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CD161+CD4+ T cells are enriched in the liver during chronic hepatitis and associated with co-secretion of IL-22 and IFN-γ

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INTRODUCTION
Hepatitis C virus (HCV) infects 170 million people worldwide and is a major cause of chronic liver disease (Lauer and Walker, 2001). The role of T cells in control of infection has been extensively studied and is a major cause of liver disease (Lauer and Walker, 2001). The role of HCV in chronic hepatitis such that they are the dominant subtype (mean 55% of CD4+ T cells). IL-22 and IL-17 secreting CD4+ T cells were readily found in the livers of HCV+ and NASH donors, although not enriched compared to blood. There was, however, specific enrichment of a novel subset of IL-22/IFN-γ dual secretors (p = 0.02) compared to blood, a result reconfirmed with direct ex vivo analyses. These data indicate the dominance of CD161+ expressing lymphocyte populations within the hepatic infiltrate, associated with a distinct cytokine profile. Given their documented roles as antiviral and hepatoprotective cytokines respectively, the impact of co-secretion of IFN-γ and IL-22 in the liver may be particularly significant.

Keywords: CD4+ T cell, IL-22, HCV, hepatic inflammation, CD161
this, IL-22 promotes epithelial defense responses through secretion of antimicrobial molecules (Wolk et al., 2004; Wolk and Sabat, 2006; Taula et al., 2008; Eyerich et al., 2009). Thus, this is a unique and important cytokine with potent effects on tissue repair and remodeling, which has a specific role in the liver in modulating tissue injury.

In man, many Th17 cells can co-secrete IFN-γ, as well as IL-22 (Annunziato et al., 2008; Cosmi et al., 2008). Additionally, recently a subset of cells which secretes IL-22 but not IL-17 or IFN-γ, termed Th22, have been described, however, these have been considered up until now specific for the skin and whether they may play any role in the liver is unknown (Dulen et al., 2009; Eyerich et al., 2009). Overall the role of Type 17 CD4+ T cells in the liver during chronic hepatitis C has not been extensively evaluated. A recent study by the group of Rosen, looking at end-stage liver disease, did reveal enrichment of CD4+ T cells secreting IL-17 in comparison to blood in 11 patients (Puster et al., 2012), although the nature of the infiltrate at earlier stages of infection remains to be determined, and the co-expression of IL-17 or IL-22 with IFN-γ has yet to be defined.

CD161 is a C-type lectin – initially defined as an NK associated molecule – which has been linked to a liver homing phenotype of T cells in health and disease (Northfield et al., 2008; Billerbeck et al., 2010). CD161 is expressed on both CD3+CD8+ and CD3+CD4+ T cells (Takahashi et al., 2006) and recently CD161 expression has been linked to both a “Tet17” and a “Te17” phenotype in man (Cosmi et al., 2008; Billerbeck et al., 2010). CD161+ T cells expressing high levels of CD161 are highly enriched in the livers of patients with chronic hepatitis, expressing chemokine receptors associated with tissue homing in resting and inflammatory conditions (Billerbeck et al., 2010). These cells have a distinct phenotype and may secrete IL-17, IL-22, TNFα, and/or IFN-γ. Data from the study of cord blood T cells suggest that CD161 expression occurs very early and that only cells that express this molecule, and therefore the master transcription factor RORγt, are able to differentiate further into IL-22 and IL-17 secreting cell populations (Annunziato et al., 2008; Cosmi et al., 2008; Romagnani et al., 2009; Billerbeck et al., 2010). Thus, CD161+ CD4+ T cells possess enhanced potential to secrete IL-22 compared to CD161 T cells.

Given our prior data on CD8+ CD161+ T cells discussed above, we therefore characterized in a parallel way the phenotype and function of CD4+ T cells in blood of chronically HCV+ donors and controls, and analyzed the function of the infiltrate in the liver. Our data suggest that CD4+ T cells expressing CD161 are an important component of the inflammatory infiltrate in HCV and other inflammatory liver diseases. This includes a distinct IL-22/IFN-γ+ population which is specifically enriched in the liver and may be of particular functional significance.

