Disruption of Cxcr3 chemotactic signaling alters lysosomal function and renders macrophages more microbicidal

Graphical abstract

**Highlights**
- Disruption of Cxcr3 chemotactic signaling increases lysosomal gene expression
- Cxcr3 deficiency augments the microbicidal capacity of macrophages
- The lysosomal regulator TFEC counteracts the effect of Cxcr3 on microbicidal capacity
- Cxcr3 deficiency affects lysosome trafficking and prevents polarized migration

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**In brief**
Sommer et al. show that migration of immune cells is inversely linked to their bactericidal properties. Disrupting Cxcr3 chemotactic signaling affects lysosome trafficking and augments lysosomal capacity. Consequently, while reducing migration, Cxcr3 deficiency also primes immune cells for better clearance of intracellular infection.
Disruption of Cxcr3 chemotactic signaling alters lysosomal function and renders macrophages more microbicidal

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SUMMARY
Chemotaxis and lysosomal function are closely intertwined processes essential for the inflammatory response and clearance of intracellular bacteria. We used the zebrafish model to examine the link between chemotactic signaling and lysosome physiology in macrophages during mycobacterial infection and wound-induced inflammation in vivo. Macrophages from zebrafish larvae carrying a mutation in a chemokine receptor of the Cxcr3 family display upregulated expression of vesicle trafficking and lysosomal genes and possess enlarged lysosomes that enhance intracellular bacterial clearance. This increased microbicidal capacity is phenocopied by inhibiting the lysosomal transcription factor EC, while its overexpression counteracts the protective effect of chemokine receptor mutation. Tracking macrophage migration in zebrafish revealed that lysosomes of chemokine receptor mutants accumulate in the front half of cells, preventing macrophage polarization during chemotaxis and reaching sites of inflammation. Our work shows that chemotactic signaling affects the bactericidal properties and localization during chemotaxis, key aspects of the inflammatory response.

INTRODUCTION
Macrophages are specialized motile cells that mediate the innate immune response to pathogens, initiate inflammation, present antigens, regulate tissue repair, and also have diverse functions in developmental processes (Ginhoux et al., 2016). Similar to other leukocytes, macrophages differentially express chemokine receptors to sense extracellular cues that direct them to inflammatory sites (Charo and Ransohoff, 2006; Rot and von Andrian, 2004). Following chemotactic stimulation, these cells acquire a polarized phenotype characterized by clearly identifiable lamellipodia (leading edge) and a uropod (rear edge) that involves both the contractile machinery of the cell and the intracellular vesicle trafficking system (Colvin et al., 2010). Recent studies revealed that intracellular vesicular trafficking, particularly lysosomes and the secretion of exosomes, plays a role in regulating chemotaxis (Colvin et al., 2010; Sung et al., 2015; Bretou et al., 2017; Becker, 1976; del Pozo et al., 1995; Reddy et al., 2001). The Ca²⁺ release triggered by chemokine receptors induces the fusion of lysosomes with the plasma membrane at the uropod to sustain cell shape remodeling through the delivery of endomembranes and to detach the uropod (Colvin et al., 2010; del Pozo et al., 1995; Bretou et al., 2017; Becker 1976; Reddy et al., 2001; Lawson and Maxfield, 1995). Synaptotagmins (calcium-sensing vesicle-fusion proteins) and Rab GTPases are critical regulators of vesicular trafficking and lysosomal exocytosis and link the chemokine signaling-dependent Ca²⁺ flux to lysosomal function (Colvin et al., 2010; Constantin and Laudanna, 2010; Lawson and Maxfield, 1995; Colvin and Luster, 2011). Processes linking cell motility and lysosomal function are only partially understood, and the effect of chemokine signaling on lysosomal function during inflammatory processes in vivo remains largely unknown.

Lysosomes are acidic membrane-bound organelles, rich in hydrolytic enzymes that mediate the catabolism of various macromolecules (Luzio et al., 2014; De Duve et al., 1955). In addition to their function as digestive organelles, lysosomes have emerged as signaling platforms and as critical regulators of cell metabolism, homeostasis, plasma membrane repair, survival, and immune defense (Martina et al., 2014; Settembre et al., 2013; Lawrence and Zoncu 2019; Zoncu et al., 2011). The mammalian/mechanistic target of rapamycin complex 1 (mTORC1), a kinase complex anchored to the lysosomal membrane, is a key regulator of lysosomal function (Martina et al., 2012; Settembre et al., 2012). The serine/threonine kinase mTOR phosphorylates the master gene of lysosomal biogenesis TFEB (transcription factor EB) to prevent its translocation to the nucleus (Sardiello et al., 2009; Palmieri et al., 2011; Verastegui et al., 2000). TFEB is a member of the basic helix-loop-helix leucine zipper family of transcription factors that bind to the CLEAR (coordinated lysosomal expression and regulation) elements (GTCACGTGAC) in the promoter regions of autophagic and lysosomal genes.
Figure 1. Disruption of Cxcr3.2 signaling transcriptionally induces genes related to lysosomal function and intracellular vesicle trafficking

(A) Principal-component analysis (PCA) of Cxcr3.2 mutant (Cxcr3.2−/−) and WT (Cxcr3.2+/+) transcriptomes. PCA analysis was performed in R on variance-stabilizing transformed (vst) data, using the DESeq2 plotPCA command.

(B) Volcano plot of Cxcr3.2 mutant versus WT differentially expressed genes. Genes are classified and color-coded by cellular compartment annotation. Compartment annotations were obtained from http://geneontology.org according to the GO cellular component and from KEGG pathways.

(C) Distribution of upregulated (yellow) and downregulated (blue) genes, classified by compartment as above. Lysosomal, Golgi, and peroxisome-related genes are more commonly upregulated in Cxcr3.2 mutant macrophages.

