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Is Strongyloides Stercoralis Hyperinfection Induced by Gglucocorticoids a Result of Both Suppressed Host Immunity and Altered Parasite Genetics?

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Is *Strongyloides stercoralis* hyperinfection induced by glucocorticoids a result of both suppressed host immunity and altered parasite genetics?

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ABSTRACT

The gastrointestinal (GI) nematode *Strongyloides stercoralis* (*S.s.*) causes human strongyloidiasis, a potentially life-threatening disease that currently affects over 600 million people globally. The uniquely pernicious aspect of *S.s.* infection, as compared to all other GI nematodes, is its autoinfective larval stage (L3a) that maintains a low-grade chronic infection, allowing undetectable persistence for decades. Infected individuals who are administered glucocorticoid therapy can develop a rapid and often lethal hyperinfection syndrome within days. Hyperinfection patients often present with dramatic increases in first- and second-stage larvae and L3a in their GI tract, with L3a widely disseminating throughout host organs leading to sepsis. How glucocorticoid administration drives hyperinfection remains a critical unanswered question; specifically, it is unknown whether these steroids promote hyperinfection through eliminating essential host protective mechanisms and/or through dysregulating parasite development. This current deficiency in understanding is largely due to the previous absence of a genetically defined mouse model that would support all *S.s.* life-cycle stages and the lack of successful approaches for *S.s.* genetic manipulation. However, there are currently new possibilities through the recent demonstration that immunodeficient NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice support sub-clinical infections that can be transformed to lethal hyperinfection syndrome following glucocorticoid administration. This is coupled with advances in transcriptomics, transgenesis, and gene inactivation strategies that now allow rigorous scientific inquiry into *S.s.* biology. We propose that combining in vivo manipulation of host immunity and deep immunoprofiling strategies with the latest advances in *S.s.* transcriptomics, *piggyBac* transposon-mediated transgene insertion, and CRISPR/Cas-9-mediated gene inactivation will facilitate new insights into the mechanisms that could be targeted to block lethality in humans with *S.s.* hyperinfection.

1. Introduction

The human parasitic nematode *Strongyloides stercoralis* infects over 600 million people worldwide, typically in tropical and developing countries [1,2], although transmission also occurs in rural parts of Australia, the United States, and other developed countries [3,4]. People become infected via direct skin penetration by infective third-stage larvae (iL3) that are present in fecally-contaminated soil [5] (Fig. 1). Infections initially may result in a skin rash, cough, dyspnea, diarrhea, constipation, weight loss, and/or abdominal pain, but then typically become sub-clinical aside from intermittent abdominal pain [6]. Chronic infections often go undiagnosed because first-stage larvae (L1) released in feces may be present in variable or low numbers and are also difficult to identify with commonly used parasitological approaches such as the fecal float technique [7].

In contrast to other human parasitic nematodes that cannot replicate inside the host, *S. stercoralis* typically forms low numbers of autoinfective third-stage larvae (L3a) that permit the parasite to complete its life cycle within the host and allow infections to persist for decades [5] (Fig. 1). Strikingly, when people who have maintained a sub-clinical

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; GI, gastrointestinal; HTLV-1, human T-cell lymphotropic virus type 1; IL, interleukin; ILC2s, type 2 innate lymphoid cells; iL3, infective third-stage larvae; L1, first-stage larvae; L3a, autoinfective third-stage larvae; MPA, methylprednisolone acetate; NSG, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ.

