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Understanding mechanisms underlying the pathology of immune reconstitution inflammatory syndrome (IRIS) by using animal models

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Abstract

Purpose of Review—Despite the increasing number of clinical reports on immune reconstitution inflammatory syndrome (IRIS), mechanistic understanding of IRIS is still largely limited. The main focus of this review is to summarize animal studies, which were performed to better understand the cellular and molecular mechanisms underlying the pathology of IRIS.

Recent Findings—Three IRIS animal models have been reported. They are Mycobacterial IRIS (M-IRIS), cryptococcal IRIS (C-IRIS) and *Pneumocystis*-IRIS. M-IRIS animal model suggested that, rather than lymphopenia itself, the failure to clear the pathogen by T cells results in excessive priming of the innate immune system. If this happens before T cell reconstitution, hosts likely suffer IRIS upon T cell reconstitution. Interestingly, T cells specific to self-antigens, not only pathogen-specific, could drive IRIS as well.

Summary—The mechanism to develop IRIS is quite complicated, including multiple layers of host immune responses; the innate immune system that detects pathogens and prime host immunity, and the adaptive immune system that is reconstituted but hyper-activated particularly through CD4⁺ T cells. Animal models of IRIS, although there are still small numbers of studies available, have already provided significant insights on the mechanistic understanding of IRIS.

Keywords

IRIS; *Cryptococcus neoformans*; *Mycobacterium avium*; *Pneumocystis*; Immune reconstitution; Opportunistic infection

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Compliance of Ethical Standards

Conflict of interest Nupur Aggarwal, William Barclay and Mari Shinohara declare that they have no conflicts of interest.

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INTRODUCTION

Reconstitution of the immune system is usually a protective response, reflecting recovery of the system. However, immune reconstitution after a period of immunosuppression in immunocompromised patients can trigger overwhelming inflammatory responses when the immunocompromised patients have previously acquired opportunistic infections (OI) by bacteria, fungi, and viruses during immune suppression. This condition is termed immune reconstitution inflammatory syndrome (IRIS). IRIS have been observed during reversal of immune suppression or the immunocompromised state, as in case of HIV patients undergoing anti-retroviral therapy (ART), withdrawal of iatrogenic immune suppression in transplant patients, and immune system recovery after chemotherapy [1–3]. IRIS was also identified upon withdrawal of natalizumab (α 4 integrin antagonist inhibiting T cell migration in the CNS) in multiple sclerosis patients who had JC virus infection in the CNS, as known as progressive multifocal leukoencephalopathy (PML)-IRIS [4]. There have been already many articles available on IRIS. Yet, a majority of the articles are from clinical settings, and mechanistic understanding of IRIS is still greatly limited. Therefore, although we start the following sections on IRIS in humans, particularly in C-IRIS, the primary focus of this review is on mechanistic aspects of IRIS mainly based on IRIS animal models.

IRIS IN HUMANS

1. IRIS in clinical settings

IRIS is classified into two categories. One is “paradoxical” IRIS, characterized by an exacerbation of a previously diagnosed and treated OI. The other is “unmasking” IRIS, characterized by an excessive inflammatory response to an undiagnosed (subclinical) OI [5, 6]. Multiple risk factors predispose the onset of IRIS, including low CD4⁺ T cell count (<50 cell/ μ l), the presence of OI and cancer before the commencement of ART [6].

