High PD-1 expression and suppressed cytokine signaling distinguish T cells infiltrating follicular lymphoma tumors from peripheral T cells

June H. Myklebust$^{1,2}$, Jonathan M. Irish$^{1,3,4}$, Joshua Brody$^{1,5}$, Debra K. Czerwinski$^{1}$, Roch Houot$^{1,6}$ Holbrook E. Kohrt$^{1}$, John Timmerman$^{7}$, Jonathan Said$^{8}$, Michael R. Green$^{1}$, Jan Delabie$^{9}$, Arne Kolstad$^{10}$, Ash A. Alizadeh$^{1}$ and Ronald Levy$^{1}$

$^1$Department of Medicine, Oncology Division, Stanford University, Stanford, CA, USA

$^2$Department of Immunology, Institute for Cancer Research, Oslo University Hospital, Oslo, and Centre for Cancer Biomedicine, University of Oslo, Norway

$^3$Department of Microbiology & Immunology, Baxter Laboratory of Genetic Pharmacology, Stanford University, Stanford, CA, USA

$^4$Department of Cancer Biology, Vanderbilt University School of Medicine, Nashville, USA

$^5$Lymphoma Immunotherapy Program, Mount Sinai School of Medicine, New York, USA

$^6$Service d’Hématologie Clinique, Centre Hospitalier Universitaire de Rennes & INSERM U917, Université de Rennes 1, Rennes, France

$^7$Division of Hematology & Oncology, Department of Medicine, University of California, Los Angeles, Los Angeles, USA

$^8$Department of Pathology & Laboratory Medicine, University of California, Los Angeles, Los Angeles, USA

$^9$Cancer Clinic, Department of Pathology, Oslo University Hospital, Oslo, Norway

$^{10}$Cancer Clinic, Department of Oncology, Oslo University Hospital, Oslo, Norway

**Corresponding author:** Dr. Ronald Levy M.D., Professor of Medicine, Chief, Division of Oncology, 269 Campus Drive, CCSR 1105, Stanford University Medical Center, Stanford, CA 94305-5151; Tel (650) 725-6452; Fax (650) 725-1454; Email: levy@stanford.edu
Key Points

- FL TILs have reduced cytokine signaling

Abstract

Defects in T cell function in cancer patients might influence their capacity to mount efficient anti-tumor immune responses. Here we identified highly reduced IL-4-, IL-10- and IL-21-induced phosphorylation of STAT6 and STAT3 in tumor infiltrating T cells (TILs) in follicular lymphoma (FL) tumors, contrasting other non-Hodgkin lymphoma TILs. By combining phospho-protein specific flow cytometry with several T cell markers, we identified that CD4⁺CD45RO⁺CD62L⁻ FL TILs were largely non-responsive to cytokines, in contrast to the corresponding autologous peripheral blood subset. We observed differential expression of the inhibitory receptor PD-1 in FL TILs and peripheral blood T cells. Furthermore, CD4⁺PD-1⁺ FL TILs, containing T_FH and non-T_FH cells, had lost their cytokine responsiveness, whereas PD-1⁻ TILs had normal cytokine signaling. However, this phenomenon was not tumor-specific, since tonsil T cells were similar to FL TILs. FL tumor cells were negative for PD-1 ligands, but PD-L1⁺ histiocytes were found within the T-cell rich zone of the neoplastic follicles. Disruption of the microenvironment and in vitro culture of FL TILs could restore cytokine signaling in the PD-1⁺ subset. Since FL TILs in vivo likely receive suppressive signals through PD-1, this provides a rationale for testing PD-1 Ab in combination with immunotherapy in FL patients.
Introduction

Follicular lymphoma (FL) is characterized by the t(14;18) translocation which results in overexpression of BCL2, an anti-apoptotic protein. Patient clinical outcomes are markedly heterogeneous, and FL can transform into diffuse large B-cell lymphoma (DLBCL), a more aggressive malignancy.

