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Q4 Review

3 Zebrafish Models to Study Inflammasome-
4 Mediated Regulation of Hematopoiesis

O6 Q5 Lola Rodríguez-Ruiz,¹ Juan M. Lozano-Gil,¹ Christophe Lachaud,² Pablo Mesa del Castillo,³
6 María L. Cayuela,³ Diana García-Moreno,^{1,*} Ana B. Pérez-Oliva,^{1,*} and Victoriano Mulero ^{1,*}

7 **Hematopoiesis is a complex process through which immature bone marrow**
8 **precursor cells mature into all types of blood cells. Although the association of**
9 **hematopoietic lineage bias (including anemia and neutrophilia) with chronic**
10 **inflammatory diseases has long been appreciated, the causes involved are**
11 **obscure. Recently, cytosolic multiprotein inflammasome complexes were shown**
12 **to activate inflammatory and immune responses, and directly regulate hemato-**
13 **poiesis in zebrafish models; this was deemed to occur via cleavage and inactiva-**
14 **tion of the master erythroid transcription factor GATA1. Herein summarized are**
15 **the zebrafish models that are currently available to study this unappreciated role**
16 **of inflammasome-mediated regulation of hematopoiesis. Novel putative thera-**
17 **peutic strategies, for the treatment of hematopoietic alterations associated with**
18 **chronic inflammatory diseases in humans, are also proposed.**

20 **Hematopoietic Lineage Bias in Chronic Inflammation**

21 Hematopoiesis is the complete process of blood cell formation occurring during embryonic develop-
22 ment and adulthood. In vertebrates, this process involves two phases. First, during embryonic develop-
23 ment, the **primitive hematopoietic wave** (see [Glossary](#)) is transitory, and the erythroid and
24 **myeloid** progenitor cells lack **pluripotency** [1]. In the second phase, the **definitive hematopoietic**
25 **wave** [2,3], which occurs at later stages of development, hematopoietic stem cells (HSCs) emerge
26 and give rise to all types of blood cells in adult organisms [4]. HSC maturation involves the differenti-
27 ation of lymphoid [T, B, and natural killer (NK) cells], myeloid (granulocytes and macrophages), and
28 erythroid (megakaryocytes, erythrocytes) cell lineages [5–7]. Several signaling pathways orchestrate
29 this complex process, by means of different transcription factors regulating the maturation, prolifera-
30 tion, and differentiation of hematopoietic cells. The transcription factors **GATA1** and **SP11**
31 (also known as PU.1), are essential and present a cross-inhibitory relationship [8,9] ([Figure 1](#)):
32 GATA1 regulates erythroid differentiation [10], and SP11 regulates myeloid differentiation [11]. How-
33 ever, there are many controversies regarding the factors that may be responsible for terminal erythroid
Q7 and myeloid differentiation, and the involvement of the **HSC niche** in these processes [12], given that
35 many unknown pathways seem to play a role in this complex biological process [10,13]. For example,
36 while GATA1 in hematopoietic precursors directs commitment to the erythroid–megakaryocyte line-
37 age [14], the overexpression of GATA1 in erythroid cells inhibits their terminal differentiation in mouse
38 [15]. In addition, GATA1 expression and activity increase in the initial stage of erythroid differentiation,
39 but they both decrease in later stages to allow terminal erythroid differentiation in mouse and human
40 [16]. All these complex pathways might have important clinical implications, since hematopoietic lin-
41 eage bias, mainly **neutrophilia** and anemia, are associated with an increased incidence of disease,
42 at least for 30 pathologies with prominent inflammatory components, including infection, rheumatoid
43 arthritis, atherosclerosis, inflammatory bowel diseases, kidney and pulmonary diseases, autoimmune
44 conditions, neurodegenerative diseases, and cancer [17]. In this review, the general structure and
45 function of mammalian inflammasomes, and the specificities of macrophage and neutrophil
46 inflammasomes (which are the best characterized), are summarized. Then follows a discussion on

Highlights

The activation of the Nlrp1a inflammasome in mouse hematopoietic stem and progenitor cells (HSPCs) triggers their pyroptotic death and prolonged leukopenia upon chemotherapy or viral infection.

The NLRP3 inflammasome drives HSPC clonal expansion and pyroptosis in myelodysplastic syndrome patients.

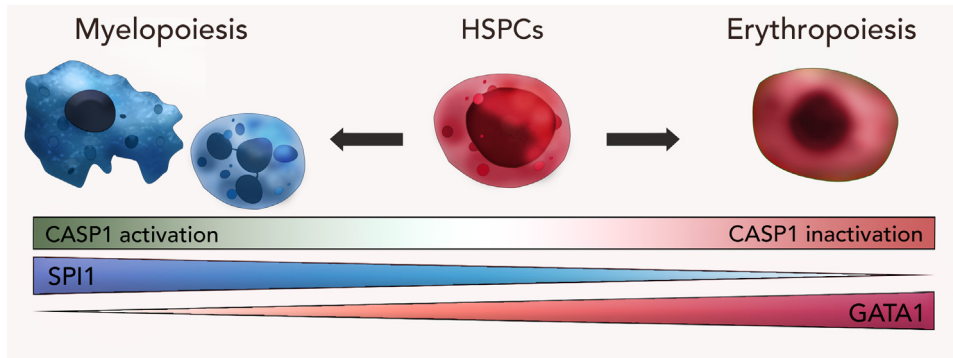
Emerging evidence from zebrafish models suggests that the canonical inflammasome autonomously regulates the erythroid–myeloid cell fate decision of HSPCs, and terminal erythroid differentiation through the cleavage of Gata1. This mechanism appears to be evolutionarily conserved since pharmacological inhibition of caspase 1 (Casp1) in mouse hematopoietic stem cells (HSCs) results in a rapid increase in Gata1 protein, leading to erythroid differentiation.

Pharmacological inhibition of the canonical inflammasome rescues zebrafish disease models of neutrophilic inflammation, anemia associated with chronic diseases, and Diamond–Blackfan anemia.