IRIS is a diverse disease, and its manifestation varies depending on the type of pathogen involved. The very first case of IRIS was reported in an Australian cohort of immunodeficient HIV patients infected with *Mycobacterium avium* complex (MAC), consisting of *M. avium* and *M. intracellulare*, and treated by the Zidovudine therapy [7]. (MAC rarely causes disease in individuals with a normal immune system.) It is, however, currently known that most common pathogens found in IRIS are *Mycobacterium tuberculosis* (i.e., M-IRIS) and a fungus *Cryptococcus neoformans* (i.e., C-IRIS). Other pathogens include *Pneumocystis*, *Histoplasma* (fungi), as well as viruses, such as Varicella zoster virus, herpes simplex virus, Kaposi’s sarcoma herpes virus, hepatitis virus, cytomegalovirus, and JC virus [8–11]. Since OI often starts from inhalation of pathogens ubiquitous in the environment, pulmonary OI could set a stage of IRIS development in immunocompromised patients. Pathogens that cause pulmonary IRIS includes *M. tuberculosis*, MAC, *C. neoformans*, and *Pneumocystis jirovecii*, with clinical manifestations such as pneumonitis, pulmonary infiltrates, lymphadenopathy, cough, and dyspnea [12, 13]. IRIS associated with *P. jirovecii* appears as unmasking or paradoxical with the worsening of pre-existing *Pneumocystis* pneumonia (PCP). Pulmonary C-IRIS may present as pneumonitis or as cavitary or nodular lesions [13, 14]

Multiple cytokines and chemokines are known to interplay in the pathophysiology of IRIS and could serve as biomarkers for IRIS, in which elevated levels of C- reactive protein (CRP), interleukin (IL)-6, IL-12, TNF α , interferon (IFN)- γ are often observed [15]. Particularly due to high levels of IL-12, TNF α , IFN γ , Th1 responses are considered to associate with IRIS. Other studies suggested that the imbalance between anti-inflammatory T regulatory cells (T_{regs}) and pro-inflammatory Th17 cells may also play a role in IRIS [16, 1, 17]. However, albeit inflammatory, the role of Th17 cells, in IRIS is still largely elusive.

2. *Cryptococcus*-associated IRIS (C-IRIS) in AIDS patients

As an opportunistic fungal pathogen, *C. neoformans* is one of the major pathogens infecting AIDS patients. *C. neoformans* evades the host immune system with a polysaccharide capsule, coating the surface of spores to decrease immunogenicity of the fungus [18]. The infection with *C. neoformans* may be latent, and clinical symptoms may not emerge until the initiation of ART. Several case studies have shown that C-IRIS affects 15–50% of HIV-infected patients with a prior diagnosis of cryptococcosis as paradoxical IRIS [19–24]. The onset of C-IRIS after initiating ART varies from 3 months to 3.5 years [25–27]. A common manifestation of C-IRIS is cryptococcal meningitis (CM), characterized by CNS complications [28]. Clinical presentation of meningitis includes a headache, visual disturbance, vomiting, intracranial cryptococcoma [29–31], increased intracranial pressure [32–34], optical disk swelling [35], and cranial nerve lesions [36, 23]. Non-neurological presentation of C-IRIS involves lymphadenopathy, multifocal disease and pneumonitis, and soft tissue disease [25, 28]. CM causes 15 % of the entire AIDS-related mortality [37].

Several risk factors for C-IRIS are suggested. For example, early initiation of ART (within 14 days of OI treatment) and high HIV loads before ART predispose patients to develop C-IRIS, while another C-IRIS study reported no association between the timing of ART initiation and diagnosis [38]. In addition, as IRIS in general, the C-IRIS incident also correlates with low CD4⁺ T cell counts before ART and high CD4⁺ T cell counts after ART [39, 40, 21, 23]. C-IRIS is known to be associated primarily with Type-1 immune responses [41], characterized by enhanced expression of interferon (IFN)- γ and tumor necrosis factor (TNF)- α , particularly by IFN γ -expressing T helper cells, or Th1 cells [15, 42, 43]. IFN γ activates macrophages and enhances host responses against *C. neoformans* [44, 45].

Development of C-IRIS goes through three phases; pre-ART, early IRIS (immediate after the reconstitution of the immune system) and IRIS [32, 46]. Distinct populations immune cells and their mediators dominate at different stages of IRIS. During the pre-ART phase, the failure to elicit appropriate immune responses, *e.g.*, compromised immune system, results in inefficient clearance of the fungus and predisposes patients to develop C-IRIS [12]. In CM, abnormal cytokine profiles of cerebrospinal fluids (CSF) correlate to early mortality of patients [30, 47].

