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In Vivo Interstitial Migration of Primitive Macrophages Mediated by JNK-Matrix Metalloproteinase 13 Signaling in Response to Acute Injury

Yong Zhang,*† Xue-Tao Bai,* Kang-Yong Zhu,† Yi Jin,*† Min Deng,† Huang-Ying Le,*† Yan-Fang Fu,*† Yi Chen,*† Jiang Zhu,* A. Thomas Look,§ John Kanki,§ Zhu Chen,*¶ Sai-Juan Chen,2*†¶ and Ting Xi Liu2*†‡¶

Interstitial cell migration through extracellular matrix is a hallmark of the inflammation response, tumor invasion, and metastasis. We have established a stable zebrafish transgenic line expressing enhanced GFP under the lysozyme C promoter for visualizing and measuring primitive macrophage migration in vivo. We show that tissue-resident primitive macrophages migrate rapidly through extracellular matrix to the site of acute injury induced by tail transection. Mechanistically, the specific inhibition of JNK, but not p38 and ERK, dramatically abolished the chemotactic migration in a dose-dependent manner, suppressing the trauma-induced recruitment of phosphorylated C-Jun transcription factor to proximal AP-1 sites in the promoter of matrix metalloproteinase 13 (mmp13), a gene specifically expressed in primitive macrophages during embryogenesis and required for the interstitial migration. Furthermore, dexamethasone suppressed the trauma-induced JNK phosphorylation and macrophage migration accompanied by simultaneous up-regulation of mmp-1, a well-known phosphatase capable of inactivating phosphorylated JNK. The results indicate that the JNK-Mmp13 signaling pathway plays an essential role in regulating the innate immune cell migration in response to severe injury in vivo. The Journal of Immunology, 2008, 181: 2155–2164.

Innate immune cell recruitment from the microcirculation to the injured tissue is a multistep process consisting of cell tethering, rolling, adhesion, traversing of vascular endothelial cells (diapedesis), and subsequent interstitial migration through extracellular matrix (ECM) (1, 2). Members of selectin, integrin, and chemokine receptor family have been demonstrated to be essential for the early steps of inflammatory cell recruitment (1–4), while constant remodeling of interstitial ECM proteins, such as collagen, fibronectin, vitronectin, laminin, sulfated proteoglycans, and recently mindin, plays a critical role in the late chemotactic migration through ECM (5, 6).

Intracellular signaling pathways mediating the innate immune cell migration remain relatively unclear. Toll-like receptors, chemokine receptors, and other pattern recognition receptors are known to recognize and respond to distinct inflammatory stimuli/factors (e.g., cytokines TNF, IL-1, IFN-γ, chemokines CCL-2 and –3, migration inhibitory factor, and anaphylatoxin complement 5a) by initiating intracellular signaling cascades that lead to the activation of the PI3K–akt murine thymoma viral oncogene homolog and the MAPKs (7–11). MAPK signal transduction cascades are well conserved from yeast to mammals (12, 13). The most extensively studied groups of vertebrate MAPKs to date are JNK, ERK, and p38 MAPK, which are activated by dual phosphorylation at neighboring threonine and tyrosine residues in the activation loop (14). Dephosphorylation of either residue by MKP-1/DUSP1, a threonine/tyrosine dual-specificity phosphatase, results in inactivation of MAPKs (14–16). Although the roles of MAPKs in innate immune responses, such as respiratory burst activity, granular exocytosis, adherence, survival, and cytokine production, have been well documented (14), the precise intracellular mechanism by which MAPKs contribute to the interstitial macrophage migration through ECM in vivo following severe injury is not fully understood.

Innate immune cells differentially use membrane-bound and secreted proteases, as well as glycosaminoglycan-degrading enzymes to remodel and penetrate ECM barriers, while migrating through the tightly intertwined basal lamina (17). Given the important function of secreted proteases of the matrix metalloproteinase (MMP) family in ECM remodeling and innate immune cell recruitment, the upstream signaling pathways that transcriptionally

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3 Abbreviations used in this paper: ECM, extracellular matrix; MMP, matrix metalloproteinase; hptt, hours post tail-transection; DN-JNK, dominant-negative JNK; dex, dexamethasone; GR, glucocorticoid receptor.

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regulate MMP gene expression to promote chemotactic macrophage migration remains to be investigated in vivo (5, 17, 18).

Deregulated migration of macrophages has been implicated in a variety of human disorders, such as inflammatory and autoimmune diseases and tumor metastasis (19–21). Identification of signaling molecules that lead to innate immune cell recruitment is essential for selective blocking of leukocyte recruitment to the site of inflammation, thus improving clinical outcome (4, 21). However, the importance of utilizing an in vivo physiological model for real-time visualization and quantification of tissue macrophage movement and the dissection of underlying molecular mechanisms have only begun to be realized (22).

Zebrafish has become a powerful vertebrate model for genetic studies of human development and diseases (23–26). The model offers several unique advantages, including large numbers of embryos per clutch, rapid and external embryonic development, and optical clarity, which allows direct visualization of organogenesis and inflammatory responses and host-pathogen interactions in vivo, revealing unexpected aspects of leukocyte behavior (27). Previous studies have demonstrated that the primitive macrophages originate from the anterior lateral plate mesoderm at 16–18 h post fertilization (hpf) during early embryogenesis and emigrate toward the yolk sac to enter the circulation (28, 29).

