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Duox1-Derived H2O2 Modulates Cxcl8 Expression and Neutrophil Recruitment via JNK/c-JUN/AP-1 Signaling and Chromatin Modifications

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DUOX1-derived hydrogen peroxide (H2O2) and CXCL8 are two key neutrophil chemoattractants. H2O2 is critical at the early phase, whereas CXCL8 plays a key role in the late phases of recruitment, but the crosstalks between the two phases in vivo remain unknown. In this study using zebrafish, we report that H2O2 also contributes to neutrophil recruitment to injuries at the late phase as it induces Cxcl8 expression in vivo through a JNK/c-JUN/AP-1 signaling pathway. However, Erk and NF-κB signaling were not involved in this crosstalk. Strikingly, H2O2 also promotes cxc18 expression through modulation of histone 3 lysine 4 trimethylation, histone 3 lysine 9 acetylation, and histone 3 lysine 9 trimethylation levels at its promoter. These results explain how early H2O2 signal regulates neutrophil recruitment at all phases, directly via Lyn oxidation or indirectly by modulating cxc18 gene expression, via the activation of redox-sensitive signaling pathways, and further point out H2O2/DUOX1 as a key drug target for anti-inflammatory therapies. The Journal of Immunology, 2015, 194: 000–000.

Inflammation is a major host defense process that occurs postinfection, injury or tissue stress and malfunction, and it involves the interplay of several molecular and cellular mediators so as to restore tissue homeostasis (1–3). Regardless of their controversial role in wound healing, neutrophils are the first leukocytes to be recruited to wounds (4, 5). Release of hydrogen peroxide (H2O2) from wounded tissues functions as an early signal for this neutrophil recruitment (6, 7), which is later on mediated by the action of chemokines, with CXCL8 being one of the most important ones in this respect (8–10).

The formation of a tissue gradient of H2O2 upon injury has been shown in zebrafish (6, 7, 11) and also in mice models (12). This gradient is not only an early signal for neutrophil recruitment, but can also influence later phases of wound healing, such as tissue regeneration (11, 12). Additionally, it has been shown in vitro that H2O2 is able to modulate the function of redox-sensitive transcription factors (13, 14) and to influence the gene expression of proinflammatory molecules, such as IL-1β or CXCL8 (15–18). Recently, we have further reported that Duox1-derived H2O2 is involved in NF-κB activation in wound healing (19). Importantly, we have also found that CXCL8 zebrafish homologs, Cxcl8-l1 and Cxcl8-l2, are crucial for neutrophil recruitment upon wounding and that they are able to dictate neutrophil behavior and to influence later tissue regeneration in zebrafish larvae (10). As both H2O2 and Cxcl8 gradients mediate neutrophil recruitment, we hypothesized that their action could be concerted, in vivo, with H2O2 regulating Cxcl8 expression and function. Using zebrafish larval tail fin injury as a model, we report in this work that early wound-induced H2O2 is able to modulate Cxcl8l2 expression and that this crosstalk involves P38 and Jnk signaling pathways as well as the phosphorylation of the transcription factor AP-1. Moreover, we also show that H2O2 modulates histone 3 lysine 4 trimethylation (H3K4me3), histone 3 lysine 9 acetylation (H3K9ac), and histone 3 lysine 9 trimethylation (H3K9me3) levels as well as p-c-JUN binding to the cxc18-l2 gene promoter. Altogether these results explain, at least in part, how early H2O2 signal can modulate different neutrophil recruitment phases, directly via Lyn oxidation or indirectly by modulating cxc18-l2 gene expression. Overall, this work contributes to a better understanding of how an early signal as H2O2 can regulate the onset of the inflammatory response in wound healing and further identifies H2O2 as a promising drug target for anti-inflammatory therapies.

