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H-2g, a Glucose Analog of Blood Group H Antigen, Mediates Mononuclear Cell Recruitment via Src and Phosphatidylinositol 3-Kinase Pathways

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Objective. **Monocyte recruitment by proinflammatory cytokines is a hallmark of rheumatoid arthritis (RA). Lewis^{y-6} and H (Le^{y}/H) are blood group antigens up-regulated on RA synovial endothelium. We have previously shown that both soluble Le^{y}/H and a glucose analog of H, H-2g, are angiogenic and mediate leukocyte–endothelial adhesion via induction of intercellular adhesion molecule 1. We hypothesized that soluble Le^{y}/H plays an important role in monocyte recruitment in RA.**

Methods. **We examined the role of H-2g in monocyte chemotaxis in vitro. We used an RA synovial tissue (ST)–SCID mouse chimera model to evaluate the role of H-2g in monocyte recruitment in vivo. We used Western blots to examine signaling molecules activated by H-2g in monocytes.**

Results. **H-2g induced human monocyte migration in vitro, which was mediated by Src and phosphatidylinositol 3-kinase (PI 3-kinase), since inhibitors and antisense oligodeoxynucleotides (ODNs) of Src and PI 3-kinase significantly decreased H-2g–induced monocyte migration ($P < 0.05$). H-2g significantly increased mononuclear cell (MNC) homing in vivo into an RA ST–SCID mouse chimera ($P < 0.05$). Transfection of MNCs with Src antisense ODNs blocked H-2g–induced MNC recruitment into the RA ST–SCID mouse chimera. Additionally, H-2g induced marked phosphorylation of protein kinase CαI/βII (PKCαI/βII), Src, IκBα, and Akt in monocytes. Src, Akt, and NF-κB were shown to be downstream targets of PKCαI/βII, since an inhibitor of PKCαI/βII reduced H-2g–mediated phosphorylation of Src, Akt, and NF-κB in monocytes.**

Conclusion. **These data suggest that H-2g may be a novel mediator of monocyte recruitment in chronic inflammatory diseases like RA.**

Leukocyte adhesion and recruitment are critical steps in the pathogenesis of rheumatoid arthritis (RA) (1,2). We have developed a monoclonal antibody (mAb), termed 4A11, which detects the glycoconjugates H-5-2 and Lewis^{y-6} (Le^{y}/H). Le^{y}/H is cytokine inducible on human endothelial cells (ECs) (3). Soluble Le^{y}/H and a glucose analog of blood group H antigen, 2-fucosyllactose, termed H-2g, mediate angiogenesis and leukocyte–endothelial adhesion via up-regulating endothelial intercellular adhesion molecule 1 (ICAM-1) (3–5). Activities of Le^{y}/H are mimicked by H-2g, and H-2g–induced angiogenesis is abrogated by mAb 4A11 (4).

Glycoconjugates play an important role in cell adhesion and leukocyte trafficking at sites of inflammation as well as in angiogenesis (6). Nguyen et al have identified a role for sialyl Le^{x} in capillary morphogenesis (7). Maly et al found that mice deficient in the enzymes that generate sialyl Le^{x} have a partial defect in neutrophil accumulation in response to an acute inflammatory stimulus (8).

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In the present study, we examined the role of H-2g in leukocyte ingress at sites of chronic inflammation. We determined the signaling mechanisms by which H-2g recruits monocytes in vitro and in vivo. We evaluated its role in leukocyte migration in an RA synovial tissue (ST)–SCID mouse chimera. H-2g induced phosphorylation of protein kinase CαI/βII (PKCαI/βII), Akt, and NF-κB in monocytes. Our data suggest that therapies targeting H-2g, such as mAb 4A11, may be beneficial in treating chronic inflammatory diseases such as RA.

MATERIALS AND METHODS

Isolation of human monocytes and chemotaxis in vitro. Monocytes were isolated from normal human peripheral blood (PB) as described previously (9–11). Cell viability determined by trypan blue exclusion was >98%, and purity was >80%. Monocyte chemotaxis was performed using 48-well modified Boyden chambers (Neuro Probe, Cabin John, MD) as described elsewhere (10,11). Hanks' balanced salt solution (HBSS) and fMLP (100 n*M*) served as negative and positive controls, respectively.