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Q1 Figure 1. Regulation of HSPC Lineage Decision Choice by the Canonical Inflammasome in Zebrafish and Mouse. Activation of the inflammasome in HSPCs results in the cleavage and inactivation of GATA1, promoting increased amounts of SPI1 that promotes myeloid differentiation. Conversely, inhibition of the inflammasome in HSPCs results in an increased GATA1 amount that promotes erythroid differentiation [30]. Abbreviation: CASP1, caspase 1; HSPCs, hematopoietic stem and progenitor cells.

47 the main advantages of using the zebrafish to model inflammation and inflammasomes, describing
 48 how this model has highlighted the importance of inflammasomes in hematopoiesis. Indeed, this
 49 model may help explain the hematopoietic lineage bias that has been associated with chronic inflam-
 50 mation in humans, revealing novel candidate therapeutic targets for future investigation in some of
 51 these diseases. (See Box 1.)

52 Inflammasomes

53 **Inflammasomes** act as sensors of the innate immune system in vertebrates, and promote in-
 54 flammation in response to various stimuli, including infection, tissue damage, or metabolic dys-
 55 regulation. They are multiprotein complexes formed by a receptor, the adaptor protein
 56 **apoptosis-associated speck-like protein containing a CARD (ASC)**, and an effector that
 57 interacts through homotypic interactions [18]. Recently, the **guanylate-binding protein (GBP)**
 58 family was found to also form a part of these multiprotein complexes in humans, mice, and
 59 zebrafish [19–22]. Inflammasomes act by activating inflammatory **caspsases**, namely **caspase**
 60 **1 (CASP1) (canonical inflammasomes)**, and CASP4, CASP5 (human), and Casp11 (mouse)
 61 **(noncanonical inflammasomes)** [18].

62 Inflammasome sensors are grouped, depending on their structure, into nucleotide-binding do-
 63 main **(NOD)–like receptors (NLRs)**, absent in melanoma 2 **(AIM2)–like receptors (ALRs)**,
 64 or **pyrin** [18]. When a specific stimulus is detected, the sensor interacts with and promotes the
 65 oligomerization of ASC, which forms **specks** and is able to recruit and activate inflammatory

b0.2 Box 1. Anemia-Associated with FMF and CAPS

b1.3 Familiar Mediterranean Fever (FMF) and cryopyrin-associated periodic syndrome (CAPS) are two inflammatory diseases
 b1.4 driven by germ-line mutations in genes encoding inflammasome components. FMF is the most common monogenic
 b1.5 autoinflammatory disease worldwide, affecting an estimated 150 000 patients [88]. More than 60% of FMF patients
 b1.6 develop anemia that is associated with iron status rather than inflammation, assayed as plasma IL-6 concentrations
 b1.7 [89]. Similarly, iron-resistant hypochromic anemia has also been associated with CAPS [90]. It is tempting to speculate that
 b1.8 overactivation of the inflammasome in HSPCs could directly promote anemia by enforcing erythropoiesis, explaining the
 b1.9 usual iron-refractory nature of these diseases, but this remains to be robustly investigated. Furthermore, released Asc
 b1.10 specks are able to propagate inflammation in mouse models of CAPS and are found in the serum of patients with CAPS,
 b1.11 but not with FMF [60,61]. Therefore, macrophage-derived ASC specks may be taken up by HSPCs, activating
 b1.12 their inflammasome and further enhancing myelopoiesis at the expense of erythropoiesis, which might explain the iron-
 b1.13 resistance anemia-associated with CAPS (see Clinician's Corner and Outstanding Questions) (Figure 2).

66 caspases. For a long time, CASP1 autoproteolysis was deemed to be triggered by the proximity
67 of its active subunits p20 and p10 [23]; however, this view was recently challenged in an elegant
68 study [24]. The authors demonstrated in mouse models that the dominant species of active
69 CASP1 dimers, were in fact full-length p46 dimers and a transient species, p33/p10. In addition,
70 p33/p10 autoprocessing occurred with kinetics specified by the inflammasome size and the cell
71 type, resulting in the release of p20/p10 species, which became unstable, and protease activity
72 was terminated [24]. Therefore, the inflammasome also incorporates an intrinsic self-limiting
73 mechanism that ensures timely CASP1 deactivation [24].

74 Caspase dimerization and auto-proteolytic maturation lead to the processing and secretion
75 of the proinflammatory cytokines interleukin-1 β (IL-1 β) and IL-18 [25], and the induction of
76 a form of programmed cell death called **pyroptosis** [18]. Pyroptosis leads to the loss of
77 membrane integrity, which in turn, mediates the release of intracellular contents into the
78 extracellular fluid [26]. Both IL-1 β /18 release and pyroptosis are mediated by **Gasdermin D**
79 (**GSDMD**) [27,28].

80 In addition to its role in infection and inflammation, inflammasomes are also important in maintaining
81 basic cellular functions, such as autophagy, metabolism, cellular proliferation, gene transcription,
82 and tumorigenesis [29]. Recent findings have also indicated that inflammasomes play an essential
83 role in the regulation of hematopoiesis in zebrafish, mouse, and human [30,31,32–35]. This is
84 relevant, as uncontrolled inflammasome activation has led to certain human autoimmune and
85 metabolic disorders, underscoring the importance of gaining a better understanding of this
86 process in physiological and pathological contexts [30].

87 Cell Type-Specific Inflammasomes

88 Neutrophils and monocytes/macrophages belong to the myeloid lineage originating from a com-
89 mon granulocyte–monocyte progenitor (GMP), which arises from the differentiation of HSCs [7].
90 Neutrophils are the most abundant circulating leukocytes (50–70%) in most mammals, released
91 from the bone marrow (BM) upon CXCL8, CXCL1, and CXCL2 chemokine signaling [36]. In a
92 similar way, monocytes are released from the BM upon CCL2 and CCL7 signaling, via CCR2
93 [37]. Subsequently, they are extravasated by signals emanating from the **activated endothelium**
94 close to inflamed tissues [38–40]. The production of neutrophils increases during infection or
95 inflammation in a process called ‘danger mobilization’ or ‘emergency hematopoiesis’, mainly
96 triggered by the hematopoietic cytokine **granulocyte colony-stimulating factor (G-CSF)** [41].