FL outcome is strongly influenced by the immune cell microenvironment. Gene expression profiling has identified a clinically relevant gene expression signature possibly representing an immune response to the tumor.1 Furthermore, the immune cell composition of the FL tumor microenvironment is important as high numbers of tissue infiltrating macrophages correlated with poor outcome in patients receiving chemotherapy regimens,2 but not in patients also receiving the monoclonal antibody Rituximab.3,4 Several observations further support the hypothesis of an immune suppressive microenvironment in affected lymph nodes (LN). These LN have increased numbers of T regulatory cells (Tregs),5,6 and purified FL lymphoma B cells can induce the conversion of conventional CD4+ T cells into FoxP3 positive Tregs.5-8 Most studies have found a positive correlation between the number of infiltrating Tregs and favorable outcome,9-11 although some report opposite findings.12 However, follicular localization of Tregs was then found to be associated with poor overall survival and high risk of transformation.13 A recent study further implied that tumor infiltrating T cells (TILs) from FL biopsies had impaired immunologic synapse formation.14

Phospho-flow cytometry analysis has emerged as a powerful tool to analyze intracellular signaling events in complex populations of cells, because of its ability to simultaneously discriminate cell types based on surface marker expression, and to assess the activation status of intracellular proteins.15-18 We recently used this method and identified a new lymphoma subset in FL patients, the lymphoma negative prognostic (LNP) subset, with abnormal B cell antigen receptor (BCR) signaling.19 Strikingly, the prevalence of this lymphoma cell subset in patient’s tumor at the time of diagnosis, prior to any treatment, was negatively associated both with the response to initial chemotherapy and with overall survival. The patients’ T-cell responses were also important, as patients with high IL-7-induced phosphorylation of STAT5 in TILs had a better outcome.19 We therefore expanded upon this observation by interrogating the responsiveness of FL TILs to a variety of effector cytokines in comparison to TILs from healthy donors and other B-cell malignancies.
Here, using phospho-flow cytometry, we found that FL TILs had distinctively reduced signaling responses to several cytokines, including interleukin (IL)-4, IL-10 and IL-21. We identified that CD4^+ CD45RO^+ CD62L^- T cells, the major T cell subset in FL LN, was largely unresponsive to cytokines, exemplified by decreased IL-4-induced phosphorylation of STAT6. This was not a general feature of these cells, since the majority of CD4^+ CD45RO^+ CD62L^- T cells in peripheral blood could respond to IL-4 stimulation. Furthermore, we showed that the non-responsive FL TILs are characterized by high expression of the inhibitory receptor PD-1, a potential therapeutic target.
Materials and methods

Human samples

All specimens were obtained with informed consent in accordance with the Declaration of Helsinki. Normal human peripheral blood and human non-Hodgkin lymphoma specimens were obtained from patients at the Stanford University Medical Center, Stanford, CA with informed consent, according to an IRB-approved protocol or with informed consent from the Norwegian Radium Hospital, Oslo, Norway according to a Regional Ethic Committee (REK)-approved protocol (REK# 2.2007.2949). Tonsils and autologous peripheral blood samples were obtained from children undergoing tonsillectomy at Stanford Hospital, with informed consent, according to an IRB-approved protocol. All samples were processed to mononuclear cells (MNC) by Ficoll gradient centrifugation (Ficoll-Paque™ PLUS, GE Healthcare, NJ, USA), and cryopreserved in liquid nitrogen. In several cases, FL LN fragments were incubated with collagenase/DNAse solution for 60 minutes at 37°C during preparation of MNC suspensions. An overview of the non-Hodgkin’s lymphoma patient samples is given in Supplementary Table S1, and the normal control samples in Supplementary Table S2.