Materials and Methods

Zebrafish husbandry

All experiments with live animals were performed using protocols approved by the European Union Council Guidelines (86/609/EU), the Spanish RD 53/2013, and the Bioethical Committee of the University of Murcia (approval 537/2011). Fertilized zebrafish eggs were obtained from natural spawning of wild type (obtained from the Zebrafish International Resource Center), the transgenic (Tg(mp5x:gfp)114) (20), and the Tg(luc:DsRED2)mu50 (21) lines held at our facilities following standard husbandry practices. Animals were maintained in a 12-h light/dark cycle at 28.5°C.
**Morpholino and mRNA injections**

Antisense (AS) and sense RNAs for the dominant-negative form of Duox1 (DN-Duox1) (19) (300 pg/egg) and morpholinos (MOs) (7, 10) were mixed in microinjection buffer (0.5×Tango buffer and 0.05% phenol red solution) and microinjected into one-cell-stage embryos using a microinjector (Narishige) (0.5–1 nl per embryo). The same amounts of RNA and MOs were used in all experimental groups. Splice-blocking MOs used in this study were the following: for cxcl8-l2, 5′-TTAGTATCTGCTTACCCTCATTGGC-3′ (10); for lyn, 5′-TCAGACAGCAAATAGTAATCACCTT-3′ (10); for duox1, 5′-AGTGAATTAGAGAAATGCACCTTTT-3′ (7); for p53, 5′-GCACCATTGCATTTCAGAAATGG-3′ (7), and for lta4h, 5′-AGCTAGGGTCTGAAACTGGAGTCAT-3′ (22).

**FIGURE 1.** H2O2 modulates different neutrophil recruitment phases. Zebrafish one-cell wild-type or Tg(lyz:DsRED2) were microinjected with mRNA Duox1 AS, sense (DN-Duox1), standard control (MO StdC), and/or MO cxcl8-l2. Tail fin larvae were amputated at 3 dpf in all groups. For H2O2 labeling, larvae were incubated for 30 min with 50 mM acetypentafluorobenzene sulphonyl fluorescein in 1% DMSO in embryo medium. (A) Representative overlay images of brightfield and red channels of wounded larvae tail fins 30, 90, 240, and 360 mpw. (B) Counts of fluorescent neutrophils at the site of injury from 30 to 360 mpw. Statistically significance differences among groups are indicated by different letters (a p < 0.05 just for samples with DN-Duox1, b p < 0.001). (C) Counts of fluorescent neutrophils at the site of injury at 30 and (D) 360 mpw. (E) Representative images of H2O2 production. (F) Wound fluorescence intensity quantification of wounded larvae tail fins at 30 mpw labeled with acetypentafluorobenzene sulphonyl fluorescein. The DN-Duox1 was able to significantly decrease H2O2 production in wounded tail fins. All data are represented as means ± SEM. The p values were calculated using two- or one-way ANOVA and Bonferroni multiple comparison test (**p < 0.01, ***p < 0.001). Scale bars, 100 μm. auf, arbitrary units of fluorescence.
Tail fin wounding

Tail fin amputation was performed, as previously described (10), in wild-type, Tg(lyz:DsRed2)pw20 or Tg(mpx:gfp)1143 d postfertilization (dpf) larvae.

Pharmacological treatments

All drug treatments were performed using the bath immersion method. Briefly, 3 dpf wild-type or Tg(mpx:gfp)114 larvae were incubated for 15 min at 28°C in the presence or absence of each of the following drugs from Sigma-Aldrich: 100 μM PD98059 (11), 10 μM SB220025 (23), 50 μM SP600125 (24), 50 mM NaI (25), 1 μM wortmannin (26), and 20 μM PP2 (11) diluted in embryo medium. After tail fin amputation, larvae were maintained in the corresponding treatments until experimental procedure.

H2O2 imaging

H2O2 imaging using 50 mM acetyl-pentafluorobenzene sulphone fluorescein, a live cell dye, was used, as previously described (19). Imaging was made 30 min postwounding (mpw).

Zebrafish tail tissue sample collection and gene expression analysis

At 3 dpf, tail tissue sample collection was performed, as previously described (10). For gene expression analysis, total RNA was extracted from tail tissue with TRIzol reagent and purified with RNAquous Micro Kit, total RNA purification system (Ambion), and treated with DNase I, amplification grade (1 U/μg RNA; Invitrogen). The SuperScript III RNase H- reverse transcriptase (Invitrogen) was used to synthesize first-strand cDNA with oligo(dT)18 primer from 1 μg total RNA at 50°C for 50 min. Real-time PCR was performed with an ABI Prism 7500 instrument (Applied Biosystems) using SYBR Green (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min 60°C, and 15 s at 95°C. Gene expression was normalized against rps11 (32) using the bicinchoninic acid protein assay reagent (Fierce) using BSA as a standard. Cell extracts (50 μg protein) were analyzed on 15% SDS-PAGE and transferred for 50 min at 200 mA to nitrocellulose membranes (Bio-Rad). The blots were probed with a purified mouse mAb generated against recombinant zebrfish Cxcl8-l2 (GeneScript) at 1:2500 and developed with ECL reagents (GE Healthcare), according to the manufacturer’s protocol.