In this study, we establish a precise quantitative assay in the transparent tail of the TG(lyz: enhanced GFP (EGFP)) transgenic embryos to investigate the physiological role of the MAPK signaling pathway and downstream factors that regulate the interstitial primitive macrophage migration. Our results indicate that chemotactic migration of primitive macrophages in response to acute injury is predominantly dependent on the JNK-Mmp13 signaling pathway, which can be efficiently suppressed by dexamethasone-mediated up-regulation of mkp-I. The TG(lyz:EGFP) transgenic line provides an invaluable tool not only to dissect the molecular pathways underlying cellular migration critical to innate immunity and tumor invasion, but also for screening therapeutic compounds to treat human disorders associated with deregulated macrophage chemotaxis and migration.

Materials and Methods

Plasmid construction

A bacterial artificial chromosome clone containing the zebrafish lyz genomic sequence was used as a template to amplify a 4.1 Kb genomic fragment upstream of the lyz translational start codon by PCR with the following primers: AAGTGCTCGAGCAGCTATATCCGCTATCC TAT (forward; XhoI site in boldface) and GGAATTCATATGATC TTATTATC (reverse; CdiI site in boldface). The promoter PCR product was digested with XhoI and CdiI, and cloned into an I-SceI-containing PBSK plasmid vector upstream of either EGFP, DsRed2, or human dominant-negative JNK coding sequence and a SV40 polyadenylation site, containing PBSK plasmid vector. The CMV promoter-driven human wild-type JNK and dominant-negative JNK were also cloned into the I-SceI-containing PBSK plasmid vector.

Generation of TG(lyz:EGFP) transgenic lines

The zygotic EGFP plasmid was prepared with endotoxin-free midiprep kit (Promega). Microinjection was performed in one-cell stage embryos with 2 nl of injection solution containing 50 pg/nl of DNA, 0.5 × I-SceI buffer, and 0.5 units/μl I-SceI meganuclease (New England Biolabs). Injected embryos were raised to sexual maturity (F0 founders) and crossed to wild-type lyz:DsRed2, or human dominant-negative JNK were also cloned into the I-SceI-containing PBSK plasmid vector.

Time-lapse analysis of living embryos with confocal microscope

For time-lapse analysis, the embryos were anesthetized in egg water supplemented with 0.1 mg/ml tricaine. The Zeiss LSM510 laser scanning confocal microscope equipped with Plan-Neoflur ×100/0.3 or ×200/0.5 objective lens was used to detect the EGFP fluorescence. Zeiss LSM Pascal software was used to generate movies as a MOV format.

Flow cytomery analysis and cytology

Embryos (60 hpf) were dissected in 0.9 × PBS and 5% FBS, and digested with 1 × trypsin/EDTA (Life Technologies) for 15 min at 37°C. Single-cell suspension was obtained by centrifugation at 400 × g for 5 min, washed twice with PBS, and passed through a 40-μm nylon mesh filter. Fluorescence-activated cell sorting was performed with MoFlo FACS (DakoCytometry) to obtain homogeneous EGFP+ cells, which were subsequently subject to cytospin preparations (600 × g for 5 min), followed by Wright-Giemsa staining (Sigma-Aldrich) for morphological analysis.

Whole-mount in situ hybridization and TUNEL assay

Whole-mount in situ hybridization was performed as described previously (25). For two-color in situ hybridization, the EGFP antisense RNA probe was labeled with digoxigenin (Roche), while antisense probes against lyz, mpo, t-actin, and mmp13 were labeled with fluorescein (Roche). The purple color was developed first with NBT/BCIP (Vector Laboratories) as a substrate, followed by the development of red color with Fast Red tablets (Roche). Simultaneous detection of EGFP protein and mRNA transcripts were performed as described previously (25, 29). Whole-mount TUNEL assay was performed as described previously (26).

Tail transection and pharmacological treatment

SP600125, PD098059, and SB203580 were purchased from Calbiochem. These compounds were prepared in DMSO to make stock concentrations of 20 mM and stored at −20°C. Mifepristone (RU-486) was purchased from Cayman Chemical and dexamethasone was purchased from Sigma-Aldrich. For drug treatment, at least five TG(lyz:EGFP) embryos at 60 hpf were arrayed into an individual well of six-well plate in embryo medium at 28.5°C in the dark. Embryos were treated with either indicated concentrations of drugs or vehicle only (0.1% DMSO) as a control. After 2 h pretreatment, embryos were subject to tail transection with a sterile scalpels at the same anatomic site posterior to the end of tail circulation, resulting in consistent macrophage recruitment with the number between 33 ± 5. The dynamic behavior and quantification of chemotactic migration were assessed by fluorescent stereomicroscope (Zeiss Luminar V12 stereomicroscope equipped with an AxioCam MRCS digital camera and AxioVision Rel.4.5.4). All experiments were performed three times with separate batches of embryos.

