Infections due to carbapenem-resistant Klebsiella pneumoniae have emerged as a global threat due to its wide-spread antimicrobial resistance. Transplant recipients and patients with hematologic malignancies have high mortality rate suggesting host factors in susceptibility. We developed a model of pulmonary infection using ST258 C4, KPC-2 clone, which are predominant Klebsiella pneumoniae carbapenemase (KPC)-producing bacteria, and demonstrated that $\text{Rag}^2^{-/-}\text{Il2rg}^{-/-}$ mice, but not wildtype C57BL/6 or $\text{Rag}^2^{-/-}$ mice, were susceptible to this opportunistic infection. Using single-cell RNA-seq in infected $\text{Rag}^2^{-/-}$ mice, we identified distinct clusters of $\text{Ifng}^+$ NK cells and $\text{Il17a}^+, \text{Il22}^+$, and inducible T-cell costimulatory molecule (ICOS)$^+$ group 3 innate lymphoid cells (ILCs) that were critical for host resistance. As solid organ transplantation is a risk factor, we generated a more clinically relevant model using FK506 in wildtype C57BL/6 mice. We further demonstrated that immunotherapy with recombinant IL-22 treatment ameliorated the ST258 pulmonary infection in both FK506 treated WT mice and $\text{Rag}^2^{-/-}\text{Il2rg}^{-/-}$ mice via hepatic IL-22ra1 signaling. These data support the development of host directed immunotherapy as an adjunct treatment to new antibiotics.
Host immunology and rationale immunotherapy for carbapenem-resistant *Klebsiella pneumoniae* infection

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Abstract

Infections due to carbapenem-resistant *Klebsiella pneumoniae* have emerged as a global threat due to its wide-spread antimicrobial resistance. Transplant recipients and patients with hematologic malignancies have high mortality rate suggesting host factors in susceptibility. We developed a model of pulmonary infection using ST258 C4, KPC-2 clone, which are predominant *Klebsiella pneumoniae* carbapenemase (KPC)-producing bacteria, and demonstrated that *Rag2*\(^{-/-}\)Il2rg\(^{-/-}\) mice, but not wildtype C57BL/6 or *Rag2*\(^{-/-}\) mice, were susceptible to this opportunistic infection. Using single-cell RNA-seq in infected *Rag2*\(^{-/-}\) mice, we identified distinct clusters of *Ifng*+ NK cells and *Il17a*, *Il22*, and inducible T-cell costimulatory molecule (ICOS)+ group 3 innate lymphoid cells (ILCs) that were critical for host resistance. As solid organ transplantation is a risk factor, we generated a more clinically relevant model using FK506 in wildtype C57BL/6 mice. We further demonstrated that immunotherapy with recombinant IL-22 treatment ameliorated the ST258 pulmonary infection in both FK506 treated WT mice and *Rag2*\(^{-/-}\)Il2rg\(^{-/-}\) mice via hepatic IL-22ra1 signaling. These data support the development of host directed immunotherapy as an adjunct treatment to new antibiotics.
Introduction

*Klebsiella pneumoniae* (*K. pneumoniae*) is one of the leading pathogens in healthcare-associated infections worldwide. The emergence of carbapenem-resistant *K. pneumoniae* has been globally recognized as a threat due to its rapid increase and limited therapeutic options (1-3). *K. pneumoniae* consists approximately 90% of carbapenem-resistant Enterobacteriaceae (CRE), and 92% of carbapenem-resistant *K. pneumoniae* produce the carbapenemase in the U.S., which is occupied almost entirely by sequence type 258 (ST258) (4, 5). Carbapenemases are the major cause of expanding resistance, whose genes can easily be transmitted by plasmids, and often confer the resistance to other classes of antimicrobials including fluoroquinolones and aminoglycosides (6). ST258 is identified by multi-locus sequencing of housekeeping genes and has obtained resistance to almost all classes of antibiotics (7). Clinically, this multidrug-resistant pathogen has represented a significant and continued threat to patients, especially in patients with high prevalence of prior hospitalizations, and a discharge to long-term care setting (8, 9), and is often associated with a high mortality rate (10). In a multicenter study in New York/New Jersey hospitals, 50% of patients with CRE bacteremia had cancer or past-history of transplantation implicating host factors as important risk factors for the infection (4). Interestingly, recent epidemiology have reported a close relationship between ST258 infection and solid organ or stem cell transplant recipients (1).

As further evidence of the opportunistic nature of ST258, this pathogen has been reported to be virtually avirulent for immunocompetent animals and highly susceptible to serum killing in vitro (11). Understanding of the immunological mechanisms of this opportunistic infection is indispensable in exploring counter-measures against this infection and would allow for the development of innovative treatments. Importantly, due to the limitations of small molecule antibiotics, alternative therapies should be considered. For example, various reports suggest that using antibody for the enhancement of complement mediated bactericidal activity
(7, 12) may be effective against this pathogen. Interestingly, Xiong et al. reported that there are differing requirements for ST258 versus more virulent strains of *K. pneumoniae*. For ST258 infection, the role of CCR2+ monocytes were essential whereas neutrophils were dispensable (13).

We initially took a genetic approach using *Rag2<sup>−/−</sup>*<sup>Il2rg<sup>−/−</sup> mice and *Rag2<sup>−/−</sup>* mice to understand critical host factors for ST258 infection. Single-cell RNA sequencing (scRNAseq) revealed that *Rag2<sup>−/−</sup>* mice were able to recruit an IFN<sub>γ</sub><sup>+</sup> NK cell population and ICOS<sup>+</sup> IL-17A<sup>+</sup> IL-22<sup>+</sup> group 3 ILCs (ILC3), and both populations were required for resistance to the infection in the *Rag2<sup>−/−</sup>*<sup>Il2rg<sup>−/−</sup> background. We next developed a clinically relevant model using FK506, a drug used to manage transplant rejection, and found that this drug renders wild type C57BL/6 mice susceptible to ST258 C4 infection and was associated with reduction in *Il17a*, *Il22*, and *Ifng* gene expression in the lung. Finally, we confirmed the capability of IL-22:Fc to rescue both the genetic and pharmacological model through IL-22ra1 signaling in liver. Thus, these data show that lymphoid cell populations expressing type 1 and type 17 cytokines mediate host resistance to infection and that recombinant IL-22 can improve host defense against this opportunistic infection via hepatic IL-22ra1 signaling.
Results