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infection for years are administered glucocorticoids (e.g., the prodrug prednisone, prednisone's biologically active metabolite prednisolone, dexamethasone, or methylprednisolone acetate (MPA)), the number of L3a often rapidly increases, which penetrate through the gut and migrate through the host, resulting in additional parasitic adults that in turn produce increasing large numbers of L3a; this uncontrolled increase in parasite numbers, termed hyperinfection, can quickly overwhelm the host [8–10] (Fig. 2). Hyperinfection is typically accompanied by disseminated strongyloidiasis, when larvae extend beyond the normal migratory route (i.e., skin, lung, and gastrointestinal tract; see Fig. 1) and enter other vital organs (e.g., brain, kidney, and liver). The outcomes of this increased migration and tissue damage include sepsis, shock, meningitis, disseminated intravascular coagulation, renal failure, and/or respiratory failure. In untreated patients, hyperinfection results in nearly 100% mortality. Mortality rates can still exceed 70% even with aggressive anthelminthic treatment [11], which is largely due to sepsis that results from enteric bacteria carried into multiple organs by migrating larvae [6]. While there are several types of conditions that may promote hyperinfection, such as human T-cell lymphotropic virus type 1 (HTLV-1) coinfection, organ transplantation, or Cushing's disease [5,12], the overwhelming majority of hyperinfection and disseminated strongyloidiasis cases are thought to occur following glucocorticoid administration [6,13]. The recent use of glucocorticoids as common treatment for COVID-19 patients has made hyperinfection a serious acute clinical care problem [14]. The anthelminthic drug of choice to treat uncomplicated *S. stercoralis* infections is ivermectin. However, ivermectin has limited efficacy against L3a and its efficacy for preventing mortality during hyperinfection varies case by case [6,15]. Surprisingly, the mechanisms by which glucocorticoids cause the rapid induction of hyperinfection remain entirely unknown.

2. *S. stercoralis* life cycle: autoinfection as a gateway to hyperinfection

In the mammalian host, *S. stercoralis* parasitic females reside in the crypts of the small intestine where they reproduce by mitotic parthenogenesis [16] (Fig. 1E). Eggs laid by the parasitic female in the host mucosal tissue hatch almost immediately, resulting in post-parasitic L1 in the host small intestine that can be either female or male [5]. In uncomplicated infections, post-parasitic larvae excreted in the feces are predominantly rhabditiform (short, tri-lobed pharynx used for feeding) L1 [17] (Fig. 1A). However, through mechanisms that remain unclear, a small percentage of female L1 rapidly molt into L2 and further into the L3a stage by the time the larvae reach the large intestine, which allows for re-infection of the host prior to elimination in the feces, thereby maintaining chronic parasitism [18] (Fig. 1F). L3a, which are all female, typically autoinfect by penetrating the bowel mucosa or the perianal skin [5] (Fig. 1B, red). L3a may follow a similar migratory route within the host as iL3 that enter through the skin—i.e., they are flushed into the lungs by the circulatory system, get lodged in the pulmonary capillaries, break out into the alveolar airspace, and then migrate up the trachea where they are thought to be coughed up, and swallowed to enter the gastrointestinal (GI) tract (Fig. 1C, D). In the small intestine, iL3 molt into the L4 stage followed by development into adult-stage parthenogenic females [5,16] (Fig. 1E). However, the rapid amplification of parasite numbers in hyperinfective bursts suggests other migration