Reagents

Recombinant human (rh) Interleukin-4 (IL-4), rh IL-7, rh IL-10 and rh IL-21 were from eBioscience (San Diego, CA) and were used at a final concentration of 20 ng/mL. Antibodies from Becton Dickinson (BD; San Jose, CA) were used to detect surface expression of CD3 (clone UCHT1), CD4 (clone RPA-T4), CD5 (clone L17F12), CD8 (clone SK1), BTLA/CD272 (clone J168-540.90.22), PD-1/CD279 (clone EH12.1), CTLA4/CD152 (clone BNI3), CD57 (clone NK-1), CD62L (clone Dreg 56), CD274/PD-L1 (clone MIH1), CD273/PD-L2 (clone MIH18), CD69 (clone L78), CD27 (clone L128), CD28 (clone CD28.2), CD45RO (clone UCHL1), CD20 (extracellular, clone L27) and CXCR5 (clone RF8B2). Detection of FoxP3 was performed by staining the samples with antibodies from BD to detect CD25, CD8, CD45RO, CD20, CD4 and CD3, prior to fixation and permeabilization according to the eBioscience protocol, and then staining with FoxP3 (clone PCH101 from eBioscience). The following antibodies from BD were used to detect phosphorylated STAT3(Y705) (clone 4/P-STAT3), STAT5(Y694) (clone 47) and STAT6(Y641) (clone 18), and these were used in combination with the following antibodies from BD: CD20 cytoplasmic tail (clone H1), BCL2 (clone 6C8), CD5 (clone L17F12), CD3 (clone UCHT1), CD4 (clone RPA-T4), CD8 (clone SK1), CD62L (clone Dreg 56), CD45RO (clone UCHL1) and PD-1/CD279 (clone EH12.1), whereas ICOS/CD278 (clone C398.4A)
was from Biolegend. Antibodies from BD were used to detect production of IL-4 (clone 8D4-8) and IL-21 (3A3-N2.1).

**Activation of signaling and phospho-Flow cytometry**

Activation of signaling and detection of phospho-proteins were performed as previously described.$^{16;18;19}$ Briefly, the samples were thawed, and 1-5 million cells were used for flow cytometry based live/dead discrimination and immunophenotyping. Signaling was analyzed in the remaining sample that were allowed to rest for 30 minutes at 37°C, before redistribution at 200 µL per well into v-bottomed 96 well plates and given another 20 minutes rest. Signaling was activated by IL-4, IL-7, IL-10 or IL-21 for 15 minutes at 37°C. Paraformaldehyde (PFA) at a final concentration of 1.6 % was added to stop signaling and incubated for 5 minutes at RT, followed by permeabilization in >90% freezer-cold methanol. At this point, the samples could be stored at -80 °C, before further processing. After rehydrating the cells by washing by centrifugation 2 times in PBS, the cells were “barcoded” as previously described,$^{20}$ using the fluorescent esters; Pacific Blue and Pacific Orange (Molecular Probes, Life Technologies), see Supplementary methods. After barcoding, all samples from one patient were combined into 1 tube, and stained with antibodies for 30 min in the dark at RT. In experiments where phosphorylation in distinct T cell subsets was measured, more markers were included and barcoding of cells were skipped. To detect cytokine-induced activation in T_{FH} cells, FL LN specimens were stimulated with cytokines, fixed with PFA, and then stained with anti-CXCR5 Alexa647 (BD, clone RF8B2). After staining, the cells were washed and permeabilized in >90% methanol, followed by staining with the other Abs as described earlier. The samples were then collected on a LSR II flow cytometer (Becton Dickinson, CA, USA). Data was analyzed using Cytobank Software (www.Cytobank.org).