We performed monocyte chemotaxis in vitro to investigate the signaling mechanisms involved in H-2g–induced monocyte migration using the chemical signaling inhibitors PP2 529573 (Src inhibitor), LY294002 (phosphatidylinositol 3-kinase [PI 3-kinase] inhibitor), and pertussis toxin (G protein inhibitor). All inhibitors were purchased from Calbiochem (San Diego, CA) except for pertussis toxin, which was purchased from Sigma (St. Louis, MO). Inhibitors were used at 10 μM concentration except for pertussis toxin, which was used at 500 ng/ml. Concentrations of fMLP and signaling inhibitors were based on optimal concentrations used previously, and the chemical signaling inhibitors are not toxic at the concentration used in this study (4,9,11,12). Monocyte chemotaxis assays were performed with H-2g and fMLP with and without inhibitors as described (10,11). To confirm our results, monocytes were transfected with Src and PI 3-kinase sense and antisense oligodeoxynucleotides (ODNs) for 48–72 hours, and chemotaxis was performed. ODN sequences of signaling intermediates used were as follows: for PI 3-kinase, sense ATGAGTGCTGAGGGGTACCAGTAC and antisense GTACTGGTACCCCTCAGCACTCAT; for Src, sense ATGGGGAGCAGCAAGAGCAAGCCC and antisense GGGCTTGCTCTTGCTGCTCCCCAT.

Mononuclear cell (MNC) homing in human RA ST–SCID mouse chimeras. Human ST was obtained from RA patients undergoing joint replacement. SCID mice (ages 4–6 weeks) were purchased from the National Cancer Institute. Anesthetized mice received 1 RA ST graft ($\sim 0.7 \times 0.7 \times 0.5$ cm) in the back, and the wound was sutured.

After 4 weeks of engraftment, isolated human PB MNCs were dye-tagged with fluorescent PKH26 (Sigma) (10,12). Each mouse was injected intravenously with dye-tagged MNCs (5×10^6). Simultaneously, we administered an intragraft injection of H-2g (10 μM). Mice were euthanized after 48 hours and grafts were removed. Cryosections (8–10-μm thick) were examined for cell homing under fluorescence. Migrated labeled MNCs were quantified in grafts by counting the total number of cells in 24 tissue sections per group of mice. The number of high-power fields (hpf) counted was dependent upon the size and composition of the graft and corresponding tissue section, counting all the labeled cells in the entire tissue section. Approximately 30–60 hpf were counted per tissue section obtained from each transplant. Phosphate buffered saline (PBS) served as a negative control. The experimental controls were the same as those used in a previously reported study testing the effects of H-2g and CXCL16 in MNC recruitment in ST-SCID mouse chimeras (10).

Monocytes were transfected with fluorescein isothiocyanate (FITC)–labeled ODNs to NF-κB for 24 hours to determine the transfection efficiency with lipofectin (Invitrogen, Carlsbad, CA). To evaluate the role of signaling intermediates in H-2g–induced MNC recruitment in vivo, MNCs were transfected with Src sense or antisense ODNs for 72 hours using lipofectin (9,10). The RA ST–SCID mouse chimera model was used with transfected MNCs, as described above (10,12). ST grafts were harvested after 48 hours, and cryosections were analyzed for migrated fluorescent MNCs.

Cell lysis and immunoblotting. Cell lysis and immunoblotting were performed as described previously (4,11). Monocytes were stimulated with H-2g (200 n*M*) for various time periods. To determine the role of signaling inhibitors, monocytes were preincubated with chemical inhibitors for 1 hour before stimulating with H-2g for 15 minutes. Monocytes transfected with PKCα or Src sense or antisense ODNs were stimulated with H-2g for 15 minutes. Western blotting was then performed, and blots were probed with phosphospecific antibodies (Cell Signaling Technology, Beverly, MA). Blots were stripped and reprobed for total PKCα, Src, Akt, and IκBα to verify equal loading. The ODN sequences of PKCα used were TCGGGGGGGACCATG (sense) and CATGGTCCCCCCCGA (antisense). After transfection, monocytes were stimulated with H-2g (200 n*M*) for 15 minutes, and cell lysates were collected. To determine transfection stability, we transiently transfected monocytes with Src sense and antisense ODNs for 24, 48, and 72 hours and stimulated monocytes with H-2g for 15 minutes.

Statistical analysis. Student's *t*-tests were performed, and *P* values less than 0.05 were considered significant. Data represent the mean $\pm$ SEM of $\geq$3 experiments.