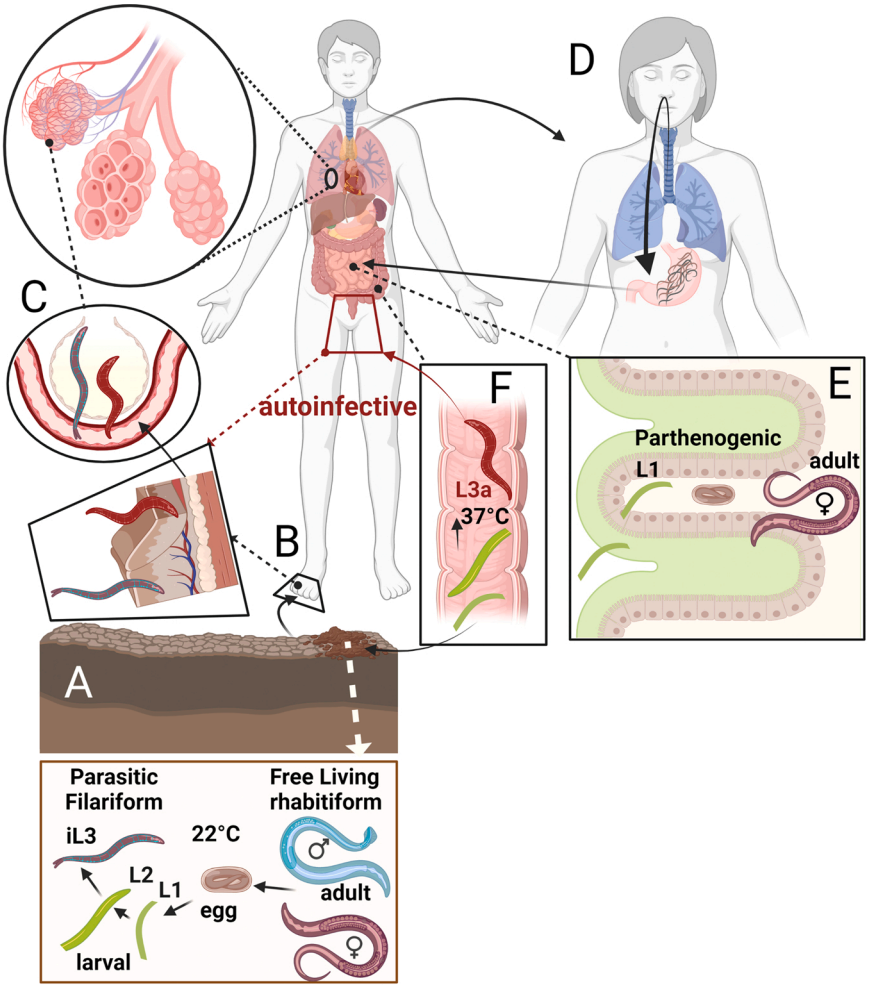

Fig. 1. *Strongyloides stercoralis* life cycle. (A) Fecally-contaminated soil contains non-parasitic free-living male and female rhabditiform nematodes that reproduce sexually and lay eggs, which invariably develop into filariform infective third-stage larvae (iL3, gray). (B) These iL3 penetrate the intact skin of a host (human, primates, dog, Mongolian gerbil, or NSG mouse) to find their way into the circulation. (C) They transit through the lungs, (D) then are coughed up and swallowed. (E) In the gastrointestinal tract they undergo two molts and develop into mature adult females, which parthenogenically reproduce to produce eggs in the mucosa, which hatch immediately. Typically, first-stage larvae (L1, green) are shed in the feces. (F) Some of the post-parasitic larvae can mature into autoinfective third-stage larvae (L3a, red) capable of penetrating the large intestine and host's skin around the anal tissue, perpetuating the parasitic stage of the life cycle in the same host (B-F repeated). L1 shed in the feces can enter the soil and undergo four additional molts to re-populate the free living sexually dimorphic stages (and the cycle continues from A).