Quantitative Western analysis

Embryos were deyolked as described previously (30). Embryos were homogenized in lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 10% glycerol, and 0.1% Triton X-100) containing protease inhibitor cocktail and phosphatase inhibitor (Roche). Protein lysates were separated on a 1.5% agarose gel. The primer sequences are: Forward: 5′/H11001 and Reverse: 5′/H11003

Tail transection and pharmacological treatment

Tail transection and pharmacological treatment

Morpholino knockdown and microinjection

Morpholino antisense oligonucleotides were purchased from Gene Tools. The sequences were as the following: mmp13 morpholino: GCAGGCTTTC ATGGTTGGCTTCTC/TCTC/mmp13 5′-mismatch: GCAcGTTCGTTAcTtTtGgTTGCTTCT and nephrosin morpholino: ACCCAAGCATGATGATGAGCAC/ACK, Morpholinos were diluted to different concentrations with Danieu’s buffer (30). Whole-mount TUNEL was performed as described previously (25, 29). Whole-mount TUNEL assay was performed as described previously (26).

Tail transection and pharmacological treatment

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Single-embryo RT-PCR

Single-embryo RT-PCR was performed as described (26). PCR conditions were as follows: 50°C, 30 min; 95°C, 10 min; 30 cycles of 94°C, 30 s; 58°C, 30 s; 72°C, 1 min; then 72°C, 10 min. PCR products were separated on a 1.5% agarose gel. The primer sequences are: mmp13 (Forward: 5′- AATCCCTCTTTTTCCGGCAAACACCGG-3′ and Reverse: 5′-CTCGGA
TTCTTCTTCAAGCCGATAGTAA-3' (mkp-1) Forward: 5'-TGAAGACACCT ACAAGGTACAACAT-3' and Reverse: 5'-CCGGGACAATCACTATAAACA CGTCGGCAAAA-3' and β-actin (Forward: 5'-CCGGAGCATGAGGG TGAT-3' and Reverse: 5'-CCGGATCCAGACGGAGGTAT-3').

Embryonic chromatin immunoprecipitation (E-ChIP)

E-ChIP was performed as previously described with modifications (31). In brief, for each immunoprecipitation, ~50 tail-transected embryos at 60 hpf were enzymatically dechorionated and then fixed in 1.85% formaldehyde in 1× embryo medium for 20 min at room temperature. Glycine (0.125 M) was added to quench the formaldehyde, and the embryos were homogenized in swelling buffer (25 mM HEPEs (pH 7.8), 1.5 mM MgCl2, 10 mM KCl, 0.5% Nonidet P-40, 1 mM DTT, and protease inhibitors), and incubated for 20 min on ice. Nuclei were collected by centrifugation, resuspended in nuclei lysis buffer (20 mM Tris-HCl (pH 8.0), 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 0.1% Na deoxycholate, 150 mM NaCl, and protease inhibitors), then incubated for 30 min and sonicated on an ice bath. Sonication conditions were optimized to give fragments of ~300 to 700 bp. Lysate was incubated with Protein-A Sepharose 4B with 40 μl per ml lysate (Amersham Biosciences) for 2 h at 4°C, then centrifugation at 2000 rpm for 5 min. The supernatant was incubated with 5 μg of the rabbit anti-phospho-C-Jun Ab (Cell Signaling Technology), with rotation overnight at 4°C. Thirty μl Protein-A Sepharose 4B per IP (equilibrated as above) were added and rotated for 1 h at 4°C. The beads were centrifuged at 4°C and washed with washing buffer (as described in the ChIP assay kit protocol; Upstate Biotechnology). Bound complexes were eluted from the beads at room temperature with vortexing 10 min in elution buffer. Cross links were reversed for 4 h at 65°C and the chromatin was purified by treatment with RNase A, followed by proteinase K digestion and phenol: chloroform:isoamyl alcohol extraction. The ChIP experiments were conducted two times with separate batches of embryos. PCR products were separated with a 1.5% agarose gel and band intensities were quantified with Quality One software (Bio-Rad). Primer sequences were: AP-1 site I (Forward: 5'- ACAGTGGCAAAAATAAACAAATAA-3' and Reverse: 5'-CCACT AATTAAGCAAGGAATAAT-3'); AP-1 site II (Forward: 5'-GGCGTGCAAAA ATTAACAAAGGA-3' and Reverse: 5'-GGCGCAAAATCTATAAAACA GGT-3'); and AP-1 site III (Forward: 5'-CTGGGTATGAAAAGGTTGTA TAGT-3' and Reverse: 5'-CCTGAAAGGGAGTTGTGGATAA-3').

Statistical analysis

Data were presented as mean values ± SD. Statistical analysis was performed using the SigmaStat software package (Systat Software). The probability level for statistical significance was p < 0.05. Statistics of macrophage migration were evaluated with one-way ANOVA, and Dunnett’s test was utilized for calculation of p values.