### MATERIALS AND METHODS

#### PATIENTS AND HEALTHY DONORS

Hepatitis C virus infected patients, patients with non-viral hepatitis, and healthy normal donors were enrolled in this study after informed consent: the patient characteristics of the liver biopsy study cohorts are tabulated in Table 1. All those studied – both HCV+ and non-alcoholic steatohepatitis (NASH) cohorts – were undergoing liver biopsies for diagnostic reasons related to their normal clinical care as in previous analyses (Billerbeck et al., 2010). For healthy donor studies, peripheral blood mononuclear cells (PBMCs) from groups of up to 12 HCV-negative, low risk healthy controls were analyzed. A further set of HCV+ patients and intrahepatic lymphocytes (IHL) from explants from five donors undergoing liver transplant were used for the comparative study in Figure 3 and this information is briefly summarized in the relevant legend. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institutions’ human research committees.

#### CELL PREPARATION

Peripheral blood mononuclear cells, liver cells, and liver-derived cell lines were prepared as previously (Spangenberg et al., 2005).

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### Table 1 | Subject characteristics for in vitro analysis.

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Briefly, for the isolation of intrathoracic lymphocytes (IHL) the liver tissue was washed twice in 3 ml of phosphate buffered saline (PBS) containing 1% FCS prior to a careful homogenization through a 70 μm cell strainer (BD Falcon). The homogenized cell suspension was incubated with magnetic microbeads coated with anti-CD4 (Dynal, Oslo, Norway) for 30 min at 4°C. CD4+ T cells bound to the microbeads were isolated from the cell suspension by using a magnetic bead concentrator (Dynal). Periperal CD4+ T cells were isolated from 4 × 10^7 PBMC by the same experimental approach. The purity of each T cell subset was confirmed by FACS analysis and was always >95%. Isolated intrahepatic and peripheral CD4+ T cells were resuspended in 1 ml of complete medium and cultured in a 48-well plate (Greiner, Frickhausen, Germany) in the presence of 100 U/ml human rIL-2 complete medium and cultured in a 48-well plate (Greiner, Frickhausen, Germany) in the presence of 100 U/ml human rIL-2. Non-parametric tests, Mann–Whitney and Wilcoxon were used throughout for statistical analysis.

LYMPHOCYTE STAINING AND ANALYSIS

Lymphocyte staining was performed on whole blood as previously (Northfield et al., 2008; Bengsch et al., 2007), using antibodies as indicated below. Lymphocyte staining was performed on whole blood as previously. Intracellular cytokine staining after PMA/Ionomycin stimulation was performed as described below. For intracellular cytokine staining after PMA/Ionomycin stimulation we noted high expression of CXCR3, a chemokine receptor linked to homing to inflammatory sites (49%) although CD161+CD4+ T cells did not express CXCR6 – a key chemokine receptor associated with intracellular expression of the liver sinusoids (Geissmann et al., 2009). We also confirmed that circulating CD4+CD161+ cells analysed ex vivo expressed the cytokine receptor IL-23R, high levels of the IL-18Ra and increased levels of RORyt expression compared to CD161+ cells (data not shown) similar to data from CD8+CD4+ T cells (Billerbeck et al., 2010). To further characterize this subset of T cells for features relevant to liver homing, we next analyzed expression of relevant chemokine receptors in relation to CD161. We found co-expression of CCR4 (45%), as well as CCR2 (39%, Figures 2A,D). We noted high expression of CXCR3, a chemokine receptor linked to liver homing. We also noted high expression of CXCR3, a chemokine receptor linked to liver homing in the liver sinusoids (Geissmann et al., 2009; Sato et al., 2009; Figures 2B,C). We also confirmed that circulating CD4+CD161+ cells displayed an "effector memory" phenotype (CD45RA-CD62L+, CCR7+) and CD62L+CCR7+, CD28+CD69+, perforin+, Granzyme B+ without evidence of exhaustion (PD-1+, data not shown).

These data confirm and further define some important functional capacities of the CD161+ T cell subset. The cells express relevant chemokine receptors for liver homing during inflammation and relevant cytokine receptors and transcription factors for IL17/IL-22 induction and maintenance. The CD161+CD4+ T cell fraction also includes IL-22+ cells (Billerbeck et al., 2010). In terms of other effector/memory markers, these CD4+ T cells displayed an "effector memory" phenotype (CD45RA-CD62L+, CCR7+) and CD62L+CCR7+, CD28+CD69+, perforin+, Granzyme B+ without evidence of exhaustion (PD-1+, data not shown).

