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Effect of PD-1:PD-L1 in Invariant Natural Killer T Cell Emigration and Chemotaxis Following Sepsis

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Abstract

Invariant natural killer T-cells (iNKT) are a subset of T-cells that play a regulatory role in sepsis. Following cecal ligation and puncture (CLP), iNKT cells emigrate from the liver and into the circulation and peritoneum in a manner dependent upon co-inhibitory molecule Programmed Cell Death Receptor 1 (PD-1). We hypothesized that the effect of PD-1 on iNKT cell emigration was dependent upon the direct PD-1:PD-L1 interaction and that PD-1 and PD-L1 would play a role in chemotaxis and chemokine receptor expression. Adoptive transfer of Vybrant-labelled wild type (WT) cells showed the donor iNKT cells migrated from the liver to the peritoneum following CLP, but PD-L1 deficient donor iNKT cells did not. In a chemotaxis assay, WT iNKT cells chemotaxed to CXCL12, but PD-1 and PD-L1 deficient iNKT cells did not. Using flow cytometry to evaluate chemokine receptor expression, peritoneal iNKT expression of CXCR4 increased following CLP in the WT, PD-1, and PD-L1 deficient animals, and CXCR6 increased in the WT and PD-1 deficient animals. In conclusion here we document that the hepatic emigration of iNKT cells following CLP to the peritoneum appears dependent upon the direct PD-1:PD-L1 interaction, however, while PD-1 and PD-L1 appear to play a role in chemotaxis, this is unlikely a reflection of iNKT cell chemokine receptor expression changes.

Keywords

Sepsis; iNKT; cecal ligation and puncture; chemotaxis; PD-1; PD-L1

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Introduction

Survival from sepsis is dependent upon control and regulation of this immune and inflammatory response. This includes a vital role of cell migration to the site of the infection. Although our understanding of the control of immune cell migration in general, is growing, there remains a paucity of information pertaining to the regulation of key innate regulatory immune cells. Invariant Natural Killer T-cells (iNKT-cells) are innate lymphocytes that are emerging as key regulators of the immune response to a variety of diseases, including cancer, autoimmune disorders, as well as sepsis(1-3). Specifically, we have demonstrated a role for hepatic iNKT cells in modulating both the systemic response to sepsis as well as the phagocytic response(1, 4).

iNKT cells comprise approximately 30% of liver lymphocytes. It has been postulated that they serve as patrolling surveillance cells in the liver, and this patrolling activity may be controlled by chemokines following infection(5, 6). Intra-hepatic trafficking has been noted following a sterile liver injury(7). Following polymicrobial peritoneal sepsis, hepatic iNKT cells are noted to become activated, migrate from the liver leading to a decline in total number of hepatic iNKT-cells, and appear in the peritoneal cavity(1).

Immune cell trafficking, including lymphocyte homing and recirculation, is noted to be modulated by a variety of chemokines(8, 9). These molecules are noted to play a variety of roles in a range of physiological and pathologic processes, including iNKT-cell functional modulation. CXCR3 is noted to be expressed on, among others, activated T-cells and natural killer cells, and has recently been shown to play a role in arresting iNKT-cell migration(6). CXCR4, with the ligand CXCL12, can potentially act through suppression of inflammation, and has been shown to mediate survival following sepsis(10). CXCR6 appears to play a potentially protective role in aiding directed iNKT-cell migration(11). Further, CXCR6 induces accumulation of iNKT-cells to the liver following peripheral stimulation(5, 12). The mechanism controlling iNKT-cell migration, and potential role of specific chemokines in response to a distant source of sepsis remains unclear.