Image acquisition and processing

For each assay, 3 dpf larvae were imaged in three independent experiments. Images were taken from wounded larvae mounted as described above using a Leica MZ16F fluorescence stereo microscope equipped with green and red fluorescent filters, whereas the animals were maintained in their agar matrices at 28.5°C. Mean fluorescence was measured in wounded epithelial cells, and the corresponding background values of unwounded larvae epithelial cells were subtracted.

Whole-mount immunofluorescence

At 3 dpf, larvae were fixed overnight at 4°C in 4% paraformaldehyde after tail fin injury at 0, 1, or 5 h postwounding (hpw). Ab labeling was performed as previously described (27). The following primary Abs were used: mouse mAb against zebrfish Cxcl8-l2 at 1:100; rabbit anti-human phospho-P38 (MA5-15177; Thermo Scientific) at 1:200 (28); mouse anti-human phospho-JNK (MA5-15228; Thermo Scientific) at 1:100 (29); mouse anti-human phospho-ERK1 plus ERK2 (ab50011; Abcam) at 1:300 (11); anti-p-c-JUN (3270; Cell Signaling) at 1:100 (30, 31); and Alexa Fluor 594- and Alexa Fluor 488-conjugated secondary Abs (Life Technologies) at 1:500.

Images were acquired using a Leica MZ16F fluorescence stereo microscope for DN-Duox1 or using a Zeiss Axiowit200 fully motorized, inverted, wide field fluorescence microscope using a NA 0.8/20× objective, in z-stack mode for cxc91-l2 morphants experiment. All data were processed using Image J (http://rsb.info.nih.gov/ij/).

Bioinformatic promoter analysis

The software MatInspector Matrix library version 8.0 (www.genomatix.de) was employed for promoter analysis (32, 33). The DNA sequence upstream of the predicted start codon (1.6 kb) was extracted for each gene from the Ensembl database (zebrafish, Zv9 assembly; human, GRCh37). DNA sequences were analyzed using matrixes optimized for

FIGURE 2. Duox1-derived H2O2 modulates Cxcl8-l2 expression both at the transcript and protein levels after wounding. Zebrafish one-cell Tg(mpx:gfp)114 larvae were microinjected with mRNA Duox1 AS or sense (DN-Duox1). Tail fin larvae were amputated at 3 dpf in all groups. (A) mRNA levels of cxc91-l2 were determined by quantitative PCR in tail fin tissue at 0 and 1 hpw (40 tail fins per time point). Gene expression was normalized against rps11 and expressed as fold change compared with transcript expression levels of 3 dpf tail fin tissue from unwounded larvae (0 hpw). Each bar represents the mean ± SEM of triplicated samples. The p values were calculated using two-way ANOVA with Bonferroni multiple comparison test; ***p < 0.001. (B) Representative images of red (ZICxcl8-l2) channel of whole-mount immunofluorescence of zfCxc91-l2 at 5 hpw. (C) Wound fluorescence intensity quantification in wounded larvae tail fins at 5 hpw. DN-duox1 form is able to significantly decrease Cxcl8-l2 protein levels in wounded tail fins. All data are represented as means ± SEM. The p values were calculated using Mann–Whitney test (***p < 0.001). Scale bars, 100 μm. au, arbitrary units of fluorescence.
FIGURE 3. Lyn is important for early neutrophil recruitment and for cxcl8-l2 gene expression. Zebrafish one-cell wild-type or Tg(mpx:GFP)114 were microinjected with control (MO StdC), lyn (MO lyn), and/or MO cxcl8-l2. Tail fin larvae were amputated at 3 dpf in all groups. (A) cxcl8-l2 mRNA levels were determined by quantitative PCR in tail fin tissue at 0 and 1 hpw (40 tail fins per time point). Gene expression was normalized against rps11 and expressed as fold change compared with transcript expression levels of 3 dpf tail fin tissue from unwounded larvae (0 hpw). Each bar represents the mean ± SEM of triplicated samples. The p values were calculated using two-way ANOVA with Bonferroni multiple comparison test; *p < 0.05. (B) Representative overlay images of brightfield and green channels of wounded larvae tail fins 30, 90, 240, and 360 mpw. (C) Counts of fluorescent neutrophils at the site of injury from 30 to 360 mpw. Statistically significance differences among groups are indicated by different letters (acpxlyn, p < 0.01). (D) Counts of fluorescent neutrophils at the site of injury at 30 and (E) 360 mpw. The lyn morpholino significantly decreased early neutrophil recruitment to wounds, and cxcl8-l2 significantly reduced neutrophil recruitment in both early and late phases. All data are represented as means ± SEM. The p values were calculated using one- or two-way ANOVA with Bonferroni multiple comparison test (***p < 0.001). Scale bars, 100 μm.
general core promoter elements of vertebrates, and enhancer elements were used specifically for NF-κB, STAT, IFN regulatory factors, and AP-1. Matrix matches with a similarity score >0.80 were considered.