RESULTS

H-2g induces monocyte chemotaxis in vitro. H-2g induced monocyte migration in the picomolar range in a dose-dependent manner, showing significance at 100 p*M* ($P < 0.05$), suggesting that H-2g was potently chemotactic for human monocytes (Figure 1A). H-2g increased migration of monocytes 2.3-fold at 100 p*M* and 4.5-fold at 100 μM compared with HBSS ($P < 0.05$).

Src and PI 3-kinase mediate H-2g–induced monocyte migration in vitro. H-2g–induced monocyte migration was mediated by Src and PI 3-kinase, while G proteins were not involved, suggesting that Src and PI 3-kinase play an important role in H-2g–mediated

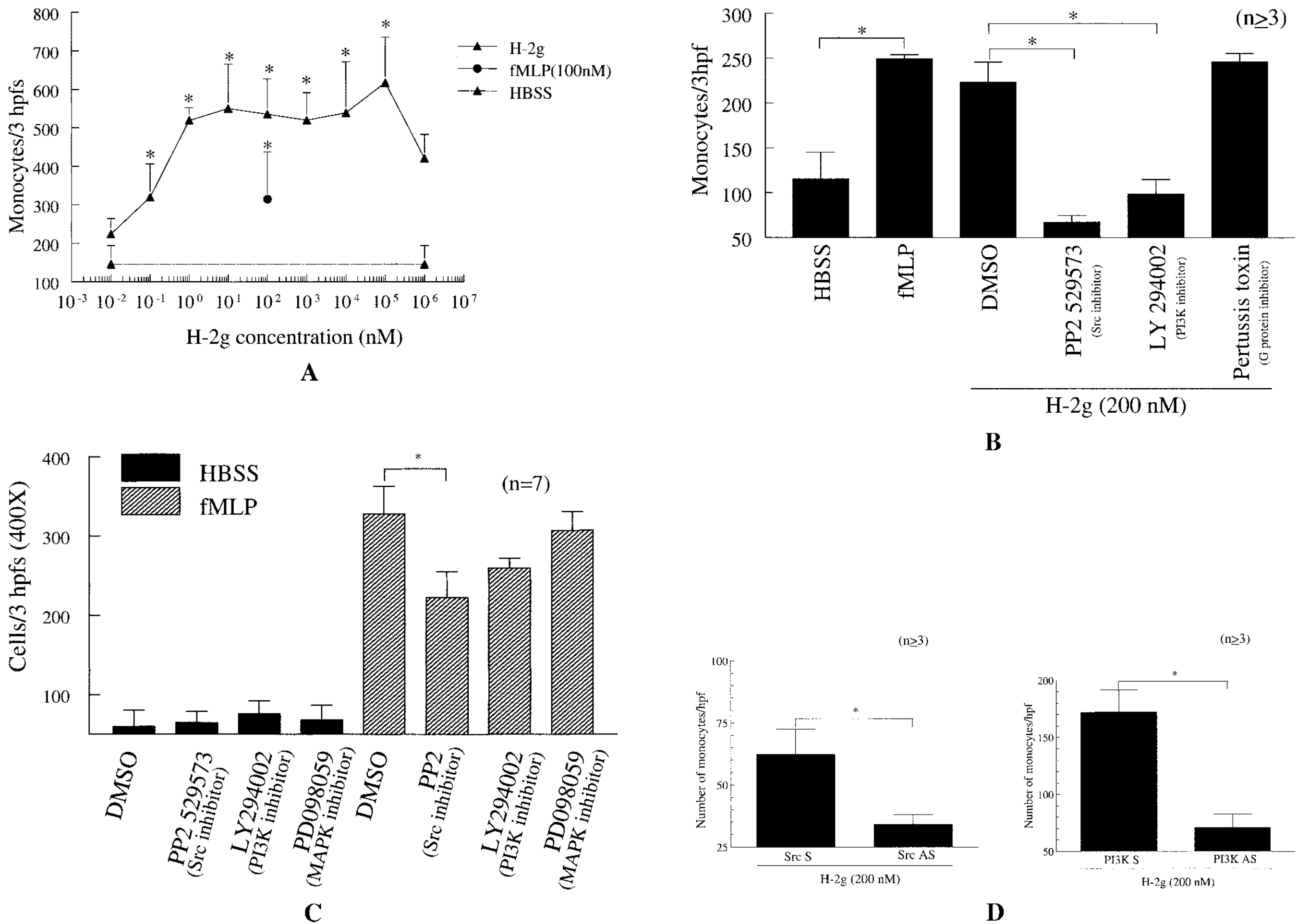

Figure 1. A, Induction of monocyte chemotaxis in vitro by H-2g. H-2g induced a concentration-dependent increase in human monocyte chemotaxis when monocytes were incubated with H-2g for 1.5 hours. H-2g–induced monocyte migration was greater than that exhibited by the positive control, fMLP (100 n*M*). Data represent the mean and SEM of 3 individual experiments. Each experiment was repeated at least 3 times, and there were 4 replicate wells in each experimental group. Three high-power fields (hpf) at 400× magnification were counted in each replicate well, and results are expressed as monocytes/3 hpfs. * = $P < 0.05$ versus Hanks' balanced salt solution (HBSS). **B,** H-2g–induced monocyte migration inhibited by inhibitors of phosphatidylinositol 3-kinase (PI 3-kinase [PI3K]) (LY294002) and Src (PP2 529573). Monocytes were incubated with signaling inhibitors (10 μM) for 15 minutes, and in vitro chemotaxis was performed with H-2g. Data represent the mean and SEM of ≥3 individual experiments. HBSS served as a negative control. All inhibitors were present