97 Neutrophil and macrophage inflammasomes sense infection and sterile inflammation, through
98 canonical and noncanonical pathways [39,42,43]. Both cell types express primarily a wide range
99 of NLRs and ALRs, and are the first cells to participate in the inflammatory process [44] (Table 1).
100 Nonetheless, there are differences between inflammasomes in these cells, enabling the complex
101 and specific regulation of cellular processes. Although they show similar amounts of NOD-, LRR-,
102 and pyrin domain-containing protein 3 (NLRP3) and NOD-, LRR-, and CARD domain-containing
103 protein 4 (NLRC4), the expression of NLRP12 is higher in mouse neutrophils than in macrophages
104 [45]. The activation of NLRC4 in human macrophages occurs through specific Gram-negative
105 bacteria components [46,47]. Pyroptosis is induced in macrophages by ASC-independent
106 NLRC4 signaling, or ASC-dependent NLRP3 and AIM2 inflammasome activation, facilitating the
107 elimination of intracellular pathogens [48]. Even though NLRC4 or AIM2 signaling has also been
108 observed in human and mouse neutrophils, they do not seem to induce pyroptosis [45]. Thus, neu-
109 trophils are quite resistant to pyroptosis and intracellular infection by bacteria [45]. Accordingly, they
110 also engage in sustained release of IL-1 β , thus amplifying neutrophil recruitment and activation [45].
111 Furthermore, when human neutrophil inflammasomes are activated by canonical agonists, IL-1 β ,

Glossary

Activated endothelium:

proinflammatory and procoagulant state of endothelial cells lining the lumen of blood vessels allowing extravasation of leukocytes.

AIM2-like receptors (ALRs):

intracellular inflammasome receptors detecting the presence of DNA in cytosol.

Apoptosis-associated speck-like protein containing a CARD (ASC):

key adaptor protein required to activate the inflammasome.

BM hypoplasia: type of hypoplastic anemia.

Canonical inflammasome: effector is CASP1 (mammals) or Caspa (zebrafish).

Caspase a (Caspa): functional homolog of CASP1 in zebrafish.

Caspase 1 (CASP1): inflammatory caspase involved in processing IL-1 β , IL-18, and GSDMD.

Caspases: family of cysteine proteases that cleave after aspartate residues (Asp).

Chronic obstructive pulmonary disease (COPD): type of obstructive lung disease characterized by long-term breathing problems and poor airflow.

Complement activation: the initiation of a series of reactions involving the complement proteins of plasma that may result in either the elimination of the pathogenic organism or amplification of the complement cascade effect.

Cryopyrin-associated periodic syndrome (CAPS): group of rare autoinflammatory diseases characterized by IL-1 β -mediated systemic inflammation; caused by gain-of-function mutations in the *NLRP3* gene.

CRISPR-Cas technology: a genome editing technique used to modify the genomes of living organisms.

Definitive hematopoietic wave: the latter of two waves of hematopoiesis where HSPCs are generated.

Diamond-Blackfan anemia:

congenital anemia caused by inefficient translation of GATA1.

Familial Mediterranean fever (FMF):

autoinflammatory disease caused by mutations in the *MEFV* gene, encoding pyrin.

Gasdermin D (GSDMD): substrate of inflammatory caspases; mediates pyroptosis.

GATA1: transcription factor regulating the expression of genes involved in erythropoiesis and thrombopoiesis.

112 but not IL-1 α and IL-18, is released, indicating that IL-1 β and IL-18 protein release is differentially
113 regulated in human neutrophils [42].

114 The NLRP3 inflammasome is involved in inflammatory and metabolic disorders as well as host
115 defense [44]. Although, it is also present in neutrophils, it acts differently. NLRP3 signaling in neu-
116 trophils is only activated through soluble agonists, but it can be triggered by soluble and particu-
117 late, or crystalline agonists in mouse macrophages [49]. In addition, NLRP3 activation leads to
118 different cellular outcomes in macrophages and neutrophils. Both NLRP3/CASP1-dependent
119 and NLRP3/CASP8-dependent inflammasomes lead to pyroptosis and apoptotic cell death in
120 mouse macrophages, respectively, but not in neutrophils [50–52].

121 Regarding **hematopoietic stem and progenitor cells (HSPCs)**, the expression/activation of
122 inflammasomes has received little attention. Nevertheless, the genes encoding the main compo-
123 nents of the NLRP3 and NLRP1 inflammasome, including NLRP3, ASC, and CASP1, are
124 expressed in human and mouse HSPCs [31,34]. Similarly, nascent HSPCs in zebrafish embryos
125 have been reported to express Asc [53]. Therefore, each cell type shows different inflammasome
126 components, activation mechanisms, and effector functions.

127 **Inflammasomes in Diseases**

128 Numerous data from mouse models, supported by human clinical data, strongly support the
129 role of the inflammasome in the initiation and/or progression of diseases, with a strong impact
130 on public health; these include certain metabolic disorders and neurodegenerative diseases
131 [54]. However, the strongest evidence supporting a key role for inflammasomes in human
132 pathology comes from numerous genetic association studies in a variety of diseases [55], con-
133 sistent with their importance in the regulation of inflammation and tissue repair. The most con-
134 vincing evidence comes from point mutations in a coding amino acid of the inflammasome
135 sensors *MEFV* (coding for pyrin), *NLRP3*, and *NLRP1*, which lead to **familial Mediterranean**
136 **fever (FMF)**, **cryopyrin-associated periodic syndrome (CAPS)**, and skin inflammatory
137 and cancer susceptibility syndromes, respectively, highlighting the clinical significance of
138 these proteins [54,56–59]. Furthermore, two recent studies demonstrated the presence of
139 ASC specks in the serum of patients with chronic inflammatory disorders such as **chronic**
140 **obstructive pulmonary disease (COPD)** and CAPS, and these might be considered as bio-
141 markers of active disease [60,61]. It is speculated that extracellular specks might also contribute
142 to amplifying inflammation, as well as neutrophilia and anemia in human chronic inflammatory
143 disorders such as these; it is possible that they might do so by altering the erythroid–myeloid fate
144 decision of HSPCs [31] when reaching the BM, but this remains to be demonstrated (see Clinician’s
145 Corner) (Figure 2, Key Figure).