Results

Establishment and characterization of TG(zlyz:EGFP) transgenic zebrafish

To generate a transgenic zebrafish that expresses EGFP in primitive macrophages, we microinjected one cell stage embryos with a transgenic plasmid construct expressing EGFP under the control of a 4.1 Kb genomic fragment upstream of the translational start site of zebrafish lysozyme C (zlyz) (glycogen Fig. 1A). The lysozyme C is a marker for macrophage differentiation in mammals (32), and expressed in the primitive macrophages of zebrafish embryos (33). Five transgenic founders from 30 adults were identified that produced offspring that expressed the EGFP transgene within 36 hpf. All of the transgenic founders produced embryos that expressed EGFP at detectable levels by fluorescence microscopy (data not shown). However, individual founder line demonstrated different levels of EGFP expression. For example, comparison of the two transgenic lines designated as zlyz:EGFP I and zlyz:EGFP II indicate that the zlyz:EGFP I line showed more EGFP+ cells at 22 hpf than the zlyz:EGFP II line that had fewer EGFP+ cells arising later at 24 hpf (data not shown). The zlyz:EGFP I line is hereafter referred to as TG(zlyz:EGFP), and was used for all subsequent studies described in this report. So far, the stable transmission of the EGFP transgene has been maintained for five generations. EGFP-expressing cells in the TG(zlyz:EGFP) were readily detected as early as 22 hpf within the head and trunk tissue mesenchymes (hereafter referred to as tissue-resident or interstitial macrophages; Supplemental movies 1 and 2) close to the ALPM (data not shown). By 24 hpf, these EGFP+ cells spread over the yolk sac close to the duct of Cuvier or circulation valley (data not shown), and

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FIGURE 1. Generation of TG(zlyz:EGFP) transgenic zebrafish line and characterization of EGFP+ cells. A, Restriction map of the zlyz:EGFP construct used to establish transgenic lines. pA, SV40 polyadenylation site. B, Distribution of fluorescent macrophages in the TG(zlyz:EGFP) transgenic embryo at 48 hpf. C, Colocalization studies of EGFP with lyz mRNAs by confocal analyses at 36 hpf, using an anti-EGFP Ab and antisense lyz mRNA as probes. More than 98% of cells coexpressed both EGFP protein and lyz mRNA in the anterior yolk sac (upper panels) and the ICM (bottom panels). D, Double in situ hybridization with both EGFP and lyz anti-sense mRNAs as probes in the head and tail at 36 hpf (red arrows). E: Wright-Giemsa staining and morphological characterizations of FACs-purified EGFP+ cells from 60 hpf embryos.
subsequently was dispersed throughout the head mesenchyme and the intermediate cell mass (ICM) at 48 and 72 hpf (Fig. 1B and data not shown), and the developing pronephric glomerulus at 120 hpf (data not shown), when a few EGFP\(^+\) cells (15–30) circulating within the head and tail vasculature were observed (referred to as circulating macrophages; Supplemental movies 1 and 2). This expression pattern is consistent with the endogenous expression of zebrafish lyz RNA transcripts (33), and the expression of EGFP cells in the recently reported lyz:EGFP zebrafish transgenic line (34).

Colocalization analysis by confocal microscopy using an anti-EGFP Ab and an antisense zebrafish lyz mRNA probe revealed that >98% of cells expressed both EGFP protein and lyz mRNA in the anterior yolk sac and the ICM at 36 hpf (Fig. 1C). This result was confirmed by double whole-mount in situ hybridization with both EGFP and lyz antisense RNA as probes (Fig. 1D, red arrows) at the same stage. The EGFP also colocalized with l-plastin, another marker for primitive macrophages (33, 35) at 36 hpf (data not shown), but not with the majority of mpo-expressing cells on the yolk sac of 24 hpf embryos (data not shown). mpo-positive cells, but not EGFP\(^+\) cells, were observed in the ICM at 24 hpf before the onset of circulation (data not shown). Fluorescent macrophages were isolated by flow cytometry from 60 hpf embryos, cytospun, and stained with Wright-Giemsa for morphological analysis. This purified cell population was homogeneous exhibiting morphological characteristics of promonocytes and/or macrophages (e.g., high nucleus-cytoplasm ratio, kidney-shaped nuclei, and ground glass appearance) (Fig. 1E, black arrowheads). One cell exhibited vacuoles and ingested debris in the cytoplasm (black asterisk), while three were undergoing apoptosis (red asterisks), and one was undergoing mitosis (black arrow). Taken together, these results suggest that the EGFP\(^+\) cells represent a population of primitive macrophages during early myeloid development, although the possibility that the EGFP also label the neutrophils in later stages cannot be excluded, as observed in previous literature (34, 36).