Programmed Cell Death Receptor 1 (PD-1) is a key checkpoint molecule, that combined with ligands PD-L1 and PD-L2, are classically thought to control T-cell response to antigens(13, 14). PD-1 plays a controlling role in the immune response in both experimental sepsis as well as in patients with sepsis. In critically ill septic patients, acute lung injury mortality was associated with increased PD-1 expression on circulating T-cells. An association was also noted between APACHE II scores and expression of PD-1(15). In murine sepsis models, PD-1 affect pulmonary lymphocyte trafficking as well development of acute lung injury(16). Following stimulation, iNKT cells upregulate PD:1 and PD:L1 expression(17, 18) and the emigration of hepatic iNKT cells following peritoneal sepsis is dependent upon PD-1(1). Furthermore, a link between PD-1 and CXCL12/CXCR4 has been described in cancer(19, 20). Thus, we sought to elucidate whether PD-1-dependent iNKT emigration also depends upon direct PD-L1 ligation, and whether PD-1 and/or PD-L1 play a role in iNKT chemotaxis and chemokine receptor expression following CLP.

Materials and Methods

Adoptive Transfer Studies

iNKT harvesting and isolation—Livers from WT and PD-L1 ^{-/-} mice were harvested using a collagenase non-parenchymal cell isolation protocol as previously described(21). 4 livers were harvested per recipient mouse. iNKT cells were purified, labeled with Vybrant™ CM-Dil from Invitrogen, and purity of the sample was assessed as previously described(1).

iNKT cell transplant and sepsis model— 2.5×10^5 labeled iNKT cells were tail vein injected into WT recipients. 24 hours after tail vein injection, the WT recipients were subjected to either cecal ligation and puncture (CLP) or sham procedure (Sham) as previously described(22). The peritoneal fluid was spun down and resuspended. Flow cytometry was done on the peritoneal fluid samples to assess for presence of the Vybrant stained iNKT cells. Percentage of total cell population as well as absolute cell counts were measured. All animal experiments were reviewed and approved by our Institutional Animal and Use Committee.

Chemotaxis Assay

iNKT cells were harvested from the livers of Male C57BL/6J, PD-1 ^{-/-} and PD-L1 ^{-/-} mice using a collagenase-free non parenchymal cell isolation protocol and purified as described above. In a Neuro Probe Micro Chemotaxis chamber, iNKT cells were chemotaxed to a solution of 50nM CXCL12 versus control media over 90 minutes at 37C in 5%CO₂ in a protocol adapted from Campbell(23). Chemotaxed cells were stained, counted with light microscopy and the average number over 20 high powered fields presented.

Chemokine receptor analysis

WT, PD-1 ^{-/-}, or PD-L1 ^{-/-} mice were subjected to either sham or CLP as described above. 24 hours after, the mice were sacrificed and liver and peritoneal samples were collected and prepared as described above. Using flow cytometry(1), numbers and percentage of iNKT cells (as detected by GalCer pre-loaded CD1d tetramers conjugated to APC) expressing CXCR3, CXCR4, and CXCR6 were assessed.

Statistics

For the adoptive transfer studies and chemokine receptor analysis, ANOVA by rank (Dunn's) was carried out. For the chemotaxis assays, t-tests as well as rank-sum test were used. Sigmaplot 12.5 was used and significance was set at $p < 0.05$. All data is presented as mean $\pm$ standard error (SE).

Results

Adoptive Transfer Studies

With respect to the i.v. injected-cells, there was a significant influx of Vybrant™ labeled WT-iNKT-cells into the peritoneal cavity following sepsis compared with sham as both a percentage and absolute numbers of cells (1.16% vs. 0.03%, $p < 0.05$; 6.24×10^4 vs. 0.03×10^4 cells, $p < 0.05$; $n = 4-7$ animals/group). However, following CLP when compared with

sham, the number of labeled PD-L1 $-/-$ iNKT i.v. injected-cells failed to migrate to (increase in) the peritoneal cavity, again as both a percentage and absolute numbers of cells (0.14% vs. 0.04%, $p>0.05$; 0.10×10^4 vs. 0.04×10^4 cells, $p>0.05$; $n=4-7$ animals/group). When looking only at mice subjected to CLP, there was a significant influx of i.v. injected-labelled WT-iNKT-cells into the peritoneal cavity as compared to their i.v. injected PD-L1 $-/-$ iNKT-cell counterparts, as a percentage, but not absolute numbers of cells (1.16% vs. 0.14%, $p<0.05$; 6.24×10^4 vs. 0.10×10^4 cells, $p>0.05$; $n=4-7$ animals/group). These findings suggest that the migration of iNKT cells is dependent upon the direct effects of PD-L1 on PD-1 (Figure 1).

