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SHORT REPORT

H-2g, a glucose analog of blood group H antigen, mediates monocyte recruitment in vitro and in vivo via IL-8/CXCL8

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Objective: Monocyte (MN) recruitment is an essential inflammatory component of many autoimmune diseases, including rheumatoid arthritis (RA). In this study we investigated the ability of 2-fucosyllactose (H-2g), a glucose analog of blood group H antigen to induce MN migration in vivo and determined if H-2g-induced interleukin-8 (IL-8/CXCL8) plays a role in MN ingress in RA.

Methods: Sponge granuloma and intravital microscopy assays were performed to examine H-2g-induced in vivo MN migration and rolling, respectively. MNs were stimulated with H-2g, and the production of IL-8/CXCL8 was assessed by enzyme-linked immunosorbent assay and quantitative polymerase chain reaction. Lastly, in vitro MN migration assays and an in vivo RA synovial tissue severe combined immunodeficiency mouse model were used to determine the role of IL-8/CXCL8 in H-2g-induced MN migration.

Results: In vivo, H-2g induced significantly greater MN migration compared to phosphate buffered saline. Intravital microscopy revealed that H-2g mediates MN migration in vivo by inducing MN rolling. In addition, H-2g induced MN production of IL-8/CXCL8, a process that was dependent on Src kinase. Moreover, we found that H-2g mediated MN migration in vitro, and in vivo migration was inhibited by a neutralizing anti-IL-8/CXCL8 antibody.

Conclusion: These findings suggest that H-2g mediates MN recruitment in vitro and in vivo (in part) via IL-8/CXCL8.

Keywords: inflammation, rheumatoid arthritis, chemokine, migration

Introduction

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by synovial hyperplasia, neovascularization, and inflammation. Many chemokines are upregulated in RA serum and synovial fluid.[1] Interleukin-8 (IL-8/CXCL8) is a member of the CXC chemokine family that is abundantly expressed by key pathogenic cell types in RA, including fibroblasts, endothelial cells, and macrophages.[2–5] IL-8/CXCL8 promotes neutrophil and monocyte (MN) migration by upregulating the expression of adhesion molecules.[6] These cells perpetuate the disease by producing a variety of chemokines, angiogenic factors, and inflammatory mediators.[7]

In addition to chemokines, other types of mediators have chemotactic properties. We have previously shown that 2-fucosyllactose (H-2g), a soluble analog of the blood group H antigen, directly mediates leukocyte recruitment both in vitro and in vivo.[8] Here, we sought to investigate if H-2g induces MN migration in vivo, and to determine if H-2g mediates MN recruitment via IL-8/CXCL8.

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Materials and methods

Patients and controls

Peripheral blood (PB) samples were collected from healthy volunteers. RA synovial tissue (ST) samples were obtained from patients undergoing synovectomy or total joint replacement. The study was approved by the University of Michigan Institutional Review Board.

Animals

All experiments were performed with approval from the University of Michigan Committee on Use and Care of Animals.

Sponge granuloma model

We performed a mouse sponge model to evaluate MN recruitment in vivo as previously described.[9] Briefly, phosphate-buffered saline (PBS) or H-2g-treated polyvinyl sponges were inserted subcutaneously into C57BL/6 mice (National Cancer Institute at the National Institutes of Health, Bethesda, MD). Simultaneously, human PB MNs, dye-tagged with PKH26 (Sigma-Aldrich, St Louis, MO), were injected intravenously. After 48 hours, mice were euthanized, sponges removed, and MNs isolated. Cytospins were performed and dye-tagged cells were counted by a blinded observer.

Intravital microscopy

Intravital microscopy was performed as previously described using C57BL/6 mice (National Cancer Institute at the National Institutes of Health, Bethesda, MD).[10] H-2g (100 μM) or PBS was injected intravenously 2 hours before executing intravital microscopy (three mice per group).[11] Three to five videos were taken per mouse. Rolling leukocytes were defined as leukocytes that rolled at a velocity slower than red blood cells.

Isolation of human MNs

MNs were isolated from the PB of normal healthy volunteers as previously described.[8] The viability of MNs determined by trypan blue exclusion was found to be >98%, and the purity was >90%.