In the pre-ART phase, reduced levels of IFN γ and TNF α , as well we IL-6 and IL-8, were found in CSF from CM patients who later developed C-IRIS [30, 46, 48]. Here, lower counts of CD4⁺ T cells and total lymphocytes were also reported in CM patients who developed IRIS, compared to those who did not develop IRIS [49–51]. Also, pre-ART serum samples from IRIS patients had low levels of G-CSF, GM-CSF, and VEGF, compared to those who

did not develop IRIS [46, 32, 30]. G-CSF and GM-CSF are required for effector functions of macrophages and neutrophils [52]. At the same time, levels of IL-4 are enhanced in pre-ART sera from C-IRIS patients [32]. IL-4 promotes Th2 immune response and shifts T helper cell differentiation away from Th1. As a result, impaired IFN γ production potentially promotes fungal growth by allowing the proliferation of *Cryptococcus* particularly within macrophages, which are activated by IFN γ [46, 53, 54].

During the early IRIS phase, *i.e.*, immediately after immune re-constitution, sera from patients showed enhanced levels of proteins such as; C-reactive protein (CRP), D-dimer (a fibrin degradation product), IL-6, IL-1RA, IL-13, and G-CSF [32]. Elevated expression of these molecules before the third phase is considered to indicate risk for cryptococcal meningeal IRIS (CM-IRIS) [32].

The IRIS phase is marked by a cytokine storm, where immune responses associated with Type-1 cytokines. IFN γ , IL-6, and TNF α levels are increased in sera [46] and CSF [32, 30] of IRIS patients. In addition to IL-6, CRP was also elevated in serum as CRP is stimulated by IL-6 [55]. In sum, enhanced Type-1 responses are considered to be involved in the pathogenesis of C-IRIS.

Findings from these clinical studies have set the stage for experimental IRIS models, which are discussed in the following sections.

BASIC MECHANISM OF IRIS – UNDERSTANDING FROM ANIMAL MODELS

1. Animal models of IRIS

The development of IRIS requires three distinct components: (1) a compromised immune system, which allows (2) the establishment of an OI, priming innate immunity, and (3) immune reconstitution followed by overreacting immunity, particularly by CD4⁺ T cells. To recapitulate these steps in animal models, a few similar approaches are used (Fig. 1). To create an environment in the animal that results in an impaired (or absent) T cell response, mice with genetic defects in T cell development are used, as in the *Tcra*^{-/-} and *Rag1*^{-/-} mouse lines. These mice are then infected with the pathogen of choice and given an adoptive transfer of CD4⁺ T cells to reconstitute T cells (Fig. 1). These experiments are controlled by comparing readouts to infected animals that do not receive T cell transfers and to uninfected mice that receive only T cell transfers. A rapid weight loss and upregulation of inflammatory cytokines, IFN γ in particular, after T cell transfer have generally been observed [56–59] (Fig. 1). Due to the importance of CD4⁺ T cells in IRIS, many immunological analyses of these models focus on the role and phenotype of CD4⁺ T cells.

2. M-IRIS

The experiments described in this section rely on the *Tcra*^{-/-} mice infected with *M. avium*. – In mice with M-IRIS, CD4⁺ T cells were highly activated (based on CD69) but their proliferation rate was comparable or even lower than mice with T cell transfer without infection [56••]. Thus, T cell counts in mice with M-IRIS were actually lower than mice without previous infection in peripheral blood mononuclear cells (PBMCs), spleen, liver, lung, and bone marrow (BM) [56••]. With the M-IRIS model, the differential role of