Chemotaxis Studies

Using unlabeled cells in the subsequent experiments, wild type iNKT cells preferentially chemotaxed towards CXCL12, but not to medium (16.9 vs. 4.4 cells/high powered field, $p<0.05$ $n=5$ animals/group). PD-1 $-/-$ and PD-L1 $-/-$ iNKT cells did not preferentially chemotax towards CXCL12 when compared to medium (4.9 vs. 5.4 and 10 vs. 5.4 cells/high powered field respectively, $p>0.05$, $n=5$ animals/group). These findings suggest that the chemotaxis of iNKT cells is dependent upon PD-1 and PD-L1 (Figure 2).

Chemokine receptor analysis

Wild type hepatic iNKT cells from mice that were subjected to CLP as compared to sham, displayed no difference in expression of CXCR3 ($20.4\% \pm 11.1$ vs $19.7\% \pm 7.3$; $p>0.05$) CXCR4 ($39.3\% \pm 17.2$ vs $32.6\% \pm 18.9$; $p>0.05$) or CXCR6 (12.0 ± 10.7 vs 10.6 ± 5.9 ; $p>0.05$). However within the peritoneal cavity, CLP, compared to sham, induced an increase in the number of wild type peritoneal iNKT cells expressing CXCR4 ($52.6\% \pm 15.3$ vs $26.0 \pm 12.0\%$; $p<0.05$) and CXCR6 ($52.0\% \pm 15.0$ vs $31.1\% \pm 10.2$; $p<0.05$). There was no difference in CXCR3 expression on septic wild type peritoneal iNKT compared to sham ($11.7\% \pm 5.3$ vs $17.1\% \pm 12.3$; $p>0.05$). PD-1 $-/-$ hepatic iNKT cells from mice that were subjected to CLP as compared to sham, displayed no difference in expression of CXCR3 ($23.6\% \pm 13.9$ vs $30.4\% \pm 18.1$; $p=0.46$), CXCR4 ($47.3\% \pm 21.7$ vs $34.3\% \pm 21.2$; $p=0.35$), but did show upregulation of CXCR6 ($14.7\% \pm 6.2$ vs $5.8\% \pm 4.5$; $p=0.02$). Again, within the peritoneal cavity, CLP, compared to sham, induced an increase in the number of PD-1 $-/-$ peritoneal iNKT cells expressing CXCR4 ($47.9\% \pm 11.7$ vs $20.4\% \pm 8.7$; $p<0.05$) and CXCR6 ($42.7\% \pm 17.4$ vs $17.3\% \pm 5.3$; $p<0.05$). Again, there was no difference in CXCR3 expression on septic PD-1 $-/-$ peritoneal iNKT compared to sham ($12.0\% \pm 5.4$ vs $15.4\% \pm 1.9$; $p>0.05$). PD-L1 $-/-$ hepatic iNKT cells from mice that were subjected to CLP as compared to sham, displayed no difference in expression of CXCR3 ($29.3\% \pm 11.3$ vs $28.9\% \pm 8.6$; $p=0.94$), CXCR4 ($39.8\% \pm 26.3$ vs $41.9\% \pm 27.2$; $p=0.93$), and CXCR6 ($19.2\% \pm 12.4$ vs $24.3\% \pm 12.1$; $p=0.43$). Again, within the peritoneal cavity, CLP, compared to sham, induced an increase in the number of PD-L1 $-/-$ peritoneal iNKT cells expressing CXCR4 ($45.6\% \pm 17.2$ vs $19.0 \pm 2.8\%$; $p=0.02$) and no difference in CXCR3 expression ($20.5\% \pm 6.8$ vs. $15.6\% \pm 3.8$; $p=0.12$). In contrast to the WT and PD-1 $-/-$ animals, there was no difference in PD-L1 $-/-$ peritoneal iNKT cell expression of CXCR6 following CLP ($41.1\% \pm 13.5$ vs $37.3\% \pm 13.4$; $p=0.60$). However, although peritoneal CXCR4 and CXCR6 expression levels were elevated following sepsis, this elevation was independent of PD-1 or PD-L1. When comparing across strains, following CLP, there was difference between wild type, PD-1 $-/-$