**Il2rg-dependent cells are required for host resistance to ST258 Infection**

To determine host factors that are required for host resilience to this infection, we developed a model of pulmonary infection using ST258 strain C4, a KPC-2-producing clone which was isolated from bronchoalveolar lavage fluid of a lung transplant patient in 2010 (BioSample: SAMN06445930; SRA: SRS2000639). Rag2\(^{-/-}\)Il2rg\(^{-/-}\) mice, which lack T, B, NK and innate lymphoid cells, showed substantially greater bacterial burdens in the lung compared to wild type C57BL/6 and Rag2\(^{-/-}\) mice at a dose of \(10^6\) CFU ST258 C4 (Figure 1A). Moreover, these mice showed greater bacterial dissemination to the liver (Figure 1B) and spleen (Figure 1C), and a significant trend toward increased mortality (Figure 1D) and weight loss (Figure 1E). There were no significant differences between C57BL/6 mice and Rag2\(^{-/-}\) mice, which suggested that adaptive immunity was not required for initial ST258 C4 pulmonary infection within the observed period. Based on these data, we designed an experiment where we infected resistant Rag2\(^{-/-}\) and susceptible Rag2Il2rg\(^{-/-}\) mice with \(10^6\) CFU ST258 C4, euthanized them at 12 hours and harvested lung tissue for bacterial burden and the other lobe for RNA extraction. At this time point, there were no differences in CFU (Figure S1) so any differences in gene expression would not be due to differences in lung bacterial burdens. Bulk RNAseq analysis showed that Rag2Il2rg\(^{-/-}\) mice had substantial reduction of genes associated with NK cells (Ifng, Gzma, Gzmb, Gzmk) and ILC3 (Il17a, Il17f, Il22) (Figure 1F, Table 1), and significantly downregulated KEGG pathways including cytokine-cytokine receptor pathways (Table S1A). These mice also showed several overexpressed genes (Figure 1F, Table 2), and pathway analysis also elucidated upregulated KEGG pathways. (Table S1B). To confirm the down regulated genes at the single cell level we performed scRNAseq in infected Rag2\(^{-/-}\) mice which showed distinct clusters of Il17a\(^{+}\), Il22\(^{+}\), and Icos\(^{+}\) ILC3 (Figure 2A) and Ifng\(^{+}\) NK cells (Figure 2B) confirming that these cytokines are expressed by distinct cell populations in the lung. As ICOS expression
was largely restricted to the ILC3 cluster, we confirmed that ICOS surface expression was detectable in ILC3 cells in the ST258 infection model (Figure 2C, 2E). Moreover, we observed an increase of Rorgt+ cells in the lung 12 hours post ST258 C4 infection in Rag2−/− mice by FACS (Figure 2D, 2F). RNAseq data was also confirmed by real time RT-PCR on ILCs and NK cells in naïve Rag2−/− and infected Rag2−/− mice. These data demonstrated a significant increase in lung Il17a (Figure S2A), Il17f (Figure S2B), Il22 (Figure S2C), Ifng (Figure S2D), and Rorc (Figure S2E) gene expression in infected Rag2−/− mice. Additional analysis of the scRNAseq data demonstrated that Icos+Il17a+ (Figure S3A) cell cluster also expressed Il22 (Figure S3B), Ccr6 (Figure S3C), Rora (Figure S3D), and Rorc (Figure S3E). We also detected Ncr1 (Figure S3F) expression in a subgroup of Il17a+Rorc+ cells but most of the Ncr1 expression was clustered in the NK cell cluster. The relative lack of Ncr1 expression might be due to a lack of transcriptome coverage in the ICL3 population (14). Also, we could detect an ILC2 population in infected Rag2−/− mice (Figure S3G), while the increase of GATA3 producing cells in lung of infected Rag2−/− mice was not observed by FACS (Figure 2D, 2G). Thus, Il2rg dependent ILC3 and NK cells might be essential for the ST258 C4 infected Rag2−/− mice.

**Requirement of NK cells and group 3 ILCs for host resistance to ST258 infection**

To determine the relative contributions of NK cells versus ILC3 to host resistance, we first depleted NK cells in Rag2−/− mice with anti-NK1.1 antibody. Anti-NK1.1 antibody administration 2 hours prior to infection completely depleted NK cells in the lung as measured by Klrb1c gene expression (Figure 3A) and reduced Ifng gene expression (Figure 3B) to essentially undetectable levels. NK cell depletion had no effect on the expression of Il17a, Il22, or Rorc (Figure 3C, D, and E). Interestingly, NK cell depletion did not perturb bacterial clearance (Figure 3F). These data suggested that NK cells were dispensable for host defense against ST258 C4 K. pneumoniae pulmonary infection. As mentioned above, mining our scRNAseq data we
determined that ICOS expression was confined largely to the ILC3 cluster and absent in the NK cell cluster (Figure 2A, 2B). To determine if ICOS was required for ILC3 activation we administered 500μg of a neutralizing anti-ICOS antibody via intraperitoneally 2 hours prior to ST258 C4 pulmonary infection. Neutralization of ICOS substantially reduced Il17a, Il17f, and Il22 expression (Figure 4A, 4B, 4C) but resulted in retained expression of Klrb1c and Ifng gene expression (Figure 4D, 4E), demonstrating an essential role of ICOS in the ILC3 response but not the NK cell response. However, like NK1.1 depletion, anti-ICOS antibody did not perturb bacterial clearance (Figure 4F). Anti-ICOS treated animals showed a small decrease in lcos gene expression by real time RT-PCR (Figure S4A), and thus in addition to neutralization, anti-ICOS mAb may also affect the number of ICOS+ cells. To determine if anti-ICOS affected ILC2 activation, we assessed Il5 and Prg2 gene expression in whole lung. In contrast to reduced Il17a and Il22 expression with in vivo anti-ICOS mAb, neutralization of ICOS did not affect Il5 gene expression (Figure S4B), or Prg2 gene expression (a marker of eosinophils, Figure S4C) (15). Moreover FACS analysis showed no decrement of IL-5+ICOS+ cells (Figure S4D, S4F) in infected lungs of anti-ICOS treated Rag2−/− mice. In contrast, IL-17A+ICOS+ cells were decreased (Figure S4E, S4G), suggesting the anti-ICOS mAb selectively suppressed ILC3 cells at this time point in infection. As we observed a reduction in lcos gene expression in the lungs of infected anti-ICOS treated Rag2−/− mice, we hypothesized that ICOS may provide a proliferative signal to ILC3 cells in the model. Ex vivo cultures of lung ILC cells (obtained 12 hours post infection in Rag2−/− mice) with recombinant ICOSL showed enhanced proliferation of lung ILC3 (Rorgt+ cells, Figure 4G, 4J), but no effect was observed in lung ILC2 cells (GATA3+ cells, Figure 4H, 4K) or lung ILC1 cells (NK1.1+ cells, Figure 4I, 4L).

However, when both anti-NK1.1 and anti-ICOS antibody were administered in this infection model, we observed reduction of gene expression in both type 17 (Figure 5A, 5B, 5C) and type 1 (Figure 5D, 5E) cytokines, resulting in exacerbation in lung infection (Figure 5F).
These data suggest a cooperativity of NK cells and ICOS+ ILC cells in ST258 clearance. In addition to Ifng, the scRNAseq data showed that NK cells also express Xcl1 and Ccl5, which was confirmed by real-time RT-PCR (Figure 5G, 5H). CCL5 can be chemotactic for dendritic cells (16), and XCL1 has been also reported to have some activity on neutrophils (17), so these chemokines may have an additional role in host immunity in this model.