**In vitro culture of FL specimens**

FL LN specimens were cultured in vitro at 5 million/mL in the presence of mouse IgG1 anti-PD-1 Ab (Biolegend, clone EH12.2H7; used at a final concentration of 10 µg/mL) or mouse IgG1 isotype control Ab (Biolegend; clone MG1-45; used at a final concentration of 10 µg/mL) for various times as indicated, before the cells were washed 2 times in PBS by centrifugation and then re-stimulated with or without IL-4 for 15 minutes. IL-4-induced phosphorylation of STAT6 in various T cell subsets was measured by phospho-flow cytometry as described above.
**Immunohistochemistry**
Staining for PD-L1 in paraffin sections was performed using mAb 5H1 (provided by Dr. Lieping Chen, Johns Hopkins University) at BioPillar Laboratories (Monmouth Junction, New Jersey) using previously described methods.

**Statistics**
Mann-Whitney U test or two-tailed t-test for paired or unpaired samples were applied as specified in figure legends, to determine the level of statistical significance, using SPSS 16.0 (SPSS Inc., IL, USA). Data were considered statistical significant at $p < 0.05$. 
Results

Tumor infiltrating T cells in FL biopsies have suppressed effector cytokine signaling

Previous studies indicated that tumor infiltrating T cells (TILs) in FL patient samples had dysfunctional T cell receptor (TCR) signaling and impaired capacity for immunological synapse formation. Therefore, we studied their response to effector cytokines in an initial cohort of 14 FL patients. For comparison, we studied TILs from 12 DLBCL patients, 19 mantle cell lymphoma (MCL) patients, and 14 chronic lymphocytic leukemia (CLL) patients, as well as blood samples from 6 healthy donors. Lymphoma LN specimens or peripheral blood mononuclear cells (PBMC) were stimulated with cytokines for 15 minutes and induced phosphorylation was measured by phospho-flow cytometry as previously described. TILs were identified as CD3+CD5−CD20− cells (Figure 1A). STAT6 was phosphorylated by IL-4 and STAT3 by IL-21 in the majority of PBMC T-cells from a healthy donor. In contrast, a lower fraction of TILs from the FL LN specimen were able to respond (Figure 1A). TILs from the majority of FL specimens had a strikingly low cytokine-induced phosphorylation of STATs, compared to DLBCL, MCL and to PBMC T cells from healthy donors (Figure 1B-E). The effect was most prominent for IL-4-induced phosphorylation of STAT6, with a relative median fold induction of p-STAT6 of 0.79 in FL TILs, compared to 1.96, 1.86, 2.39 and 2.18 in TILs from DLBCL, MCL, CLL and healthy T-cells, respectively (Figure 1B, p<0.0003). IL-10- or IL-21-induced p-STAT3 gave similar results (Figure 1C-D). IL-7-induced phosphorylation of STAT5 was more heterogeneous also within each lymphoma type, but was significantly lower in FL TILs as compared to MCL TILs (Figure 1C, p<0.04). These results indicate that FL TILs have reduced capacity to respond to important effector cytokines regulating immune responses such as IL-4, IL-7, IL-10 and IL-21.

T cell composition is skewed towards CD4+CD45RO+ T cells in FL LN biopsies

The reduced cytokine responses among FL TILs, compared to those from other B cell malignancies and normal PBMC, could be due to different T cell subset composition. Indeed, immunophenotypic analysis showed a significant higher percentage of CD4+ T cells in FL specimens, with an average of 76% CD4+ T cells out of total CD3+ T cells, as compared to 62% and 63% in DLBCL and MCL respectively (Figure 2A). Furthermore, the majority of CD4+ T cells in FL specimens expressed CD45RO (Supplementary Figure S1A), and in average accounted for 69% of all TILs in FL specimens, as compared to 45% and 51% in DLBCL and MCL specimens, respectively (Figure 2B). However, also tonsil T cells showed similar high proportion of CD4+CD45RO+ cells (Figure 2B). Malignant LN from FL patients are known to have increased percentages of Tregs compared to healthy controls. We observed here that FL specimens in average
had 15% CD4⁺CD25⁺FoxP3⁺ Tregs out of total CD3⁺ T cells, compared to less than 11% in DLBCL and MCL specimens (Figure 2C).