Chromatin immunoprecipitation

Larvae with 72 hpf previously microinjected with RNA AS or DN-Duox1 (400 per each chromatin immunoprecipitation [ChIP] biological replicate) were wounded, and at 1 h postwounding processed for ChIP using the MAGnify Chromatin Immunoprecipitation System (Life Technologies), as described before (34). ChIP and input DNA were amplified by quantitative PCR using specific primers for the 5′ upstream sequences of the different genes (Supplemental Table I). Quantitative ChIP values were normalized to 10% input, and ChIP controls were performed with mouse or rabbit anti-IgG. ChIPs were performed with at least two independent chromatin preparations. The Abs used in this assay were as follows: anti-H3K9ac (ab1791; Abcam), anti-H3K4me3 (ab1012; Abcam), anti-H3K9me3 (06–942; Millipore), anti–p-c-JUN (3270; Cell Signaling), and control mouse and rabbit anti-IgG provided by the 

FIGURE 4. SFKs, Erk, P38, and Jnk signaling pathways are crucial for Cxcl8-l2 expression and for normal neutrophil recruitment. Zebrafish 3 dpf Tg(mpx:GFP)i114 larvae were pretreated for 15 min with 100 μM PD98059, 10 μM SB220025, 50 μM SP600125, 50 nM Nai, 1 μM wortmannin, and 20 μM PP2. Tail fin larvae were amputated in all groups; larvae were maintained with inhibitors during all the procedures. (A) cxcl8-l2 mRNA levels were determined by quantitative PCR in tail fin tissue at 0 and 1 hpw (40 tail fins per time point). Gene expression was normalized against rps11 and expressed as fold change compared with transcript expression levels of 3 dpf tail fin tissue from unwounded larvae (0 hpw). Each bar represents the mean ± SEM of triplicated samples. The p values were calculated using two-way ANOVA with Bonferroni multiple comparison test; *p < 0.05. (B) Representative overlay images of brightfield and green channels of wounded larvae tail fins 30, 90, 240, and 360 mpw. (C) Counts of fluorescent neutrophils at the site of injury from 30 to 360 mpw. Statistically significance differences among groups, p < 0.001. (D) Counts of fluorescent neutrophils at the site of injury at 30 and (E) 360 mpw. All inhibitors significantly decreased neutrophil recruitment to wounds in both phases. All data are represented as means ± SEM. The p values were calculated using two-way ANOVA with Bonferroni multiple comparison test (***p < 0.001). Scale bars, 100 μm.
MAGnify kit. All these Abs have been previously validated in zebrafish (29, 31, 34).

Statistical analysis

All error bars indicate SEM. A Mann–Whitney t test and one- or two-way ANOVA with Bonferroni posttest were used.

Results

$H_2O_2$ modulates different neutrophil recruitment phases

Tissue gradients of $H_2O_2$ were previously shown to be required for the early neutrophil recruitment upon wounding (6, 7, 11, 19). Previously, we have reported that, by impairing $H_2O_2$ production, DN-Duox1 significantly reduces the recruitment of neutrophils toward wounds between 30 and 90 mpw after tail fin transection of 3 dpf larvae in comparison with control AS DN-Duox1 RNA-injected larvae (19) (Fig. 1A–C, 1E, 1F). In this study, by extending this analysis up to 6 hpw, we found that expression of DN-Duox1 not only affects this early recruitment phase, but also significantly reduces neutrophil recruitment at later time points (Fig. 1A, 1B, 1D). Similar results were obtained using duox1 morphants (Supplemental Fig. 1).