Dynamic characterization and quantitative analysis of interstitial primitive macrophage migration in response to acute injury

To assess the response of fluorescent macrophages to acute injury, time-lapse confocal microscopy was first used to visualize the migratory dynamics of macrophages in tail-transected embryos in the presence of tricaine anesthesia. We always made the tail transection (served as a potential trigger to cause the release of soluble mediators or chemokines that could promote the migration of primitive macrophage) at the site close to the end of tail circulation at 60 hpf, so that the inflammatory stimulus and the number of recruited macrophages remained at a relatively constant level. Interestingly, only tissue-resident (interstitial) macrophages were rapidly recruited to the site of injury by directional movement, but no circulating macrophages were observed recruited through extravasations within the first hour post tail transection (hptt) (Supplemental movie 3) (4). To confirm the observation, we labeled and followed nineteen macrophages (indicated by Arabic numerals from 1 to 19) in the trunk mesenchyme and tracked their migration for 1 hptt (Fig. 2A–F). We found that these macrophages could be classified into three categories in terms of their migratory behaviors: 1) macrophages 7, 12, and 19 residing in the tissue dorsal to the neural tube appeared not respond to the inflammatory stimulus, remaining static throughout the time observed; 2) macrophages 2, 5, 8, 9, 10, and 18 were motile and migrated to the site of injury as single cell; 3) macrophages 1, 3, 4, 6, 11, 13, 14, 15, 16, and 17 migrated as single cell in the beginning, and then formed cell clusters. For example, macrophage 16 migrated forward to join the macrophage 17, followed by the addition of macrophage 14 (Fig. 2, B and C, white arrow), so these three macrophages were maintained as a cell cluster (designated as 17/16/14). A similar phenomenon was also observed for macrophage clusters 6/1 and 15/3/4 (Fig. 2F). During this period of observation, more resident macrophages, but not circulating macrophages, were recruited to the injured tailfin (Fig. 2B–F, red asterisks). When macrophages were recruited from the circulation later, the number of macrophages accumulated peaked at 33 ± 5 between 4 and 6 hptt (Fig. 2G). Between 6 and 8 hptt, real-time tracking of individual macrophages demonstrated that they initiated sequential retrograde migration back to the ICM mesenchyme, suggesting a resolution of the inflammatory response (Fig. 2G and data not shown). Ten hours postinjury, ~50% macrophages had exited from the injured region (data not shown).

Mmp13 is required for macrophage migration

Membrane proteases of the MMP family are known to play a crucial role in innate immune cell migration through the degradation and remodeling of ECM (17). We focused on two metalloproteinase genes, mmp13 (37) and nephrosin (38), which have been shown to be specifically expressed in primitive myelomonocytes during zebrafish embryogenesis. Zebrafish mmp13 is a member of metalloproteinase family expressed in primitive macrophages (37), and colocalized with lysozyme C in 48 hpf embryos (Fig. 3A). Mammalian homologue MMP-13 is a highly specific collagenase capable of degrading insoluble collagens type I, II, and III within ECM (17, 39). To investigate their roles in inflammatory macrophage migration in vivo, we analyzed the expression of mmp13 and nephrosin by whole-mount in situ hybridization in the transected tails of 60 hpf embryos at 0 and 6 hptt. A robust induction of mmp13 transcripts was observed in single macrophages of 6 hptt animals (Fig. 3B, arrowheads), while no obvious increase of nephrosin transcripts were observed (data not shown). Consistently, single-embryo, semiquantitative RT-PCR demonstrated a significant induction of mmp13 transcripts in 6 hptt embryos (Fig. 3C).

Morpholino-mediated knockdown is an efficient strategy to specifically block the translation of target proteins in the zebrafish embryo (26, 40). Our preliminary data indicates that mmp13 and nephrosin morpholino oligonucleotides, but not their five-mismatch control morpholino, were able to efficiently and specifically suppress the translation of reporter constructs expressing EGFP downstream of mmp13 and nephrosin 5′UTRs, which contained the morpholino-recognizing sequence (data not shown). Interestingly, a slightly curling notochord was noted in the mmp13-deficient embryos at 2.5 days post fertilization, which worsened by 4.5 days post fertilization (data not shown, 45/50). This morphological abnormality could be reminiscent of physical signs observed in the Missouri type of human spondyloepimetaphyseal dysplasia, caused by a missense mutation in the human MMP13 gene (41).

Consistent with the up-regulation of mmp13 mRNAs in migrating macrophages of 6 hptt tails, knockdown of Mmp13 resulted in a significant decrease (50% of normal) in the number of recruited macrophages, compared with five-mismatch control morpholino injected embryos at 6 hptt (Fig. 3, D and E). In contrast, knockdown of nephrosin had no any effects on macrophage migration (Fig. 3, F and G). These results suggest that Mmp13 loss may, at least in part, contribute to the macrophage migration in response to acute injury in vivo.
Transactivation of mmp13 promoter by JNK-C-Jun signaling

To study the mechanism contributing to the up-regulation of the primitive macrophage-specific mmp13 gene, we analyzed the potential transcription factor binding sites in the promoter of the mmp13 gene using TFSEARCH software (http://www.cbrc.jp/research/db/TFSEARCH.html). We identified three putative AP-1

**FIGURE 2.** Dynamic tracing and quantitation of macrophage movement in response to inflammation induced by tail transection. A–F. Nineteen macrophages in the trunk mesenchyme were labeled with Arabic numerals from 1 to 19 to uncover the migratory path of individual cell 1 h post tail transection (hptt) of 60 hpf embryo. Black arrows in A indicated the site of tail transection. Yellow dashed line denoted the location and direction of tail circulation. White arrows indicated the cellular clusters in the highly inflamed region. Red asterisks indicated that additional resident macrophages were recruited into the injured tailfin. G. Starting at 10 min posttail transection, the time-course behaviors of macrophage migration were evaluated within 8 hptt.