Enzyme-linked immunosorbent assay (ELISA)

Isolated human MNs were serum-starved for 2 hours and incubated with PP2 529573 (Src inhibitor), LY294002 (phosphatidylinositol 3-kinase [PI3K] inhibitor), PDTC (nuclear factor kappa-light-chain-enhancer of activated B cells [NFκB] inhibitor), Ro31 (protein kinase C [PKC] inhibitor), or dimethyl sulfoxide (vehicle control) for 1 hour. All inhibitors were purchased from Calbiochem (Calbiochem Novabiochem Corp, San Diego, CA) and were used at 10 μM. The MNs were then stimulated with H-2g (500 nM) in the presence of each inhibitor, based on our previous study.[8] Cell culture supernatants were collected after 24 hours and assayed using a human IL-8/CXCL8 ELISA kit (R&D Systems, Inc, Minneapolis, MN).[12]

RNA extraction and quantitative polymerase chain reaction (qPCR)

MNs were cultured as described above, RNA was extracted, and qPCR was performed as previously described.[12,13] Primers for human IL-8/CXCL8 (5′-GAGGGTTGTGGAGAAGTTTTTG-3′, 5′-CTGGCATCTTCACTGATTCTTG-3′) and human β-actin (5′-GTCAGGCAGCTCGTAGCTCT-3′, 5′-GCCATGTACGTTGCTATCCA-3′) were used. All samples were run in duplicate and the relative abundance of each gene was normalized to β-actin.

MN chemotaxis

MN chemotaxis was performed using 48-well modified Boyden chambers (Neuro Probe, Inc, Cabinjohn, MD) as previously described.[8] H-2g (500 nM) in the presence of mouse anti-human IL-8/CXCL8 antibody (R&D Systems, Inc) or an isotype-matched control antibody (R&D Systems, Inc) was added to the bottom wells of the chambers. Formyl-met-leu-phe and Hank's balanced salt solution served as positive and negative controls, respectively. Each test group was assayed in quadruplicate. Three high-power fields (hpfs; ×400) were counted in each replicate well.

Human RA ST-SCID chimeras

Severe combined immunodeficiency (SCID) mice (National Cancer Institute at the National Institutes of Health) were anesthetized, and each mouse received one RA ST graft. After 4 weeks of engraftment, human PB MNs were dye-tagged with PKH26 fluorescent dye (Sigma-Aldrich), and were injected intravenously. Simultaneously, H-2g (10 μM) with an anti-IL-8/CXCL8 antibody or an isotype control antibody (R&D Systems, Inc) was injected into the RA ST grafts. Mice were euthanized after 48 hours and grafts were removed and snap frozen in liquid nitrogen. Cryosections were examined for cell homing using a fluorescence microscope. Migrated MNs were quantified by counting

three slides per mouse, with two sections per slide, and at 6 hpfs per section.

Statistical analysis

Student's *t*-tests were performed, and *P*-values less than 0.05 were considered significant. All values presented were the mean ± standard error of the mean (SEM).

Results

H-2g induces MN migration in vivo

Sponges containing H-2g and PBS were implanted in SCID mice, and dye-tagged PB MNs were injected intravenously in the mice at the same time. In this model, leukocytes enter the sponges and form inflammatory tissue. Sponges containing H-2g recruited significantly more MNs than sponges containing PBS (Figure 1A; $P < 0.05$).

H-2g promotes migration by inducing MN rolling in vivo

To determine how H-2g induces MN migration, intravital microscopy experiments were performed. Intravital microscopy allows for the visualization of cellular processes in vivo. We found that H-2g induced a significantly greater amount of rolling leukocytes compared to PBS (Figure 1B–D; $P < 0.05$). These results suggest that the increase in MN migration in vivo is due, in part, to H-2g-increased MN rolling.

Src is required for H-2g induced MN secretion of IL-8/CXCL8

Human PB MNs were treated with H-2g and chemical signaling inhibitors, and cell culture supernatants and mRNA lysates were collected. H-2g stimulated MNs showed IL-8/CXCL8 expression at both the mRNA and protein levels (Figure 2). Moreover, H-2g-induced MN production of IL-8/CXCL8 was significantly decreased in the presence of a Src inhibitor (Figure 2; $P < 0.05$). In contrast, inhibitors of PKC, PI3K, and NF-κB did not inhibit H-2g-induced MN secretion of IL-8/CXCL8 (Figure 2).