Cxcl8-l2 and LTB4 are involved in early neutrophil recruitment

We have previously reported that the chemokine cxcl8-l2 expression is induced after wounding and is actually crucial for normal neutrophil recruitment and behavior under this condition (10). Interestingly, cxcl8-l2 morphants showed decreased neutrophil recruitment not just at a later phase, but also during the early recruitment phase, more specifically at 90 mpw (Supplemental Fig. 2A). In these morphants, however, $H_2O_2$ levels were unaffected (Fig. 1E, 1F). Accordingly, we further verified by Western blot (Supplemental Fig. 2B) and whole-mount immunofluorescence (Supplemental Fig. 2C) analysis, using an anti–zfCxcl8-l2 mAb, that 3dpf unwounded zebrafish larvae express a basal level of Cxcl8-l2. The specificity of the staining was confirmed by using 5 hpw cxcl8-l2 morphant larvae, which showed decreased Cxcl8-l2 protein signal at the wound margin in comparison with the controls (Supplemental Fig. 2D, 2E).

As leukotriene B4 (LTB4) has been reported to be one of the main modulators of neutrophil recruitment (35–37), we also examined the impact of inhibiting its production by knocking down leukotriene A4 hydrolase (22). We verified that leukotriene A4 hydrolase deficiency was able to significantly decrease neutrophil recruitment (Supplemental Fig. 3A). Strikingly, despite the fact that LTB4 deficiency mainly affected the early phase of neutrophil recruitment (Supplemental Fig. 3B), it did not affect $H_2O_2$ production (Supplemental Fig. 3C).

Duox1-derived $H_2O_2$ is needed for Cxcl8-l2 expression after wounding

Previously, in vitro studies have suggested $H_2O_2$ and oxidative stress as possible modulators of chemokine and cytokine production (16, 38). Notably, we noticed that the DN-duox1 recruitment curve presented a kinetics very similar to that obtained for cxcl8-l2 morphants (10) (Fig. 1B), suggesting that $H_2O_2$ might be required for normal Cxcl8 signaling in vivo. So next we decided to address whether Duox1-derived $H_2O_2$ is involved in Cxcl8-l2 signaling. We observed that, in the presence of the DN-Duox1, a significant decrease of Cxcl8-l2 gene expression (Fig. 3A).

Lyn is important for normal neutrophil recruitment at early phase and also contributes to normal cxcl8-l2 gene expression

Lyn is a Src family kinase (SFK) previously identified by others as the neutrophil redox sensor responsible for early recruitment (7). So, next, we wanted to address its involvement in $H_2O_2$-mediated Cxcl8-l2 expression. Upon tail fin transection, lyn knockdown was able to slightly diminish cxcl8-l2 gene expression in comparison with the control condition during the early phase (Fig. 3A).

FIGURE 5. Inhibition of SFK, Erk, P38, and Jnk signaling pathways does not affect $H_2O_2$ production. Zebrafish 3 dpf wild type were pretreated for 15 min with 100 μM PD98059, 10 μM SB220025, 50 μM SP600125, and 20 μM PP2. Tail fin larvae were amputated in all groups; larvae were maintained with inhibitors during all the procedures. (A) Representative images of $H_2O_2$ production. (B) Wound fluorescence intensity quantification of wounded larvae tail fins at 30 mpw labeled with acetyl-pentafluorobenzene sulphonyl fluorescein. None of the inhibitors affected $H_2O_2$ levels in wounded tail fins. All data are represented as means ± SEM. The p values were calculated using one-way ANOVA and Bonferroni multiple comparison test. Nonstatistically significant differences were observed. Scale bars, 100 μm. auf, arbitrary units of fluorescence.
suggesting that the neutrophil could contribute to this chemokine expression at the wound. Nevertheless, we observed that lyn morphants presented a significant decrease in neutrophil recruitment toward wounds before 90 mpw (Fig. 3A–C), but not at later time points (240 and 360 mpw) (Fig. 3A, 3B, 3D), consistent with the role of Lyn in the early phase of neutrophil recruitment.