**FIGURE 3.** Involvement of mmp13 in chemotactic macrophage migration. A. Double in situ hybridization of mmp13 with lyz antisense mRNA as probes at 36 hpf. Colocalization of mmp13 and lyz transcripts was observed in nearly all macrophages (left, black arrowhead) with amplified views of eye, neck, and trunk (white arrowheads). B. Whole-mount mRNA in situ hybridization analysis of mmp13 transcripts at 0 and 6 h post tail transection (hptt: h). Arrowheads indicated individual macrophages with enhanced mmp13 expression at 6 hptt. C. Semiquantitative analysis of mmp13 expression by single-embryo RT-PCR at 0 and 6 h. D–G. Time-course and quantification of macrophage migration in the transected tail (white brackets) of Mmp13-deficient embryos (D, E) and nephrosin-deficient embryos (F, G) at 0, 2, and 6 hptt. Representative results obtained from three independent experiments (n = 15). *, p < 0.01.
transcription factor binding sites (5’TGACTCA3’) within a 4.0 Kb-long promoter fragment upstream of the endogenous translational start site (Fig. 4A, indicated by I, II, and III). To test whether the phosphorylated C-Jun transcription factor directly binds to these sites, E-ChIP was performed in 60 hpf embryos at 0 and 6 hptt. Little or low level of phosphorylated c-Jun binding to AP-1 site I, II, and III, respectively, was detected at 0 hptt (Fig. 4B, lanes 1–3). However, the binding activities of phosphorylated C-Jun to sites II and III, but not site I, were robustly induced at 6 hptt compared with 0 hptt (p < 0.01) (Fig. 4, B, lanes 4–6, and C). Interestingly, the enhanced binding to sites II and III appeared to be dependent on JNK activation, because 100 μM of JNK-specific inhibitor SP600125 treatment markedly reduced the binding activity of phosphorylated C-Jun transcription factor compared with 6

FIGURE 4. In vivo binding of phosphorylated C-Jun transcription factor to the proximal AP-1 sites of mmp13 promoter is dependent on JNK activation. A, Schematic representation of the 4.0 Kb mmp13 genomic region used for E-ChIP assay. Three putative AP-1 binding sites at the positions of 3.6, 2.5 and 0.5 kb upstream of the transcriptional start site were indicated by I, II, and III, respectively. B and C, E-ChIP analysis and quantification at 0 and 6 hptt using an Ab against endogenous phosphorylated C-Jun protein in the absence or presence of 100 μM of either JNK or ERK inhibitor. D, Whole-mount mRNA in situ hybridization of mmp13 transcripts in 0 and 6 hptt tails treated without or with 20 μM of JNK inhibitor (SP600125) treatment. Arrowheads indicated individual macrophages with enhanced mmp13 expression at 6 hptt. E, Semiquantitative analysis of mmp13 expression by single-embryo RT-PCR at 0 and 6 hptt in the presence of 20 μM of either SP600125 or PD98059 inhibitor.
treatment with a higher concentration of PD98059 (200 μM) served with SB203580 incubation (Fig. 5, B, lanes 7–9, and C). No obvious changes were observed using an ERK-specific inhibitor PD98059 (100 μM) (Fig. 4, B, lines 10–12, and C). Furthermore, SP600125 treatment, but not ERK treatment, also abolished the transcriptional up-regulation of mmp13 induced by acute injury at 6 hptt as evidenced by WISH and semiquantitative RT-PCR assays (Fig. 4, D, right, and E). Taken together, the results suggest that JNK-mediated phosphorylation and activation of C-Jun plays an essential role in the transcriptional up-regulation of the mmp13 gene, which is likely to mediate the chemotactic movement of macrophages in response to injury.

Involvement of JNK, but not ERK and p38 MAPK, in primitive macrophage migration to the site of acute injury

The phosphorylation of C-Jun has been demonstrated to be a major target of the JNK signal transduction pathway (42) and because the inflammation-induced binding of phosphorylated C-Jun transcription factor to the proximal AP-1 sites of mmp13 promoter can be abolished by JNK, but not ERK, inhibitor treatment. We tested whether inhibition of JNK activity would also suppress chemotactic macrophage migration. Embryos at 60 hpf were tail transected and treated with graded concentrations (20, 50, 100, and 200 μM) of chemical inhibitors of SP600125 (JNK), PD98059 (ERK), and SB203580 (p38), while the number of recruited macrophages was recorded at 0, 2, and 6 hptt. The results showed that the JNK-specific inhibitor, SP600125, strongly suppressed macrophage migration in a dose-dependent manner (Fig. 5, A and B), while only moderate suppressive effects was observed for PD98059 treatment at high concentration (Fig. 5, C and D), and no effects were observed with SB203580 incubation (Fig. 5, E and F). Treatment with 20 μM of SP600125 decreased the number of recruited macrophages to 24% of normal level at 6 hptt (p < 0.001), while no effects were observed in the tails treated with the same dose of PD98059 and SB203580, respectively (Fig. 5, C–F). Although treatment with a higher concentration of PD98059 (200 μM) reduced the macrophage recruitment to ~50% of control, these results are questionable because high concentrations of PD98059 might have nonspecific suppressive effects on other members of the MAPK family. It was surprising that treatment with the p38 inhibitor, SB203580, did not have any effects on macrophage migration (Fig. 5, E and F), suggesting that p38 MAPK activation is not essential for macrophage migration in vivo, although it may participate in other inflammatory responses, such as cytokine production and survival (43). Notably, a few macrophages were able to migrate to the inflamed tail treated with 100 μM of SP600125 inhibitor at 6 hptt (Fig. 5A), perhaps due to either higher levels of JNK phosphorylation in these macrophages or other JNK-independent mechanisms.