(legend continued on next page)
Toll-like receptors (TLRs) triggers the release of calcium from et al., 2016; El-Houjeiri et al., 2019). Pathogen sensing through facilitates its translocation to the nucleus (Bretou et al., 2017; and activates calcineurin, which dephosphorylates TFEB and the lysosome through the MCOLIN 1 (mucolipin 1) ion channel microbial molecules (Sachdeva and Sundaramurthy, 2020).

gradually matures by acquiring lysosomal hydrolases, a pro-phages, microbes are enclosed inside a phagosome, which following their phagocytic uptake by macrophages, microbes are enclosed inside a phagosome, which gradually matures by acquiring lysosomal hydrolases, a process that goes along with acidification and production of antimicrobial molecules (Sachdeva and Sundaramurthy, 2020). However, a number of intracellular pathogens, with Mycobacterium tuberculosis as a notable example, are able to inhibit phagosome maturation and avoid lysosomal degradation (Upadhyay et al., 2018; Flannagan et al., 2015; Tuli and Sharma, 1997).

In macrophages, lysosomes are involved in pro-inflammatory, chemotactant, and antimicrobial responses (Bretou et al., 2017; Settembre et al., 2013; Pastore et al., 2016; Visvikis et al., 2014). Following their phagocytic uptake by macrophages, microbes are enclosed inside a phagosome, which gradually matures by acquiring lysosomal hydrolases, a process that goes along with acidification and production of antimicrobial molecules (Sachdeva and Sundaramurthy, 2020). However, a number of intracellular pathogens, with Mycobacterium tuberculosis as a notable example, are able to inhibit phagosome maturation and avoid lysosomal degradation (Upadhyay et al., 2018; Flannagan et al., 2015; Tuli and Sharma, 1997). Macrophage recognition of pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) primes lysosomes for pathogen degradation and chemotaxis in an mTORC1-independent manner (Bretou et al., 2017; Shen et al., 2016; El-Houjeiri et al., 2019). Pathogen sensing through Toll-like receptors (TLRs) triggers the release of calcium from the lysosome through the MCOLIN 1 (mucolipin 1) ion channel and activates calcineurin, which dephosphorylates TFEB and facilitates its translocation to the nucleus (Bretou et al., 2017; Medina et al., 2015; Tong and Song, 2015; Schilling et al., 2013). TFEB activation leads to increased phagosomal acidification and accumulation of lysosomes (Settembre et al., 2013; El-Houjeiri et al., 2019; Settembre et al., 2011). Likewise, macrophages activated by TLR sensing show accumulation of TFEB in the nucleus and induction of immune genes that are directly implicated in the inflammatory response (Pastore et al., 2016; Schilling et al., 2013). In contrast, depletion of TFEB or TFEB results in reduced cytokine and chemokine secretion (Pastore et al., 2016; Visvikis et al., 2014; Settembre et al., 2011).

Thus, the function of the lysosomal transcriptional regulators is tightly linked to macrophage migration.

In the present study, we investigated the link between chemotactic signaling and lysosomal function in vivo using a cxcr3.2 mutant zebrafish line deficient in a macrophage-attractant chemokine receptor homologous to human CXCR3 (Torraca et al., 2015). We previously showed that zebrafish larvae lacking Cxcr3.2 are more resistant to mycobacterial infection and that reduced motility of macrophages limits the tissue dissemination of mycobacteria (Torraca et al., 2015; Sommer et al., 2020). In this study, we report that RNA deep sequencing (RNA-seq) data of these macrophages revealed a dysregulation of lysosomal and Golgi-related genes. In agreement, we found that chemokine signaling disruption in macrophages was linked to increased lysosomal staining and enhanced clearance of a mycobacterial pathogen. Supporting the connection between Cxcr3 chemotactic signaling and lysosomal function, we found that expression of dominant-negative Tfec phenocopied the infection resistance of cxcr3.2 mutants, while their enhanced microbicidal capacity was counteracted by tfec overexpression. Finally, we assessed whether aberrant macrophage motility in cxcr3.2 mutants was linked to altered subcellular lysosome dynamics during chemotaxis. Indeed, we observed that cell polarization in mutant macrophages was incomplete, with lysosomes failing to shuttle between the leading and trailing edges of the cell. Taken together, these results link macrophage chemotaxis to intracellular vesicular trafficking, showing that disruption of the Cxcr3 axis induces lysosomal gene expression and renders macrophages more microbial against intracellular bacteria.

**RESULTS**

**Intracellular vesicle trafficking and lysosomal genes are upregulated when Cxcr3.2 chemotactic signaling is disrupted**

The zebrafish Cxcr3.2 chemokine receptor is a functional homolog of human CXCR3. In developing zebrafish larvae lacking the Cxcr3.2 receptor, we observed that the macrophages display reduced random motility compared to macrophages in wild-type (WT) larvae (Torraca et al., 2015). In addition, Cxcr3.2-deficient macrophages are impaired in directed migration to the receptor ligand (Cxc11aa) and to sites of infection and injury where the production of this chemokine is increased (Torraca et al., 2015; Sommer et al., 2020; Xie et al., 2019). To identify genes and biological pathways affected by the disruption of Cxcr3 signaling, we sorted macrophages from cxcr3.2 mutant and WT zebrafish larvae under non-infected conditions and subjected these to RNA-seq. Principal-component analysis (PCA) confirmed overall distinction between the cxcr3.2 mutant and WT transcriptomic profiles (Figure 1A). Differential expression analysis revealed that cxcr3.2 mutation led to the

(D) Graphical representation of induced genes exerting key functions in Golgi and lysosomal pathways. ER, endoplasmic reticulum; PTM, post-translational modification; TG, trans-Golgi.