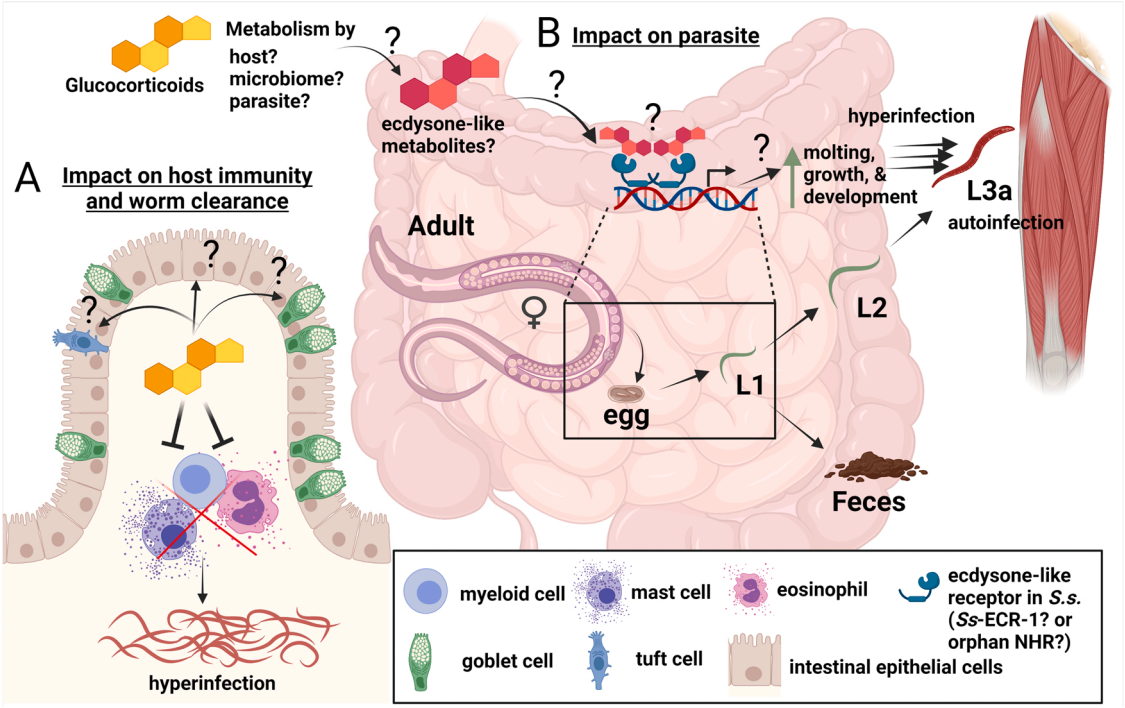

Fig. 2. Possible mechanisms of *Strongyloides* hyperinfection. Immunosuppression due to glucocorticoid signaling in the host may restrict myeloid, mast cell, and eosinophil populations that would normally limit parasite burden, contributing to hyperinfection. Glucocorticoids may also alter the ability of key epithelial effector cells (e.g., tuft and goblet cells) to aid in anti-helminthic actions. (B) Endogenous or exogenous glucocorticoids, or their metabolites, may target receptors or genes such as the ecdysone pathway that regulate development of the L1 parasite to accelerate maturation of autoinfective third-stage larvae (L3a) inside the body, thereby favoring aggressive autoinfection leading to hyperinfection. These two possibilities (A & B) are not mutually exclusive.

routes in addition to the traditionally accepted route through the lungs [19,20]; this non-canonical migratory route to the small intestine may also occur in uncomplicated infections [19]. L3a are morphologically distinct from iL3, in that L3a are both larger in diameter and shorter in length than iL3, and L3a have a single pointed tail whereas iL3 have a forked tail [21,22] (Fig. 1B). While other frequently studied *Strongyloides* species, including *S. ratti*, *S. papillosus*, and *S. venezuelensis*, have parthenogenic females that produce L1 that are excreted in the feces, none of these other *Strongyloides* species are known to undergo autoinfection. Thus, studies of hyperinfection and disseminated strongyloidiasis must be undertaken using *S. stercoralis*.

In host conditions that promote hyperinfection (e.g., glucocorticoid administration), the proportion of post-parasitic female L1 precociously developing into L3a relative to L1 exiting the host in the feces dramatically increases as does the fecundity of the parasitic female [20]. Factors contributing to the development of L3a are unclear. One hypothesis is that L3a form as a result of development at an elevated temperature, where post-parasitic L1 precociously develop into L3 inside of a 37 °C host. This is supported by the observation that for *S. stercoralis* strains biased towards heterogonic development (i.e., towards the single free-living generation), the female post-parasitic L1 recovered from the small intestine and incubated outside the host predominantly develop into filariform iL3 when cultured at 37 °C, but develop into rhabditiform L3 when cultured at 22 °C [23,24]. However, the cause of this rapid development or why L1 in the 37 °C GI tract are typically unaffected is unknown. A possible contributing factor may be that impaired GI motility due to glucocorticoid therapy permits greater time for larval development; however, chronic gastrointestinal motility disorders and constipation have not been associated with hyperinfection. A second hypothesis is that the number of L3a is intrinsically regulated as a function of parasite population density inside the host, and that hyperinfection is a result of disruption of this equilibrium [5,18]. This is supported by the experimental observation that inoculation of hosts with a large bolus of iL3 also results in hyperinfection [25]. Additionally, in other *Strongyloides* species, which do not undergo autoinfection, a strong host immune response results in post-parasitic female larvae that preferentially develop via the heterogonic route, while a weak host immune response results in homogonic (i.e., directly to iL3) development [26,27], suggesting that *S. stercoralis* post-parasitic larval development may also be influenced by the interaction between the host and the parasite.

3. Animal models and general effects of glucocorticoids

Several animal models have been used to study *S. stercoralis* hyperinfection, which typically infects humans, non-human primates, and dogs [5]. Prednisone and its derivatives are glucocorticoids routinely used in the experimental setting to elicit hyperinfection. Dogs typically control infections after the acute phase, similar to the course of human disease, but hyperinfection can be triggered by the administration of prednisolone [28]. Patas monkeys (*Erythrocebus patas*) can also be experimentally infected with *S. stercoralis* and hyperinfection triggered with prednisone [29]. Rodent models of *S. stercoralis* infection, where hyperinfection can be triggered by glucocorticoids, include both the Mongolian gerbil (*Meriones unguiculatus*) [21] and the severely immunodeficient NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mouse strain [22].