Consistently, quantitative Western blot analyses with whole-embryo lysates showed that the levels of JNK phosphorylation (p-JNK) increased ~1.5-fold at 6 hptt embryos, compared with 0 hptt embryos (p < 0.01). Pretreatment of embryos with 20 μM of JNK inhibitor SP600125, dramatically abolished the induction of p-JNK activation (Fig. 6, A and B).

The I-sceI was originally utilized in Medaka to induce stronger promoter activity in the F0 founder, and to reduce nonspecific expression and promote the stable integration of transgene into the genome (44). For example, it has been used to study the BMP signaling under the control of zmo2 promoter in zebrafish hematovascular development, which decreased the mosaicism substantially caused by transient transgenic expression and acquired a specific expression pattern in ~30% of the injected embryos (45).

To further verify the involvement of JNK signaling in the chemotactic migration of macrophage in vivo, we expressed the human dominant-negative JNK (DN-JNK) in the EGFP+ macrophages of TG(zlyz:EGFP) embryos under the control of lysozyme C promoter flanked by I-sceI sites (named as zlyz-DN-JNK). In the DN-JNK mutant, the tyrosine at 185 and threonine at 183 required for phosphorylation activity have been replaced with alanine and phenylalanine, respectively, which results in the inactivation of JNK activity (46).

We first confirmed by Western blot analysis that the human DN-JNK was able to suppress endogenous JNK phosphorylation in the zebrafish embryo after microinjection of the CMV promoter-driven DN-JNK into wild-type embryos (Fig. 6C). Consistent with the results obtained with SP600125 treatment, suppression of JNK activity by targeted overexpression of DN-JNK, resulted in a significant decrease in the number of EGFP+ macrophages in the injured tails (Fig. 6, D and E), indicating an essential role of JNK signaling in the inflammatory macrophage migration in vivo.

Glucocorticoid negatively regulates macrophage migration via Mkp-1-mediated suppression of JNK-Mmp13 signaling

The dexamethasone (dex)-glucocorticoid receptor (GR) complex is known to up-regulate the expression of MKP-1/DUSP1, a threonine/tyrosine dual-specificity phosphatase that efficiently suppresses JNK phosphorylation in murine macrophages and other cell types (43, 47, 48). Treatment of the tail-transected embryos

![Figure 6](http://www.jimmunol.org/Downloadedfrom)
with 500 μM dex significantly increased the expression of mkp-1 mRNAs at 6 hptt (Fig. 7A), along with a concomitant suppression of mmp13 up-regulation at 6 hptt induced by the inflammatory activation of JNK-C-Jun signaling (Fig. 7A). The dex treatment also suppresses the levels of phosphorylated JNK by ~22% in the tail-transected embryos at 6 hptt, without affecting the levels of total JNK protein (Fig. 7B). Furthermore, treatment of the injured embryos with different concentrations of dex resulted in a dose-dependent decrease in the number of recruited macrophages. More than 90% of macrophages lost the ability to migrate to the injured tail treated with 500 μM dex at 6 hptt (Fig. 7C and D), which can be dramatically rescued by 200 μM of mifepristone (RU-486) treatment, a specific inhibitor of GR (Fig. 7C and D). Dex-induced apoptosis is unlikely to cause the decreased macrophage migration.

**FIGURE 7.** Suppression of macrophage migration by anti-inflammatory glucocorticoid signaling. A, Semiquantitative analyses of mmp13 and mkp-1 transcripts by single-embryo RT-PCR in 0 and 6 hptt (h) embryos treated with either vehicle or 500 μM of dexamethasone. B, Quantitative Western blot analysis of p-JNK levels in 0 and 6 hptt without or with treatment of dexamethasone (500 μM). The value indicated the ratio of p-JNK to t-JNK protein. Representative results obtained from three independent experiments and the mean value of p-JNK/t-JNK in 0 hptt was represented as 1. *, p < 0.05; **, p < 0.01. C and D, Quantification and time-course analyses of macrophage migration in the tail (white bracket) of vehicle-, dexamethasone-, or dexamethasone plus RU-486-treated embryos at 0, 2, and 6 hptt (h). Representative results obtained from three independent experiments. E, TUNEL analyses of EGFP+ macrophages in the 6 hptt tails treated with either vehicle or dexamethasone (μM) using an anti-EGFP Ab (green) and TUNEL staining (red).