**CD4⁺CD45RO⁺CD62L⁻ FL TILs, but not the corresponding autologous PBMC T cells have reduced cytokine signaling capacity**

Since Tregs in average were 15% of total T cells in FL specimens, it was unlikely that reduced cytokine signaling was accounted for by this subset alone. This was confirmed when detection of p-STAT3 or p-STAT6 were combined with detection of CD4, CD25 and FoxP3, as CD4⁺CD25⁺FoxP3⁺ Tregs overall responded better than the conventional CD4⁺ T cells after cytokine stimulation, in particular to IL-10-induced p-STAT3 (Supplementary Figure S2). Next, we combined detection of T cell markers, CD45RO and CD62L with p-STATs to measure cytokine-induced signaling in CD62L⁺CD45RO⁺, CD62L⁺CD45RO⁻ and CD62L⁻CD45RO⁺ T cells (gating strategy is shown in Figure 3A). We selected FL patients whose malignant LN and autologous blood samples were both available. The largest difference in IL-4-induced p-STAT6 between FL TILs and PBMC T cells was observed for the CD4⁺CD62L⁻CD45RO⁺ subset (Figure 3A), with average FC of 0.65 and 1.44 for LN and PBMC, respectively (Figure 3B, p<0.003). IL-21-induced p-STAT3 was also significantly lower in CD4⁺CD62L⁻CD45RO⁺ FL TILs than CD4⁺CD62L⁺CD45RO⁻ FL TILs, but was not different from the corresponding PBMC T cell subset (Supplementary Figure S3). We also investigated cytokine-induced signaling in tonsil and autologous PBMC T cells from healthy donors, and found that tonsil CD4⁺CD62L⁻CD45RO⁺ and CD4⁺CD62L⁻CD45RO⁺ T cells had significantly reduced IL-4-induced p-STAT6, compared to their blood counterparts, with an average FC of 0.34 and 0.73 as compared to 1.53 and 2.01 (Figure 3C). Taken together, the reduced cytokine signaling observed in FL TILs was mainly due to highly reduced signaling capacity in CD4⁺CD62L⁻CD45RO⁺ T cells. As the frequency of this subset is elevated in FL LNs, this further potentiates the difference in cytokine signaling as compared to MCL and DLBCL TILs. However, due to the similarities in reduced cytokine signaling between FL TILs and tonsil T cells, this might represent a physiological process.

The cytokine signaling deficit in FL TILs is restricted to PD-1⁺CD4⁺ T cells, and includes Tfh cells and non-Tfh cells.

Since CD4⁺CD62L⁻CD45RO⁺ FL TILs had low cytokine signaling capacity compared to their autologous PBMC T cell counterpart, we next investigated if this difference in cytokine signaling could be explained by differential expression of the inhibitory receptors CTLA-4, BTLA or PD-1. The greatest difference was
found for PD-1, which in average was expressed on 82% of the CD4+ TILs, in comparison to 33% of CD4+ PBMC T cells in FL patients (Figure 4A and B). A similar difference was found between tonsil and PBMC CD4+ T cells in healthy donors (Figure 4B). Also BTLA was expressed in a greater fraction of CD4+ FL TILs than in PBMC CD4+ T cells, but the difference was smaller than for PD-1 and did not differ between tonsil and autologous PBMC T cells from healthy donors (Figure 4C). In contrast to the high expression of PD-1 and BTLA in CD4+ TILs, only few of them expressed CTLA-4 (Figure 4A). Furthermore, the CD4+ TILs were all positive for CD28, and the majority of them also expressed CD27 and the activation marker CD69 (Supplementary Figure S4A and Supplementary Table S3). PD-1+CD4+ TILs co-expressed CD45RO whereas a smaller fraction of them also expressed CD57 (Supplementary Figure S4B).

