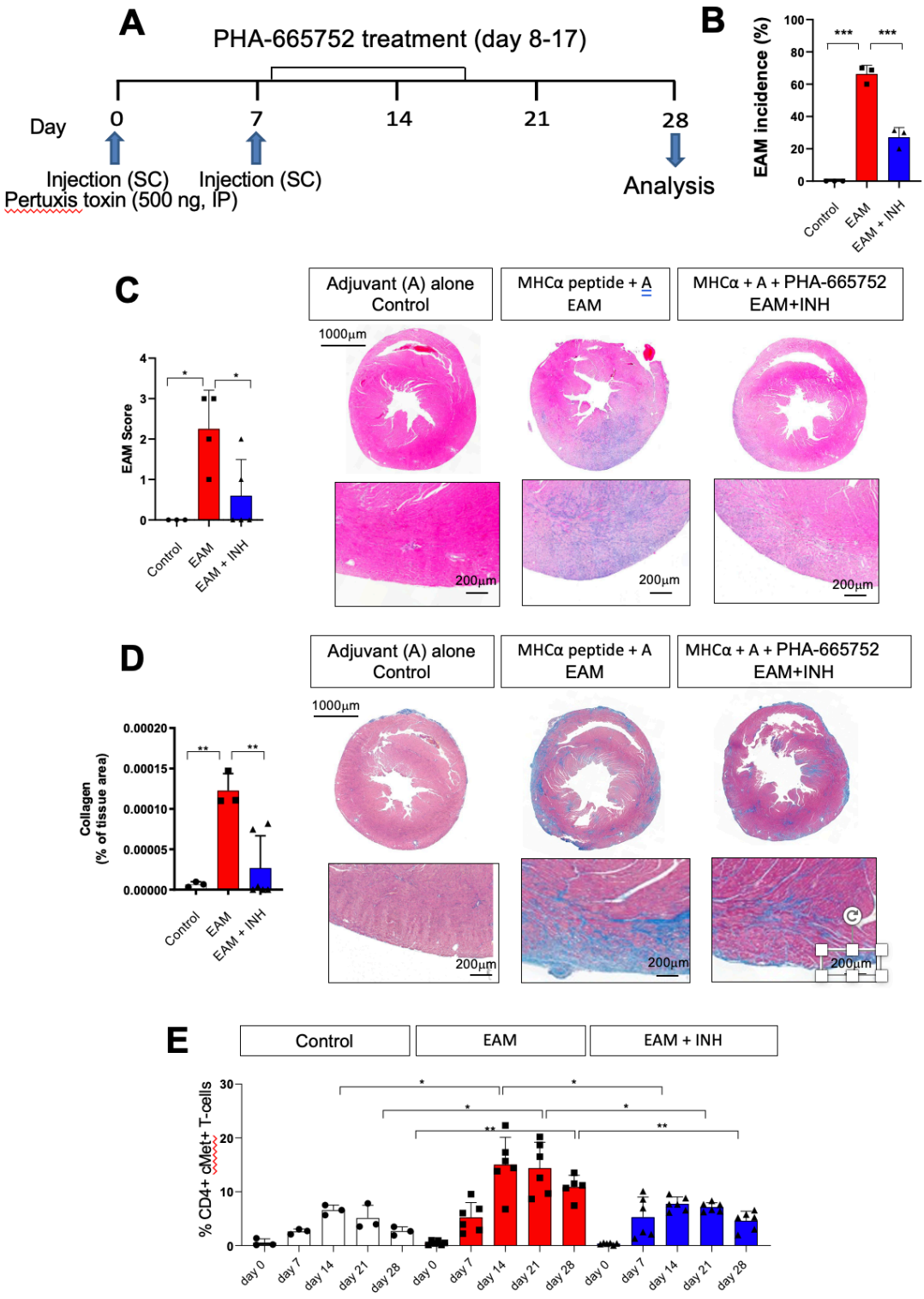
patients. Panel **E** includes representative dot-plots and summary data are shown in panels **F** and **G**.