The endogenous hormones of the adrenal system include glucocorticoids and mineralocorticoids, which are together termed corticosteroids. Whereas mineralocorticoids regulate water and electrolyte balance, glucocorticoids control body homeostasis and immune responses [30]. Importantly, only glucocorticoids have been reported to be associated with the development of disseminated strongyloidiasis and hyperinfection, although the reason for this is not clear. Glucocorticoids are endogenously produced (e.g., cortisol in humans or corticosterone in rodents) in response to inflammation and glucocorticoid receptors function as ligand-dependent transcription factors that regulate gene expression through an array of mechanisms [31]. Synthetic glucocorticoids include prednisone, prednisolone, dexamethasone, hydrocortisone

(structurally identical to endogenous cortisol), and MPA. These drugs are widely used in both human and animal medicine to suppress a wide array of inflammatory disease states [32,33]. In general, these synthetic glucocorticoids are more potent than endogenous glucocorticoids, such as cortisol, due to their enhanced pharmacokinetic and pharmacodynamic properties [34]. Like their endogenous counterparts, these drugs promote immunosuppression through mechanisms that include direct binding to glucocorticoid response elements in the DNA, either alone, or in complex with co-receptors or other transcription factors to change gene expression [32].

4. Two potential mechanisms of glucocorticoid-mediated hyperinfection

Two hypotheses exist as to why glucocorticoid treatment causes hyperinfection: 1) glucocorticoids suppress key parts of the immune system that normally keep the parasite "in check" by killing substantial numbers of larvae produced by parthenogenic females, and/or 2) glucocorticoids and/or their metabolites serve as a molecular mimic for molting hormones like ecdysone, which drive a rapid change in parasite gene expression and developmental signaling pathways, thereby causing the adult worms or L1/L2 stages to preferentially develop into L3a (Fig. 2). These two hypotheses are not mutually exclusive, and it is certainly plausible that hyperinfection and disseminated strongyloidiasis are due to a combination of both processes.

5. Glucocorticoid potential suppression of host anti-helminth immunity

Regarding the protective nature of host immunity against *S. stercoralis,* several redundant mechanisms have been identified and glucocorticoids could alter these (Fig. 2A). Complement factor C3b is involved in *S. stercoralis* killing [35]; the glucocorticoid dexamethasone reduces secretion of C3b by human macrophages [36], suggesting that glucocorticoids could reduce C3b efficacy during hyperinfection. There is a significant role for B cell production of immunoglobulins M and G that license myeloid lineages cells, including eosinophils, macrophages, and neutrophils, for antibody-dependent cellular cytotoxicity of *S. stercoralis* larvae [37,38]. The reported effects of glucocorticoids on distinct subsets of immune cells are vast and include: inhibiting lymphocyte activation, promoting lymphocyte apoptosis, and reprogramming of macrophages [32]. It is possible some or all of these effects may counteract anti-parasitic immune mechanisms. Glucocorticoids can inhibit expression of proteins required for leukocyte recruitment and trafficking to the infection site, including e-cadherin, integrins, and chemokines [31]; in the context of hyperinfection, this could limit trafficking of important effector cells to sites of parasitic infiltration. Eosinophils, macrophages, and neutrophils kill and trap larval stages of *S. stercoralis*, particularly in the context of extracellular trap formation [39,40]. Glucocorticoids can affect the formation of neutrophil extracellular traps in other inflammatory conditions and are generally inhibitory [41–43], although MPA treatment enhanced neutrophil trap formation and bacterial clearance in samples from dogs [44]. Thus, it remains to be determined how glucocorticoids affect the formation of extracellular traps in the context of *S. stercoralis* infection. While not demonstrated for *S. stercoralis*, mast cells have been shown to serve an essential role for immunity against the closely related *S. ratti* [45,46], the latter of which maintains high parasite fecundity over the course of an infection when the host is treated with glucocorticoids in contrast to declining fecundity over the course of an infection in a non-immunosuppressed host [47,48]. Glucocorticoids also hinder mast cell histamine release by suppressing Fcε receptor signaling [31]; thus, mast cells clearance could be inhibited in the context of glucocorticoid-induced hyperinfection.