SFKs, Erk, P38, and Jnk are crucial for Cxcl8s expression and normal neutrophil recruitment, but not for H2O2 production

To further understand the signaling mechanisms underlying H2O2-mediated Cxcl8-I2 expression, our next step was to unravel which signaling molecules might be implicated in this respect. For such, we made use of several pharmacological drugs to inhibit specifically distinct signaling pathways, namely SB220025 for P38 signaling; SP600125 for Jnk signaling; Nai for NF-κB signaling; wortmannin for PI3K signaling; and PP2, as a SFK inhibitor. Upon SFK inhibition or inhibition of P38, Jnk, or Erk signaling pathways, cxcl8-I2 gene expression was significantly diminished after wounding. In contrast, the Nai inhibitor failed to affect cxcl8-I2 expression (Fig. 4A).

We next addressed the effect of these pharmacological inhibitors on neutrophil recruitment in response to wounding. We observed that neutrophil recruitment was significantly reduced at both early and late recruitment phases in presence of all the inhibitors used (Fig. 4B–E). Wortmannin was in this study used as a negative control of neutrophil recruitment taken as the well-established role of Lyn in the early phase of neutrophil recruitment. Nevertheless, we observed that lyn morphants presented a significant decrease in neutrophil recruitment toward wounds before 90 mpw (Fig. 3A–C), but not at later time points (240 and 360 mpw) (Fig. 3A, 3B, 3D), consistent with the role of Lyn in the early phase of neutrophil recruitment.

H2O2 induces P38 and Jnk signaling and c-JUN phosphorylation, but not Erk signaling, in wounded tissue

After establishing that Erk, P38, and Jnk signaling were required for normal cxcl8-I2 gene expression and neutrophil recruitment, we further asked whether these effects were modulated by H2O2. For such, we next addressed whether H2O2 was able to affect the phosphorylation levels of Erk, P38, and Jnk. Immunofluorescence analysis of wounded larvae tail fins at 1 hpw showed that this was the case for P38, Jnk, and c-Jun, but not for Erk, as the expression of the DN-Duox1 reduced the levels of phospho-P38, phospho-Jnk, and phospho-c-Jun, but did not affect phospho-Erk levels in comparison with control larvae (Fig. 6).

H2O2 modulates histone 3 modifications on proximal cxcl8-I2 promoter

H2O2 is able to modulate gene expression by several modes of action (15–18). Among these, in vitro studies have suggested a possible role in modulating the acetylation/methylation status of histone core at gene promoters. To understand how H2O2 modulates Cxcl8-I2 expression, we decided to address in vivo whether H2O2 might contribute to histone 3 modifications in the cxcl8-I2 gene-proximal promoter, and more precisely at site I upon wounding in comparison with AS control larvae (Fig. 7B). These results suggest that the effect of Duox1-derived H2O2 on cxcl8-I2 gene expression upon wounding could in part be mediated by the modulation of the chromatin status at the promoter of this gene (Fig. 8).

H2O2 promotes the binding of the transcription factor AP-1 to cxcl8-I2 promoter

H2O2 has also been shown to modulate gene expression via the activation of redox-sensitive transcription factors, such as AP-1 (13, 14). Analysis of the promoter sequences of human CXCL8 and zebrafish cxcl8-I2 genes using the software MatInspector (Genomatix) allowed us to identify several binding sites for AP-1, among other known transcription factors (Fig. 7A). Of notice, this analysis further supports the absence of binding site for NF-κB in the zf cxcl8-I2 promoter (Fig. 7A), in good agreement with the lack of effect of the Nai inhibitor on the expression of this gene (Fig. 4A). A ChIP assay for p-c-Jun, one of the two AP-1 subunits, was performed using wounded tail fin tissue at 1 hwp, from zebrafish larvae microinjected with AS or DN-Duox1 mRNA. Quantitative PCR analysis for p-c-Jun bound to

![FIGURE 6. Duox1-derived H2O2 induces Jnk, P38, and c-JUN phosphorylation, but not that of Erk, in wounded tail tissue. Zebrafish one-cell stage Tg(lyz:DsRED2)fz50 were microinjected with mRNA Duox1 AS or sense (DN-Duox1). Tail fin larvae were amputated at 3 dpf. (A) Representative three-dimensional images with green (molecular target) channel of whole-mount immunofluorescence at 1 hwp. (B) Wound fluorescence intensity quantification in wounded larval tail fins. H2O2 tissue gradients induce the phosphorylation of P38, Jnk, and c-JUN, but not that of Erk at wounded tissues. All data are represented as means ± SEM. The p values were calculated using one-way ANOVA and Bonferroni multiple comparison test (*p < 0.05, **p < 0.01, ***p < 0.001). Scale bars, 100 μm, auf, arbitrary units of fluorescence; ns, nonsignificant.](http://www.jimmunol.org/Download?uri=10.4049/jimmunol.1700585)
the promoter in AS larvae compared with larvae expressing the DN-Duox1 mutant (Fig. 7C), indicating that H2O2 production enhances AP-1 recruitment to the zebrafish cxcl8-l2 gene promoter (Fig. 8).