and PD-L1^{-/-} in the elevation of CXCR4 (52.6 ± 15.3 vs 47.9 ± 11.7 vs 45.6 ± 17.2 ; $p>0.05$), or CXCR6 (52 ± 15 vs 42.7 ± 17.4 vs 41.1 ± 13.5 ; $p>0.05$).

Discussion

iNKT-cells are a subset of innate regulatory T-cells that respond to a wide variety of stimuli and are capable of producing a diversity of cytokine responses. iNKT-cells play key regulatory roles in a diversity of diseases including viral and bacterial infections, cancer surveillance and autoimmune diseases(1-3, 6). It is being increasingly demonstrated that iNKT-cells play a key role in coordinating this immune response in both experimental sepsis as well as in critically ill septic patients. iNKT-cells have been shown to be capable of modulating the inflammatory and immune responses to sepsis including modulating phagocytic function(1, 5, 24, 25).

iNKT-cells, akin to many immune cells, are known to traffic(26, 27). However many of the mechanisms driving this migration, especially as it relates to polymicrobial sepsis, remains largely unknown. It is becoming increasingly evident that iNKT-cells display patterns of tissue trafficking, homeostasis and homing distinct from conventional T-lymphocytes. Much of our current understanding of iNKT-cell migration is based on developmental studies or in response to non-infectious stimuli(7, 28). Kubes et al demonstrated localized migratory patterns of iNKT-cells in response to a sterile insult(7, 29). This alteration in iNKT-cell migration was independent of CD1d, and appeared to be related to neuroendocrine control. Further, Velazquez et al noted iNKT-cell arrest within liver sinusoids following stimulation(28). Ourselves and others have noted that iNKT-cells have an ability to migrate in response to infectious stimuli(1, 7, 24, 29).

We have previously demonstrated that, following the onset of intra-abdominal sepsis, liver iNKT-cells become activated, migrate from the liver, enter the circulation and ultimately arrive in the peritoneal cavity, the source of the sepsis(1). We noted that this migration was PD-1 dependent. PD-1 is a checkpoint molecule which plays a critical role in both experimental sepsis as well being associated with outcomes in critically ill septic patients(15). It is predominantly considered a co-inhibitory molecule located primarily on T-cells.

Ligation of PD-1 with its ligands PD-L1 and PD-L2 can induce either inhibitory or stimulatory responses(13). The exact functioning of PD-1 on iNKT-cells remains unclear. Although PD-1 engagement is critical to initial and activation, repeated iNKT-cell stimulation will over time lead to induction of anergy, a mechanism mediated by PD-1(13, 17, 18). We herein undertook to assess whether our previously noted PD-1 mediated iNKT-cell migration was dependent upon direct PD-1:PD-L1 ligation. We noted that following adoptive transfer, the emigration of iNKT cells from the liver to the peritoneum appears to require direct ligation of PD-1 by PD-L1. Typically PD-1 and PD-L1 are thought to deliver a co-inhibitory signal to iNKT cells. However, this data supports the concept that initial PD-1:PD-L1 may be stimulatory.