By combining the immune phenotype results for percent positive PD-1 cells with IL-4-induced p-STAT6+ cells in CD4+ T cells, we found PD-1 expression to be inversely correlated with IL-4-induced p-STAT6 (Figure 4D, $r^2=0.617$ and $p<0.0003$), whereas no correlation was found for BTLA expression and p-STAT6 (Figure 4E, $r^2=0.271$).

To establish a direct relationship between expression of PD-1 and capacity to respond to cytokines, we activated FL LN specimens with IL-4 and then detected expression of PD-1 and p-STAT6 in single cells by phospho-flow cytometry. We found that PD-1 hi CD4+ TILs had normal levels of IL-4-induced p-STAT6, in contrast to only a few IL-4-induced p-STAT6+ cells in the PD-1 int CD4+ subset (Figure 5A). Furthermore, calculating the FC of IL-4-induced p-STAT6 in CD4+ T cells based on the different PD-1 expression levels, PD-1 hi FL TILs had a FC of 0.33 as compared to 0.88 and 1.36 in PD-1 int and PD-1- TILs, respectively (Figure 5B). Some of these PD-1 hi cells could be T follicular helper (T FH ) cells, as they are characterized by high expression of PD-1, as well as high expression of CXCR5 and ICOS. T FH cells have emerged as a specialized CD4+ T cell lineage that provides help to B cells for their selection and differentiation into memory B and plasma cells. We first activated FL LN specimens with PMA and ionomycin, and found IL-4 and IL-21 production mainly within the CD4+PD-1 hi subset (Supplementary Figure S5), suggesting the presence of functional T FH cells. Immunophenotyping revealed that the T FH subset in FL LN in average accounted for 24.4% and 58.5% out of CD4+ and CD4+PD-1 hi TILs, respectively (Supplementary Figure S6). Therefore, to more clearly delineate the identity of the PD-1 hi cytokine-non-responsive cells, we next included anti-ICOS and anti-CXCR5 Abs in the phospho-flow assay. Gating of CD4+ TILs into 4 different subsets, based on expression of CXCR5 and ICOS, showed that 96% of T FH cells had high expression of PD-1 (Figure 5C). Note that also the majority of CXCR5+ICOS+ cells had high expression of PD-1, which
contrasted negative/low PD-1 expression in almost all CXCR5−ICOS− cells. Interestingly, the T_{fh} and CXCR5−ICOS+ subsets had the lowest IL-4-induced p-STAT6 responses with FC of 0.37 and 0.58, respectively, as compared to 1.27 in the CXCR5−ICOS− subset (Figure 5C and D). In contrast, these T cell subsets had similar IL-7-induced p-STAT5 responses in some FL LN, but not in others (Figure 5C and data not shown), suggesting inhibitory mechanisms at play, preventing phosphorylation of STAT6, but not STAT5. Repeating these experiments with tonsil specimens, showed that the same signaling features applied to the corresponding T cell populations, identified by ICOS and CXCR5 (Figure 5E). Taken together, these data indicate that the cytokine signaling deficit observed in FL TILs as well as in tonsil T cells is restricted to PD-1^{hi}CD4^{+} T cells, which included T_{fh} cells and non-T_{fh} cells.

**The cytokine signaling deficit can be restored in PD-1^{hi} TILs**

Although the high PD-1 expression in CD4^{+} FL TILs, and the highly reduced cytokine signaling observed in these T cells was not tumor-specific (i.e. also seen in tonsil T cells), the high PD-1 expression levels might negatively influence FL patients therapeutic response to immunotherapy. We therefore tested if *in vitro* culture of FL LN specimens in the presence of neutralizing anti-PD-1 could restore the cytokine signaling deficit in PD-1^{hi} TILs. FL LN specimens were pre-cultured with anti-PD-1 Ab or an isotype control Ab for 30 min, 24 hours or 48 hours before the cells were washed twice and re-stimulated with IL-4 for 15 min, followed by phospho-flow analysis. Upon increasing pre-culture time in the presence of anti-PD-1 Ab, CD3^{+} TILs showed improved IL-4-induced p-STAT6 with 68% p-STAT6^{+} cells as compared to 52% p-STAT6^{+} cells after 48h and 30 min pre-culture, respectively (Figure 6A). However, the presence of anti-PD-1 Ab did not improve cytokine signaling beyond that of the isotype control Ab (Figure 6A and B), suggesting that disruption of the microenvironment was sufficient to release the negative suppression. The cytokine signaling capacity gradually improved over time, with 48 hours pre-culture being better than 24h pre-culture, and occurred in CD4^{+} as well as in CD8^{+} FL TILs (Figure 6B).