**FK506 increases host susceptibility to ST258 infection**

The genetic models have allowed us to identify key cellular events that are critical for host resistance against ST258 *K. pneumoniae* pulmonary infection; however, they do not mimic important clinical factors that may increase host susceptibility. To develop a more clinically relevant model we investigated if FK506, a clinically used calcineurin inhibitor, could be used to model susceptibility to infection in solid organ transplant recipients, like the patient from whom this strain C4 was identified. In these experiments, C57BL/6 mice were administered 10 mg/kg FK506 via intraperitoneally 24 hours prior to ST258 infection, and every 24 hours until 24 hours before euthanasia. FK506, in contrast to vehicle control, substantially reduced the gene expression of Il17a, Il17f, Il22, Ifng (Figure 6A, 6B, 6C, 6D), and exacerbated bacterial burden in the lung 48 hours post infection (Figure 6E). To determine cellular sources of cytokines in this model, we performed scRNAseq comparing the FK506 treated C57BL/6 mice and WT mice at 24 hours post infection with 10^6 CFU ST258 C4. There was no significant difference of CFU at 24 hours, so we conducted scRNAseq at this time point to perform an unbiased comparison of gene expression. The violin plots from Seurat of scRNAseq data showed that FK506 treatment reduced both type 1 and type 17 cytokine genes including Il17a, Il17f, Il22, Ifng (Figure 6F, 6G, 6H, 6I), but not Klrb1c (the major expresser of Ifng) and Tcrg (the major expresser of Il17a, Il22) (Figure 6J, 6K). Similarly, FACS analysis also showed that the absolute number of NK cells and γδ cells at 24 hours post 10^6 CFU ST258 C4 pulmonary infection was modestly decreased in
FK506 treated C57BL/6 mice, but this was not statistically significant (Figure 6L, 6M). Additionally, the Klrb1c and Tcrg gene expression were not so changed (Figure 6N, 6O). TSNE plots from the scRNAseq data in ST258 C4 infected C57BL/6 mice with FK506 treatment showed that Ifng expression was mostly observed in NK cells (Figure S5A), whereas Il17a and Il22 expressing cells were clustered in γδ T-cells (Figure S5B). Pathway analysis was also performed, and significant KEGG pathways are shown (Table S2). To examine if FK506 also affected ILC responses, we performed an ex vivo analysis using lung ILCs from Rag2−/− mice (obtained 12 hours post infection with ST258 C4) and incubated these cells with vehicle or FK506. FK506 showed an inhibitory effect on proliferation in ILC3 (Rorgt+ cells, Figure 7A, 7D), but not in lung ILC2 cells (GATA3+ cells, Figure 7B, 7E) and ILC1 (NK1.1+ cells, Figure 7C, 7F). Moreover, FK506 suppressed the phorbol 12-myristate 13-acetate (PMA) elicited type 1, type 2, and type 17 cytokines secretion in lung ILCs ex vivo in a cell intrinsic manner (Figure 7G, 7H, 7I, 7J, 7K, 7L). In addition, we examined the effect of FK506 on myeloid cells. FK506 treatment in Rag2−/−Il2rg−/− mice showed a slight exacerbation in lung CFU, but this was not statistically significant (Figure S6A). Moreover bacterial dissemination to the liver and spleen of FK506 treated Rag2−/−Il2rg−/− mice was similar to untreated Rag2−/−Il2rg−/− mice (Figure S6B, S6C). These data suggest that the major effects of FK506 are on T-cells and ILC3 cells. In support of this, scRNAseq data in ST258 C4 infected Rag2−/− mice showed about 8.1% in Ly6g+ cells (Figure S6D) and 60.7% in CD68+ cells (Figure S6E) express Fkbp1a+ the target of FK506. In contrast, 98.7% in Rorc+ cells and 57.9% in Klrb1c+ cells expressed Fkbp1a by scRNAseq.

**IL-22 but not IFNγ rescues pulmonary immunity to ST258 infection**

To determine the potential therapeutic benefit of cytokine immunotherapy, we examined if local IFNγ or systemic IL-22 could be used as immunotherapy. Recombinant IFNγ was ineffective in
augmenting host resistance to infection (Figure 8A) despite evidence of its bioactivity as measured by CXCL10 induction (Figure 8B)(18, 19). In contrast, recombinant IL-22:Fc administration 2 hours prior to infection to FK506 treated C57BL/6 mice substantially reduced bacterial burden in the lung (Figure 8C). IL-22:Fc was also effective in reducing bacterial burden in Rag2\(^{-/-}\)Il2rg\(^{-/-}\) mice (Figure 8D). IL22ra1 is expressed on airway epithelium (20) and on hepatocytes (21). To determine if hepatocyte signaling was required we examined the effect of IL-22:Fc in mice with a conditional deletion of Il22ra1 in hepatocytes (22). In contrast to AlbCre\(^{+/−}\) x Il22ra1\(^{+/−}\) (Il22ra1\(^{+/−}\)) mice, IL-22:Fc effects on bacterial control in the lung and spleen was substantially abrogated in AlbCre\(^{+/−}\) x Il22ra1\(^{+/−}\) (Il22ra1\(^{Δ\text{HEP}}\)) mice (Figure 8E, 8F, 8G)(22, 23).

Furthermore, we performed an experiment administering 1μg IL-22:Fc via intratracheally once 30 minutes before infection on FK506 treated mice. Intratracheal administration of IL-22:Fc also ameliorated infection as measured by bacterial CFU in Il22ra1\(^{+/−}\) mice, but the efficacy again was abrogated in Il22ra1\(^{Δ\text{HEP}}\) mice, suggesting the efficacy might be attributed to the systemic circulation (Figure S7). We next assessed serum killing or growth inhibition of ST258 C4 ex vivo. We observed that serum from parenteral IL-22:Fc treated C57BL/6 mice could inhibit the growth of ST258 C4 (Figure 9A) and this effect was lost with heat inactivation consistent with complement. (Figure 9B). The bacteriostatic effect by IL-22:Fc treatment was diminished in C3\(^{-/-}\) mice compared to the WT mice (Figure 9C). With regard to these results, we could speculate the efficacy was generated partially by C3 activity. In confirmation of this, IL-22:Fc treated serum in Il22ra1\(^{+/−}\) mice showed greater C3 deposition by FACS on ST258 C4 post co-incubation ex vivo, but the efficacy was abrogated in Il22ra1\(^{Δ\text{HEP}}\) mice (Figure 9D, 9E). These data suggest the IL-22 mediated augment of the immunity to ST258 pulmonary infection is largely dependent on hepatocytes, especially via acceleration of C3 deposition.
Discussion

We show that \textit{Rag2}$^{/-}$\textit{Il2rg}$^{/-}$ mice were susceptible to ST258 C4 pulmonary infection while \textit{Rag2}$^{/-}$ and WT C57BL/6 mice were resistant to the infection. Both \textit{Rag2}$^{/-}$ and \textit{Rag2}$^{/-}$\textit{Il2rg}$^{/-}$ mice have mutations in the \textit{rag} gene and they cannot form mature B or T cell receptors (24). Additionally, \textit{Rag2}$^{/-}$\textit{Il2rg}$^{/-}$ mice also have deletions in the \textit{Il2rg} gene which causes this model to lack ILCs, NK cell, and functional receptors for many cytokines such as IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (25). Interestingly, bulk RNAseq of the ST258 C4 infected lungs shows that \textit{Rag2}$^{/-}$\textit{Il2rg}$^{/-}$ mice lack both NK cells and ILCs, and scRNAseq attests the distinct clusters of \textit{Il17a}$^{+}$, \textit{Il22}$^{+}$, and \textit{Icos}$^{+}$ ILC3 (Figure 2A) and \textit{Ifng}$^{+}$ NK cells (Figure 2B) in ST258 C4 infected lungs in \textit{Rag2}$^{/-}$ mice. Considering early in infection that the majority of IL-17 and IL-22 are produced by $\gamma\delta$ T cells and ILC3 (26, 27), there might be a substantial role for the ILC3 in protection in \textit{Rag2}$^{/-}$ mice. Similar data were reported in lung infection due to \textit{Streptococcus pneumoniae} (28); thus, ILC3 could be identified as a major producer of IL-22, possibly contributing to early host defense against these pathogens. Concomitantly, several genes in the NK chemotaxis and cytotoxicity pathway were also deficient in the \textit{Rag2}$^{/-}$\textit{Il2rg}$^{/-}$ mice and thus these cells also contribute to host resilience to this infection in \textit{Rag2}$^{/-}$ genetic background.