**Discussion**

During the last years, zebrafish has been used to uncover in vivo several aspects of neutrophil recruitment. One of the most important findings was the discovery that H2O2, one of the molecules believed to be released after wounding, actually forms a tissue gradient (6) that is primarily responsible for early leukocyte recruitment (7) and later tissue regeneration (11). Besides this great advance, we (10) and others (40, 41) have further reported the importance of Cxcl8 gradients in neutrophil recruitment and behavior in zebrafish inflammation. Altogether, these and other findings clearly support the use of zebrafish as an important animal model for the in vivo study of neutrophil functions.

Overall, these previous studies have highlighted a role for H2O2 and Cxcl8 chemokines, among other chemotactic cues, in neutrophil recruitment in zebrafish models of inflammation. A currently accepted view of neutrophil recruitment proposes that it occurs by sequential phases in response to several different inflammatory mediators, including lipids, cytokines, and chemokines (35, 37). Taking into account the expression and function of H2O2 and Cxcl8 chemokines, these cues may apparently act at different phases of neutrophil recruitment, with H2O2 being an early recruitment signal and the Cxcl8 chemokines guiding neutrophils in subsequent phases. Therefore, in this study, we decided to focus our attention more specifically on the possible crosstalk between H2O2 and Cxcl8-l2. We found that early H2O2 production by wounded epithelial cells contributes to Cxcl8-l2 induction, and that this crosstalk is able to modulate neutrophil recruitment at later time points. Notably, we also found that besides H2O2, Cxcl8-l2 and LTB4 are also involved in the early recruitment of...
neutrophils. This is not completely unexpected in view of data reported in previous studies together with the basal production of Cxcl8-l2 in zebrafish larvae found in the current study. This pre-formed Cxcl8-l2 may be stored in intracellular vesicles and rapidly released after wounding complementing H2O2 and LTB4 function during the early phase of neutrophil recruitment. Undoubtedly, the mechanisms involved in early neutrophil recruitment need to be further clarified specially in what concerns these three important players, although such goal is beyond the scope of this work.

By modulating the production and release of H2O2 by Duox1 using a DN form of this enzyme (19), we could observe that this early signal is in fact important for Cxcl8-l2 expression, both at transcript level (as observed at 1 hpw) as well as at the protein level (as observed at 5 hpw) after tail fin injury. As such, we conclude that H2O2 not only directs neutrophil recruitment at initial recruitment phases (30–90 mpw), but can also modulate the expression of Cxcl8-l2 gradients later on and, thus, can indirectly contribute to neutrophil recruitment at later phases. Although this is likely an oversimplified model given the role of other mediators, such as LTB4 and other chemokines, it represents a significant modification to the current view of the mechanisms controlling neutrophil recruitment.

Our results raise the important question of the cellular players involved in this H2O2–Cxcl8 crosstalk. In zebrafish, several cells and tissues involved in inflammatory response have been reported to be able to produce Cxcl8 (42), including skin and leukocytes such as macrophages and neutrophils. Our data show that Lyn deficiency seems to have a significant contribution on normal cxcl8-l2 gene expression, but surprisingly not on later neutrophil recruitment. Such effect might be due to the increased number of neutrophils previously reported upon lyn knockdown (7), which could compensate for reduced Cxcl8 expression. The mechanisms by which neutrophils are recruited after H2O2-induced Lyn autophosphorylation still remain unknown, but are beyond the scope of this work.