RESULTS

FUNCTION AND PHENOTYPE OF CD161+CD4+ T CELLS IN HEALTHY DONOR BLOOD

Initially, we examined the immediate effector functions of CD161+ and CD161-CD4+ T cells in healthy donors. To do this we stimulated populations taken directly ex vivo from healthy donors with PMA/Ionomycin and examined cytokine production by intracellular cytokine secretion. CD161+CD4+ T cells generated high levels of IL-17A upon stimulation, compared to CD161- cells (mean ± 7%, Figure 1A). We noted co-secretion of IL-17A and IFN-γ, as previously described for CD8+ T cells (Billerbeck et al., 2010; data not shown). Importantly, we readily identified ex vivo secretion of hepatoprotective IL-22 by a larger fraction of cells, and found this to be strongly associated with the CD161+CD4+ T cell subset (mean ± 10%; Figure 1B).

To further characterize this subset of T cells for features relevant to liver homing, we next analyzed expression of relevant chemokine receptors in relation to CD161. We found co-expression of CCR4 (45%), as well as CCR2 (30%, Figures 2A,D). We noted high expression of CXCR3, a chemokine receptor linked to homing to inflammatory sites (49%) although CD161+CD4+ T cells did not express CXCR6 – a key chemokine receptor associated with intracellular expression of the liver sinusoids (Geissmann et al., 2009). We also confirmed that circulating CD4+CD161+ cells analysed ex vivo expressed the cytokine receptor IL-23R, high levels of the IL-18Ra and increased levels of RORyt expression compared to CD161+ cells (data not shown) similar to data from CD8+CD4+ T cells (Billerbeck et al., 2010). In terms of other effector/memory markers, these CD4+ T cells displayed an "effector memory" phenotype (CD45RA-CD62L+, CCR7+) and CD62L+CCR7+, CD28+CD69+, perforin+, Granzyme B+ without evidence of exhaustion (PD-1+, data not shown).
their frequency. We found the relative reduction of CD161+CD4+ T cells in blood occurred most significantly amongst CXCR3+CD161+CD4+ T cells (Figure 3B). The expression of the other markers on CD4+ T cells analyzed, including CXCR6, was not affected by HCV infection (Figure 3B and data not shown). This feature is consistent with homing of CXCR3+ T cells to the liver as has been indicated in previous studies (Boisvert et al., 2003; Wang et al., 2004).

ANALYSIS OF CYTOKINE SECRETING CD4+ T CELLS IN THE LIVER IN HCV INFECTION

We next examined the functional capacity of CD4+ T cells derived from the liver biopsies of HCV+ persons, focusing on IL-22 and IL-17 production. Initially, as previously in our studies of CD8+ T cells, we used populations of CD4+ T cells which had been expanded in vitro using non-specific stimuli (Billerbeck et al., 2010). Exactly parallel stimulations with PMA/Ionomycin were then performed using blood and liver-derived cells (Figures 4A,B). We compared data between CD4+ and CD8+ T cell subsets.