ICOS is one of the CD28 family of costimulatory molecules and highly expressed on the activated T cell (29, 30), especially type 2 helper T cells (31) as well as ILC2 (32), contributing to the isotype switch and germinal center formation (33). However, in this infection model on the \textit{Rag2}$^{/-}$ background it is also a key regulator of ILC3. Our data are also consistent with data we recently reported that showed ICOS was highly expressed in lung ILC3s during \textit{Mycobacteria tuberculosis} infection (34), however the functional role of ICOS was not studied in that paper. Further supporting our observation that ICOS is a regulator of ILC3, recombinant ICOSL enhanced proliferation of ILC3 ex vivo (Figure 4G, 4J).
Considering the relationship between IL-17 or IL-22 producing ILC3 and IFNγ producing NK cells during innate immunity, which cell populations are responsible for the immunity against ST258 C4 pulmonary infection remains to be determined. Accumulating evidence has elucidated that IFNγ, mainly produced by activated NK cells during *K. pneumoniae* infection, plays a critical role in models of infection with more virulent carbapenemase negative strains of *K. pneumoniae* (35, 36). Moreover, several reports have shown the additional requirements of IL-17 (37, 38) and IL-22 (28, 39, 40) in these models as well. In contrast, in the model developed with ST258, NK cells by themselves were dispensable. It was only in the setting of NK cell depletion and antagonism of ILC activation using anti-ICOS antibodies that Rag2−/− mice showed abnormal bacterial clearance. Our scRNAseq data in infected Rag2−/− mice also revealed that *Icos* expression was restricted mostly to the *Il17a*, *Il22* ILC3 cluster, which was well separated from *Ifng*+ NK cells, suggesting the co-operative role resistant to ST258 infection.

Given the risk of solid organ transplantation and this infection, we investigated if calcineurin inhibitors would perturb bacterial clearance. To test this, we administered FK506 in WT C57BL/6 mice. FK506 is an immunosuppressant prevalent in the clinical setting, similar to cyclosporine A, especially for the prevention of allograft rejections in patients undergoing solid organ or stem cell transplantation. This drug inhibits the expression of IL-2 (41) and subsequent T cell proliferation (42) through blocking activation of the calcineurin pathway (43). Our data show that administration of FK506 renders WT C57BL/6 mice susceptible to ST258 infection, similar to infection in Rag2−/−Il2rg−/− mice, and real time RT-PCR elucidated the downregulation of *Il17a, Il17f, Il22* and *Ifng*; however, the cellular source of these cytokines in WT C57BL/6 mice was unclear. scRNAseq showed that although FK506 had little effect on the recruitment of NK cells or γδ T cells in the lungs, *Ifng, Il17a, Il17f*, and *Il22* expressing cells were all significantly decreased. Furthermore, *Ifng* expression was largely confined to NK cells whereas most of *Il17a*
and Il22 expressing cells mostly clustered in γδ T-cells. Interestingly local IFNγ was ineffective in augmenting bacterial clearance in FK506 treated C57BL/6 mice (Figure 8A) whereas IL-22:Fc could ameliorate the ST258 pulmonary infection in both Rag2−/−Il2rg−/− mice (Figure 8D) and FK506 treated C57BL/6 mice (Figure 8C). IL-22 primarily acts on epithelial cells to promote barrier function, such as enhancing the production of antimicrobial peptides (39). To date, there have been several reports demonstrating the effect of IL-22 in augmenting host resistance to infection in part by inducing antimicrobial proteins such as lipocalin 2 (39, 44) and by augmenting hepatic C3 expression (45, 46). Meanwhile, ST258 isolates could be killed in healthy human serum, suggesting the prominent role for complement system against ST258 bacteremia (7). Based on this, there have been some reports showing an antibody-based approach via complement-mediated bactericidal activity against this multidrug-resistant pathogen (47). We observed the effect of IL-22:Fc was abrogated in Il22ra−/−HEP mice (Figure 8E, 8F, 8G), suggesting the essential role of hepatocytes for the effect of IL-22:Fc to ameliorate the ST258 infection. Moreover, intratracheal administration of IL-22:Fc also showed efficacy in ameliorating ST258 C4 infected Il22ra1fl/fl mice, but the efficacy was abrogated in Il22ra1−/−HEP mice (Figure S7). Based on these data, hepatic IL-22ra1 signaling is essential for IL-22:Fc in this model. However, we cannot exclude a role of IL-22ra1 signaling in lung epithelium at this stage. The dominant role of hepatic IL-22ra1 in vivo is likely related to the pharmacological amount of IL-22:Fc provided, the large amount of cardiac output to the liver, as well as the fenestrated endothelium of the liver which permit greater accessibility of IL-22 to hepatocytes. One mechanism underlying IL-22’s efficacy of an antibacterial effect in Il22ra1fl/fl mice is, at least partially, augmented in complement mediated protection (Figure 9A, 9B, 9C, 9D, 9E).

While we showed detailed data in ST258 pulmonary infection, there are several limitations to our investigation. We focused on NK cell and ILC genes in this study as they were
markedly different between *Rag2*<sup>+</sup> and *Rag2Il2rg*<sup>-/-</sup> mice but we also observed downregulation of *Ccr2* and *Nos2* in these data that suggest that monocyte recruitment may also be perturbed in *Rag2Il2rg*<sup>-/-</sup> mice and these cells have previously been described as important in a higher dose infection model (13). However, we did not observe differential expression of *Ccr2* or *Nos2* in the FK506 model (in our scRNAseq data). Second, we observed a distinct population of NK cells and ILC3 by scRNAseq, and the antibodies to each population clearly downregulate the signature genes of each population. However, again, this data was generated using *Rag2*<sup>-/-</sup> mice, so the protection by NK and ILC3 cannot be necessarily applied to immunocompetent mice where most *Il17a* and *Il22* is expressed by γδ T cells. FK506 likely exacerbates infection by inhibiting these responses but this would require additional experiments to define all of the cell populations that may be affected by this drug. Third, anti-ICOS antibodies showed that *Il17a* expression was substantially reduced (Figure 4A), but *Il22* expression was only reduced by approximately 50% (Figure 4C). Considering that only IL-22:Fc could restore protective immunity in these immunocompromised hosts, this residual ICOS independent IL-22 producing population may play a key role in immunity, and that may explain why anti-NK1.1 and anti-ICOS treated mice exacerbate but not as severe as *Rag2Il2rg*<sup>-/-</sup> mice that lack IL-22 completely. This concept might be inconsistent to the prior report by Ahn, et al showing that ST258 infection was similar in WT and *Il22*<sup>-/-</sup> mice (48). However, in this study there were increases in *Il1β* as well as *Il17* gene expressions in these mice, which may compensate for the lack of IL-22. *Il22*<sup>-/-</sup> and *Reg3g*<sup>-/-</sup> mice can have overgrowth of segmented filamentous bacteria (49) (50) and the latter mice have increased pulmonary IL-1 and IL-17 responses. Thus it is possible that the increased IL-1 and IL-17 observed by Ahn, et al may be due to gut dysbiosis and may explain the lack of a robust phenotype in *Il22*<sup>-/-</sup> mice in that study. Additionally, we observed anti-IL-22 treatment in WT C57BL/6 mice exacerbated ST258 C4 pulmonary infection (Figure S8), supporting the concept that IL-22 is critical in this model. Additionally, in terms of rescuing with IL-22-Fc, we administered pharmacological doses that mediate robust STAT3 signaling in the liver and thus
any activator of hepatic STAT3 may be therapeutic in this model. Finally, we cannot currently 
exclude a role of airway epithelium as a critical target for the IL-22 response. However in vivo, 
the data show that the efficacy of IL-22 was dependent on hepatic IL-22ra1 signaling using the 
liver (22). Although IL-22 can signal to the epithelium we believe the liver may be dominant in 
vivo for the three reasons. First we believe that the liver is a critical site for the systemic route of 
IL-22 administration as the liver receives a much larger percentage of the cardiac output 
compared to the lung epithelium. Additionally hepatocytes express abundant amounts of 
Il22ra1, and thirdly the liver has a fenestrated endothelium and thus large amounts of the 
administered IL-22 are likely sequestered in the liver.