during the experiment. n = number of experiments using cells from different donors. * = $P < 0.05$. **C,** Critical role of Src in fMLP-induced monocyte migration. To contrast the signaling events of the potent monocyte chemoattractant fMLP with those of H-2g, we performed monocyte chemotaxis with fMLP in the presence and absence of various signaling inhibitors. Monocyte migration induced by fMLP was inhibited by a Src inhibitor (* = $P < 0.05$), but inhibitors of PI 3-kinase and MEK-1/2 (MAPK) did not have a role in fMLP-induced monocyte migration. Signaling inhibitors also did not affect HBSS-induced monocyte basal migration. n = number of experiments using cells from different donors. **D,** Essential roles of Src and PI 3-kinase in H-2g–induced monocyte chemotaxis in vitro. To confirm our results, monocytes were transfected with Src and PI 3-kinase sense (S) and antisense (AS) oligodeoxynucleotides (ODNs) for 48–72 hours. H-2g–induced monocyte chemotaxis was significantly decreased in monocytes transfected with Src or PI 3-kinase antisense ODNs compared with sense ODN–transfected monocytes. Data represent the mean and SEM of ≥3 individual experiments. n = number of experiments using cells from different donors. * = $P < 0.05$.

monocyte chemotaxis (Figure 1B). Signaling inhibitors of Src (PP2 529573) and PI 3-kinase (LY294002) significantly decreased H-2g–induced monocyte migration, but an inhibitor of G protein (pertussis toxin) did not ($P < 0.05$).

Src mediates fMLP-induced monocyte chemotaxis in vitro. In contrast to results obtained with H-2g, while Src plays an important role in fMLP-induced monocyte migration, since an inhibitor of Src (PP2 529573) inhibited fMLP-induced monocyte chemotaxis,