We confirmed that IL-22 and IL-17 secreting CD4+ T cells were readily detectable within liver-infiltrating lymphocyte (LIL) populations (Figures 4A,B). IL-22 secreting CD4+ T cells comprised on average 4.7% of the CD4+ T cell infiltrate, compared to 2.2% of the IL-17 secreting cells ($p = 0.02$). The levels of CD4+ T cells and CD8+ T cells secreting IL-17 in liver tissue were not significantly different from each other, while in blood, CD4+ T cells secreting IL-17 were significantly more numerous than their
FIGURE 2 | Chemokine receptor expression of CD161+ CD4+ T cells in healthy donor blood. Healthy donor blood was stained as described in methods and analyzed for expression of the following chemokine receptors: CCR6 (A), CXCR6 (B), CXCR3 (C), and CCR2 (D). Plots show gated live CD3+CD4+ lymphocytes. In each case an example is shown on the left and group data on the right, with comparison by Mann-Whitney test.
FIGURE 3 | CD161+CD4+ T cells in blood and liver in HCV infection. CD161+CD4+CD3+ T cell frequencies were examined in blood from healthy donors, HCV+ chronically infected donors and liver derived lymphocytes, studied ex vivo (A). Percentage CD161+ cells amongst CD4+ T cells in liver explants, PBMC from HCV+ donors and PBMC from healthy donors are shown. The 15 HCV+ donors studied in the analysis of PBMC were additional to those in Table 1 and comprised persistently infected donors (eight genotype 1, six genotype 3, one genotype 2), of whom five had Ishak histologic scores of 5/6 and/or clinical cirrhosis; the IHL in this study were obtained at explant for cirrhosis from HCV+ donors. Groups were compared by Mann-Whitney tests. In (B), the phenotype of CD161+CD4+CD3+ T cells in peripheral blood of HCV+ donors and healthy controls were compared as in Figures 1 and 2. Data is shown from CXCR3 and CXCR6 analyses. No significant change was seen in analyses of CD27, CD28, CD45RA/RO, CD62L, CCR6, CD103, or PD-1.

CD8+ counterparts (Figure 4A). For CD8+ T cells there was a marked enrichment of IL-17 secreting cells in liver compared to blood, but for CD4+ T cells the levels were very similar in both sites and the differences were not significant (Figure 4A). IL-17+ CD4+ populations were also not enriched in liver compared to blood but were significantly more numerous than their CD8+ counterparts in both locations (Figure 4B). Both IL-22+ and IL-17+ CD4+ T cells were markedly outnumbered by IFN-γ secreting cells (40%; both p < 0.001; Figure 4C).

To assess whether these results were restricted to HCV, we also compared a set of patients with NASH using identical protocols. Overall, remarkably similar results were obtained (Figure 4C); the frequencies and relative distributions of CD4+ T cells secreting IL-17, IL-22, and IFN-γ were very similar to those obtained in HCV+ patients, with both IL-17+ and IL-22+ populations present, but outnumbered clearly by IFN-γ+ CD4+ T cells (p < 0.001).

CO-SECRETION OF IL-22 WITH OTHER CYTOKINES BY CD4+ T CELLS IN THE HUMAN LIVER
Since we had identified IL-22-secreting CD4+ T cells within the liver infiltrates, we next addressed whether these cells co-secreted other cytokines. Previous reports have described T cells that secrete IL-22 but not IFN-γ or IL-17 (Duhen et al., 2009; Eyerich et al., 2009) in skin (Th22 cells) and we asked whether such cells exist within the liver-homing populations.

We identified clear subsets of CD4+ T cells that have a "Th22" phenotype (Figure 5A), i.e., these cells secrete IL-22, but not IFN-γ or IL-17A. However, the frequencies of IL-22 monosecretors as a fraction of IL-22 secreting cells were similar in blood and liver overall (p = 0.0018; Figure 5B). In the tissue-derived cells these populations represented more than 50% of the IL-22 population on average. Indeed, taking into account
FIGURE 4: Analysis of cytokine secreting cells in the liver of HCV+ persons and controls. (A) Liver-derived and blood-derived CD4+ and CD8+ lymphocytes were analyzed for their cytokine secretion capacity as described in methods. The fractions of cells secreting IL-17A are indicated. (B) Data for IL-22 secretion in blood and liver are indicated as for IL-17A. (C) Analysis of CD4+ T cell cytokine secretion (IFN-γ vs IL-22 and IL-17) in liver, in patients with HCV (left panel) and NASH (right panel).

those cells that additionally secreted IL-17 (i.e., triple producers; Figure 5B), this was clearly the dominant population in the liver.