Panel **H** shows representative dot plot for c-Met-expressing $CD4^+$ and $CD8^+$ T-cells from peripheral blood samples from dilated cardiomyopathy (DCM) and genetically-confirmed familial heart muscle disease (fHMD). A summary of the data, including healthy controls (HC), is shown in panel **I** with a significant Kruskal-Wallis test for both $CD4^+$ and $CD8^+$ T-cells. For $CD4^+$ T-cells there was a significant Dunn's multiple comparison test for DCM vs. HC and fHMD vs. HC, but not DCM vs. fHMD. For $CD8^+$ T-cells there was a significant Dunn's multiple comparison test for DCM vs. HC and DCM vs. fHMD but not between fHMD and HC.

J-K: Peripheral blood T-cells from AM, DCM and fHMD and were stained for markers of stem memory T-cells (SMTC, $CD3^+CD4^+CCR7^+CD45RA^+CD95^+$). Representative dot plots obtained after gating on $CD3^+CD4^+CCR7^+$ T-cells from non-familial DCM and fHMD are shown in panel J. Grouped data displaying the proportion of $CD4^+$ and $CD8^+$ SMTC cells in the peripheral blood of DCM and fHMD patient groups are shown in panel K. Statistical analysis was performed with Mann Whitney tests that were significant for the $CD4^+$c-Met^+ T-cells and $CD8^+$c-Met^+ T-cells but not c-Met^- $CD4^+$ or $CD8^+$ T-cells.

P values are highlighted as: ****$p<0.0001$, ***$p<0.001$, **$p<0.01$, *$p<0.05$ and data are represented as median ± interquartile range.

Figure 5. cMet^{+} T-cells mediate inflammation in experimental autoimmune myocarditis.