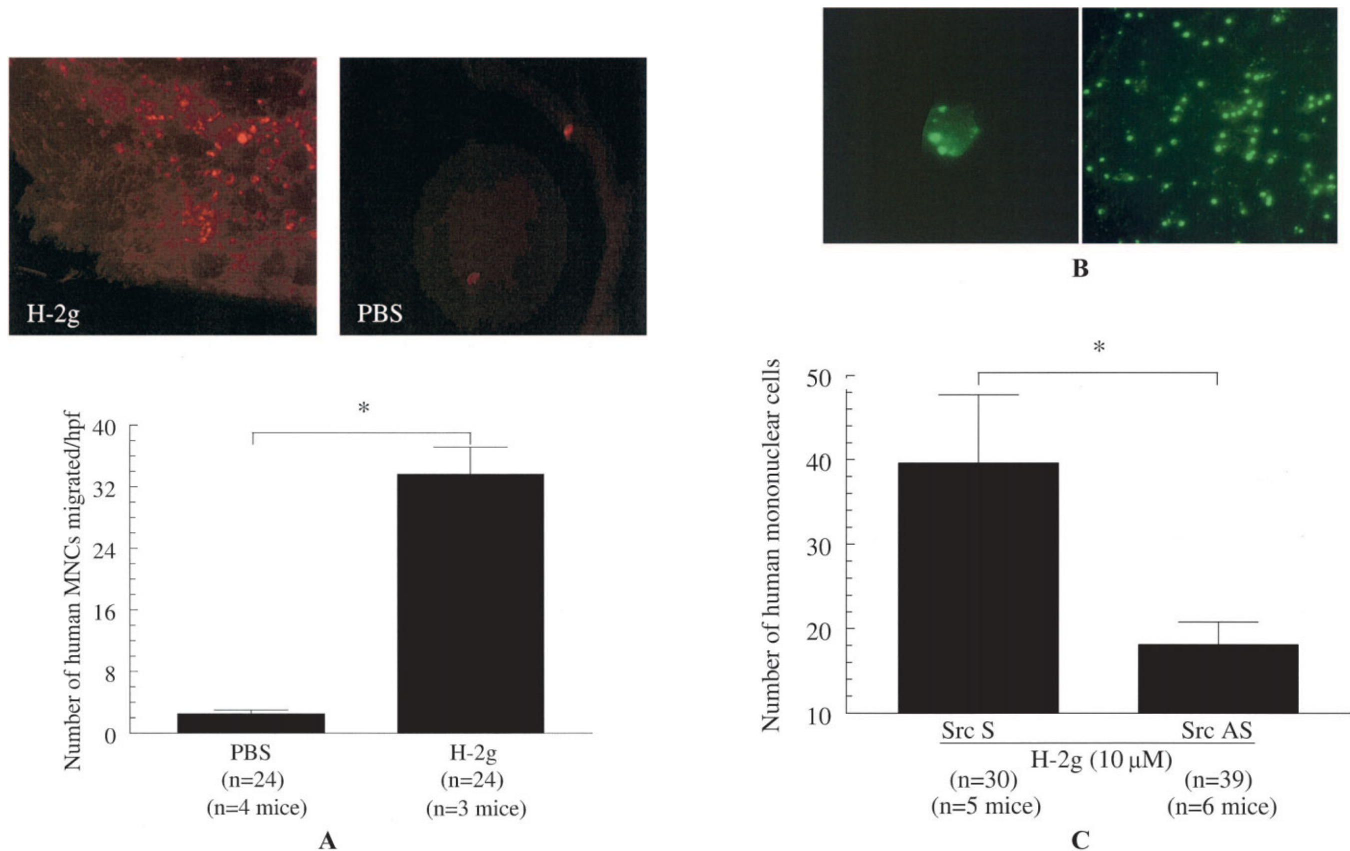

Figure 2. A, Recruitment of mononuclear cells (MNCs) by H-2g in a human rheumatoid arthritis (RA) synovial tissue (ST)–SCID mouse chimera. After 4 weeks of human RA ST engraftment in SCID mice, dye-tagged MNCs (5×10^6) were injected intravenously (IV) into each mouse. At the same time, ST grafts were injected with H-2g (10 μM/graft) or phosphate buffered saline (PBS), and grafts were harvested after 48 hours. The number of fluorescence-labeled cells was significantly higher in the grafts injected with H-2g than in those injected with PBS. The upper panel shows a representative RA ST section with dye-tagged MNCs migrated to the graft in response to H-2g, while grafts sham injected with PBS show limited MNC migration (original magnification × 100). Twenty-four tissue sections were counted per test group of animals. For the PBS group, 6 tissue sections were counted for each of 4 mice, using the same negative control group we have used previously (10). For the H-2g group, 8 tissue sections were counted for each of 3 mice. Results are expressed as the mean and SEM cells/hpf using data obtained from 24 tissue sections analyzed in each test group. $* = P < 0.05$. **B,** Transfection of human monocytes with fluorescein isothiocyanate (FITC)–labeled ODNs to NF-κB. Human monocytes were transfected with FITC-labeled ODNs to NF-κB for 24 hours (original magnification × 400 at left; × 100 at right). Transfection efficiency was >80% at 24 hours. **C,** Mediation by Src of H-2g–induced MNC migration in RA ST–SCID mouse chimeras in vivo. Human MNCs were transfected with Src sense or antisense ODNs for 72 hours. After transfection, dye-tagged MNCs were administered IV into SCID mice engrafted with human RA ST. While MNCs transfected with Src sense ODN migrated readily to RA ST in response to H-2g, Src antisense ODN–transfected MNCs did not. Data represent the mean and SEM MNCs migrated in the entire tissue sections, as described in **A**. Thirty sections were evaluated from 5 separate mice for Src sense ODNs, and 39 sections were evaluated from 6 separate mice for Src antisense ODNs. $* = P < 0.05$. See Figure 1 for other definitions.

inhibitors of PI 3-kinase and MEK-1/2 did not decrease the chemotaxis. None of the inhibitors decreased monocyte migration in response to negative control HBSS (Figure 1C).