(E and F) Expression fold change of representative lysosomal markers and transcriptional regulators of lysosomal functions of cxcr3.2 mutant and WT FACS macrophages, as determined by qPCR (E) or RNA-seq analysis (F). qPCR analysis confirmed that overall lysosomal function is increased in cxcr3.2 mutants as indicated by the upregulation of lysosomal function markers ctsl.1, atp6v1c1b, and slc36a1, whereas the expression of the lysosomal biogenesis regulators tfe3, and tfe6 remained unaltered. Three biological samples of 150–200 larvae were used, and three technical replicates were conducted. Data were analyzed using a two-tailed t test and results are shown as mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant [p > 0.05]).
downregulation of 490 genes and upregulation of 407 genes (Data S1) among different subcellular compartments (Figure 1B; Data S2). Classification of these genes by compartment showed that peroxisomal, lysosomal, and Golgi-related genes were most frequently upregulated (Figure 1C; Data S2), although only lysosomal- and Golgi-related terms were significantly differentially represented in Gene Ontology (GO) or Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, i.e., KEGG “lysosome,” “GO cellular components “Golgi-associated vesicle,” “Golgi apparatus,” “ER-Golgi intermediate compartment,” “lysosome,” “vacuole,” and GO biological process “Golgi vesicle transport” (Data S3). Differentially expressed genes related to lysosomal and Golgi function were also classified under different processes, including Golgi stacking, post-Golgi coating, endoplasmic reticulum (ER) to Golgi trafficking, Golgi post-translational modifications (Golgi-PTM), endosome-lysosome trafficking, trans-Golgi network (TGN) function, lysosomal biogenesis and maturation, and proton transport (Figure 1D). To confirm the upregulation of lysosomal genes, we ran a qPCR on marker genes ctsi.1 (lysosomal cysteine protease), atp6v1c1b (acidifies intracellular compartments), and slc36a1 (lysosomal amino acid transporter) and lysosomal regulators tf eb, tfe3, and tfec. All lysosomal markers showed upregulation comparable to those observed in the RNA-seq profile (Figures 1E and 1F). However, the expression of the lysosomal regulators was unaffected, indicating that the effects on lysosomal gene expression cannot be attributed to changes in the transcription of tf eb, tfe3b, or tfec. Collectively, our data suggest that disruption of the Cxcr3 axis induces a transcriptional increase in genes related to lysosomal function and intracellular vesicle trafficking, independently of expression changes in the regulators tf eb, tfe3b, and tfec.

**Disruption of chemotactic signaling increases lysosomal staining and microbicidal capacity of macrophages**

To assess whether altered expression of vesicle trafficking and lysosomal genes impacts lysosomal function, we assessed the microbicidal capacity of macrophages in cxcr3.2 mutant and WT embryos. We had previously shown that cxcr3.2 mutant zebrfish embryos had increased resistance to *Mycobacterium marinum*, a mycobacterium species widely used to model tuberculosis infection (Torraca et al., 2015; Ramakrishnan 2013; Ramakrishnan 2012). However, we did not address the competency of single macrophages in eliminating mycobacteria. Therefore, we infected cxcr3.2 mutant and WT embryos with the ΔERP mutant *M. marinum* strain. This strain lacks the ERP (exported repetitive protein) virulence factor that confers resistance to acidity and allows mycobacteria to replicate inside phagolysosomes (Cosma et al., 2006). In zebrafish, the response of macrophages toward ΔERP *M. marinum* has been shown to serve as an indicator of microbicidal efficacy because one can track the clearance of a stationary bacterial population by enumerating the number of bacteria in individual macrophages (Somm er et al., 2020; Clay et al., 2008; Takaki et al., 2013). Data show that cxcr3.2 mutants cleared ΔERP *M. marinum* infection more efficiently than did WT controls, as they developed fewer bacterial clusters per fish (Figure 2A) and these clusters consisted of lower numbers of bacteria per macrophage (Figure 2B). To assess whether enhanced clearance of bacteria in cxcr3.2 mutants was related to a higher phagolysosome and lysosome acidity, we injected pH-rodo *E. coli* bioparticles into the circulation of WT and cxcr3.2 mutant larvae. The pH-rodo *E. coli* bioparticles fluoresce at low pH values, and fluorescence intensity increases with acidity. In line with the RNA-seq data and augmented microbicidal efficacy, phagosomes of cxcr3.2 mutant macrophages were more acidic at 30–40 min post-injection (mpi) than WT (Figures 2C–2E). To assess whether upregulation of lysosomal genes affected the quantity of lysosomal vesicles within macrophages, we bath-exposed WT and cxcr3.2 mutant embryos to the intravital LysoTracker dye and quantified the fluorescently stained area within single macrophages. Lysosomal staining was more abundant in cxcr3.2 mutants than in WT (Figures 2F–2H). These in vivo experiments support that upregulation of lysosomal genes in cxcr3.2 mutants affects both the properties and the total area of lysosomal vesicles and acidic compartments, rendering mutant macrophages more microbicidal.