In conclusion, our findings show that both ILC3 and NK cells are associated with the 
resistance to ST258 C4 pulmonary infection. Considering the difficulty of treating this invasive 
infection due to multi-drug resistance, immunomodulatory treatment or prevention, especially IL-
22 treatment, might be considered to manage this infection.
Methods

Mice.

Wildtype C57BL/6, \(Rag2^{-/-}\) (B6.129S6-Rag2\(^{tm1Fwa}\) N12), and \(Rag2^{-/-}Il2rg^{-/-}\) (B10; B6-Rag2\(^{tm1Fwa}\) Il2rg\(^{tm1Wj}\)) mice were obtained from Taconic Farm. \(AlbCre^{+/}\times Il22ra1^{fl/fl}\) (Il22ra1\(^{fl/fl}\)) mice, \(AlbCre^{+/}\times Il22ra1^{fl/fl}\) (Il22ra1\(^{fl/fl}\ HEP) mice, and \(Il22ra1E2Acre\) mice, which is germline \(Il22ra1\) knockout mice, were inbred. All mice for experiments were six to eight weeks old male mice.

Experimental \(K.\ pneumoniae\) Infection

Carbapenemase (KPC)-producing \(K.\ pneumoniae\) strain C4, kindly provided by Yohei Doi at University of Pittsburgh, were grown in 100 mL tryptic soy broth (Difco) containing the 5 \(\mu\)g/mL meropenem (ThermoFisher Scientific) and 100 \(\mu\)g/mL carbenicillin (ThermoFisher Scientific) for 18 hours at 37°C. The culture was then diluted at 1:100 and grown for an additional 2.5 hours to reach the early logarithmic phase. The concentration of \(K.\ pneumoniae\) was determined by measuring the absorbance at 600 nm. Bacteria were pelleted by centrifugation and washed twice in cold PBS, and then resuspended to achieve the density of \(10^7\) CFU/ml. Mice were then infected with \(10^6\) CFU in 100 \(\mu\)L live \(K. pneumoniae\) by oropharyngeal aspiration-tongue pull technique (referred as intratracheal) and sacrificed at 24 hours after infection. Bacterial burden in the left lung, liver and spleen were analyzed by CFU on the homogenates. Simultaneously, right middle lobe was resected in Trizol (ThermoFisher Scientific) for RNA extraction.
Real-Time RT-PCR

RNA was isolated using Trizol reagent and cDNA was prepared using iScript reverse transcriptase master mix (Bio-Rad). Real-time RT-PCR was carried out with Bio-Rad CFX96 system using TaqMan PCR Master Mix (ThermoFisher Scientific) and premixed primers/probe sets from Thermo Fisher Scientific.

Flow cytometry and intracellular cytokine staining

Fluorophore conjugated antibodies used mouse cell analysis are as follows: anti- RORγt (Q31-378, BD Bioscience #562607), anti-GATA3 (16E10A23, Biolegend #653813), anti-NK-1.1 (PK136, Biolegend #108715), anti-IL-5 (TRFK5, Biolegend #504305), anti-CD278 (ICOS) (C398.4A, Biolegend #313529), anti-CD45.2 (104, Biolegend #109830), anti-CD127 (A7R34, Biolegend #135021), anti-Lin (17A2/ RB6-8C5/RA3-6B2/Ter-119/M1/70, Biolegend #133313), anti-IL-17A (eBio17B7, ThermoFisher Scientific #12-7177-81, TC11-18H10.1, Biolegend #506907), anti-IL-22 (1H8PWSR, ThermoFisher Scientific #46-7221-82, IL22JOP, ThermoFisher Scientific #17-7222-82), anti-TCR γδ (GL3, Biolegend #118108), anti-TCRβ Chain (H57-597, BD Bioscience #553173), and anti-IFN-γ (XMG1.2, Biolegend #505825).

Nonspecific binding was blocked using a rat anti-mouse antibody directed against the FcγIII/II receptor (CD16/CD32) (2.4G2, BD Bioscience #553141). All samples were resuspended in wash buffer and then subjected to flow cytometry analysis by using an Aurora (Cytek). For intracellular staining, each cell suspension was stimulated with phorbol 12-myristate 13-acetate (Sigma Aldrich) (PMA; 40 ng/mL) with ionomycin (Sigma Aldrich) (4 ug/mL) in 200 μl complete IMEM medium (ThermoFisher Scientific), supplemented with 10% fetal bovine serum (GE Healthcare) and 1% penicillin/streptomycin (ThermoFisher Scientific), at 37C and 5% CO2 for 6 hours, and with GolgiPlug (BD Bioscience) supplementation to the media for the last 5 hours,
and intracellular staining was performed after cell permeabilization (BD Bioscience) according to the manufacturers’ instructions. All flow cytometry data were analyzed using Flow Jo Mac, version 9.6 (Tree Star).

Bulk RNA Sequencing.

Total whole lung tissue RNA was used to perform RNA sequencing. RNA quantity and quality were assessed using NanoDrop and Agilent RNA 6000 Nano kit with Agilent 2100 Bioanalyzer instrument. Illumina TruSeq Stranded mRNA sample prep kit was used for library preparation, followed by Agilent DNA 1000 kit validation with Agilent 2100 Bioanalyzer and quantification by Qubit 2.0 fluorometer. The cDNA libraries were pooled at a final concentration 1.8pM. Cluster generation and 75 bp paired read single-indexed sequencing was performed on Illumina NextSeq 550. Raw reads were processed and mapped, then gene expression and nucleotide variation was evaluated by previously described methods (51). Data was deposited in the Gene Expression Omnibus, GEO accession number: GSE130045.

Single Cell RNA-Seq.

Mice were euthanized by exposure to CO2 and exsanguination. Lungs were isolated and minced with forceps and small scissors and digested in 2mL serum-free medium with 2 mg/mL collagenase (Sigma) and 80U/mL DNase I (Sigma) for 60 min at 37°C. Digested tissues were passed through a sterile 70 mm filter (BD Falcon) to get a single cell suspension. After washing, 2mL ACK Lysing Buffer (Life Technologies) was added into the cell suspension and let sit for 2 minutes and washed two times additionally. The whole lung single cell suspension obtained from this procedure was then tested for purity and quantity by Cellometer Auto 2000 (Nexcelom Bioscience) and found to be ready for single cell RNAseq. For single cell RNAseq analysis,
$1 \times 10^6$ cells per condition were saved as whole lung single cell population. The remaining cells were then subjected to CD45 enrichment by applying Ly-6G negative selection (Miltenyi, #130-092-332) and followed by CD45 positive selection (Miltenyi, #130-052-301). Briefly, the cells were suspended in MACS separation buffer, incubated with the anti-Ly6G-biotin followed by anti-biotin microbeads, and applied to a MACS LS separation column. The flow through was collected as the Ly6G negative (Ly6G-) population. The Ly6G- cells were then incubated with anti-CD45 microbeads and applied to a MACS MS column. After washes, the CD45 positive (CD45+) population was released from the column by flushing the column and collected into a 1.5-mL microtube. Both whole lung single cell and CD45 enriched populations were treated with 100uL TrypLE for 1 minute to dissociate single cells from any small aggregates or clusters. Cell numbers and viabilities were validated by Cellometer prior to single cell RNAseq library preparation.