classical CD4⁺ T cell subsets (Th1, Th2, and Th17) was evaluated. Mice transferred with IL-4- or IL-17A-deficient CD4⁺ T cells showed no clear difference in IRIS development from mice transferred with WT CD4⁺ T cells, suggesting that Th2 and Th17 cells may not be involved in pathogenesis or protection of M-IRIS, at least [56••] (Table 1). In contrast, IFN γ -deficient CD4⁺ T cells, however, were unable to recapitulate the severe disease with a significantly extended period of survival after the T cell transfer [56••] (Table 1). This was congruent with reduced serum levels of an inflammatory cytokine TNF α in IRIS mice receiving IFN γ -deficient CD4⁺ T cells [56••] (Table 1). These results corroborate human IRIS data suggesting the critical role of Th1 responses. Interestingly, the administration of a TNF α -neutralizing antibody to IRIS mice resulted in a more severe disease phenotype [56••]. To further complicate the involvement of Th1 cells in IRIS, selective transfers of IFN γ -producing Th1 cells from donors into infected mice did not induce any disease symptoms, while transfers of naïve CD4⁺ T cells appeared to induce the most severe disease [56••]. Collectively, this indicates that IFN γ is critical to the development of this form of IRIS but raises the possibility that mechanisms other than Th1 exist in this disease, when considering that the mice that received IFN γ -deficient CD4⁺ T cells eventually did succumb to IRIS. Indeed, in addition to IFN γ , IL-6 is highly associated with M-IRIS in human patients, as well as the mouse model of M-IRIS [57•]. Blockade of IL-6 in mouse M-IRIS delayed disease development, but does not affect the expansion of the transferred T cell populations [57•] (Table 1). Here, IL-6 from the non-hematopoietic compartment was driving disease in mouse M-IRIS, based on the result that the absence of IL-6 produced by the hematopoietic compartment did not alleviate M-IRIS by using irradiation BM chimera [57•]. The study further showed that IL-6 and IFN γ synergistically enhance MIRIS [57•].

T cell receptor (TCR) transgenic (Tg) mice were used to determine whether T cells have to be specific to *M. avium* antigens (Ags) for M-IRIS induction (Table 2). First, T cells that express irrelevant TCR, detecting ovalbumin (OVA) (*i.e.*, OT-II Tg CD4⁺ T cells), could not induce M-IRIS [56••]. However, if the T cells are specific to *M. avium* Ags (*i.e.*, P25 Tg CD4⁺ T cells), M-IRIS was induced [56••]. Thus, T cells detecting irrelevant Ags cannot induce M-IRIS. The next question was whether self Ag-specific CD4⁺ T cells could induce M-IRIS (Table 2). Male *Tcra*^{-/-} recipients transferred with male Ag-specific CD4⁺ T cells exhibited IRIS-mediated mortality, while all the female *Tcra*^{-/-} recipients survived [56••]. Thus, M-IRIS was induced by CD4⁺ T cells specific to either pathogen or host Ags.

Manifestations of IRIS are promoted by previously lymphopenic environments, and the effects of lymphopenia in proliferating T cells is well established. In M-IRIS, however, it seems that lymphopenia itself is not a prerequisite: M-IRIS was successfully induced in OT-II TCR Tg mouse recipients, which possess a normal number of CD4⁺ T cells but all of which are specific to OVA. In contrast, when M-IRIS was induced in P25 TCR Tg recipients, the disease was very mild [56••], likely due to the host's successful protective response to the pathogen before T cell transfer. The results suggested, rather than lymphopenia, the absence of T cells that can recognize *M. avium* is a factor to trigger M-IRIS.

Taken together, the results suggested that M-IRIS is triggered when hosts cannot mount pathogen-specific T cell responses before immune reconstitution and reconstituted CD4⁺ T

cells are either pathogen- or host Ag-specific. Based on the experimental study [56•], a model of the IRIS pathology was provided as: The lack of pathogen-specific CD4⁺ T cells causes uncontrolled pathogen growth and triggers excessive innate immunity, which sets a stage for hyper-responses by reconstituted T cells [60].

3. C-IRIS

As stated above, the mouse model of C-IRIS is induced similarly. It relies on the use of mice deficient in adaptive immune cells (*Rag1*^{-/-} mice) that are infected with low inocula of *C. neoformans* [59•] (Fig. 1). These mice are then transferred with CD4⁺ T cells obtained from naïve mice 3–4 weeks post-infection. Transferred T cells do not protect hosts from *C. neoformans* infection, as they do not alter fungal burden as compared to infected mice without T cell transfer. Instead, this T cell transfer increased levels of inflammatory cytokines in the serum – namely TNFα, IL-6, and IFNγ [59•]. This mouse model of C-IRIS also manifested an inflammatory phenotype in the CNS, consistent with the neurotropism of *C. neoformans* and meningitis commonly seen in such infected patients. This is typified by increased expression of a gene encoding a chemokine CXCL10 in the brain, the subsequent infiltration of CD4⁺ T cells into the CNS parenchyma, and activation of microglia [59•].