**Tfec inhibition phenocopies increased resistance of cxcr3.2 mutants to mycobacterial infection, while tfec overexpression counteracts enhanced bacterial clearance**

Having linked the cxcr3.2 mutant phenotype to increased lysosomal staining and enhanced bacterial clearing, we asked whether this phenotype could be evoked by manipulating one of the lysosomal regulators. We chose Tfec for this purpose because well-characterized molecular tools are available to modulate its function (Mahony et al., 2016). First, we used a dominant-negative version of Tlec (DN-tfec), which has been shown to inhibit the function of endogenous Tlec through competition for Tfec target sites, as DN-tfecn contains only the DNA-binding domain (Mahony et al., 2016). We injected mRNA encoding DN-tfecn at the one-cell stage to achieve ubiquitous Tfecn expression (CMV:tfecn). We then used the CMV promoter construct driving ubiquitous Tfecn expression (CMV:tfecn), larvae had a higher bacterial burden than did controls (Figures 3C and 3D; Figure S1B). We asked whether tfecn expression changes upon *M. marinum* infection, but qPCR analyses showed that *M. marinum* infection does not alter tfecn transcription (Figure S1C). Furthermore, we verified that Tfec inhibition or tfecn overexpression did not affect expression levels of cxcr3.2 (Figures S1D and S1E). To confirm whether tfecn directly affects lysosomal function in macrophages, we inhibited Tfec with the DN-tfecn construct in ΔERP *M. marinum*-infected larvae and observed that they developed fewer and smaller bacterial clusters than did controls (Figures 3E and 3F). We then used the CMV:tfecn construct to overexpress tfecn in cxcr3.2 mutants and the results showed that it counteracts enhanced bacterial clearance of cxcr3.2 mutants. In fact, tfecn overexpression in the cxcr3.2 mutants restored the bacterial numbers to a level comparable to WT, while non-injected cxcr3.2 mutants preserved...
their enhanced microbicidal capacity, showing a lower total number of bacterial clusters and a lower number of clusters larger than 10 bacteria in size (Figures 3G and 3H). Thus, we showed that manipulating Tfec levels alters the microbicidal capacity of macrophages. In contrast, Tfec overexpression or inhibition did not affect the ability of macrophages to migrate toward a site of injury in WT or \( cxcr3.2 \) mutant larvae (Figures S1F and S1G). Taken together, we conclude that inhibiting Tfec function phenocopies the increased resistance to \( M. marinum \) of \( cxcr3.2 \) mutants and that increasing Tfec levels counteracts the enhanced microbicidal properties of Cxcr3.2-depleted macrophages.

**Disruption of chemotactic signaling in \( cxcr3.2 \) mutant macrophages alters lysosome trafficking and prevents cell polarization during chemotaxis**

Chemokine signaling triggers the release of intracellular calcium to orchestrate highly dynamic cell membrane rearrangements that result in a polarized phenotype (Colvin et al., 2010; del Pozo et al., 1995). Lysosome exocytosis delivers layers of lipid membrane to sustain plasma membrane turnover and extension, and it mediates uropod detachment (Colvin et al., 2010; Bretou et al., 2017; Reddy et al., 2001). Therefore, as cells move, lysosomes shuttle between the cell front and rear (Constantin and Laudanna, 2010; Colvin and Luster, 2011). We used lysosomal localization during chemotaxis as an indicator of cell polarization. We stained transgenic (Tg) \( (mpeg1:mCherry-F) \) \( cxcr3.2 \) mutant and WT larvae with LysoTracker and divided the total macrophage area into halves to calculate the anterior-posterior ratio of LysoTracker staining. WT macrophages had recognizable leading and rear edges and lysosomes moved continuously from rear to the front (1.15:1) during chemotaxis (Figures 4A and 4C). In contrast, the leading edge and uropod of \( cxcr3.2 \) mutant macrophages were not well defined and lysosomes moved discontinuously from rear to the front (1.15:1) during chemotaxis (Figures 4A and 4C). In contrast, the leading edge and uropod of \( cxcr3.2 \) mutant macrophages were not well defined and lysosomes moved continuously from rear to the front (1.15:1) during chemotaxis (Figures 4A and 4C). In contrast, the leading edge and uropod of \( cxcr3.2 \) mutant macrophages were not well defined and lysosomes moved continuously from rear to the front (1.15:1) during chemotaxis (Figures 4A and 4C). In contrast, the leading edge and uropod of \( cxcr3.2 \) mutant macrophages were not well defined and lysosomes moved continuously from rear to the front (1.15:1) during chemotaxis (Figures 4A and 4C). 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accumulation of lysosomes and is tightly linked to aberrant macrophage chemotaxis.

**DISCUSSION**

Leukocyte chemotaxis is inextricably intertwined with the subcellular localization and exocytosis of lysosomes (Colvin et al., 2010; Bretou et al., 2017; Constantin and Laudanna, 2010; Balasubramani, 2017; Sumoza-Toledo et al., 2011). However, our understanding of the complex network of processes linking chemotaxis and lysosomal function is incomplete. We used the zebrafish model to study the conserved Cxcr3 signaling axis implicated in several inflammatory disorders to show that disrupting Cxcr3 signaling in zebrafish macrophages leads to transcriptional upregulation of lysosomal genes, increased lysosomal staining, enhanced bacterial clearance, altered lysosome trafficking, and aberrant motility. These results provide in vivo evidence linking lysosomal function to chemotactic signaling and led us to conclude that disrupting Cxcr3 chemotactic signaling primes macrophages for better clearance of intracellular infection.