When targeting SFKs (7, 11) or Erk (11, 43, 44), P38 (43), Jnk (31), and NF-kB pathways (19, 44) with specific inhibitors, we observed that almost all drug targets tested seem to be involved in
cxcl8-l2 expression. Surprisingly, inhibition of NF-kB signaling was the only condition that did not affect this chemokine expression upon wounding. Accordingly, a cxcl8-l2 gene promoter analysis confirmed the absence of binding sites for this transcription factor. Our next step was then to test whether these drug-mediated decreases in cxcl8-l2 expression would be correlated with a decrease in neutrophil recruitment. As expected, all the inhibitors that have downregulated cxcl8-l2 expression were also able to significantly decrease neutrophil recruitment, both at early and later phases. Additionally, H2O2 measurements showed no changes in its levels, indicating an upstream role for this molecule in the signaling cascade. Yoo et al. (7, 11) have previously reported that H2O2 is able to induce SFK phosphorylation upon wounding both in neutrophils and in wounded epithelia tissue, modulating this way tissue regeneration. In this study, we show that besides SFKs, H2O2 is also able to crosstalk with Cxcl8 signaling by modulating the phosphorylation levels of P38 and Jnk in wounded tissue. Additionally, phosphorylation levels of c-Jun are also modulated by the presence of H2O2. Although inhibition of Erk signaling decreased cxcl8-l2 gene expression and neutrophil recruitment, the activation of this pathway in the wounded tissue was observed to be H2O2 independent. This result is in accordance with the previous data reported by Yoo et al. (11) in which Erk phosphorylation was observed to occur as a H2O2-independent early mechanism involved in later tissue regeneration. Altogether, our findings show that the mechanism underlying H2O2–Cxc8-l2 crosstalk is partly based on the activation of P38 and Jnk signaling pathways as well as c-Jun phosphorylation.

Among other modes, H2O2 role is able to modulate gene expression by regulating several histone protein modifications (14, 18). Acetylation/Deacetylation and methylation/demethylation of histone residues at the histone core, around which DNA is coiled, are important to modulate the access of transcription factors to promoter elements and gene transcription. These nuclear histone modifications are reversible processes regulated by the following: acetyltransferases, which promote acetylation; deacetylases, which promote deacetylation; histone methyltransferases, which promote methylation; and finally, histone demethylases, which promote demethylation (13, 14). By ChIP analysis, we found that H2O2 tissue gradient regulates the acetylation and methylation status of histone 3 at lysines 4 and 9 at the cxcl8-l2 proximal promoter, after tissue injury. The levels of histone modifications obtained in the absence or presence of H2O2 tissue gradient were consistent with a role of H2O2 in inducing the transcriptional activation of cxcl8-l2.

H2O2 has also been shown to modulate gene transcription through controlling the function of redox-sensitive transcription factors such as NF-κB and AP-1 (13, 14). By performing a ChIP analysis for p-c-Jun, one of the AP-1 subunits, we observed that H2O2 modulates activated AP-1 binding to at least one of its potential binding sites in cxcl8-l2 gene-proximal promoter. Our findings are in accordance with a previous study that pointed out early Jnk signaling and Junb as essential players, both for macrophage recruitment and tail fin tissue regeneration in zebrafish (31). Additional work will be needed to better understand whether this H2O2-mediated effect occurs directly at the transcription factor level, like it happens, for example, with Lyn oxidation (7), and/or indirectly by activating factors upstream of AP-1 in the pathway, such as Jnk.

Overall, our study enabled us to conclude that H2O2 is a key regulatory factor in the in vivo wound response that crosstalks with Cxcl8 signaling by a complex mechanism involving SFKs, P38, and Jnk signaling pathways, as well as histone 3 modifications and AP-1 binding to cxcl8-l2 proximal promoter (Fig. 8). The existence of a complex signaling mechanism was previously proposed to explain how Duoap1-derived H2O2 could modulate the expression of CXCL8 in mammalian models, based on in vitro studies performed throughout the past decade. To our knowledge, our study provides for the first time in vivo evidence to support the existence of such crosstalk within an organism, the zebrafish, in the context of a particular biological response, the wound-associated inflammation. Moreover, this study unravels important issues regarding the in vivo modulation of Cxcl8 expression by H2O2. Unanswered questions will need further attention in the near future, such as, for example, how H2O2 is able to modulate P38 and Jnk signaling and, ultimately, c-Jun phosphorylation levels, or how it is able to impact on histone 3 epigenetic markers. As H2O2 does not only control the expression of the cxcl8-l2 gene, but most likely also modulates the expression and/or activity of several other proinflammatory and proresolution molecules, our studies highlight H2O2 as a major potential drug target for the development of new anti-inflammatory treatments.

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References


