Global deficiency in the inflammatory chemokine receptors 1,2,3 and 5 ameliorates atherosclerosis and selectively modulates aortic myeloid cell phenotype

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**Funding Acknowledgements:** Type of funding sources: Private grant(s) and/or Sponsorship. Main funding source(s): This work is supported by the British Heart Foundation (BHF).

**Background:** Atherosclerosis is a chronic inflammatory disease in which leukocytes traffic to the vessel wall, a process in part mediated by the inflammatory chemokine receptors (iCCRs): Ccr1, Ccr2, Ccr3 and Ccr5. iCCRs display significant ligand promiscuity and redundancy, which have hampered the definitive deduction of the isolated and collective roles of these receptors.

**Purpose:** To assess iCCR expression in human and mouse atherosclerosis and determine the contribution of global iCCR expression to atherosclerosis formation in mice.

**Methods:** We derived data from the human carotid endarterectomy biobanks, Athero-Express and Biobank of Karolinska Endarterectomy (BIKE) to investigate iCCR expression. Next, we employed fluorescent reporter mice (iREP) to track iCCR expression via flow cytometry and confocal microscopy. To ascertain the contribution of iCCRs to atherosclerosis, we utilised mice in which the complete iCcr locus incorporating Ccr1, Ccr2, Ccr3 and Ccr5 has been deleted (TACKO mice). We induced atherosclerosis using tail vein injection of AAV-8 proprotein convertase subtilisin/kexin type 9 (PCSK9) in combination with 12- or 20-weeks Western diet (WD). Plaque size and composition was assessed via histology while cytometry of time-of-flight (CyTOF) and single-cell RNA-sequencing (scRNA-seq) were used to assess cellular changes.

**Results:** By analysing scRNA-seq data from Athero-Express, CCR1 emerged as the most strongly expressed iCCR and was highly localised to macrophages. Next, by utilizing BIKE data, we discovered that CCR1, 2 and 5 expression increased in plaques compared to healthy vessels, with CCR1 and CCR5 more strongly associated with symptomatic patients compared with asymptomatic patients. In iREP mice, CCR1, 2 and 5 + cell numbers were significantly increased in the atherosclerotic aorta compared with healthy mice. Cellular expression levels were variable with CCR2 being dominant in the plaque and increasing markedly on macrophages. Following 12 weeks WD, there was a reduction in mean plaque area within the aortic sinus of TACKO mice (WT 0.20 mm\textsuperscript{2} vs. TACKO 0.10 mm\textsuperscript{2}, n=19-20, P<0.0001). Following 20 weeks WD, mean plaque area was also reduced in TACKO mice (WT 0.41 mm\textsuperscript{2} vs. TACKO 0.25 mm\textsuperscript{2}, n=9-12, P<0.01) and this was associated with a significant reduction in necrotic core area and a reduction in calcification, reflecting a shift towards a more stable plaque phenotype, as classified according to the Virmani grading system. CyTOF analysis revealed reductions in inflammatory monocytes and conventional type 2 dendritic cells in TACKO aorta accompanied by a phenotypic switch in macrophages towards a less inflammatory phenotype. Finally, scRNA-seq analysis revealed phenotypic differences in non-immune vascular cells between WT and TACKO mice.

**Conclusion:** Our results support an athero-protective, plaque-stabilizing effect of iCcr deletion with a selective reduction in inflammatory myeloid cell aortic homing.