We found a marked dysregulation of lysosomal genes in sorted macrophages of larvae lacking Cxcr3.2, the zebrafish homolog of human CXCR3. The expression of lysosomal regulators of the MITF/TFE protein family remained unaltered, in line with previous work showing that members of this protein family are regulated mostly at the posttranscriptional level (Steingrimsson et al., 2002; Yasumoto and Shibahara, 1997). The induction of lysosomal genes in cxc3.2 mutant macrophages...
was linked to increased lysosomal staining, higher phagolysosomal acidity, and enhanced clearance of mycobacteria. A previous work by Shen et al. (2016) used zebrafish to assess lysosomal clearance of apoptotic neuronal debris in RagA mutants (El-Houjeiri et al., 2019). They reported enlarged lysosomes as in cxcr3.2 depleted larvae, but low acidity and poor clearance of apoptotic debris as opposed to our observations in cxcr3.2 mutants. The RagA GTPase anchors TEBB/TFE3 to the lysosomal membrane and interacts with v-ATPases on the lysosomal membrane to acidify the lysosomal lumen (Zoncu et al., 2011; Bar-Peled et al., 2012). The absence of raga prevents Tfeb/Tfe3 anchoring and the interaction with v-ATPases, while it promotes the translocation of the transcription factors to the nucleus, arguably leading to sustained tfeb-driven induction of lysosomal genes but low intraphagosomal acidity (Shen et al., 2016; Martina and Puertollano, 2013; Dou et al., 2012; Kim et al., 2014). In contrast, the RNA-seq results of cxcr3.2 mutant macrophages showed induction of genes that could be responsible for highly acidic phagolysosomes, such as the transmembrane amino acid carrier gene slc361 and the lysosomal v-ATPase subunit c gene atp6v1c1b, a direct downstream target of Tfeb (Sardiello et al., 2009). The upregulation of ctsl.1 (Cathepsin L.1), involved in catabolic processes and the immune response,
could also be linked to enhanced clearance of bacteria in cxcr3.2 mutant macrophages.

We studied the connection between Cxcr3 chemotactic signaling and lysosomal function by modulating the activity of the lysosomal regulator Tfec and found that blocking Tfec function in WT larvae had a similar host-protective effect as the cxcr3.2 mutation. Moreover, tfec overexpression reverted the protective effect of the cxcr3.2 mutation and resulted in poor control of bacterial dissemination in WT larvae. It has been shown that lysosome signaling, which involves transcriptional regulators and Ca\textsuperscript{2+} channels, can be triggered by bacterial phagocytosis or macropinocytosis and drives the migration of immune cells besides controlling many other aspects of their function (Bretou et al., 2017; Spix et al., 2020). In our experimental setup, genetic modulations of Tfec activity determined microbicidal activity, but they did not alter wound-induced macrophage migration, indicating that lysosomal activity and the ability to respond to chemotactic cues are not inseparable properties. Nevertheless, these properties could be reciprocally linked. Macrophage migration in cxcr3.2 mutants might be impaired due to lysosome alterations larger than those inflicted by Tfec alone. Alternatively, increased lysosomal gene expression in cxcr3.2 mutants could be a result of the motility defect, which is observed even under non-infected conditions due to the production of the Cxcr3.2 ligand (Cxc11aa) at basal levels as well as at sites of injury or infection (Rougeot et al., 2019; Xie et al., 2019). It has been shown that one of the mammalian TFEC isoforms can strongly inhibit TFE3-mediated gene transactivation (Palmieri et al., 2011; Pastore et al., 2017, 2019). In agreement, we posit that zebrafish Tfec antagonizes the Tfe3-driven transactivation of lysosomal and pro-inflammatory genes and, therefore, inhibiting Tfec function leads to enhanced lysosomal function and pathogen resistance. Altogether, our results support that the highly microbicidal phenotype of cxcr3.2 mutant macrophages is associated with deregulations in lysosomal function.

Our previous work suggests that the increased microbicidal capacity of cxcr3.2 mutant macrophages is not the only factor responsible for the infection-resistant phenotype. The macrophage motility defect also contributes, as tissue dissemination of mycobacteria in zebrafish larvae depends on Cxcr3.2-dependent macrophage migration (Torraca et al., 2015; Sommer et al., 2020). The motility defect is the likely cause of the aberrant accumulation pattern of lysosomes during cell migration, which may elicit a transcriptional stress response through the lysosomal regulators, resulting in an increased size of the lysosomal compartment and altered lysosomal properties benefitting host defense. The opposite situation is observed in zebrafish models of lysosomal storage disorders, where the primary defect lies in the deficiency of hydrolytic enzymes, due to which undigested lysosomal material accumulates and disrupts vesicle trafficking and cell migration to the extent that mycobacterial infection cannot be controlled (Berg et al., 2016; Meijer and Aerts, 2016). This is a tight balance, as the outcome of mycobacterial infection is affected positively when macrophage migration is reduced to a limited extent but negatively when macrophage migration is severely impaired (Berg et al., 2016; Meijer and Aerts, 2016; Pagán et al., 2015; Volkman et al., 2010).

By time-lapse imaging we showed that Cxcr3.2-depleted macrophages are not properly polarized during chemotaxis and that lysosomes accumulate in the leading edge of the cell and rarely reach the uropod. The disruption of chemokine signaling axes CXCRI/CXCL12 and CCR2/CCL2 resulted in reduced T cell migration when synaptotagmin SYT7 and the related protein SYTLS were downregulated (Colvin et al., 2010). Taking these observations as a precedent, the disruption of the Cxcr3 axis might affect intracellular levels and distribution of intracellular chemokine receptor-induced Ca\textsuperscript{2+}, leading to ER stress and lysosome accumulation due to calcineurin-independent Tfeb translocation to the nucleus (Brady et al., 2018a, 2018b). Moreover, vesicle trafficking and lysosome exocytosis might be compromised at low intracellular concentrations, further contributing to the accumulation of lysosomes in cxcr3.2 mutant macrophages and their aberrant motility.