The host protective responses are largely driven by the prototypical type 2 cytokines interleukin (IL)− 4 and IL-5 [38]. Glucocorticoids can generally dampen signaling via cytokine receptors [31,49,50]. Type 2 innate lymphoid cells (ILC2s) generate IL-5, and this can be suppressed with glucocorticoid treatment in the context of allergic airway inflammation or in cultured airway epithelial cell lines [51]. ILC2s are also important for immunity against other helminth species [52,53]; thus, suppression of type 2 cytokine production by ILC2s in immune competent model animals may contribute to glucocorticoid-induced hyperinfection.

While the highly immunodeficient NSG mouse lacks lymphocytes (innate and adaptive populations), it still possesses several myeloid lineage subsets that may be sufficiently functional to hold parasite expansion in check prior to glucocorticoid administration [22]. These observations also suggest that myeloid cells and/or specialized epithelial cell lineages in NSG mice like tuft cells and goblet cells [54] may contribute to parasite control normally, but these functions are then lost following glucocorticoid administration. Intestinal epithelial cells maintain high immune suppressive tone by secretion of IL-10 and TGF-β [55]. Glucocorticoids up-regulate immunosuppressive cytokines like IL-10 to further inhibit immune responses [56]. Outside of their effects on hematopoietic system, glucocorticoids can have direct, receptor-mediated effects on nonhematopoietic cells like intestinal epithelia where they dysregulate mucosal barrier function, cytokine secretion, and other effector functions [57]. Thus, glucocorticoids may enhance immune-suppressive signals and membrane permeability, suppressing immune-mediated killing and allowing parasite transit into the intestinal lumen. In cultured airway epithelial cells, glucocorticoid treatment reduced IL-13-evoked goblet cell metaplasia [58]. Similarly, MPA treatment reduces goblet cell hyperplasia in an airway inflammation model [59]. Other glucocorticoids such as dexamethasone inhibit expression of a mucus-related mucin protein Muc5A in an airway-derived cell culture system [60]. Although not explicitly tested in the gastrointestinal tract, it is possible that changes in goblet cell hyperplasia or mucus production may contribute to glucocorticoid-induced *S. stercoralis* hyperinfection. The effects of glucocorticoid signaling on tuft cell function or expansion has not been directly studied. Thus, it is possible that intestinal tuft cells and goblet cells are perhaps important effector cells that restrict adult or larval development within the GI tract [54]. In fact, the intestinal immune landscape of the NSG mouse is poorly defined; therefore, there is a clear need for systematic immunoprofiling studies to be conducted in the context of *S. stercoralis* infection that leverage single-cell RNA sequencing, immunofluorescence microscopy, and multiparameter flow cytometry along with adoptive transfer strategies to clearly define the key immune cells and determine whether they are found in the hematopoietic or non-hematopoietic system.

6. Glucocorticoid metabolites as effectors of parasite development

Alternatively, a second long-standing hypothesis is that hyperinfection results from modification of glucocorticoids into ecdysone-like agonists that over-activate ecdysteroid-responsive genes in the parasite [61], which increases the rate of larval development, frequency of L3a, and/or rate of L3a migration through the host (Fig. 2B). In support of this second hypothesis, glucocorticoid-driven hyperinfection can be avoided in both the NSG mouse and Mongolian gerbil models by administration of the DAF-12 nuclear hormone receptor ligand Δ7-dafachronic acid, which reduces the number of L3a and host mortality [15,22]. Although the mechanisms responsible for hyperinfection in an immunocompromised host remain ill-defined, the NSG mouse lacks both innate and adaptive lymphocytes and only develops hyperinfection after glucocorticoid administration, which implies that perhaps glucocorticoids directly affect parasite biology in addition to impacting host physiology.

It is possible that glucocorticoids trigger hyperinfection by either up-regulating, mimicking, or being modified into steroid hormones that

increase or alter *S. stercoralis* development (Fig. 2B). It has been proposed that glucocorticoids could be modified into ecdysteroids (e.g., ecdysone or 20-hydroxyecdysone), which subsequently increase the rate of larval production and development, thereby driving hyperinfection [18]. Evidence supporting this hypothesis includes: an increase in the reproductive output of *S. stercoralis* parasitic females in gerbils treated with MPA [21], an increase in the proportion of larvae developing into L3a in both gerbils and NSG mice treated with MPA [21,22] and, an increase in the rate of *S. stercoralis* development, evidenced by the precocious development of free-living adult males in the large intestine of gerbils treated with MPA [62]. However, the increased rate of both larval production and development in MPA-treated animals may also be an indirect effect of immunosuppressing the host; but if this were strictly the case, it is unclear how immunosuppression of the host would cause post-parasitic L1 to precociously develop into L3a.