Src and PI 3-kinase antisense ODNs inhibit H-2g–induced monocyte chemotaxis in vitro. To confirm the data obtained with chemical signaling inhibitors, we transfected monocytes with sense or antisense ODNs of Src and PI 3-kinase. Initially, to determine transfection efficiency, we transfected monocytes with FITC-bound ODNs to NF-κB and found the transfection efficiency to be >80% (Figure 2B). MNC viability was 80–85% after transfection with Src or PKCα ODNs for 72 hours, as determined by MTT assay (data not shown), consistent with viability after transfection of monocytes with other (ERK-1/2) ODNs (10). H-2g–induced monocyte migration was significantly decreased by antisense ODNs of Src and PI 3-kinase compared

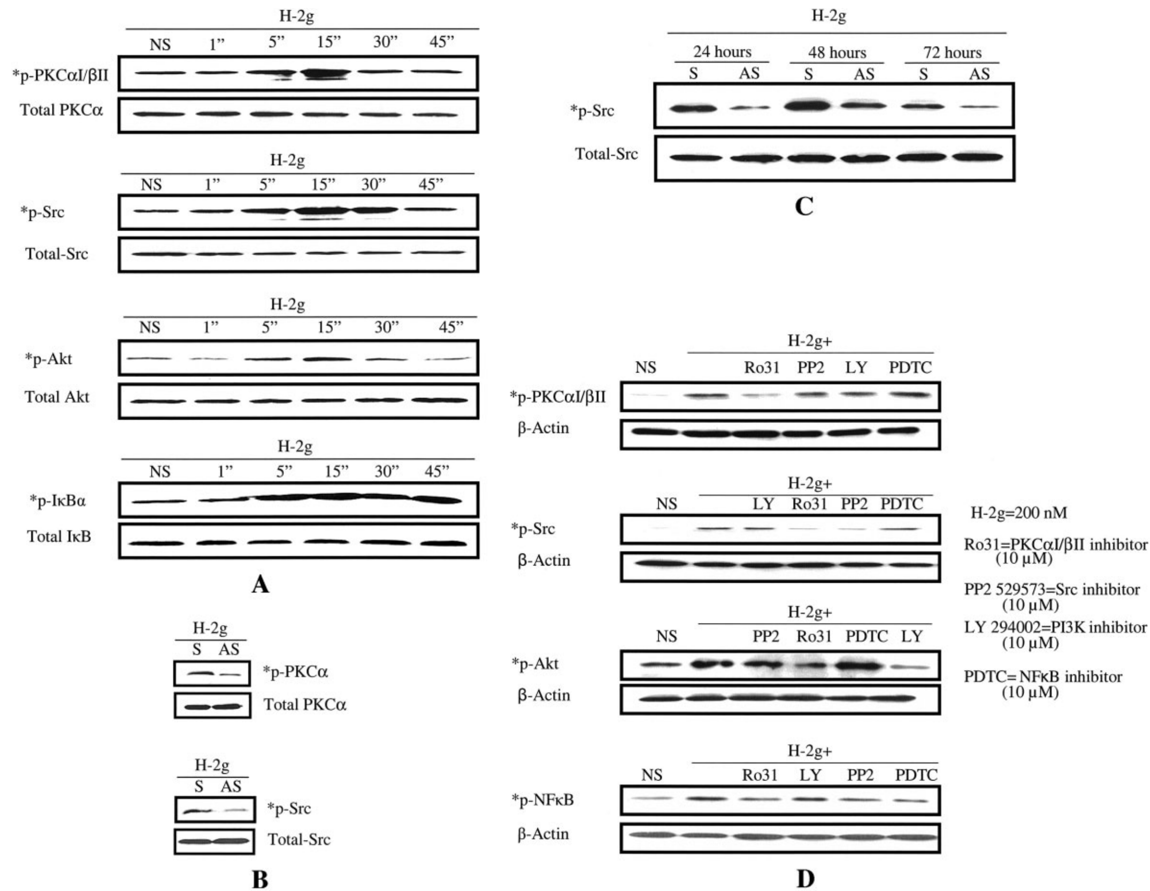

Figure 3. **A,** Induction by H-2g of phosphorylation of human monocyte protein kinase CαI/βII (PKCαI/βII), Src, Akt, and IκBα. Monocytes were stimulated with H-2g (200 n*M*) for 1, 5, 15, 30, or 45 minutes. H-2g induced a marked increase in PKCαI/βII, Src, Akt, and IκBα phosphorylation in a time-dependent manner compared with unstimulated (NS) cells. Each blot represents 1 of 3 experiments using blood obtained from different donors. **B,** Transient transfection of monocytes with PKCα and Src sense and antisense ODNs. Monocytes were transfected with PKCα or Src sense and antisense ODNs for 48–72 hours before stimulation with H-2g for 15 minutes. Cell lysates were collected and Western blotting was performed. PKCα and Src antisense ODNs markedly reduced H-2g–induced phosphorylation compared with sense ODN–transfected monocytes. Blots were reprobed for total PKCα and Src to verify equal loading. **C,** Transient transfection of monocytes with Src sense and antisense ODNs for various time periods. The transient transfection of monocytes with Src sense and antisense ODNs remained effective for 72 hours, as determined by decreased H-2g–induced phospho-Src in monocytes transfected with Src antisense ODNs. **D,** Upstream location of H-2g–induced PKCαI/βII relative to Src, Akt, and NF-κB. H-2g–induced Src, Akt, and NF-κB are the downstream targets of PKCαI/βII, since the inhibitor of PKCαI/βII decreased H-2g–induced phosphorylation of Src, Akt, and NF-κB. Src and PI 3-kinase/Akt pathways do not cross talk with each other, since the Src inhibitor, PP2 529573, did not decrease H-2g–induced phosphorylation of Akt and vice versa. All inhibitors (10 μM) were present in the medium during monocyte stimulation with H-2g for 15 minutes. Each blot represents 1 of 2 experiments using blood obtained from different donors. PDTC = pyrrolidine dithiocarbamate (see Figure 1 for other definitions).