For 10x single cell RNAseq assay, 5000 live cells per sample were targeted by using 10x Single Cell RNAseq technology provided by 10x Genomics (10X Genomics Inc, CA). Briefly, viable single cell suspensions were partitioned into nanoliter-scale Gel Beads-In-EMulsion (GEMs). Full-length barcoded cDNAs were then generated and amplified by PCR to obtain sufficient mass for library construction. Following enzymatic fragmentation, end-repair, A-tailing, and adaptor ligation, single cell 3’ libraries comprising standard Illumina P5 and P7 paired-end constructs were generated. Library quality controls were performed by using Agilent High Sensitive DNA kit with Agilent 2100 Bioanalyzer and quantified by Qubit 2.0 fluorometer. Pooled libraries at a final concentration of 1.8pM were sequenced with paired end single index configuration by Illumina NextSeq 550. Cell Ranger version 2.1.1 (10x Genomics) was used to process raw sequencing data and Loupe Cell Browser (10x Genomics) to obtain differentially expressed genes between specified cell clusters. In addition, Seurat suite version 2.2.1 (51, 52) was used for further quality control and downstream analysis. Filtering was performed to
remove multiplets and broken cells and uninteresting sources of variation were regressed out. Variable genes were determined by iterative selection based on the dispersion vs. average expression of the gene. For clustering, principal-component analysis was performed for dimension reduction. Top 10 principal components (PCs) were selected by using a permutation-based test implemented in Seurat and passed to t-SNE for clustering visualization.

Proliferation Assay

Single lung cell suspensions from 6-8 male Rag2−/− mice 12 hours post infection with ST258 C4 can be acquired following the digestion protocol as described above, and then lung ILCs were purified using Percoll (Sigma Aldrich) gradient centrifugation. CellTrace™ Violet (CTV) labeling was performed as manufacturer described (ThermoFisher Scientific). CTV-labeled lung ILCs (5 × 10^4/well) were co-incubated with ICOSL (HK5.3, Bio X Cell #BE0028, 1.5 μg/mL) for 24 hours and analyzed for FACS. Finally, we examined the effect of FK506 (50 ng/mL) by co-incubation on the proliferation of ICOSL-activated ILCs.

Antimicrobial Assay

To assay bacteriostatic activity, diluted serum from isotype control or IL-22:Fc treated WT C57BL/6 mice, or conditioned medium was incubated with K. pneumoniae in a 96-well plate and growth kinetics were analyzed over 9 h in a heat-controlled shaking microplate reader and read at an optical density of 600 nm (OD600) every hour (22). This procedure was repeated using heat inactivated serum (56C, 30min). For C3 binding assay, a total of 10^5 CFU ST258 C4 was incubated for 20 min at 37°C (no CO2) with 5% diluted serum from isotype control or IL-22:Fc treated WT C57BL/6 mice, and then performed FACS analysis (23).
Antibody Administration

In some experiments, mice were injected via intraperitoneal with 500μg anti-ICOS antibody (7E.17G9, Bio X Cell #BE0059), 250μg anti-NK1.1 antibody (BE0036, Bio X Cell #50562112), IgG2a isotype control (2A3, Bio X Cell #BE0089), or IgG2b isotype control (LTF-2, Bio X Cell #BE0090) once 2 hours before bacterial challenge, and injected via intratracheally with 50μg anti-IL-22 mAb (R&D #AF582) 30 minutes before bacterial challenge.

Cytokine Immunotherapy

For pharmacological model of Rag2^{-/-}Il2rg^{-/-} mice, 10mg/kg FK506 was administered in C57BL/6 mice, AlbCre^{-/-} x Il22ra1^{fl/fl} (Il22ra1^{fl/fl}) mice, or AlbCre^{-/-} x Il22ra1^{fl/fl} (Il22ra1^{-/-HEP}) mice intraperitoneally 24 hours prior to intratracheal challenge with 10^6 ST258 C4 strain. FK506 were administered every 24 hours until 24 hours before euthanasia. These mice were euthanized at 48 hours after infection. To examine the efficacy of cytokine rescue, we administered 4μg IL-22:Fc (Generon, Shanghai, China) intraperitoneally 2 hours before, 1μg recombinant murine IFNγ (Peprotech) or 1μg IL-22:Fc intratracheally 30 minutes before bacterial challenge.

Statistics

Graphs were generated and statistical significances were analyzed using GraphPad Prism8, and the used statistical tests are defined in the figure legends. P values of pairwise comparisons between two normally distributed groups were performed by simple 2-tailed unpaired student’s t-test, while other analyses for unequal variance were determined by Mann-Whitney U test or Welch’s t-test. We used one-way ANOVA with Tukey’s multiple comparison test or Kruskal-
Wallis with Dunn’s multiple comparison test for multiple groups comparisons. Values are represented as means ± SEM. $P$ values are annotated as follows (*) $\leq$ 0.05, (**) $\leq$ 0.01, (***) $\leq$ 0.001, and (****) $\leq$ 0.0001.

Study approval

All mice were housed in accordance with the NIH guidelines and in specific pathogen-free rooms within animal facilities at Tulane University (New Orleans). All mice experiments were approved by the Tulane IACUC.

Author contributions

Conceptualization: N.I., I.S., and J.K.K; Methodology: N.I., A.W., K.S., and J.K.K.; Validation: N.I. and J.K.K; Formal Analysis: N.I.; Investigation: N.I., I.S., A.W., K.S., and J.K.K.; Resources: J.K.K.; Writing – Original Draft: N.I.; Writing – Review & Editing: N.I., I.S., A.W., J.M., K.S., and J.K.K.; Visualization: N.I. and A.W.; Funding Acquisition: J.K.K.

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Declaration of interests

The authors declare no competing interests.

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Figure 1. *Rag2*/*Il2rg*−/− mice are susceptible to the ST258 C4 pulmonary infection and lack type1 and type3 cytokine expression.

C57BL/6 mice, *Rag2*−/− mice and *Rag2*/*Il2rg*−/− mice were infected intratracheally with 10⁶ CFU ST258 C4 and euthanized at 24 hours. Bacterial CFU in (A) lungs, (B) liver, and (C) spleen are representative as box and whisker plots showing median, first and third quartiles, and maximum and minimum values (n = 8, two independent experiments). Significant differences are designated by using Kruskal-Wallis test followed by Dunn’s multiple comparisons test. **, P < 0.01 (D) Kaplan–Meier survival curves of mice infected with 10⁷ CFU ST258 C4. Significant differences were designated by log-rank test (n = 5, two independent experiments). **, P<0.01 versus C57BL/6 and *Rag2*−/− mice. (E) Weight change for comparing C57BL/6 mice, *Rag2*−/− mice and *Rag2*/*Il2rg*−/− mice were also observed (n = 4-5, two independent experiments). Significant differences are designated by using one-way ANOVA followed by Tukey’s multiple comparisons test. ****, P < 0.0001 versus C57BL/6 and *Rag2*−/− mice. (F) Volcano plot of
differentially expressed genes in lungs between infected \textit{Rag2}^{-/-} versus \textit{Rag2}^{-/-}\textit{Il2rg}^{-/-} mice 12 hours post infection (\(n = 3\)).
Figure 2. scRNAseq analysis reveals ICOS+ NK cells and ICOS+ group 3 innate lymphoid cells in the lung post infection.