To further assess a potential mechanism associated with this PD-1 mediated iNKT-cell migration, we undertook an assessment of the possible role of chemokine receptor expression alterations. NKT, both variant and invariant require both survival signal and growth factors for peripheral maintenance. Much of NKT-cell functioning is driven by CD1d ligation(2, 30). However, unlike thymic NKT cells, non-thymic NKT-cells do not require ongoing CD1d interaction for homeostasis, proliferation or survival in their tissue beds(30). Rather, the cytokine milieu as well as the tissue locale in which the iNKT-cells are found, liver versus spleen, may be just as important in influencing iNKT-cell function(3, 27, 30, 31).

CXCR4, and its ligand CXCL12, play a potent role in lymphocyte chemoattraction. CXCL12 is noted to be released from sites of injury inducing recruitment of lymphocytes. An *in vitro* chemotaxis assay with CXCL12, a chemoattractant of leukocytes and iNKT-cells(23, 32), yielded directed migration of iNKT-cells towards CXCL12. Although this appears to differ from findings noted by Hornung et al who reported only weak migration to CXCL12, they utilized naïve cultured cells that underwent single antibody stimulation(32), far different from the milieu found in polymicrobial sepsis. This directed migration that we observed in wild type mice was not seen in either the PD-1^{-/-} or the PD-L1^{-/-} mice, further noting that PD-1 may play an early stimulatory role in iNKT-cell activity.

These observations raised the further question of whether iNKT-cell migration was due to changes in iNKT-cell chemokine receptor expression. Therefore, we specifically assessed CXCR3, which has been shown to play a role in NKT-cell migration(33) and hepatic iNKT-cell arrest and clustering(6) as well as CXCR4 and CXCR6 which induce directional tissue targeted migration, and survival, of iNKT-cells(5, 10-12).

Overall, there was very little difference in the chemokine expression among hepatic iNKT-cells following sepsis, whereas iNKT-cells which migrated to the peritoneal cavity were noted to elevated expression of both CXCR4 and CXCR6. The only hepatic change noted was an increase in CXCR6 expression on PD1^{-/-} hepatic iNKT-cells. CXCR6 has been shown to mediate iNKT-cell accumulation, clustering and arrest within the liver in several inflammatory conditions(5, 12). It is known that in the iNKT cells display a baseline population of patrolling iNKT cells that derive from the liver, emigrating into the blood, to tissue beds, and track back to the liver. Following a stimulus, hepatic iNKT-cells are noted to arrest and cluster within one hour of the stimulus(5, 28), potentially awaiting directional signaling to induce targeted migration(25, 33, 34). This is in keeping with our previous observation that iNKT-cell numbers were unchanged at 4 hours following stimulation, but migration was noted at 12 hours(4). If the iNKT-cell stimulus is localized to the liver, then further migration is not required and iNKT-cells are noted to cease patrolling(24, 28). If the site of infection or inflammation is distant to the liver, such as in CLP, then secondary, CXCR4 and CXCR6 directed migration, potentially mediated via PD-1, is required to disperse the iNKT-cells to source of sepsis or inflammation(11, 26, 32, 33).

This is further supported by the finding that iNKT-cells that have migrated to the peritoneal cavity do display increased CXCR4 and CXCR6 expression, implying that iNKT-cells that are recruited to the source of the infection do so under the direction of directed trafficking

signaling. This is in keeping with the observation of Thomas et al who noted elevated levels of CXCR4 expression on iNKT-cells indicative of a homing or patrolling profile(27). A potential interaction between PD-1 and CXCL12/CXCR4 has been described in pancreatic tumor sensitivity to immunotherapy and cancer regression(19, 20). However, the changes we observed in CXCR4 and CXCR6 expression appeared independent of PD-1:PD-L1, since receptor expression on iNKT-cells was elevated across all strains. Since PD-1 plays a role in modulating chemotaxis to CXCL12, the ligand for CXCR4, but not expression of the receptor, we postulate that PD-1 is mediating downstream effects linking receptor expression and iNKT-cell migration.