ANALYSIS OF EX VIVO SECRETION OF IL-22 IN BLOOD AND LIVER DERIVED CD4+ T CELL POPULATIONS

To address further the significance of these results a further set of analyses were performed ex vivo on liver-derived and blood-derived cells from a further 10 patients. Ex vivo derived cell populations were those isolated fresh from blood or tissue and then subjected to PMA/Ionomycin stimulation and intracellular cytokine staining without any further expansion in vitro. Using these freshly obtained cell populations we observed comparable patterns of response to those obtained with cultured cells. Firstly, we observed readily detectable levels of IL-22+ cells
Kang et al. Intrahepatic IL-22 secreting T cells

FIGURE 5 | Analysis of IL-22 secreting cells and polyfunctionality in blood and liver derived lymphocytes. Liver derived lymphocytes and PBMCs from HCV+ donors expanded in vitro were analyzed in parallel for expression of cytokines IL-17A, IL-22 and IFN-γ in response to PMA/Ionomycin stimulation as described in methods. In (A) an example of staining is shown. IL-22+ CD4+ T cells were gated upon and co-secretion of IL-17A and IFN-γ was assessed. In (B) the fraction of cells secreting IL-22 either alone or in combination with the other cytokines is displayed and compared (paired Student’s t-test). A significant enrichment of liver-derived IFN-γ/IL-22 co-secreting cells is observed.

DISCUSSION

These data provide a novel insight into the composition of the inflammatory infiltrate in chronic liver disease. Despite the clear evidence from animal models, we do not yet know to what extent IL-22 secreting T cells participate in different stages of human liver disease, their impact in tissue, or to what extent measurement of such cells in blood or liver may vary or correlate with clinical outcome. These data represent an important step in the direction of defining the significance of this relevant cytokine in chronic hepatitis.

Although we have focused on HCV, since this is a major clinical problem, a comparable profile was observed in non-viral inflammation. Indeed, since previous data have shown that there is...
We focused on the phenotype and function of CD161+ CD4+ T cells in health and disease for two reasons. Firstly, CD161 has been linked to LIL populations in humans, as in this paper and in our previous work, which showed enrichment on antigen-specific CD8+ T cells specific for hepatotropic viruses (Northfield et al., 2008), as well as on bulk CD4+ T cell populations (Foster et al., 2012). Secondly, it has been recently shown that Th17 populations express CD161, using an expression microarray approach.
Acosta-Rodriguez, E. V., Rivino, L., et al., 2011; Foster et al., 2012), although their overall role in disease progression is still not clear. Foster et al. (2012) addressed the impact of IL-22 on IL-17A to date has not been shown to have a major influence on outcome in liver disease models (Zenevics et al., 2007). In contrast, however, there is a dominant hepatoprotective role of IL-22 in vivo. Both blockade of IL-22 and genetic knockout of IL-22 revealed a major effect of this cytokine in the ConA hepatitis model (Zenevics et al., 2007). IL-22 has a major influence on hepatocyte biology through triggering via the IL-22R. IL-22 may protect directly against hepatic damage since it is known to promote proliferation and limit apoptosis (Wolk et al., 2004; Zenevics et al., 2007; Dambacher et al., 2008; Park et al., 2010). We have further analyzed the impact of IL-22 on gene expression patterns in human hepatocyte lines in vitro and confirm these data, indicating that the major up-regulated gene families relate to cell division (Ramatamurthy et al., manuscript in preparation). This is also consistent with the proven impact of IL-22 overexpression in vivo in promoting tumor growth (Park et al., 2010).

Recent studies have addressed whether type 17 cells may be identified in the liver during chronic hepatitis B and C (Zhang et al., 2010). We have further analyzed the impact of IL-22 on gene expression patterns in human hepatocyte lines in vitro and confirm these data, indicating that the major up-regulated gene families relate to cell division (Ramatamurthy et al., manuscript in preparation). This is also consistent with the proven impact of IL-22 overexpression in vivo in promoting tumor growth (Park et al., 2010).

We attempted to correlate the frequencies of cytokine secreting T cells with clinical parameters in our patient groups. Analysis of correlation with disease stage, ALT, or viral load did not reveal any significant relationship for CD4+ T cells expressing IL-17, IL-22, or IFN-γ. Although we did not find an association between Th17 or Th22 levels in tissue and clinical state in our study, one caveat is that most patients had relatively mild disease, and there were very few with severe levels of fibrosis. The impact of these cells in long term disease progression (of HCV infection and other inflammatory diseases of the liver), therefore, requires future longitudinal studies.