scRNAseq was performed 12 hours post infection with $10^6$ CFU ST258 C4 on 6-8 week old male $Rag2^{-/-}$ lung cells using CD45 selection after depletion of Ly6G+ cells. ($n = 2$). TSNE plots of scRNAseq data revealed that $Rag2^{-/-}$ mice have a distinct group 3 ILC population with preferential expression of (A) ICOS and $Ii17a$, whereas $Ifng$ was primarily confined to the (B) NK cells cluster. Representative FACS plots and the quantification showed (C, E) surface ICOS expression in both naïve and infected lung. Rorgt+ ILC3 (D, F) in the lung increased post infection whereas GATA3+ ILC2 (D, G) cells decreased. Flow gating strategies for Rorgt+ or GATA3+ cells are conducted as CD45+CD127+Lin-. Data are presented as mean ± SEM ($n = 4$, two independent experiments). Significant differences are designated using unpaired t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. 
Figure 3. Depletion of NK cells with antibody reveals these cells are dispensable for host resistance to ST258 infection

6-8 week old male Rag2−/− mice were administered 250μg anti-NK1.1 antibody or isotype control once 2 hours prior to intratracheal challenge with 10^6 CFU ST258 C4 strain and euthanized 24 hours post infection. (A) Klrb1c, (B) Ifng, (C) Il17a, (D) Il22, and (E) Rorc mRNA expression in lung, and then (F) Lung CFU 24 hours post infection are shown. Data are presented as mean ± SEM (n = 4-5, data shown representative of two separate experiments). Significant differences are designated using one-way ANOVA followed by Tukey's multiple comparisons test. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
Figure 4. Effect of ICOS blockade on group 3 ILCs during lung ST258 C4 infection

6-8 week old male Rag2⁻/⁻ mice were administered 500μg anti-ICOS or control once 2 hours prior to intratracheal challenge with 10⁶ CFU ST258 C4 strain, and euthanized 24 hours after infection. Gene expression in lung of (A) Il17a, (B) Il17f and (C) Il22 were reduced, but not (D) Ifng or (E) Klrb1c. (F) CFU in the lung was not affected by anti-ICOS. Data are presented as mean ± SEM (n = 5, two independent experiments). Significant differences are designated using one-way ANOVA followed by Tukey’s multiple comparisons test. *, P < 0.05; **, P < 0.01.

Ex vivo effects of ICOSL (1.5 μg/mL) on (G, H) innate lymphoid cells and (I) NK cell proliferation (as assayed by dye dilution) obtained from Rag2⁻/⁻ mice 12 hours post infection (n = 4, two independent experiments). Flow gating strategies are conducted as CD45⁺CD127⁺Lin⁺ Rorgt⁺ for Rorgt⁺ cells, CD45⁺CD127⁺Lin⁻ GATA3⁺ for GATA3⁺ cells, and CD45⁺CD127⁺Lin⁻ NK1.1⁺ for NK1.1⁺ cells. Representative histograms are showing (G) Rorgt⁺ cells, (H) GATA3⁺ cell, and (I) NK1.1⁺ cells gated. Percentage of dye diluted (J) Rorgt⁺ cells, (K) GATA3⁺ cell, and (L) NK1.1⁺ cells are shown. Significant differences are designated by using unpaired t-test. **, P < 0.01.
Figure 5. Dual blockade of ICOS and NK cells exacerbates lung infection.

6-8 week old male \textit{Rag2}^{-/-} mice were administered both 500\,µg anti-ICOS and 250\,µg anti-NK1.1 or isotype control once 2 hours prior to intratracheal challenge with \(10^6\) CFU ST258 C4 strain, and euthanized 24 hours after infection. Dual blockade reduced mRNA expression of (A) \textit{Il17a}, (B) \textit{Il17f}, (C) \textit{Il22}, (D) \textit{Klrbc} and (E) \textit{Ilfn}, and significantly increased CFU in the lung (F), \((n = 9-10)\). Data are pooled from three independent experiments. Additionally, (G) \textit{Xc1} and (H) \textit{Ccl5} mRNA expression in the naïve \textit{Rag2}^{-/-}, infected \textit{Rag2}^{-/-} and infected \textit{Rag2}^{-/-} pretreated with 250\,µg anti-NK1.1 mAb via intraperitoneally once 2 hours prior to infection are shown \((n = 5, \text{data shown representative of two separate experiments})\). Data are presented as mean ± SEM. Significant differences are designated by using one-way ANOVA followed by Tukey’s multiple comparisons test unless (F), while Mann-Whitney U test is used for the comparison of (F). *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\); ****, \(P < 0.0001\).
Figure 6. Pharmacological model of host KPC susceptibility with FK506 mimics the susceptibility in Rag2⁻/⁻ Il2rg⁻/⁻ mice

6-8 week old male C57BL/6 mice were treated with vehicle control or 10mg/kg FK506 via intraperitoneally 24 hours prior to intratracheal challenge with 10⁶ ST258 C4 strain. FK506 was administered every 24 hours until 24 hours before euthanasia. Lungs were harvested 48 hours post infection. (A) Il17a, (B) Il17f, (C) Il22, (D) Ifng, (N) Klrbc1c and (O) Tcrg mRNA expression, and (E) CFU in the lungs of naïve C57BL/6, infected C57BL/6, and infected C57BL/6 mice treated with FK506 are shown (n=4, two independent experiments). Expression of mRNA was normalized to the constitutive expression of Hprt mRNA, and data are presented as mean ± SEM. The absolute number of (L) lung NK cells and (M) lung γδ cells were also examined by FACS 24 hours post pulmonary challenge in 6-8 week old male C57BL/6 mice treated with vehicle control or FK506. Data are presented as mean ± SEM (n=5, two independent
experiments). Significant differences are designated by using one-way ANOVA test followed by Tukey’s multiple comparisons test. *, P < 0.05, ***, P < 0.001, ****, P < 0.0001. CFU was compared by using Mann-Whitney U test. ***, P < 0.001; versus vehicle control.

6-8 week old male FK506 treated wild type C57BL/6 mice were infected with 10^6 CFU ST258 C4 strain via intratracheal and 24 hours after inoculation lungs were harvested to analyze single cell RNA sequencing. 10mg/kg FK506 was administered via intraperitoneally 24 hours prior to intratracheal challenge, and every 24 hours until 24 hours before euthanasia. The violin plots from Seurat of scRNAseq data showed FK506 treatment affected to reduce various cytokine gene expression such as (F) Il17a, (G) Il17f, (H) Il22, (I) Ifng, but not (J) Klrbc and (K) Tcrg.
Figure 7. FK506 suppresses ILC function in vitro