146 From another angle, the presence of neutrophils in atherosclerotic plaques correlates with the
147 instability of these plaques, and a poor prognosis in atherosclerosis; this instability has also been
148 associated with the formation of **neutrophil extracellular traps (NETs)** and inflammasome
149 activation, since NETs have been detected in atherosclerotic lesions and arterial thrombi in humans
150 and mice, and can induce the activation of endothelial cells, antigen-presenting cells, and platelets
151 [62,63]. Moreover, both types of cellular death, pyroptosis and NETosis, need GSDMD cleavage;
152 however, while mouse neutrophils use inflammasome-dependent mechanisms to cleave GSDMD
153 and activate NETosis, human neutrophils use neutrophil elastase to do so [64,65]. Altogether,
154 these observations suggest that chronic activation of macrophage and neutrophil inflammasomes
155 can disrupt homeostasis in an organism and potentially contribute to inflammatory diseases; as
156 such, inflammasomes might represent promising therapeutic targets for restoring homeostasis in
157 a variety of pathologies, but this evidently remains to be further investigated.

Guanylate-binding proteins (GBP): family of GTPases induced by IFN- γ and regulating inflammasome activation.

Granulocyte colony-stimulating factor (G-CSF): glycoprotein that stimulates the BM to produce granulocytes and stem cells, releasing them into the bloodstream.

Hematopoietic stem and progenitor cells (HSPCs): cells from which differentiated blood cells originate.

HSPC niche: anatomical location in which HSPCs reside and self-renew.

Inflammasomes: cytosolic multiprotein oligomers of the innate immune system responsible for the induction of inflammation.

Leukopenia: low white blood cell count.

Morpholino: a DNA oligonucleotide used to modify gene expression through blocking mRNA splicing or translation.

Myelodysplastic syndrome: group of cancers in which immature blood cells in the bone marrow do not mature.

Myeloid: cells including neutrophils, monocytes, macrophages, myeloid dendritic cells, and mast cells.

Myeloperoxidase: peroxidase abundant in neutrophil lysosomes.

Neutrophil extracellular traps

(NETs): network of extracellular fibers composed primarily of DNA from neutrophils that bind pathogens and allow their clearance.

Neutrophilia: high number of neutrophils in the blood.

Neutrophilic dermatitis: group of inflammatory skin disorders comprising sterile inflammation and neutrophilic skin infiltration.

NOD-like receptors (NLRs): inflammasome receptors detecting pathogens and cytosolic signals of cell stress or tissue damage.

Noncanonical inflammasome: effector is human CASP4 and CASP5, or murine Casp11.

Pluripotency: capacity to self-renew by dividing and developing into the three primary germ cell layers of the early embryo and therefore, into all cells of the adult.

Primitive hematopoietic wave: the first of the two waves of hematopoiesis. It is transitory and gives rise to erythrocytes and myeloid cells (macrophages and neutrophils) during early embryonic development.

PYRIN: inflammasome receptor sensing modifications in the activity of the small GTPase RhoA.

Q12

158 **The Zebrafish: A Unique Model for Studying Hematopoiesis and Inflammation**

159 The zebrafish has become an exceptional model organism in many fields of biomedical research.
160 It is the only vertebrate model that has the main advantages of invertebrates (e.g., the fruit fly and
161 *Caenorhabditis elegans*), including: (i) high fecundity (between 200 and 300 eggs per female can
162 be obtained each week), and the embryonic development is rapid and external; (ii) the embryos
163 are transparent, allowing the development and tracking of their cells in tissues and organs
164 using optical and fluorescent methods; and (iii) the animal is suitable for high throughput chemical
165 and genetic screenings [66]. In addition, its genome is completely sequenced and shares 70% of
166 its genes with humans, where up to 82% of them are known to be relevant/causative of human
167 diseases [67]. This high genetic homology allows transgenesis studies with human genes and en-
168 ables the *in vivo* study of gene regulation and function.

169 Zebrafish have been used as a research model to study hematopoiesis [68]. Although hemato-
170 poiesis in zebrafish occurs in sites that are distinct from other vertebrates (e.g., zebrafish HSCs
171 reside in the kidney marrow), the genetic programs controlling hematopoiesis are conserved


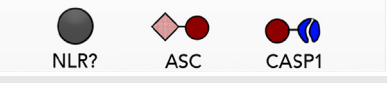
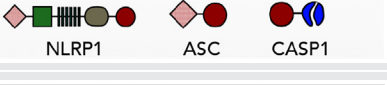
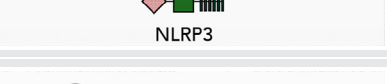
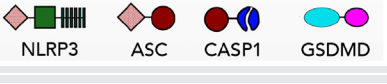

Pyroptosis: highly inflammatory form of programmed cell death promoted by inflammasomes.

Specks: large protein complex of ASC formed after inflammasome activation.

SPI1: transcription factor regulating the expression of genes involved in myelopoiesis and B lymphocyte development.