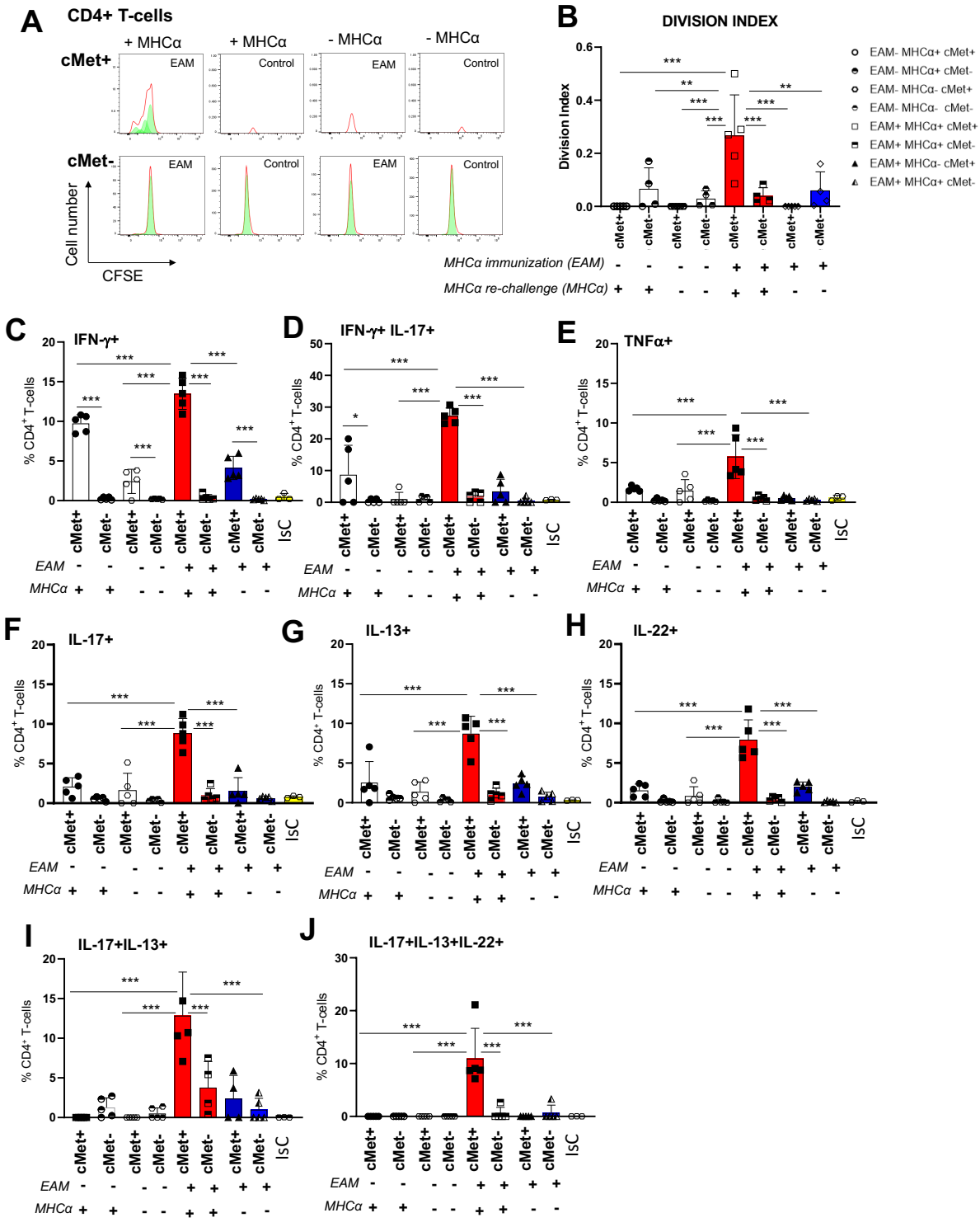


To induce autoimmune myocarditis, Balb/cAnN male mice were immunized with Murine cardiac Myosin Heavy Chain (MHCα) peptide (RSLKLMATLFSTYASADR) as described in Methods. As shown in the protocol summarized in panel **A**, some mice received i.p. injections of the cMet inhibitor PHA-665752 (500µg/ml; $EAM^{+}INH$) from day 8 to day 17 after immunization. As a control, a group of mice received adjuvant alone (Control). Panel **B** shows EAM incidence 28 days after the first immunization. The development of inflammatory infiltrates and collagen deposition were assessed by HE (C) and Masson's Trichrome staining (D) of the heart 28 days after immunization. Column graphs show disease scores obtained as described in Methods. Representative images at 20X and 40X magnification are shown on the right-hand side of each panel. Statistical analysis was performed with one-way ANOVA. N=3.

E: Tail vein blood was sampled on the same mouse on the indicated time points. The measurement on different days were taken from the same mouse. The % of CD44high, $CD4^{+}$ $cMet^{+}$ T-cells was determined by flow cytometry. Statistical analysis was performed with repeated measures two-way ANOVA test.

P values are highlighted as: ***$p<0.0005$, **$p<0.005$, *$p<0.05$ and data are represented as median ± interquartile range.

Figure 6. Specificity and functional characteristics of cMet⁺ T-cells in EAM.



Balb/cAnN male mice were immunized with murine MHCα peptide (RSLKLMATLFSTYASADR). Fourteen days after the second immunization, mice were sacrificed and $CD4^+$ T-cells from heart-draining LNs re-stimulated in vitro with autologous splenocytes, stimulatory anti-CD28 antibody and MHCα peptide. Controls included mice treated with adjuvant alone. The white bars correspond to the negative controls, where mice were not immunized nor rechallenged with MHCα peptide (i.e. EAM-, MHCα-). Red and blue bars correspond to immunized mice (i.e., EAM+) with and without MHCα peptide rechallenge (red bars: MHCα+, blue bars: MHCα-), respectively.

A-B: Prior to stimulation, some T-cells were labeled with CFSE. Cells were harvested and analyzed 5 days later for CFSE dilution and $cMet^+$ expression. Representative histograms are shown in panel A. In panel B, the division index measured in samples from 5 animals are shown (N=2)

C-J: Production of the indicated cytokines by $cMet^+$ and $cMet^-$ T-cells was measured 6 hours after re-stimulation with MHCα peptide by intracellular staining.

In the experimental design, two independent variables were applied on the same level (i.e. EAM+/-, and MHCα+/-) in non-independent samples. Downstream of EAM immunization and MHCα peptide rechallenge cMet-positive and -negative populations were discriminated through flow cytometry evaluation. A mixed-effects ANOVA test was used for statistical analysis, to account for the fact that some samples are paired and correlated.

P values are highlighted as: ***$p<0.001$, **$p<0.01$, *$p<0.05$ and data are represented as median ± interquartile range.