In conclusion, our results in the zebrafish tuberculosis model support that disruption of Cxcr3 chemokine signaling affects intracellular vesicle trafficking in macrophages, preventing them from acquiring a polarized phenotype and migrating toward inflammatory foci while rendering them more microbicidal. It remains to be studied whether altered lysosome function also impacts other leukocytes using the Cxcr3 axis. Especially T cells and neutrophils are of interest in this respect, because altered behavior of these cells has been associated with better control of mycobacterial infection in cxcr3 mutant mice (Chakravarty et al., 2007; Seiler et al., 2003). Our work contributes to further our understanding of chemotaxis as a complex process that incorporates various physiological processes and integrates different extracellular cues. It emphasizes the importance of vesicle trafficking during chemotaxis and transcriptional and posttranscriptional regulation of lysosome function in immunity. Intravital imaging of zebrafish enabled us to show that there is a direct link between chemokine signaling and lysosomal function that enhances the microbicidal properties and primes macrophages for a better intracellular defense.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Ethics statement
  - Zebrafish lines
  - Zebrafish embryo and larva handling
- **METHOD DETAILS**
  - FACS, RNA extraction, and cDNA synthesis
  - RNA-Seq analysis
  - Quantitative PCR analysis
  - Assessment of microbicidal capacity
  - Acidification assessment using pHrodo
  - LysoTracker staining of acidic compartments
Systemic infection with Mycobacterium marinum
- tfec overexpression and Tfec inhibition
- Lysosome localization within macrophages

QUANTIFICATION AND STATISTICAL ANALYSIS
- Quantitative PCR analysis
- RNA-Seq data analysis
- Assessment of microbialic capacity
- Acidification assessment using pH-rodo
- LysoTracker staining of acidic compartments
- Systemic infection with M. marinum
- Lysosome localization within macrophages

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.109000.

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AUTHOR CONTRIBUTIONS

F.S. designed and performed experiments, analyzed the data, and wrote the manuscript. V.T. designed and performed experiments and analyzed data. A.E.T.V. and Y.X. contributed to the experimental work. J.W. wrote the script approved the final version. All authors commented on the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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DAVID Gene Functional Classification Tool: A novel biological module-centric algorithm to functionally analyze large gene lists. Genome Biol. 8, R183.


STAR METHODS

KEY RESOURCES TABLE

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial and virus strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycobacterium marinum M-strain: mCherry</td>
<td>van der Sar et al., 2004</td>
<td>N/A</td>
</tr>
<tr>
<td>Mycobacterium marinum ΔERP: mWasabi</td>
<td>Takaki et al., 2013</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Critical commercial assays</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRNAsasy mini kit</td>
<td>QIAGEN</td>
<td>217004</td>
</tr>
<tr>
<td>SMARTer Universal Low Input RNA Kit for Sequencing</td>
<td>Clontech</td>
<td>634938</td>
</tr>
<tr>
<td>iTaq Universal SYBR Green Supermix</td>
<td>BioRad</td>
<td>1725120</td>
</tr>
<tr>
<td>LysoTracker Green DND-26</td>
<td>Thermo Fisher Scientific</td>
<td>L7526</td>
</tr>
<tr>
<td>pHrodo E. coli BioParticles conjugate for phagocytosis</td>
<td>Invitrogen</td>
<td>P35361</td>
</tr>
<tr>
<td><strong>Deposited data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw and analyzed RNA sequencing data</td>
<td>This paper</td>
<td>GEO: GSE149942; Data S1, S2, and S3</td>
</tr>
<tr>
<td><strong>Experimental models: Organisms/strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zebrafish: AB/TL</td>
<td>ZIRC Zebrafish International Resource Center</td>
<td>ZL1/ZL86</td>
</tr>
<tr>
<td>Zebrafish: Tg(cxcr3.2−/− mpeg1: mCherry-F)</td>
<td>Torraca et al., 2015; Bernut et al., 2014</td>
<td>ZFIN: u6044/ ump2</td>
</tr>
<tr>
<td>Zebrafish: Tg(cxcr3.2+/+ mpeg1: mCherry-F)</td>
<td>Torraca et al., 2015; Bernut et al., 2014</td>
<td>ZFIN: u6044/ ump2</td>
</tr>
<tr>
<td><strong>Oligonucleotides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>qPCR primers</td>
<td>This paper</td>
<td>Table S1</td>
</tr>
<tr>
<td>Recombinant DNA</td>
<td>Mahony et al., 2016</td>
<td>N/A</td>
</tr>
<tr>
<td>pcDNA3.1/V5-His-CMV-tfec</td>
<td>Thermo Fisher Scientific (This paper)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Software and algorithms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Java script for “Lysosomal distribution”</td>
<td>This paper</td>
<td><a href="https://sites.imagej.net/Willemsejj/">https://sites.imagej.net/Willemsejj/</a></td>
</tr>
<tr>
<td>gprofiler</td>
<td>Reimand et al., 2016</td>
<td><a href="https://biit.cs.ut.ee/gprofiler">https://biit.cs.ut.ee/gprofiler</a></td>
</tr>
<tr>
<td>DAVID bioinformatics tools</td>
<td>Huang et al., 2007a, 2007b; Sherman et al., 2007</td>
<td><a href="https://david.ncifcrf.gov">https://david.ncifcrf.gov</a></td>
</tr>
<tr>
<td>PANTHER</td>
<td>Mi et al., 2010</td>
<td><a href="http://geneontology.org">http://geneontology.org</a>; <a href="http://www.pantherdb.org/">http://www.pantherdb.org/</a></td>
</tr>
</tbody>
</table>

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Prof. dr. Annemarie H. Meijer (a.h.meijer@biology.leidenuniv.nl).

Materials availability
Plasmids used can be provided by the lead contact.
**Data and code availability**

Newly generated RNaseq data (Data S1, S2, and S3) are available at the Gene Expression Omnibus database under accession number GSE149942. The Java script for the “Lysosomal distribution” Fiji/ ImageJ plugin can be used following the link: https://sites.imagej.net/Willemsejj/.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Ethics statement**

Zebrafish were handled in compliance with guidelines from the Zebrafish Model Organism Database (http://zfin.org), the EU Animal Protection Directive 2010/63/EU, and the directives of the local animal welfare committee of Leiden University (License number: 10612). All experiments were performed on larval stages before the free feeding stage, which do fall under animal experimentation under EU legislation.