Since the presence of anti-PD-1 Ab during *in vitro* pre-culture induced downregulation of PD-1 expression as determined by PD-1 Ab staining (Supplementary Figure S7), simultaneous detection of PD-1 and p-STAT6 could only be determined in cells pre-cultured in the presence of control Ab. When dividing the CD4^{+} TILs from the control Ab pre-culture condition into 3 different subsets based on PD-1 expression levels, we discovered that IL-4-induced p-STAT6 was improved mainly in the PD-1^{hi} subset (Supplementary Figure S8). Whereas IL-4-induced p-STAT6 slightly decreased after *in vitro* pre-culture for 48 hours in the PD-1^{−} subset, p-STAT6 increased in the PD-1^{hi} subset from 0.33 to 0.86 (Figure 6C,
After 48h pre-culture, there was a 36% decrease in the frequency of PD-1\textsuperscript{hi} CD4\textsuperscript{+} T cells (n=5), as well as of T\textsubscript{\textsc{fh}} cells (n=3), but an increase in PD-1\textsuperscript{-} CD4\textsuperscript{+} T cells, suggesting selective cell death among PD-1\textsuperscript{hi} cells. Repeating the 48h pre-culture experiments with tonsil specimens also showed that cytokine signaling was improved mainly in the PD-1\textsuperscript{hi} subset (Figure 6D), again showing similarities between FL TILs and tonsil T cells. There was also a trend for improved cytokine signaling in FL T\textsubscript{\textsc{fh}}, CXCR5\textsuperscript{hi}ICOS\textsuperscript{-} and CXCR5\textsuperscript{-}ICOS\textsuperscript{hi} cells, although this did not reach statistical significance (Supplementary Figure S9).

Immunophenotypic analysis of the FL LN specimens prior to in vitro culture, showed that less than 1% of the cells expressed PD-L1 or PD-L2, and CD20\textsuperscript{+} tumor cells were found to be negative (Supplementary Fig S10). Importantly, while PD-L1\textsuperscript{+} cells were not readily detectable in these cryopreserved tumor cell suspensions used in all the cytokine signaling experiments and in vitro cultures, immunohistochemical staining of 7 cases of FL revealed varying numbers of PD-L1\textsuperscript{+} histiocytes within the T-cell rich zone of the neoplastic follicles (Figure 7). Some of these were colocalized with interfollicular PD-1\textsuperscript{hi} T cells (Figure 7, E and F). Reasoning that these PD-L1\textsuperscript{+} histiocytes might be tightly adherent to the fibrous interfollicular stroma, we attempted to recover these cells from several FL LN specimens by digestion of tumor fragments with collagenase and DNase during preparation of cell suspensions. However, even under these conditions, PD-L1\textsuperscript{+} cells were not detectable by flow cytometry in recovered cell suspensions (data not shown), providing an explanation for why the presence of anti-PD-1 blocking Ab had no effect on FL TILs cultured in vitro over time (Figure 6). In conclusion, the highly reduced cytokine signaling responses identified in PD-1\textsuperscript{hi} CD4\textsuperscript{+} FL TILs could at least partially be restored upon disruption of the microenvironment and in vitro co-culture when the PD-L1\textsuperscript{+} histiocytes are no longer present, therefore suggesting that FL TILs in vivo receive suppressive signals through PD-1.
Discussion