6-8 week old male *Rag2*<sup>−/−</sup> mice were infected with 10<sup>6</sup> CFU ST258 C4 strain via intratracheal, and twelve hours after inoculation lungs were harvested and enriched for innate lymphoid cells for analyzing via flow cytometry. The lung ILCs were incubated for 24 hours with or without ICOSL (1.5 μg/mL) and FK506 (50 ng/mL) (n = 4). After that flow cytometry was performed. Flow gating strategies are conducted as CD45<sup>+</sup>CD127<sup>+</sup>Lin<sup>−</sup> Rorgt<sup>+</sup> for Rorgt<sup>+</sup> cells, CD45<sup>+</sup>CD127<sup>+</sup>Lin<sup>−</sup> GATA3<sup>+</sup> for GATA3<sup>+</sup> cells, and CD45<sup>+</sup>CD127<sup>+</sup>Lin<sup>−</sup> NK1.1<sup>+</sup> for NK1.1<sup>+</sup> cells. Representative histograms showing (A) Rorgt<sup>+</sup> cells, (B) GATA3<sup>+</sup> cell, and (C) NK1.1<sup>+</sup> cells gated. Percentage of dye diluted in (D) Rorgt<sup>+</sup> cells, (E) GATA3<sup>+</sup> cell, and (F) NK1.1<sup>+</sup> cells are shown. Moreover, we examined to see if FK506 could suppress the production of cytokine in ILCs activated by phorbol 12-myristate 13-acetate (PMA; 40 ng/mL) with ionomycin (4 μg/mL) for 24 hours in vitro (n = 4). The supernatant was assessed for (G) IL-2, (H) IL-4, (I) IL-5, (J) IL-13, (K) IL-17A and (L) IFN-g by Luminex. Data are presented as mean ± SEM, and representative data are shown of two separate experiments. Significant differences are designated by using ANOVA followed by Tukey's multiple comparisons test. *, P < 0.05, **, P < 0.01, ***, P < 0.001, ****, P < 0.0001.
Figure 8. IL-22:Fc treatment rescued ST258 pulmonary infection in susceptible mice

(A) 1μg IFNγ was administered via intratracheal injection once 2 hours prior to infection in FK506 treated C57BL/6 mice, and lung CFU was measured 48 hours after ST258 infection (n = 4). (B) CXCL10 induction was analyzed in lung tissue after IFNγ administration (n = 4). (C) 4μg IL-22:Fc was administered via intraperitoneal injection once 2 hours prior to infection in FK506 treated C57BL/6 mice, and lung CFU was measured 48 hours after ST258 infection (n = 4). (A,
B, C) Significant differences are designated by using Kruskal-Wallis test followed by Dunn’s multiple comparisons test. *, P < 0.05. (D) 4μg IL-22:Fc was administered via intraperitoneal injection once 2 hours prior to infection in *Rag2*<sup>−/−</sup>/*Il2rg*<sup>−/−</sup> mice, and lung CFU was measured 24 hours after ST258 infection (*n* = 7). Significant differences are designated by using Mann-Whitney U test. (E, F, G). Box and whisker plots show median, first and third quartiles, and maximum and minimum values (A, C, D). 4μg IL-22:Fc was administered via intraperitoneal injection once 2 hours prior to infection in FK506 treated C57BL/6, *AlbCre<sup>i</sup> x Il22ra1<sup>fl/fl</sup>* (*Il22ra1<sup>fl/fl</sup>) and *AlbCre<sup>i</sup> x Il22ra1<sup>fl/fl</sup> (Il22ra1<sup>−/HEP</sup>) mice for comparing to C57BL/6 mice treated with PBS or FK506 (*n*=5), and euthanized 48 hours after infection. Data are presented as mean ± SEM. Significant differences are designated by using ANOVA followed by Tukey’s multiple comparisons test. **, P < 0.01, ***, P < 0.001. All data are shown representative of two separate experiments.
Figure 9. IL-22:Fc mediated bacteriostatic activity are partially dependent on complement produced by hepatocyte

(A) 4μg IL-22:Fc treated serum for 24 hours in WT C57BL/6 mice augmented bacteriostatic activity against ST258 C4, and (B) the bacteriostatic effect proved to be heat labile by using heat inactivated serum. (C) The bacteriostatic effect by IL-22:Fc treatment was diminished in C3−/− mice more than WT mice. Lastly, 4-8 week old male Il22ra1fl/fl mice and Il22ra1ΔHEP mice were treated with 4μg IL-22:Fc via intraperitoneally for 24 hours and blood were collected. (D, E) IL-22:Fc treated serum in Il22ra1fl/fl mice results in more deposition of C3 binding to ST258 C4 post co-incubation with 10^5 CFU ST258 C4 in vitro, but the effect was abrogated in Il22ra1ΔHEP mice. Data are presented as mean ± SEM (Representative data are shown of two separate experiments.) Significant differences are designated by using ANOVA followed by Tukey’s multiple comparisons test. †, P < 0.05 (vs IL-22:Fc treated C3−/− mice), ****, P < 0.0001 (vs medium, WT, C3−/− mice).
Table 1. Significantly downregulated gene datasets in infected *Rag2^-/-Il2rg^-/-* mice compared to *Rag2^-/-* mice.

Bulk RNA sequencing of lung cells was performed 12 hours post infection with 10^6 CFU ST258 C4 on 6-8 week old male *Rag2^-/-* and *Rag2^-/-Il2rg^-/-* mice.

| Gene   | ID    | Log (FC) | p-value   | Gene   | ID    | Log (FC) | p-value   |
|--------|-------|----------|-----------|--------|-------|----------|-----------|
| *Il22* | 50616 | -3.074   | 1.080e-08 | *Ilng* | 3458  | -9.096   | 1.960e-41 |
| *Il17f*| 112744| -5.596   | 3.020e-18 | *Cxcr6*| 10863 | -6.552   | 7.030e-07 |
| *Il2rb*| 3560  | -7.951   | 3.020e-08 | *Ccr8* | 1237  | -8.475   | 1.820e-16 |
| *Il2rg*| 3561  | -4.143   | 9.470e-58 | *Gzma* | 3001  | -8.489   | 1.090e-94 |
| *Il7r* | 3575  | -2.617   | 2.430e-12 | *Gzmb* | 3002  | -6.971   | 6.970e-69 |
| *Il17a*| 3605  | -6.252   | 1.110e-12 | *Gzmk* | 3003  | -6.180   | 4.570e-05 |
| *Il5*  | 3567  | -6.159   | 4.531e-05 |        |       |          |           |
Table 2. Significantly upregulated gene expressions in infected $Rag2^{-/-}Il2rg^{-/-}$ mice compared to $Rag2^{-/-}$ mice

Bulk RNA sequencing of lung cells was performed 12 hours post infection with $10^6$ CFU ST258 C4 on 6-8 week old male $Rag2^{-/-}$ and $Rag2^{-/-}Il2rg^{-/-}$ mice.

| Gene  | ID    | Log (FC) | p-value  | Gene  | ID    | Log (FC) | p-value  |
|-------|-------|----------|----------|-------|-------|----------|----------|
| Padi4 | 374   | +2.159   | 6.574e-13| Serpina1a | 85480 | +2.340   | 3.328e-09 |
| Apoa2 | 650   | +1.834   | 3.608e-10| Serpina1b | 4208 | +1.652   | 4.832e-10 |
| Ttr   | 3560  | +2.243   | 6.947e-16| Serpina1c | 8993 | +1.520   | 4.458e-10 |
| Apoa1 | 9518  | +2.244   | 3.711e-07| Serpina1d | 2244 | +8.549   | 2.533e-33 |
| Abtb2 | 6376  | +1.796   | 2.990e-17| Serpina1e | 4057 | +2.213   | 3.825e-10 |
| Rasd1 | 2920  | +1.809   | 1.988e-07| Gc     | 14473 | +4.514   | 1.000e-06 |
| Hlx   | 2921  | +1.815   | 5.128e-08| Fgb    | 110135 | +8.549   | 0.000001 |
| Rag2  | 3084  | +2.352   | 5.663e-08|        |       |          |          |

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