**Zebrafish lines**

The wt fish line used in this study is AB/TL. The homozygous mutant (cxcr3.2/--/) and homozygous wild-type (wt) siblings (cxcr3.2+/+) of the cxcr3.2^{hu6044} allele were crossed into the Tg(mpeg1:mCherry-F)ump2 background to visualize macrophages (Bernut et al., 2014; Torraca et al., 2015).

**Zebrafish embryo and larva handling**

Zebrafish larvae and eggs were stored at 28.5°C in egg water (60 µg/ml Instant Ocean sea salts and 0.0025% methylene blue) and anesthetized with 0.02% buffered tricaine, (3-aminobenzoic acid ethyl ester; Sigma Aldrich, St. Louis, MO, USA) before infections and imaging. Larvae were kept in E2 medium (15 mM NaCl; 0.5 mM KCl, 1.0 mM MgSO4, 150 μM KH2PO4, 50 μM Na2HPO4, 1mM CaCl2; 0.7 mM NaHCO3) for a minimum of 6h prior and during experimental procedures involving pH-rodo and LysoTracker. For confocal imaging, larvae were kept in egg water containing 0.003% PTU (1-phenyl-2-thiourea, Sigma Aldrich) to prevent pigmentation.

**METHOD DETAILS**

**FACS, RNA extraction, and cDNA synthesis**

For RNaseq experiments, three biological samples of 150-200 6dpf Tg (mpeg1:mCherry-F cxcr3.2/--/ and cxcr3.2+/+) larvae were dissociated for FACS following the procedure described in Rougeot et al. (2014). For qPCR analysis on sorted cells, three biological samples of 100-200 Tg (mpeg1:mCherry-F cxcr3.2/--/ and cxcr3.2+/+) 5dpf larvae were used. For both procedures, RNA was extracted using the miRNeasy mini kit (QIAGEN) according to the manufacturers' instructions. For RNaseq, the synthesis of cDNA was done using the SMARTer Universal Low Input RNA Kit for Sequencing (Clontech) following the manufacturer’s guidelines. For qPCR analysis, cDNA was generated using the iScript cDNA Synthesis Kit (Bio-Rad).

**RNA-Seq analysis**

Illumina RNaseq, mapping and counting of reads was performed as described previously (Rougeot et al., 2019). Analysis of mapped reads was done with the DESeq2 bioinformatics package (https://bioconductor.org/packages/release/bioc/html/DESeq2.html) (Love et al., 2014). Before data processing, lowly expressed genes (< 50 total reads) were filtered. Genes with a p.adj < 0.05 and|log2(fold change)| > 0.5 cut off were selected for gene ontology analyses (Data S1 and S2). Correspondence between human and zebrafish orthologs was determined through g:profiler (https://biit.cs.ut.ee/gprofiler) and manually curated (Reimand et al., 2016). The significantly affected KEGG pathways were determined by submitting the predicted human orthologs of the significantly regulated zebrafish genes to DAVID bioinformatics tools (https://david.ncifcrf.gov) (Huang et al., 2007a,b; Sherman et al., 2007) (Data S3). The significantly affected Gene Ontology (GO) terms were determined by submitting the predicted human orthologs of the significantly regulated zebrafish genes to PANTHER (Mi et al., 2010). Raw data are deposited in the Gene Expression Omnibus database under accession number GSE149942.

**Quantitative PCR analysis**

For qPCR analyses on cxcr3.2 expression, three batches of 10 ABT/TL larvae injected with DN-tfec, CMV:tfec or PBS each, were collected in QIAzol lysis reagent (QIAGEN). Similarly, 3 batches of infected and non-infected AB/TL larvae were collected to assess tfec induction upon infection. Reactions were run on a Myq Single-Color Real-Time PCR Detection System (Bio-Rad) using iTaq Universal SYBR Green Supermix (Bio-Rad). Three technical replicates were done for every biological sample. The cycling conditions were: 3 min pre-denaturation at 95°C, 40 denaturation cycles for 15 s at 95°C, annealing for 30 s at 60°C (for all primers), and elongation for 30 s at 72°C. We used the housekeeping gene ppiab (peptidylprolyl isomerase Ab) for whole larvae, and eif5 for sorted macrophages. Primer sequences can be found in Table S1.
Assessment of microbicidal capacity

To determine the microbicidal capacity of zebrafish larval macrophages, embryos were infected with 200 CFU of the attenuated strain, ΔERP-M. marinum-mWasabi (Cosma et al., 2006). Larvae were infected in the blood island (BI) with 1 nL of a ΔERP-M. marinum-mWasabi single-use glycerol stock and microinjected at 28 hpf as previously described (Sommer et al., 2020). Infected larvae were fixed with 4% paraformaldehyde (PFA) at 44 hpi, mounted in 1.5% low-melting-point agarose (SphaeroQ, Burgos, Spain) and bacterial clusters were quantified under a Zeiss Observer D16.32 laser scanning confocal microscope (Carl Zeiss, Sliedrecht, the Netherlands) using a Capochromat 63x/1.20 W Corr UV-VIR-IR objective (Carl Zeiss, Sliedrecht, the Netherlands).

Acidification assessment using pHrodo

cxcr3.2 mutant and wt larvae were injected with 1 nL of E. coli pHrodo E. coli BioParticles conjugate for phagocytosis (Invitrogen) at 28-37 hpf into the blood island and imaged over the circulation valley at 30-45 minutes post-injection (mpi). In all cases, the same area was imaged by mounting anesthetized larvae in 1.5% low-melting-point agarose and imaged with Plan-Neofluar 40x/0.9 Imm corr objective on a Zeiss Observer D16.32 laser-scanning confocal microscope (Carl Zeiss, Sliedrecht, the Netherlands).