with sense ODNs, confirming that Src and PI 3-kinase play an important role in H-2g–induced monocyte migration (Figure 1D).

H-2g recruits MNCs to human ST in an RA ST–SCID mouse chimera. We used an RA ST–SCID mouse chimera to examine H-2g–induced MNC migration in vivo. ST grafts injected with H-2g showed a significant increase in MNC migration compared with negative control (PBS) ($P < 0.05$). This increase was 8-fold higher compared with PBS, suggesting that H-2g is a very potent chemotactic stimulus for MNCs in vivo (Figure 2A).

Src antisense ODNs inhibit H-2g–induced MNC recruitment into RA ST–SCID mouse chimeras. Transfection with antisense ODNs resulted in significant reduction of MNC migration in vivo compared with sense ODN–treated controls in response to H-2g in an

RA ST–SCID mouse chimera (Figure 2C). These results show that targeting H-2g–mediated signaling pathways may influence MNC migration to RA ST.

H-2g activates phosphorylation of PKCαI/βII, Src, Akt, and IκBα in monocytes. H-2g induced phosphorylation of PKCαI/βII, Src, and Akt in monocytes at 1 minute, with peak expression between 5 minutes and 30 minutes, which subsided at 45 minutes. IκBα phosphorylation, a measure of NF-κB phosphorylation and nuclear translocation, started at 5 minutes, with peak expression between 15 minutes and 45 minutes (Figure 3A).

H-2g–induced PKCαI/βII in monocytes is upstream of Src, Akt, and NF-κB. Antisense ODNs of Src and PKCα decreased H-2g–induced phosphorylation of Src and PKCαI/βII compared with that in sense ODN–transfected monocytes, suggesting a knockdown of activated kinases by antisense ODN transfection (10) (Figure 3B). We found a marked decrease in H-2g–induced phospho-Src in monocytes transfected with Src antisense ODNs compared with monocytes transfected with Src sense ODNs. The effect of transient transfection lasted for 24–72 hours (Figure 3C).

H-2g activates a number of signaling pathways in monocytes, including PKCαI/βII, Src, Akt, and NF-κB. We next studied the upstream and downstream targets of these signaling intermediates. PKCαI/βII was upstream of Src, Akt, and NF-κB, since an inhibitor of PKCαI/βII (Ro-31-8425) abrogated the H-2g–induced phosphorylation of all 3 molecules (Figure 3D).

DISCUSSION

We have demonstrated that H-2g induces ICAM-1 and leukocyte–endothelial cell adhesion via JAK-2/STAT, PI 3-kinase/Akt, and ERK-1/2 signaling pathways (4). H-2g induces angiogenesis both directly and by inducing basic fibroblast growth factor and vascular endothelial growth factor (VEGF) via activating endothelial cell PI 3-kinase, JAK-2, and NF-κB signaling intermediates (3,5).