The phenotype of the transferred CD4⁺ T cells in C-IRIS closely matches that of the T cells in M-IRIS: No definitive differences in proliferation compared to a simple T cell transfer to naïve immunodeficient mice, but an increase in the activation marker, CD69, only in the IRIS condition [56•, 59•]. Distinct from M-IRIS, however, the absence of IFNγ in transferred CD4⁺ T cells did *not* alter the disease severity, shown as a comparable survival curve to that of WT CD4⁺ T cell-transferred recipients [59•, 56••] (Table 1). The hosts transferred with *Ifng*^{-/-} T cells also did not show a statistically significant reduction in serum TNFα and IL-6 levels, as well as the proportion of active T cells, shown as CD69-positive [59•] (Table 1). The result suggests that, as described in mouse M-IRIS, particularly the role of IFNγ in IRIS is not definitive, although it is still possible that titrations of inocula, numbers of transferred T cells, and the length of the pre-IRIS period could change the outcomes.

4. *Pneumocystis* IRIS

Pneumocystis carinii is a fungal pathogen that commonly infects chronically-infected HIV patients and can induce IRIS. *Pneumocystis* IRIS is the first characterized animal IRIS among all kinds of animal IRIS. The first discovery was reported in early 90's and describing severe lung inflammation, and morbidity in Severe Combined Immune Deficient (SCID) mice reconstituted with WT bone marrow (BM) cells [61]. It turned out that the mice had a spontaneous infection by *P. murina*. The report did not use the word "IRIS" yet, but it can be considered as an animal case of "unmasking IRIS." (Although the article [61] described the fungus as *P. carinii*, the current nomenclature defines *Pneumocystis* infecting mice as *P. murina* [62].) Indeed, it appears that even animals in specific pathogen-free (SPF) facilities could have spontaneous *Pneumocystis* infection, though largely subclinical in most of the cases [63], because *P. murina* is a common environmental microbe and not included in the list of SPF pathogens. However, severely lymphopenic mice cannot clear *P. murina* and the infection could become apparent under certain circumstances [61, 63]. In the study with

BM cell transfer [61], *P. murina* was not cleared even after the inflammation resolved, in contrast to the other mouse models of IRIS. *Pneumocystis* IRIS in SCID mice were found to be induced by CD4⁺ T cells, not CD8⁺ T cells, similar to the other mouse models of IRIS [64•, 65] (Fig. 1). More recently, *Rag2*^{-/-} mice were used to study *Pneumocystis* IRIS [66•, 58] and displayed increased levels of IFN γ , as well as TNF α and chemokines, CCL2 and CCL5, in the bronchoalveolar lavage fluid during IRIS [58]. Interestingly, however, the deficiency of IFN γ receptor (IFNGR) in the *Rag2*^{-/-} hosts was actually detrimental to *Pneumocystis* IRIS, characterized by poor function and immune cell infiltration in the lung, in addition to significantly high levels of IFN γ in the lung homogenates [66•] (Table 1). The result is interpreted that, although IFN γ is considered to be a pathological cytokine in IRIS, IFNGR signaling in recipients is actually protecting hosts. As suggested in the mouse M-IRIS model [60], it is possible that IFNGR in myeloid cells are critical to control pathogen loads and to prevent IRIS before immune reconstitution. The study suggested that IFN γ and IFNGR somehow mediate the involvement of Foxp3-positive CD8⁺ T cells, which are considered to be a suppressor (*i.e.*, anti-inflammatory) population, in restricting pathogenic outcomes of IRIS [66•]. It will be interesting if adoptive transfer of Foxp3⁺CD8⁺ T cells would ameliorate *Pneumocystis* IRIS. In addition, it will be also of interest to know whether Foxp3⁺CD4⁺ regulatory T cells are involved in controlling IRIS.