LysoTracker staining of acidic compartments

2-day-old cxcr3.2 mutant and wt larvae were incubated for 1-2 h with 10 μM LysoTracker Green DND-26 (Invitrogen) in E2 medium. Larvae were anesthetized following the staining and rinsed 3 times for 5 min each with E2 medium and tricaine. Images of live macrophages were acquired with a Plan-Neofluar 40x/0.9 Imm corr objective on a Zeiss Observer D16.32 laser-scanning confocal microscope (Carl Zeiss, Sliedrecht, the Netherlands).

Systemic infection with Mycobacterium marinum

M. marinum M-strain expressing the fluorescent marker mCherry was grown and prepared freshly for injection as described in Takaki et al. (2013). Embryos were systemically infected with 300 CFU of M. marinum-mCherry (van der Sar et al., 2004) by microinjection into the blood island at 28 hpf. Bacterial burden was quantified based on fluorescence, a well-established approach for assessing mycobacterial infection in zebrafish larvae with advantages over colony forming units determination (Takaki et al., 2013; Adams et al., 2011; Stoop et al., 2011; Stirling et al., 2020). Infected larvae were imaged under a Leica M165C stereo-fluorescence microscope at 4 days post-infection, and the bacterial burden was determined using a dedicated pixel counting program (Stoop et al., 2011).

tfec overexpression and Tfec inhibition

An expression construct pcDNA3.1/V5-His TOPO-CMV/tfec (Mahony et al., 2016) was injected into the yolk at 0 hpf to overexpress the gene in wt and cxcr3.2 mutant larvae. The CMV promoter is this construct drives ubiquitous expression of transgenes in zebrafish, including expression in uninfected or infected macrophages, relevant to this work (van der Vaart et al., 2014; Zhang et al., 2020; Massud et al., 2019). Overexpression levels were verified by qPCR analysis. Tfec function was inhibited by injecting mRNA encoding DN-tfec in wt larvae at 0 hpf to achieve ubiquitous expression in developing embryos. DN-tfec mRNA was transcribed from a pCS2+ vector using the SP6 mMachine kit (Ambion). The inhibition of Tfec function by DN-tfec was verified through qPCR on kitlgb, a downstream target of Tfec (Mahony et al., 2016).

Lysosome localization within macrophages

Time-lapse images of LysoTracker stained macrophages of 3-day-old cxcr3.2 mutant and wt larvae (5 larvae per genotype) were acquired 1 after tail-amputation every 30 s for 1 h. Larvae were mounted in 1.5% low-melting-point agarose and microscopy was done using a Leica TCS SP8 MP confocal microscope (Leica Microsystems). Data were analyzed using a Fiji/ImageJ homemade plugin “Lyosomal distribution” (https://sites.imagej.net/Willemsejj/). The plugin divides the total area of single macrophages in half and quantifies the proportion of LysoTracker staining in each part of the cell in every time-frame.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantitative PCR analysis

The data were analyzed with the 2 – ΔΔCt method. Results are shown as mean ± SEM (ns p > 0.05, * p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001). A One-way ANOVA was used to test for significance of the sorted macrophages data and results are plotted as mean ± SEM (ns p > 0.05, * p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001). For cxcr3.2 expression and tfec induction on whole larvae, we used a two-tailed t test and plotted the results as mean ± SEM (ns p > 0.05, * p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001).

RNA-Seq data analysis

Gene enrichment analysis criteria were Fisher Exact test or False Discovery Rare (FDR) < 0.05 (for DAVID or PANTHER respectively), number of affected genes ≥ 10, fold enrichment ≥ 1.5. The complete data analysis can be found in Data S1, S2, and S3.
Assessment of microbicidal capacity
We used a Mann-Whitney test to analyze the overall bacterial burden of the pooled data of 2 independent replicates of 12-15 fish each, where data are shown as mean ± SEM. A Kolmogorov-Smirnov test was used to analyze the distribution of bacterial cluster sizes (ns p > 0.05, * p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001).

Acidification assessment using pH-rodo
Fluorescence intensity was assessed using FIJI/ ImageJ quantification tools and data were analyzed using a two-tailed t test. Results are shown as mean ± SEM (ns p > 0.05, * p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001). Results are expressed as % relative to the wt control (100%).

LysoTracker staining of acidic compartments
To quantify LysoTracker staining within macrophages, the mean intensity of LysoTracker overlapping with mpeg1:mCherry-F signal was measured using FIJI/ImageJ quantification tools. Data were analyzed using a two-tailed t test. Results are shown as mean ± SEM (ns p > 0.05, * p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001). Results are expressed as % relative to the wt control (100%).

Systemic infection with M. marinum
Data were analyzed using a two-tailed t test. Results are shown as mean ± SEM (ns p > 0.05, * p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001) and combined data of 3 independent replicates of 20-30 larvae each.

Lysosome localization within macrophages
The images with labeled macrophages were used to identify the macrophages within the 3D-stack. To reduce background signal for the segmentation the images were blurred using a Gaussian blur with a size of 3 by 3 pixels. Subsequently they were converted to binary images using the Li Thresholding method (Li and Lee, 1993), using a minimum size of 15 pixels. The regions of interest (ROI) obtained over the entire 3D stack were used to analyze the original data, the Gaussian blurred image were only used for segmenting. Each ROI was subsequently split into a top part, and a bottom part by fitting an ellipse over the entire ROI and then splitting it over the short axis. For each ROI in the z stack the intensity at that stack position was measured both in the original macrophage image, as well as in the LysoTracker labeled image. Additionally, the ratio of these intensities was calculated. By analyzing the ROIs separately for each stack position, we made sure there is no overlapping information from cells above or below the cell of interest interfering with our analysis. Finally, the data were organized by cell and by fish and analyzed with a two-tailed t test and a Mann-Whitney test, respectively. Data are shown as mean ± SEM (ns p > 0.05, * p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001).