Transcription activator-like effector nucleases (TALENs): restriction enzymes that can be engineered to cut specific sequences of DNA.

t1.1 **Table 1. Components and Effector Functions of the Main Canonical Inflammasomes in HSPCs, Erythroid Progenitors, and Mature Myeloid Cells**
t1.2 **(Macrophages and Neutrophils) in the Indicated Species^{a,b}**

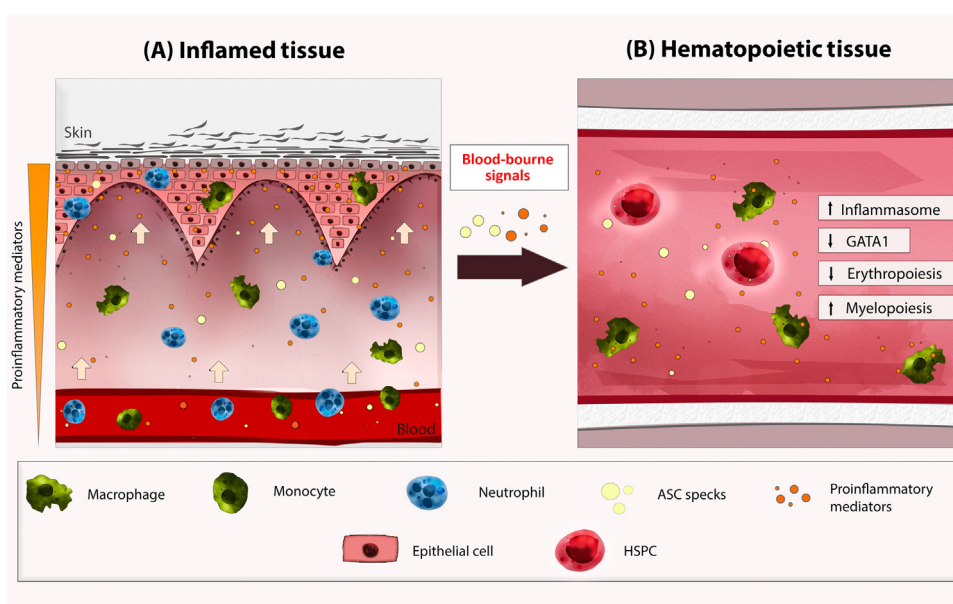
t1.3	Canonical inflammasomes				
t1.4	Cell type	Components	Effector functions	Models	Refs
t1.6	HSPCs		GATA1 cleavage	Zebrafish	[31]
t1.7			GATA1 cleavage	Human Mouse	[31]
t1.8			Pyroptosis	Mouse	[32]
t1.9			HSPC mobilization and engraftment	Mouse	[33–35]
t1.10	Mature myeloid cells		IL-1 β and IL-18 cleavage Pyroptosis	Human Mouse	[19]
t1.11			IL-1 β and IL-18 cleavage Pyroptosis	Human Mouse	[19]



t1.12 ^aAbbreviations: ASC, apoptosis-associated speck-like protein containing a CARD; CARD, caspase activation and recruitment domains; CASP1, caspase 1; GBP, guanylate-binding proteins; GSDMD, Gasdermin D; HSPCs, hematopoietic stem and progenitor cells; NLRs, nucleotide-binding domain-like receptors; PYD, pyrin domain.
t1.13 ^bThe domains of each inflammasome component are shown.
t1.14
t1.15

Key Figure

Proposed Model of the Inflammasome in Hematopoiesis and the Subsequent Effects of Released ASC Specks in Chronic Inflammatory Diseases



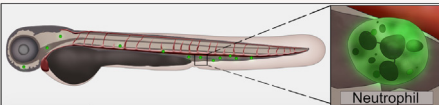
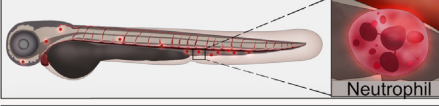
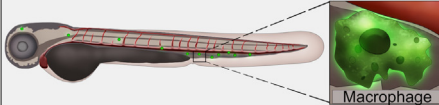
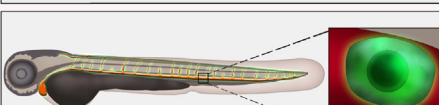
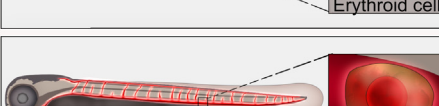
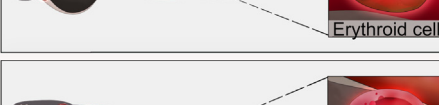
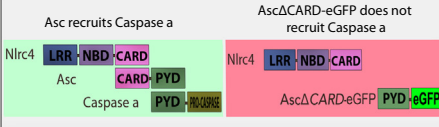
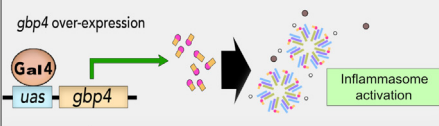
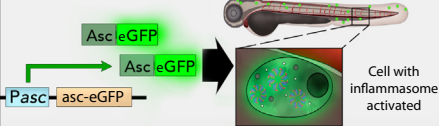
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Figure 2. (A) In this model, based largely on zebrafish studies, macrophages and neutrophils are recruited to inflamed tissues, for example the skin, where they are activated by different proinflammatory signals. Activation of the inflammasome results in ASC speck release into the circulation. (B) Blood-borne specks that can reach the hematopoietic tissue (bone marrow in mammals and kidney marrow in zebrafish), are taken up by HSPCs or resident macrophages, and activate their inflammasome, leading to IL-1 β /IL-18 release, GATA1 cleavage, reduced erythropoiesis, and increased myelopoiesis [30,60,61]. Abbreviations: ASC, apoptosis-associated speck-like protein containing a CARD; HSPCs, hematopoietic stem and progenitor cells; IL, interleukin.

172 between zebrafish and mammals, including humans, making zebrafish a clinically relevant model
 173 system [4]. The earlier mentioned advantages, together with the construction of detailed genomic
 174 databases, have allowed a series of genetic approaches to be used in this model for the study of a
 175 variety of blood disorders, such as congenital and acquired anemia, neutrophilia, and leukemia
 176 [69]. Zebrafish has also consolidated its position as a powerful animal model in the fields of immu-
 177 nity, inflammation, and infection [70,71]. It has contributed to improving our knowledge of human
 178 infections, such as tuberculosis which is difficult to model *in vivo* in mammals [72]. In addition, the
 179 optical transparency of the early life stages of zebrafish offers unprecedented possibilities for
 180 studying the activation and dynamics of inflammasomes *in vivo* [73].