IL-8/CXCL8 is required for H-2g-induced MN migration in vitro

In vitro chemotaxis assays were performed to determine if IL-8/CXCL8 plays a role in H-2g-induced MN migration. MN migration to H-2g was significantly decreased in the

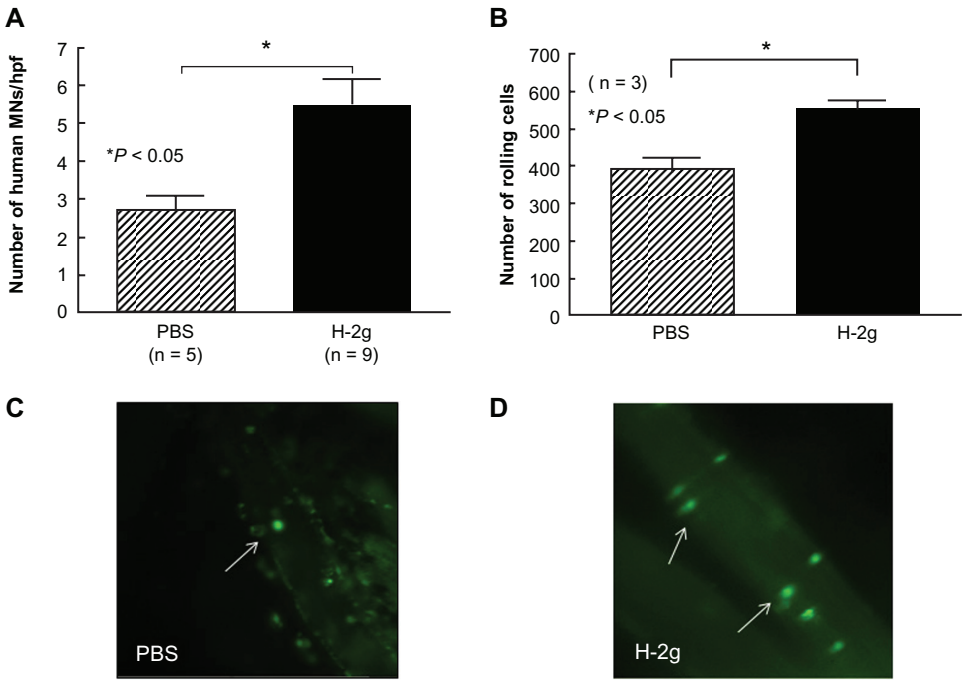

Figure 1 H-2g induces MN migration and leukocyte rolling in vivo. (**A**) H-2g or PBS was applied to sponges, which were then inserted into C57BL/6 mice. The sponges were removed, sectioned, and MN were counted. (**B**) C57BL/6 were injected intravenously with either H-2g or PBS, and the amount of rolling leukocytes was assessed using intravital microscopy. Representative photographs are shown for (**C**) PBS- and (**D**) H-2g-treated mice.

Notes: Arrows indicate rolling MNs. For both experiments, n = the number of mice; means are presented with SEM; differences were determined using the Student's *t*-test; and *P*-values less than 0.05 were significant *$P < 0.05$.

Abbreviations: MN, monocyte; H-2g, 2-fucosyllactose; PBS, phosphate-buffered saline.

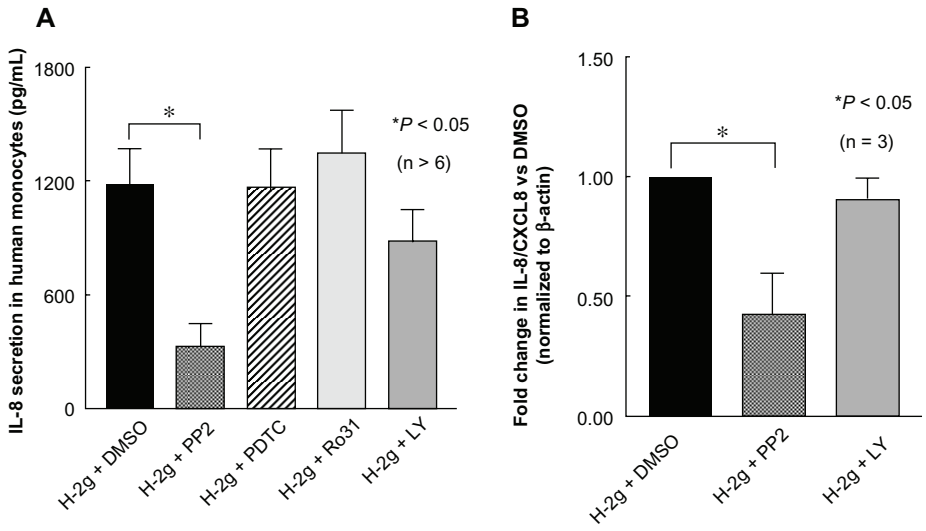

Figure 2 Src is required for H-2g induced MN production of IL-8/CXCL8. (**A**) Isolated MNs were cultured in the presence of H-2g with DMSO (vehicle control), PP2 529573 (Src inhibitor), PDTC (NF-κB inhibitor), Ro31 (PKC inhibitor), or LY294002 (PI3K inhibitor). Cell culture supernatants were collected and subject to an ELISA to determine the amount of IL-8/CXCL8. (**B**) Isolated MNs were cultured in the presence of H-2g with DMSO (vehicle control), PP2 529573 (Src inhibitor), or LY294002 (PI3K inhibitor).