While many parasitic nematode species have a gene encoding an ecdysone receptor and can utilize ecdysone signaling to regulate development and molting [63–67], the *Caenorhabditis elegans* genome does not contain a gene encoding an ecdysone receptor [68] nor does this species appear to produce endogenous ecdysteroids [69], suggesting ecdysone signaling is not universal in nematodes. Recently, a homolog of the ecdysone receptor, *Ss-ecr-1*, was identified in *S. stercoralis* [62]. *Ss-ecr-1* transcripts are up-regulated in free-living females and developing larvae [62], a pattern similar to insects, suggesting that *S. stercoralis* utilizes ecdysone signaling to regulate development. Whether this endogenous signaling can be altered by exogenous ligands remains unknown in *S. stercoralis*, although it is likely, given that several ecdysone receptor agonists and antagonists regulate *Brugia malayi* development [70]. Additionally, it is unknown whether the endogenous ligand of *Ss*-ECR-1 is ecdysone/20-hydroxyecdysone, one of the other 537 ecdysteroids that have been characterized [71], or some other undescribed ecdysteroid.

A key piece of this hypothesis is that glucocorticoids must be metabolized into molecules that can activate the ecdysone receptor in the parasite. It is unlikely that the glucocorticoids themselves have a direct effect on the parasite, as they are structurally dissimilar to ecdysone [72] and MPA does not inhibit *S. stercoralis* post-parasitic L1 development into iL3 at 37 °C [22]. It is unknown whether glucocorticoids can be metabolized into ecdysteroids, either by the host, the parasite, or the gut microbiome. While many of the metabolites of prednisolone and MPA have been characterized in humans [73,74], it is unknown whether any of these can activate the ecdysone receptor. In the parasite, glucocorticoid metabolism would presumably be carried out by cytochrome P450 enzymes [75]. Since all 26 cytochrome P450-encoding genes have been identified in *S. stercoralis* [24] and can be expressed in insect cells [15], it should thus be possible to identify any parasite cytochrome P450 enzyme(s) potentially involved in this process.

It is also possible that glucocorticoids or their metabolites regulate *S. stercoralis* development through processes independent of ecdysone signaling. The *S. stercoralis* genome encodes multiple orphan nuclear hormone receptors [62,76], which likely have various roles in controlling parasite development—potentially including L3a development. The signaling of these nuclear hormone receptors could presumably be altered by the presence of exogenous ligands. Indeed, the sole nuclear hormone receptor with a characterized ligand in *S. stercoralis*, *Ss*-DAF-12, regulates L3a development [15,22]. Recent work has delineated the biosynthetic pathway for the steroid hormone Δ7-dafachronic acid, which is the endogenous ligand for *Ss*-DAF-12, in *S. stercoralis* and demonstrated that it can act synergistically with ivermectin to control hyperinfection in the gerbil model [15].

Exogenous administration of Δ7-dafachronic acid inhibits the development of *S. stercoralis* L3a in both the gerbil and NSG mouse models of glucocorticoid-mediated hyperinfection [15,22]. Whether this is the result of Δ7-dafachronic acid acting on the parasitic females and/or the post-parasitic larvae remains unknown. Since dafachronic acid decreases, rather than increases, the number of post-parasitic larvae, it is unlikely that Δ7-dafachronic acid is acting solely on the L3a. Three possible scenarios are that Δ7-dafachronic acid acts on 1) the parasitic female to decrease larval output; 2) post-parasitic L1 to promote development toward free-living larvae; or 3) post-parasitic L2 or L3a in the lumen of the intestine and prematurely activates them, thereby abrogating their ability to penetrate host tissue. The hypothesis that Δ7-dafachronic acid promotes development of post-parasitic L1 into free-living larvae is supported by the fact that Δ7-dafachronic acid inhibits the formation of L3i when post-parasitic larvae are cultured at 37 °C [24], promotes development of a second free-living generation [24,77], and activates iL3—evidenced by feeding [77], resulting in changes in gene expression that are similar to host-adapted L3, which are termed L3 + [78].