In this study, we demonstrate that H-2g is a novel cytokine-like mediator of monocyte chemotaxis in vitro and in vivo. We found that H-2g induced human monocyte chemotaxis in a concentration-dependent manner in vitro. H-2g–induced monocyte migration was significantly decreased by inhibitors of Src and PI 3-kinase. H-2g–induced monocyte migration was also inhibited by antisense ODNs of Src and PI 3-kinase, confirming chemical inhibitor data. This suggests that Src and PI 3-kinase are essential in H-2g–induced monocyte migration, but a G protein inhibitor, pertussis toxin, had no effect.

To test the direct functional role of H-2g in leukocyte migration in vivo and in relation to a disease, we used a human RA ST–SCID mouse chimera. This model has been used by us and other investigators to examine the role of different cytokines in leukocyte ingress (10,12). In the RA ST–SCID mouse chimera, H-2g–induced MNC recruitment was significantly higher compared with PBS. This suggests that H-2g may play a critical role in chronic inflammatory diseases by mediating leukocyte ingress.

We also used the RA ST–SCID mouse chimera to elucidate the role of signaling molecules in MNC homing by H-2g in an in vivo environment. Transfection of MNCs with antisense ODN directed against Src resulted in a significant decrease in H-2g–mediated cell recruitment to engrafted RA ST compared with MNCs transfected with Src sense ODN. These data suggest that blockade of signaling intermediates important in H-2g–mediated MNC recruitment may abrogate H-2g–induced MNC ingress into inflammatory tissue.

PKC is composed of a family of 11 isoenzymes that participate in T cell proliferation, differentiation, and angiogenesis (13,14). PKC inhibitors were shown to diminish arthritis in rat adjuvant-induced arthritis (13). Because of those studies, we investigated whether H-2g activated PKCαI/βII phosphorylation in monocytes. H-2g induced phosphorylation of monocyte PKCαI/βII isoforms in a time-dependent manner, with a maximum response at 15 minutes.

Src kinases play an important role in RA by participating in cell adhesion and leukocyte migration (9,11,15). H-2g activates Src phosphorylation in monocytes in a time-dependent manner. H-2g–induced Src phosphorylation was inhibited by a PKCαI/βII inhibitor, suggesting that PKCαI/βII is upstream of Src. Transfection of monocytes with PKCα and Src antisense ODNs markedly reduced PKCαI/βII and Src phosphorylation. Similarly, we showed that ERK-1/2 MAPK antisense ODN transfection significantly decreased ERK-1/2 phosphorylation in monocytes (10). The effect of transient transfection lasted for 72 hours, as we observed a marked decrease in H-2g–induced phospho-Src in monocytes transfected with antisense ODNs compared with monocytes transfected with sense ODNs.

H-2g induces phosphorylation in monocytes of the PI 3-kinase effector, Akt, in a time-dependent manner. Inhibitors of both PKCαI/βII and PI 3-kinase decrease Akt phosphorylation, suggesting that PKCαI/

βII is upstream of PI 3-kinase, since Akt is a downstream target of PI 3-kinase. Our data are concordant with those of Gliki et al, who demonstrated that VEGF induces PKC-dependent angiogenesis, and also that PI 3-kinase/Akt is a downstream target of PKCα and PKCδ in human umbilical vein ECs (14).

NF-κB is an inducible transcription factor and is a downstream target of PKC, PI 3-kinase, and JAK-2/STAT signaling pathways (5,9,15). H-2g–induced NF-κB is a downstream target of PKC, since it is inhibited by a PKC inhibitor. The NF-κB/Rel transcription factors are present in the cytosol in an inactive state complexed with the inhibitory IκB proteins. Activation occurs via phosphorylation of IκBα followed by degradation, resulting in the release and nuclear translocation of active NF-κB. Phosphorylation of IκBα is essential and is an excellent marker for release of active NF-κB.

In conclusion, these data suggest that H-2g and signaling intermediates induced by H-2g play an important role in inflammation. H-2g may prove to be a potential therapeutic target for the treatment of chronic inflammatory diseases characterized by persistent leukocyte recruitment, such as RA.

AUTHOR CONTRIBUTIONS

Dr. Amin had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Amin, Ruth, Koch.

Acquisition of data. Amin, Ruth, Haas, Pakozdi, Mansfield, Haghshenas.

Analysis and interpretation of data. Ruth, Haas, Pakozdi, Mansfield, Haghshenas, Koch.

Manuscript preparation. Amin, Koch.

Statistical analysis. Amin.

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