Notes: mRNA was collected, cDNA was prepared, and qPCR was performed using primers specific for IL-8/CXCL8 or actin. For both experiments, n = the number of replicates; means are presented with SEM; differences were determined using the Student's *t*-test; and *P*-values less than 0.05 were significant.*$P < 0.05$.

Abbreviations: H-2g, 2-fucosyllactose; MN, monocyte; IL-8/CXCL8, interleukin-8; DMSO, dimethyl sulfoxide; PKC, protein kinase C; ELISA, enzyme-linked immunosorbent assay; qPCR, quantitative polymerase chain reaction.

presence of a neutralizing anti-IL-8/CXCL8 antibody when compared to H-2g induced migration with an isotype control antibody (Figure 3A; $P < 0.05$). This result suggests that IL-8/CXCL8 mediates H-2g-induced MN migration.

IL-8/CXCL8 is required for H-2g induced MN migration to RA ST in vivo

We employed an RA ST-SCID mouse chimera to determine if IL-8/CXCL8 plays a role in H-2g-induced MN migration in vivo. RA ST grafts treated with H-2g and an isotype control antibody showed a significant increase in MN migration compared to those treated with PBS ($P < 0.05$) (Figure 3B–D). In comparison, treating RA ST grafts with H-2g and a neutralizing anti-IL-8/CXCL8 antibody resulted in a significant decrease in MN migration compared to those treated with H-2g with an isotype control antibody ($P < 0.05$). Collectively, these results suggest that H-2g mediates MN migration in vitro and in vivo via IL-8/CXCL8.

Discussion

MN migration is a key step in the pathogenesis of RA. Here we investigated the ability of H-2g to induce MN migration in vivo and tested the hypothesis that H-2g mediates MN migration, in part, via IL-8/CXCL8. We first showed that H-2g induces monocyte migration in vivo in a sponge model. Our data also demonstrated that H-2g increases the ability of MNs to roll along vessels. As rolling adhesion is the first step in the leukocyte migration cascade, these findings further implicate H-2g as a mediator of MN migration.

We then determined if MNs stimulated with H-2g secrete IL-8/CXCL8. Previous studies have shown that the stimulation of MNs with lipopolysaccharides increases the production of IL-8/CXCL8, a potent MN chemoattractant.[14] We found that IL-8/CXCL8 production occurs in MNs treated with H-2g. This process was dependent on Src, but not NF-κB, PKC, or PI3K. Src family kinases have previously been shown to be required for MN production of IL-8/CXCL8 following stimulation by lipopolysaccharides and other mediators.[15,16] Previously, we demonstrated that H-2g induces the phosphorylation of Src, PKC, Akt, and IκBα in MNs, and that H-2g-mediated MN chemotaxis in vitro is dependent on Src and PI3K.[8] Our data now suggest that Src is required for H-2g-induced MN production of IL-8/CXCL8 and further illustrates the importance of Src in MN migration.

After observing that H-2g stimulates MN production of IL-8/CXCL8, we explored the relationship between H-2g and IL-8/CXCL8 with respect to MN chemotaxis.[6] We found that MN chemotaxis in response to H-2g is significantly