CONCLUSION

Over the period, there have been several reports on IRIS triggered by undergoing retroviral therapy, immune reconstitution, or withdrawal of immunosuppressant; irrespective of underlying pathogens. Nevertheless, the lack of understanding of the IRIS pathology makes it difficult to establish methods to predict, diagnose, and treat the disease. Many questions cannot be answered by clinical observations alone and require animal models. For example, to clarify the IFN γ conundrum, the IRIS pathology needs to be dissected to cell type-specific and pathogen-specific experimental settings. It will also be important to ask an anti-inflammatory aspect of host system during IRIS because the majority of studies has been focusing on a pro-inflammatory aspect of IRIS. In addition, although IRIS could be systemic, each pathogen has its tropism to certain organs. Each organ or tissue provides its unique microenvironment, which greatly impacts tissue-resident cells. Thus, depending on pathogens and their tropism, distinct immune responses are likely to occur. Since a majority of IRIS occurs under iatrogenic settings, it is possible that more cases of IRIS emerge in future. The effort to dissect the mechanism of IRIS has just started, and there are limited reports yet to be available. Due to the complexity of IRIS, animal models are essential to progress our understanding of the pathology of IRIS.

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Figure 1. Protocols and outcomes of three mouse IRIS models. The upper panel indicates experimental protocols of mouse IRIS. The bottom panel shows results from M-IRIS [56, 57], C-IRIS [59], Pneumocystis-IRIS [66].

Table 1

Results from IRIS experiments using cytokine- or cytokine receptor-deficient T cells and mice

M-IRIS	<ul style="list-style-type: none"> • <i>Ifng</i>^{-/-} CD4⁺ T cells → <i>Tcra</i>^{-/-} recipient [56, 57] Mortality and weight loss delayed, dead by day 57 [56] Reduced serum IFN-γ, TNF-α, and nitrate [56] IL-6 similar [57] • <i>Il4</i>^{-/-} or <i>Il17a</i>^{-/-} CD4⁺ T cells → <i>Tcra</i>^{-/-} recipient [56] Mortality and weight loss similar • WT CD4⁺ T cells → <i>Tcra</i>^{-/-} recipient+ anti-IL-6 neutralizing antibody [57] Mortality and weight loss delayed IFN- γ similar
C-IRIS	<ul style="list-style-type: none"> • <i>Ifng</i>^{-/-} CD4⁺ T cells → <i>Rag1</i>^{-/-} recipient [59] Weight loss and granulomatous inflammation in liver similar IL-6, TNF-α, CD69 similar Reduced serum IFN-γ No up-regulation of MHC-II on microglia
<i>Pneumocystis</i>-IRIS	<ul style="list-style-type: none"> • WT CD4⁺T cells → <i>Rag2</i>^{-/-}<i>Ifngr</i>^{-/-} recipients [66] Slightly higher fungal burden Weight loss milder Higher respiratory rates and pulmonary function deficits Higher inflammatory cell infiltration in the lung Higher IFN-γ in lung homogenates CD8⁺Foxp3⁺ Treg cells absent • WT CD4⁺T cells → <i>Il4ra</i>^{-/-}<i>Rag2</i>^{-/-} recipients [66] Weight loss milder Pulmonary function deficits similar Inflammatory cell infiltration in the lung similar IFN-γ in lung homogenates similar CD8⁺Foxp3⁺ Treg cells present

All the observations are comparative to relevant controls.

References are indicated in square brackets.

Table 2Results from IRIS experiments using antigen-specific CD4⁺ T cells

TCR Tg CD4 ⁺ T cells	→ <i>Tcra</i> ^{-/-} recipients [56]
OTII (OVA sp.)	No mortality (by day 10) No weight loss
P25 (Pathogen Sp.)	Lethal Higher serum nitrate and TNF-α. Higher inflammatory monocytes, neutrophils in peripheral blood and lungs
Marilyn (Male Ag Sp.)	♂ - Wasting, lethal Reduced IFN-γ Higher nitric oxide production ♀ - No mortality (by day 15)

All the observations are comparative to relevant controls.