CXCR3, a chemokine receptor expressed preferentially on NK, NKT and T-cells, has been shown to be important in the migration of T-cells in general(34), and more recently iNKT-cells. CXCR3 mediated lymphocyte trafficking has been reported in response to viral infections, malignancies, and auto-immune diseases(35), and recently in response to bacterial infections. However, the data pertaining to the role of CXCR3 in iNKT-cell trafficking remains conflicting. We herein found no effective difference in wild type, PD-1^{-/-} or PD-L1^{-/-} mice with respect to iNKT-CXCR3 expression in either liver or peritoneal cavity following CLP. This is in contradistinction to work by Lee et al which demonstrated that with *Borrelia*-based models of infection, hepatic iNKT cells show a CXCR3-dependent arrest and decreased velocities at sites of antigen presentation(6), while Tsutahara et al noted a significant role for CXCR3 in distal migration(33). However, our findings are in keeping with those reported by Herzig et al(34) who noted no significant difference in CXCR3 expression on iNKT-cells in response to polymicrobial peritoneal sepsis. We believe that these differences may be explained by the fact that CXCR3 plays a strong role in lymphocyte trafficking in response to viral infection, as well as the fact that iNKT-cells are known to respond differentially to intra- versus extra-cellular bacteria. Thus, we believe that CXCR3 may play a muted role in iNKT-cell trafficking in response to a polymicrobial infection.

Further, our data supports the concept that peripherally activated iNKT-cells traffic initially through the liver. However, in the absence of secondary signaling, potentially through PD-1, these activated iNKT-cells may remain trapped in the liver unable to continue directed migration to the peritoneal cavity.

Conclusion

Following CLP, iNKT-cell activation and mobilization is dependent upon the direct ligations of PD-1 and PD-L1. Ongoing direct PD-1:PD-L1 interaction and stimulation allows for the iNKT cells to mobilize out of their cluster state, emigrate into circulation, and then into the peritoneum. These iNKT cells exhibit increased CXCR4 and CXCR6 potentially aiding in the directed and targeted migration. PD-1 deficiency leads to decreased emigration out of the liver and, thus, no accumulation of peritoneal iNKT-cell. However, further work is needed to address downstream pathways between receptor expression and cell migration. To our knowledge, this is the first study examining the effects of PD-1 and PD-L1's on iNKT chemotaxis from the liver to the peritoneum following CLP. Given our growing appreciation of the importance of iNKT-cells in mediating survival from sepsis, coupled with the clinical

use of PD-1 blocking antibody, this may provide a potential therapeutic target in the management of sepsis.

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Abbreviations

CLP	Cecal ligation and puncture
iNKT	Invariant natural killer T-cells
PD-1	Programmed Cell Death Receptor 1
PD-L1	Programmed Death-Ligand 1
PD-L2	Programmed Death-Ligand 2
WT	Wild type

Figure 1. Adoptively transferred wild type Vybrant™-labelled iNKT cells were noted to migrate to the peritoneal cavity following CLP. However, iNKT-cells from PD-L1-/- mice failed to migrate to the peritoneal cavity
(Mean $\pm$ SE; * $p < 0.05$; $n = 4-7$ animals/group).

Figure 2. Wild type iNKT cells were noted to display chemotaxis towards CXCL12. However, no preferential chemotaxis towards CXCL12 was noted with either PD-1^{-/-} or PD-L1^{-/-} iNKT-cells (Mean $\pm$ SE; * $p < 0.05$; n=5 animals/group)

Figure 3. (A-C). Lack of PD-1 or PD-L1 gene expression (with the exception of CXCR6 on PD-L1 ^{-/-} mice) was not noted to effect the frequency iNKT chemokine receptor⁺ cells irrespective of impact of CLP

A) Percentage of peritoneal iNKT cells expressing CXCR3. **B)** Percentage of peritoneal iNKT cells expressing CXCR4. **C)** Percentage of peritoneal iNKT cells expressing CXCR6 (Mean $\pm$ SE; *p<0.05; n=6-8 animals/group).