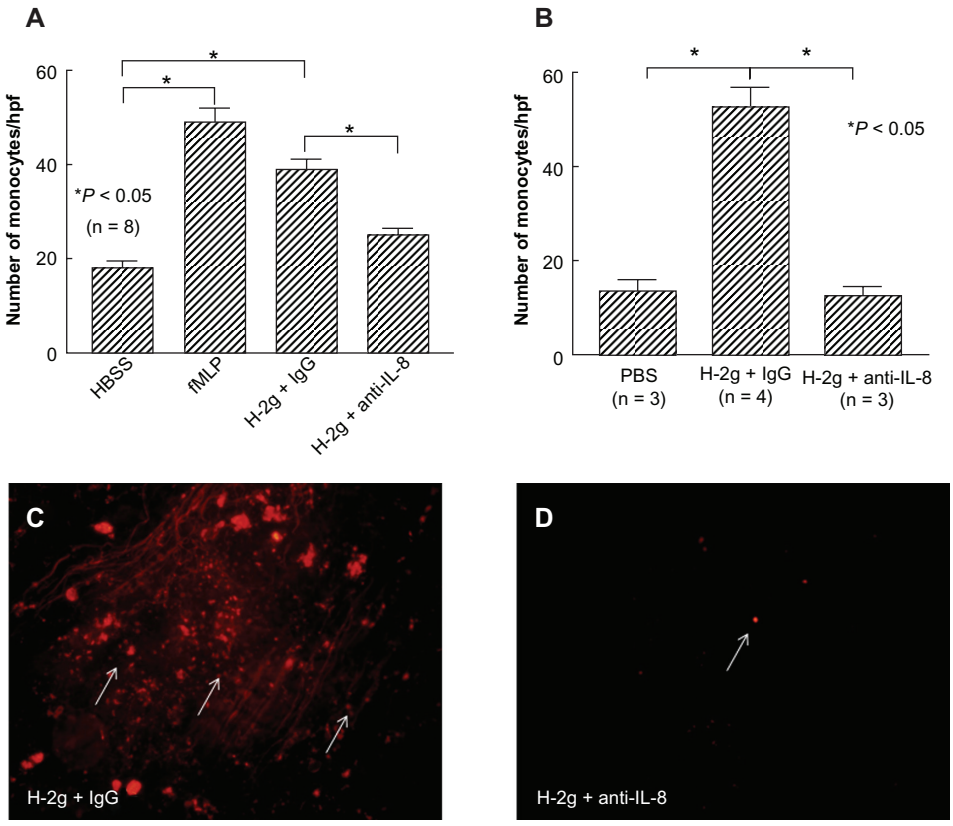

Figure 3 H-2g mediates in vitro and in vivo MN chemotaxis via IL-8/CXCL8. (**A**) MN chemotaxis assays were performed using a modified Boyden chamber. MN migration was determined in response to H-2g (500 nM) with either neutralizing anti-IL-8/CXCL8 antibody or an isotype matched IgG control. PBS and formyl-met-leu-phe were used as negative and positive controls, respectively. Three hpfs were counted per well and the assay was performed in quadruplicate; n = the number of replicates. Means are presented with SEM, differences were determined using the Student's *t*-test. (**B**) SCID mice were engrafted with RA ST and 4 weeks later fluorescence-labeled MNs were injected intravenously. At the same time, RA ST grafts were injected with H-2g (100 μM) and either a neutralizing anti-IL-8/CXCL8 antibody or an isotype matched IgG control. After 48 hours, the mice were sacrificed, ST was removed and sectioned, and migrated MNs were counted. Six ST sections were analyzed per mouse and 6 hpfs were counted per section. Means are presented with SEM; differences were determined using the Student's *t*-test; and n = the number of mice. Representative photographs of RA ST graft sections treated with either H-2g + IgG (**C**) or H-2g + anti-IL-8/CXCL8 (**D**) are shown at 200×.

Notes: Arrows indicate MNs.*$P < 0.05$.

Abbreviations: H-2g, 2-fucosyllactose; MN, monocyte; IL-8/CXCL8, interleukin-8; HBSS, Hank's balanced salt solution; IgG, immunoglobulin-G; PBS, phosphate-buffered saline; hpfs, high power fields; SCID, severe combined immunodeficiency; RA, rheumatoid arthritis; ST, synovial tissue.

impaired with the addition of an anti-IL-8/CXCL8 antibody. Collectively, these findings implicate H-2g as both a direct and an indirect mediator of MN migration.

Following this observation, we utilized an RA ST-SCID mouse chimera model to determine if H-2g induces MN migration in vivo.[8,17] Here we found that MN migration to RA ST was significantly reduced in the presence of neutralizing anti-IL-8/CXCL8 antibody compared to an isotype control. These results are consistent with our in vitro findings and suggest that IL-8/CXCL8 plays a significant role in H-2g induced MN migration in vivo.

MNs are primary mediators of inflammation in RA. We have previously shown that H-2g promotes inflammation by upregulating intercellular adhesion molecule-1 on endothelial cells.[18] Here we demonstrated that H-2g promotes MN migration, in part, via the induction of IL-8/CXCL8. These results further implicate H-2g as a potential therapeutic target in chronic inflammatory diseases like RA.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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