7. Future Directions

Given the advent of powerful immunoprofiling techniques including multiparameter flow cytometry and single cell RNA-Seq, it will be quite feasible to elucidate the immune cells in the NSG mouse (including tuft cells and goblet cells) that are present prior to, and subsequently reduced by, glucocorticoid administration. Likely candidates include the myeloid subsets (neutrophils, eosinophils, and M2 macrophages) that work in concert with extracellular trap formation and complement to trap and kill larval stages. Curiously, even myeloid subsets (e.g., macrophage and dendritic cells) in NSG mice are known to bear abnormalities. Given the migratory route taken by L3a during autoinfection (i.e., small intestine, large intestine, lung), evaluation of local immune infiltrates of different granulocyte and mononuclear cells induced by parasites in mucosal tissues is critical for understanding the key mechanisms that hold parasites in check. It is possible that following glucocorticoid administration, these hematopoietic effector cells, in combination with mucosal epithelial cells (i.e., tuft cells and goblet cells), are rendered ineffective for larval killing/control. It is intriguing to speculate that in addition to eliminating host mechanisms that limit worm survival, glucocorticoids could also impact parasite biology in ways that render worms resistant to killing. Although transcriptome profiles for most *S. stercoralis* developmental stages have been completed using RNA-Seq [62,76,78], the transcriptomic profiles of L3a and parasitic females within a host undergoing hyperinfection are unknown. Having these transcriptomic profiles would almost certainly bring insights into the potential mechanisms governing hyperinfection. Comparative studies with *S. ratti*, in which glucocorticoid treatment maintains parasite fecundity over the course of an infection but does not result in hyperinfection [26,27], may also be informative in identifying glucocorticoid-responsive genes that may drive autoinfection in *S. stercoralis*. Recent advances in single worm RNA-Seq [79] should also allow researchers to determine whether populations of parasitic females and post-parasitic larvae have uniform or heterogeneous transcriptomic profiles. Studies employing single worm RNA-Seq of parasites both pre- and post-glucocorticoid administration in an amenable rodent model like the NSG mouse or Mongolian gerbil could reveal whether L3a are generated by all or only a subpopulation of parasitic females and whether glucocorticoids bias L1 development towards L3a relative to iL3.

Once the putative glucocorticoid-sensitive regulatory factors in the *S. stercoralis* transcriptome that control L3a development have been identified, one would then have the capability to rigorously test their biological role(s) by either selectively editing these genes using CRISPR/Cas9 or over-expressing these genes using the *piggyBac* transposon system [80]. Advances in CRISPR/Cas9 mutagenesis methodologies have allowed researchers to create targeted deletions in multiple *S. stercoralis* genes, including *Ss-unc-22* [81], *Ss-tax-4* [82], *Ss-daf-12* and *Ss-dip-1* [83], and *Ss-cyp22a9* [15], and a targeted insertion in *SSTP_0000742500* (a homolog of *rol-6*) [84], each of which resulted in specific observable phenotypes. However, utilizing CRISPR/Cas9 to target genes necessary for L3a formation presents several significant challenges, including: 1)

the likely need to create a stable transgenic line of edited parasites rather than simply targeting iL3 in the post-free-living generation, a process which will require passaging edited worms through a suitable mammalian host, and 2) the possibility that genes controlling L3a formation may also be required for other stages of *S. stercoralis* development. Although daunting, the opportunity to understand the mechanisms by which L3a develop and the mechanisms controlling glucocorticoid-driven hyperinfection should not be overlooked, given the life-threatening nature of this disease and its increased prevalence amid the COVID 19 pandemic. These factors, combined with the lack of effective treatment for patients with severe strongyloidiasis makes this topic a considerable importance for human health in both the developed and developing world.

Adoptive transfer protocols, in vivo cell depletion, and deep immunoprofiling strategies in the NSG mouse model system combined with advances in *S. stercoralis* transcriptomics, *piggyBac* transposon-mediated transgene insertion, and CRISPR/Cas-9-mediated gene inactivation will permit the elucidation of the mechanisms controlling *S. stercoralis* hyperinfection in animal models and thus potentially lead to new interventions that reduce its lethal impact on human populations.

CRediT authorship contribution statement

D.R.H. contributed to the conceptualization, funding acquisition, review and editing. J.D.C.S contributed to the conceptualization, data curation, writing of original draft, review and editing, H.L.R. contributed to the conceptualization, data curation, writing of original draft, review and editing, D.A. contributed to the conceptualization, data curation, review and editing of this manuscript.

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