**FIGURE 8.** Model of JNK-Mmp13 signaling in macrophage migration in response to acute injury induced by severe trauma. Trauma-induced phosphorylation of JNK resulted in the activation of transcriptional factor C-Jun. Phosphorylated C-Jun binds to the proximal AP-1 sites of mmp13 promoter, to up-regulate mmp13 gene expression, which in turn, at least in part, contributes to the chemotactic migration of macrophages. GC-GR transcriptionally activates the mkp-1 gene, which has been shown to efficiently inactivate the phosphorylated JNK by dephosphorylation, thus, negatively regulate JNK-Mmp13 signaling and suppress macrophage migration.
recruitment, indicated by an absence of TUNEL staining in the recruited macrophages (Fig. 7E). Taken together, the results suggest that evolutionarily conserved GR-Mkp-1 signaling negatively regulates the chemotactic macrophage migration by inactivating JNK-C-Jun signaling and suppressing the up-regulation of mmp13, which may provide novel insight into the anti-inflammatory mechanisms of glucocorticoid hormone, one of the most widely used drugs in clinical practice.

Discussion

The transgenic line TG(zlyz:EGFP) that uses a longer 11.0 kb promoter fragment (34), suggesting that key regulatory elements are located within the 4.1 kb of genomic region. In both transgenic lines, the EGFP expression is first detectable by fluorescence microscope at 22 hpf, and coexpressed with lysozyme C and l-plastin on the anterior yolk sac and P-ICM at 36 hpf, but not with mpo at 24 hpf. The highly purified EGFP+ cells from TG(zlyz:EGFP) embryos at 60 hpf exhibit the classical morphology of monocytes and/or macrophages, suggesting that the TG(zlyz:EGFP) line marks populations of primitive macrophages, as does the lyc:EGFP line (34). However, we cannot rule out the possibility that in the TG(zlyz:EGFP) line, some neutrophil granulocytes can express EGFP at later stages of development, including larval and adult tissues, as have been reported in the lyc: EGFP zebrafish kidney (34), and the bone and blood marrow of the lyc:EGFP knock-in mice (36).

The MAPK signaling cascades regulate diverse cellular activities including proliferation, differentiation, and survival in innate immune cells (14, 49). However, due to the difficulty of monitoring in vivo cell migration in an intact animal (34), it is unclear whether MAPKs or downstream signaling molecules contribute to macrophage migration through the ECM in response to severe injury. By precisely controlling the size of tail transection at 60 hpf, we established an assay that allows us to reliably quantify the number of recruited macrophages and dissect the molecular pathways underlying inflammatory macrophage migration. We found that the number of recruited macrophages was relatively consistent averaging 33 ± 5 cells in 6 hptt of the injured tails, compared with <5 macrophages at 0 hptt. Using this assay, we demonstrate that the JNK molecule, but not ERK or p38 MAPK, is a critical factor mediating primitive macrophage migration in vivo. Activation of ERK1/2 and p38 MAPK secondary to Mmp13 activation in human fibroblasts (50) and mouse macrophages (43) may be important for macrophage proliferation, survival, and cytokine production, as previously reported, but do not appear to be required for chemotactic migration of primitive macrophages.

Recent studies in zebrafish have shown that an intact microtubule organization, which may be downstream of MAPK signaling (51), is critical for normal macrophage chemotaxis toward a laser-induced injury on the yolk sac (52). In this study, we show that the mmp13, a macrophage-specific metalloproteinase gene, is a downstream target of JNK activation. Phosphorylation of JNK induced by tail transection resulted in the phosphorylation of C-Jun, and subsequent binding to the proximal AP-1 sites of mmp13 promoter, at least in part, contributing to the inflammatory macrophage migration. The importance of Mmp13 metalloproteinase in innate immune cell recruitment has been documented recently. For example, hepatic neutrophil recruitment is reduced in Mmp-13-deficient mice (53), and the Gr-1"CD11b" myeloid cells express high levels of Mmp13 and Mmp14 to enhance migration and tumor metastasis (54), suggesting an important function for Mmp13 in ECM remodeling and cellular movement.

We also provide evidence that dexamethasone, one of the most widely used anti-inflammatory agents in clinical practice, is able to suppress macrophage migration, through the transcriptional up-regulation of mmp1-1, a well-documented phosphatase capable of inactivating phosphorylated JNK and ERK. The results suggest novel cellular and molecular mechanisms for dexamethasone in the regulation of innate immune responses. We propose a model in which the activation of JNK-C-Jun-Mmp13 signaling following severe tissue injury promotes the interstitial macrophage migration within ECM, which can be negatively regulated by the GR-mediated Mkp-1 signaling pathway (Fig. 8).

Provided the highly evolutionary conservation of signaling pathways in innate immunity between zebrafish and human (24, 55), the transgenic line reported in this study and methodologies we have established provide an excellent in vivo model for dissecting cell migratory pathways and identifying therapeutic small molecules against unfavorable cellular migration associated with autoimmune diseases, tumor progression, and metastasis (19, 56, 57).

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Disclosures

The authors have no financial conflict of interest.

References