181 Gain (mRNA) and loss of function experiments in embryos [morpholinos, transcription
 182 activator-like effector nucleases (TALEN) and CRISPR-Cas technologies] are relatively
 183 simple to perform, enabling the study of the role of specific molecules, including those involved
 184 in the inflammasome, and these roles have been better deciphered using different stimuli and
 185 pathogens [71]. In addition, zebrafish has allowed the analysis and visualization of different
 186 types of immune cells, including neutrophils and macrophages; indeed, the assembly of
 187 inflammasomes has been studied using transgenic zebrafish lines that are either already available,

188 or easy to generate [74]. Among the numerous transgenic lines available to study hematopoiesis
189 and inflammasomes, the following should be mentioned (Figure 3): Tg(mpx:eGFP) [75] and Tg(lyz:
190 dsRed) [76], which express fluorescent eGFP or dsRed driven by the neutrophil specific

Transgenic line	Description	Visual representation
Tg(mpx:eGFP)	eGFP expression under the neutrophil specific promoter myeloperoxidase. Neutrophils labelled with green fluorescence.	
Tg(lyz:dsRED)	dsRED expression under the neutrophil specific promoter lysozyme. Neutrophils labelled with red fluorescence.	
Tg(mpeg1:eGFP)	eGFP expression under the macrophage specific promoter macrophage expressed gene 1. Macrophages labeled with green fluorescence.	
Tg(lcr:eGFP)	eGFP expression under the erythroid specific globin regulatory sequence locus control region. Erythroid cells labeled with green fluorescence.	
Tg(gata1:dsRED)	dsRED expression under the specific promoter erythroid transcription factor Gata1. Erythroid cells labeled with red fluorescence.	
Tg(runx1:GAL4)	GAL4 expression under the hematopoietic stem and progenitor cell specific promoter with the aim of using GAL4/UAS system to label specifically HSPCs.	
Tg(UAS:ascΔCARD-eGFP)	Tissue-specific inhibition of inflammasome using the GAL4/UAS system.	 <p>Asc recruits Caspase a</p> <p>AscΔCARD-eGFP does not recruit Caspase a</p>
Tg(uas:gbp4)	Tissue-specific activation of inflammasome using the GAL4/UAS system.	 <p>gbp4 over-expression</p> <p>Inflammasome activation</p>
Tg(asc:asc-eGFP)	Knock-in transgenic line in which the endogenous Asc is fused with eGFP.	 <p>Asc-eGFP</p> <p>Cell with inflammasome activated</p>

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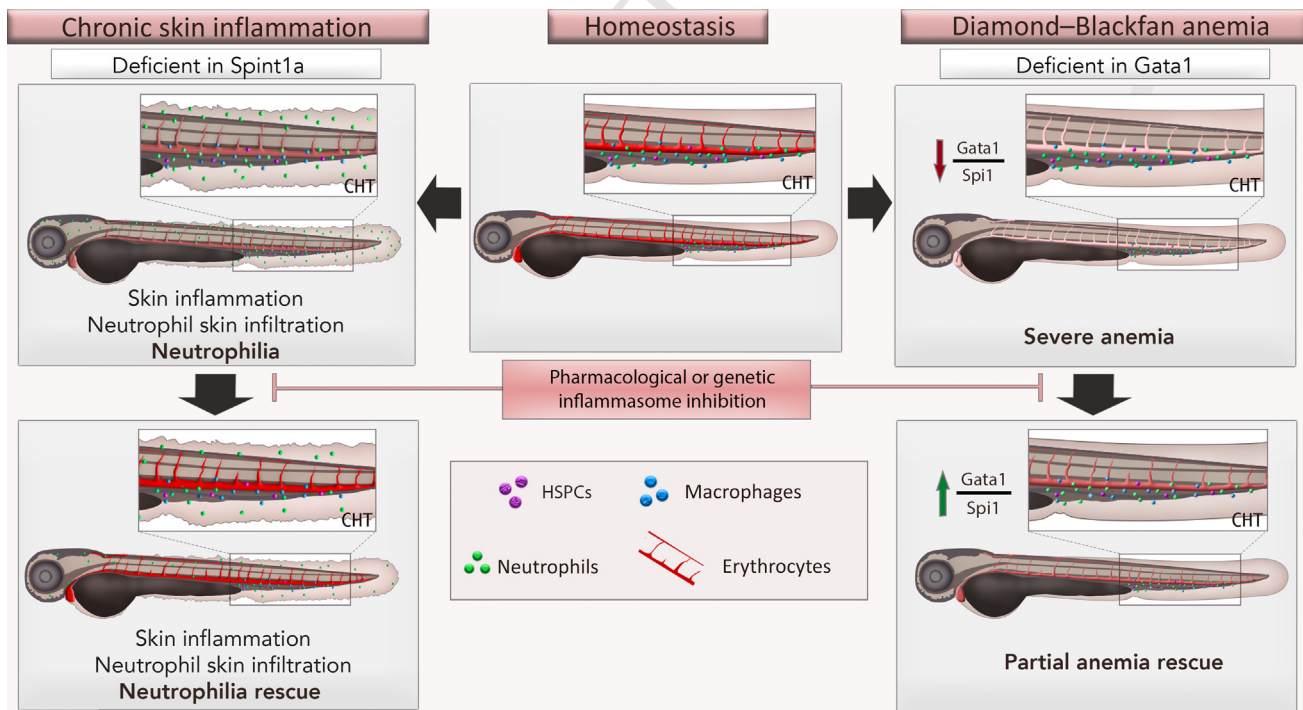
Figure 3. Zebrafish Transgenic Lines in the Study of Inflammation and the Inflammasome in Hematopoiesis. Specific hematopoietic cell types and the visualization of the inflammasome assembly in real time have been feasible using these lines [30,73]. Chemical and genetic screening methods are also amenable in zebrafish, sharing certain advantages of invertebrate models for high throughput screening and *in vivo* imaging, such as high fecundity, optical transparency, and rapid and external development. Abbreviations: ASC, apoptosis-associated speck-like protein containing a CARD; HSPCs, hematopoietic stem and progenitor cells; PYD, pyrin domain.