Interestingly, there do appear to be subsets of “Th22-like” cells in the human liver. This subset has been described by the group of Lanzavecchia; differentiation appears to be driven by IL-6 and TNFα, and may also be triggered by plasmacytoid dendritic cells (Duhen et al., 2009). These cells have been shown to express CCRI, CCRII, and CCR10. CCR6 has been linked to mucosal surveillance and Th17 differentiation (Schuitzer et al., 2003; Azcuta-Rodriguez et al., 2007; Liu and Rohowsky-Kochan, 2008; Eyerich et al., 2009). Thus, the features of the Th22 subset extend beyond the skin and are likely to include other relevant epithelial tissues and certainly the liver.

The most interesting finding of our study was the consistent enrichment of IL-22/IFN-γ co-producing CD4+ T cells in liver. Their in vivo function was not elucidated in this study, but interestingly, IL-22R expression may be up-regulated by IFN-γ (Wolk et al., 2004). This implies that the IFN-γ/IL-22 secreting cell population may have a particularly relevant role in this tissue, potentially augmenting the impact of IL-22. IFN-γ itself clearly has important pro-inflammatory and antiviral properties in HCV. Additionally there appear to be many other cytokines and chemokines which may be co-secreted with IFN-γ or IL-22 by the CD161+ T cell population (Fleming et al., manuscript in preparation) and, therefore, further description of the true functional potential of the IL-22/IFN-γ-secreting CD4+ T cell subset in health and disease is an important future goal.

In summary, we have analyzed the CD4+ T cell infiltrate in the liver in chronic HCV, focusing on CD161 expression and associated cytokine secretion. Within liver tissue we identified a population of CD4+ T cells which are consistently enriched and which co-secrete IL-22 and IFN-γ. Both these cytokines are of likely significance in the pathogenesis of human liver disease. To what extent these cells play a role in long-term evolution of disease and whether modulation of the appropriate pathways could influence this process, are important questions for the future.

ACKNOWLEDGMENTS

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We attempted to correlate the frequencies of cytokine secreting T cells with clinical parameters in our patient groups. Analysis of correlation with disease stage, ALT, or viral load did not reveal any significant relationship for CD3+ T cells expressing IL-17, IL-22, or IFN-γ. Although we did not find an association between Th17 or Th22 levels in tissue and clinical state in our study, one caveat is that most patients had relatively mild disease, and there were very few with severe levels of fibrosis. The impact of these cells in long term disease progression (of HCV infection and other inflammatory diseases of the liver), therefore, requires future longitudinal studies.

Interestingly, there do appear to be subsets of “Th22-like” cells in the human liver. This subset has been described by the group of Lanzavecchia; differentiation appears to be driven by IL-6 and TNFα, and may also be triggered by plasmacytoid dendritic cells (Duhen et al., 2009). These cells have been shown to express CCRI, CCRII, and CCR10. CCR6 has been linked to mucosal surveillance and Th17 differentiation (Schuitzer et al., 2003; Azcuta-Rodriguez et al., 2007; Liu and Rohowsky-Kochan, 2008; Eyerich et al., 2009). Thus, the features of the Th22 subset extend beyond the skin and are likely to include other relevant epithelial tissues and certainly the liver.

The most interesting finding of our study was the consistent enrichment of IL-22/IFN-γ co-producing CD4+ T cells in liver. Their in vivo function was not elucidated in this study, but interestingly, IL-22R expression may be up-regulated by IFN-γ (Wolk et al., 2004). This implies that the IFN-γ/IL-22 secreting cell population may have a particularly relevant role in this tissue, potentially augmenting the impact of IL-22. IFN-γ itself clearly has important pro-inflammatory and antiviral properties in HCV. Additionally there appear to be many other cytokines and chemokines which may be co-secreted with IFN-γ or IL-22 by the CD161+ T cell population (Fleming et al., manuscript in preparation) and, therefore, further description of the true functional potential of the IL-22/IFN-γ-secreting CD4+ T cell subset in health and disease is an important future goal.

In summary, we have analyzed the CD4+ T cell infiltrate in the liver in chronic HCV, focusing on CD161 expression and associated cytokine secretion. Within liver tissue we identified a population of CD4+ T cells which are consistently enriched and which co-secrete IL-22 and IFN-γ. Both these cytokines are of likely significance in the pathogenesis of human liver disease. To what extent these cells play a role in long-term evolution of disease and whether modulation of the appropriate pathways could influence this process, are important questions for the future.

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