Q2

218 promoters **myeloperoxidase** and lysozyme, respectively; Tg(mpeg1:eGFP) [77], with labeled
 219 macrophages stemming from activation of the *mpeg1* promoter; and Tg(lcr:eGFP) [78] which
 220 has green fluorescent erythrocytes due to the expression of eGFP, driven by a globin regulatory
 221 sequence. Moreover, another two lines can be used to study erythropoiesis: Tg(gata1:DsRed)
 222 [79], which allows visualization of erythroid cells because they express dsRed driven by the
 223 *gata1* promoter; and Tg(runx1:GAL4) [80] with its labeled HSPCs. Finally, the knock-in line Tg
 224 (asc:asc-eGFP) allows real-time single cell visualization of inflammasome assembly [31], and
 225 the lines Tg(uas:asc Δ CARD-eGFP) and Tg(uas:gp4) allow the respective inhibition or activation
 226 of the inflammasome in specific cell types [64].

227 The Zebrafish Model Sheds Light on the Importance of the Inflammasome in 228 Hematopoiesis

229 As mentioned, a relationship between inflammasome activation and hematopoiesis has been
 230 suggested for several medical conditions; for instance, hematopoietic lineage bias has been as-
 231 sociated with chronic inflammatory diseases. However, the mechanisms and signaling pathways
 232 involved are unknown. One of the first pieces of evidence for this was the demonstration that
 233 human GATA1 could be cleaved *in vitro* by many caspases, and *in vivo* by caspase-3 in
 234 human erythroid precursors [81,82]. Furthermore, the activation of Nlrp1a in mouse HSPCs trig-
 235 gers pyroptotic death of these cells and **leukopenia** in the absence of inflammation, while in pe-
 236 riods of hematopoietic stress induced by chemotherapy or viral infection, NLRP1a activation
 237 caused prolonged cytopenia, **BM hypoplasia**, and immunosuppression [31]. In addition, the



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Figure 4. Zebrafish Disease Models for Studying Hematopoiesis. In homeostasis conditions, embryonic hematopoiesis takes place in the caudal hematopoietic tissue (CHT) 2–3 days postfertilization (middle panel). The Spint1a-deficient line (*spint1a*^{h22177g/h22177g}) is an excellent model for studying hematopoietic lineage bias associated with chronic inflammation, since it leads to neutrophilia which can be visualized (left panel). The Gata1 amorphant animal is an amenable model of Diamond-Blackfan anemia. In both models, inhibition of the inflammasome can rescue hematopoietic lineage bias [30]. Abbreviation: HSPCs, hematopoietic stem and progenitor cells.

238 NLRP3 inflammasome is required for the optimal release of mouse HSPCs from the BM in
 239 response to purinergic signaling, **complement activation**, and G-CSF administration [32,33].
 240 Notably, transplantation experiments in mouse have shown that NLRP3 knockout, either in
 241 HSPCs or in BM stromal cells, results in impaired HSPC homing and engraftment [34]. Further-
 242 more, the NLRP3 inflammasome has been found to drive clonal expansion and pyroptotic cell
 243 death in **myelodysplastic syndrome** patients [35] (see Clinician's Corner). Accordingly, it has
 244 been demonstrated that, while the canonical inflammasome is dispensable for HSPC emergence
 245 in zebrafish larvae, it autonomously regulates HSPC differentiation in homeostatic conditions at
 246 two different levels: erythroid–myeloid cell fate decision and terminal erythroid differentiation
 247 [30] (Figure 1 and Table 1). Thus, the genetic or chemical inhibition of different inflammasome
 248 components, {e.g., Gbp4 [Tg(uas:gbp4)], Asc Tg(uas:ascΔCARD-eGFP)}, or **caspase a**
 249 (**Caspa**), (the zebrafish functional equivalent of pharmacological inhibition of mammalian
 250 CASP1), resulted in a decreased number of neutrophils and macrophages accompanied by a
 251 concomitant increase in erythrocyte counts in larvae, while conversely, the forced expression of
 252 both Asc and Caspa simultaneously by mRNA injection, increased neutrophil numbers [30].
 253 These results were further confirmed by neutrophil ablation experiments, achieved by treating lar-
 254 vae, which express the bacterial nitroreductase in neutrophils with the prodrug metronidazole;
 255 pharmacological inhibition of CASP1 fully impaired neutrophil recovery after ablation. Further
 256 analysis showed that the emergence of HSPCs in *Tg(runx1:GAL4)* was not affected, suggesting
 257 that the inflammasome could regulate HSPC differentiation [30]. Mechanistically, inflammasome
 258 activation in HSPCs resulted in reduced Gata1 protein amounts, enforcing myelopoiesis and
 259 inhibiting erythropoiesis. The mechanism seems to be evolutionarily conserved, since the phar-
 260 macological inhibition of CASP1 in HSCs from SPI1-eYFP and GATA1-mCherry reporter mice
 261 rapidly increased Gata1 protein amounts, without affecting those of Spi1, resulting in increased
 262 erythroid differentiation at the expense of myeloid differentiation (Figure 1) [30]. In addition, ery-
 263 throid differentiation of the human erythroleukemic line K562 was also impaired upon inhibition
 264 of CASP1, but not CASP4, suggesting a role for the canonical inflammasome in terminal erythroid
 265 differentiation, by dampening GATA1 protein amounts [30]. *In vitro* experiments confirmed the
 266 ability of human recombinant CASP1 to cleave GATA1 at residue D300 [30]. Taken together,
 267 these results support a scenario in which GATA1 protein amounts are fine-tuned by the canonical
 268 inflammasome in HSPCs, to regulate the erythroid–myeloid decision, and simultaneously in ery-
 269 throid progenitors, to regulate their terminal differentiation. Furthermore, the inflammasome and
 270 macrophage-derived IL-1 β appear to be required for the expansion of HSPCs of zebrafish em-
 271 bryos, assayed by whole-mount *in situ* hybridization of the HSPC markers *runx1/cmyb* and *Tg*
 272 (*cd41:eGFP*) larvae [58]. Further studies are required, however, to elucidate the signals responsi-
 273 ble for activating the inflammasome in HSPCs in homeostasis, as well as the inflammasome com-
 274 ponents involved (see Outstanding Questions).

275 This close relationship between GATA1 and CASP1 is relevant at a clinical level, since bias of the
 276 hematopoietic lineage has been associated with a higher incidence of diseases with an inflamma-
 277 tory component, such as infection, rheumatoid arthritis, atherosclerosis, inflammatory bowel dis-
 278 eases, kidney and pulmonary diseases, autoimmune pathologies, neurodegenerative diseases,
 279 and certain cancers [17]. An example of this group of diseases is **neutrophilic dermatosis**,
 280 characterized by the accumulation of neutrophils in polymorphic cutaneous lesions of patients
 281 [83]. A zebrafish line deficient in the serine peptidase inhibitor, Kunitz type 1a (Spint1a), has
 282 been used as a model of this disease, since it shows neutrophilia, skin lesions, and high skin neu-
 283 trophil infiltration [84,85]. Accordingly, inhibition of the canonical inflammasome with the CASP1
 284 inhibitor Ac-YVAD-CMK in this model, restored Gata1 amounts and decreased the number of
 285 neutrophils, even though both skin lesions and neutrophil infiltration were not affected [30]
 286 (Figure 4).

Clinician's Corner

Alterations in inflammasome activity might have important clinical implications, since hematopoietic lineage bias has been associated with certain chronic diseases with prominent inflammatory components, including infections, rheumatoid arthritis, atherosclerosis, inflammatory bowel diseases, kidney and pulmonary diseases, autoimmune conditions, neurodegenerative diseases, and cancer [17,83,91].

Emerging evidence suggests that the inflammasome may play a key role in a variety of human diseases, including FMF, CAPS, and skin inflammatory and cancer syndromes, which are driven by gain-of-function mutations in MEFV, NLRP3, and NLRP1, respectively [54,56–59]. As many FMF and CAPS patients develop iron-resistant hypochromic anemia, inflammasome overactivation in HSPCs might contribute to anemia, which might explain its iron-refractory nature, although this warrants further investigation.

NLRP3 and ASC specks are present in the serum of CAPS and COPD patients, and might be considered as putative biomarkers of active disease [60,61]. These specks might contribute to amplifying not only inflammation, but also neutrophilia and anemia, after being taken up by HSPCs, activating their inflammasome, and promoting hematopoietic lineage bias through GATA1 cleavage and inactivation. This also requires further testing.

287 Another disease in which inhibition of the canonical inflammasome might be considered a puta-
288 tive therapeutic target for further exploration is **Diamond–Blackfan anemia (DBA)**, a hereditary
289 disease in which erythropoiesis is impaired due to reduced GATA1 protein amounts [86]. We
290 modeled DBA in zebrafish by reducing Gata1 amounts using a specific morpholino [87]. Pharma-
291 cological inhibition of Caspa with Ac-YVAD-CHO in this DBA model was able to partially rescue
292 anemia and to increase Gata1 and to decrease Spi1 protein amounts [30] (Figure 4). Taken
293 together, pharmacological inhibition of the inflammasome rescued neutrophilic inflammation
294 and anemia in these zebrafish disease models [30]; this suggested that the inflammasome can
295 play a major role in hematopoietic lineage bias during certain chronic inflammatory diseases
296 and DBA, at least stemming from zebrafish models. Nevertheless, these findings certainly merit
297 further attention in identifying novel druggable targets for potential therapeutic interventions in
298 some of these conditions (see Clinician’s Corner).

299 Concluding Remarks

300 The zebrafish model has unique advantages for biomedical research and, herein discussed is its
301 relevance for designing studies to better understand the roles and mechanisms of inflammasome
302 activation in hematopoiesis. The availability of models of chronic inflammatory diseases, including
303 neutrophilic dermatosis and rare diseases such as DBA, provides an excellent and unique tool to
304 rapidly identify novel treatments for some of these pathologies, and furthermore, for screening
305 clinically approved drug libraries that may facilitate relatively rapid repurposing. In addition, pow-
306 erful CRISPR-Cas9 technology allows the rapid generation of candidate pathogenic mutations,
307 which can even be analyzed in the F0 generation without the need for generating time-
308 consuming deficient lines, to confirm their pathogenicity, and target or lead drug screening (see
309 Clinician’s Corner). Finally, it is essential to find the specific inflammasome(s) involved in hemato-
310 poiesis to develop more specific treatments for the hematopoietic lineage bias associated with
311 chronic inflammatory disorders (see Outstanding Questions). The possibility of *in vivo* tracking
312 of hematopoietic cells, together with the inflammasome tools available, including reporters to
313 visualize its assembly in real time, can make this model complementary to other models of verte-
314 brate species, to study the crosstalk between hematopoiesis and inflammation in general, and
315 the inflammasome in particular.

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Outstanding Questions

What signals are responsible for activating the inflammasome in HSPCs? What is the cellular source of these signals – do they have a stromal or hematopoietic origin?

Are the signals activating the HSPC inflammasome the same in steady state and inflammatory conditions?

What are the components of the HSPC inflammasome? Does ASC form specks upon activation of the HSPC inflammasome? Which kind of specks? Are these specks able to activate GSDMD?

What are the respective contributions of IL-1 β /IL-18 release, pyroptotic cell death, and GATA1 cleavage to HSPC and erythroid precursor differentiation?

Besides IL-1 β , IL-18, GSDMD, and GATA1, are other proteins cleaved by the inflammasome in HSPCs in steady state and inflammatory conditions?

Since activated macrophages can release specks that activate bystander cells, are these specks able to reach the BM and modulate the activation of the HSPC inflammasome in inflammatory conditions?

In what ways can zebrafish models significantly complement other animal models to further elucidate many of these questions?

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