An important hallmark of cancer progression is the ability of the malignant cells to evade immune recognition. How the immune microenvironment impacts follicular lymphoma disease progression and outcome is not clearly understood. However, several observations support the hypothesis that T cells infiltrating FL tumors are dysfunctional, possibly mediated by the malignant lymphoma B cells. In this study, we found that effector cytokine-induced activation of signaling pathways, as measured by phosphorylation of STATs, was highly reduced in T cells infiltrating FL tumors. This contrasted normal cytokine-induced signaling in T cells infiltrating MCL and DLBCL tumors. By combined detection of inhibitory receptors, T cell markers and p-STATs post cytokine activation, we identified the cytokine signaling deficit to be restricted to PD-1Hi CD4+ TILs. Reduced cytokine signaling in PD-1Hi TILs were due to the presence of functional Tfh cells, and the presence of other CD4+ T cell subsets, identified as CXCR5-ICOS+ cells. The latter subset might be truly exhausted T cells and also showed high expression of PD-1. PD-L1+ histiocytes were found in the T-cell rich areas between follicles, suggesting that these cells may deliver the negative message via PD-L1 to PD-1Hi T cells. We also observed a striking similarity in cytokine signaling response between FL TILs and tonsil T cells, suggesting that FL TILs might mimic normality/ongoing immune activation, in contrast to other NHL like MCL and DLBCL.

Detection of CXCR5 and ICOS, together with detection of p-STAT6 post IL-4 activation, showed additional heterogeneity within PD-1Hi FL TILs, as this included IL-4-low-responsive, but otherwise functional Tfh cells, in addition to low-responsive CXCR5ICOS+ cells. The majority of the CXCR5ICOS+ cells also had high expression of PD-1, which contrasted negative/low PD-1 expression in almost all CXCR5ICOS- cells with normal IL-4-pSTAT6 response. TILs in FL tumors have previously been shown to be dysfunctional as compared to peripheral blood T cells. CD4+ and CD8+ TILs were shown to have impaired T cell immunological synapse formation with APCs, and subsequent impaired TCR-signaling responses.14 Peripheral blood T cells exhibited this defect in patients with leukemic-phase disease. However, this T cell dysfunction was not specific to FL TILs as TILs in DLBCL LN had the same defect.14 This finding contrasted the highly reduced cytokine signaling responses in FL TILs that we have identified, as this was specific to FL tumors and was not observed in TILs in DLBCL and MCL tumors. On the contrary, reduced cytokine signaling in FL TILs might be physiologic, as we observed very similar signaling features in comparable CD4+ T cell subsets present in tonsils. The features of FL TILs might resemble ongoing immune activation. This is supported by our observation that FL TILs are skewed toward an effector memory (T_em) phenotype, and with the expression of CD69, suggests that these cells are activated...
antigen-experienced T cells. Therefore, the skewed T cell subset composition in FL TILs towards CD4^+CD45RO^+CD62L^- T cells could be caused by chronic antigenic stimulation that drives naive T cells into T_{EM}. The identity of such auto-antigens are not clear, although microenvironmental lectins can bind to surface immunoglobulins (Ig), due to unusual high mannosylation of Ig in FL. Contained within the T_{EM} subset is the specialized T_{FH} subset. T_{FH} cell differentiation is skewed in FL tumors, similar to what is found in reactive LN as well as in tonsils. In this respect, viral persistence and prolonged T cell receptor stimulation was found to redirect CD4^+ T cell differentiation away from T_{H1} response toward T_{FH}. Together, this suggests that these tissues have common inflammatory and immune activation signals which modulate the immune microenvironment.

By the combined detection of p-STAT6 with several T cell markers and PD-1, we showed that the highly reduced effector cytokine signaling in FL TILs was confined to PD-1^hiCD4^+ TILs, whereas PD-1^CD4^+ T cells within the same samples had normal levels of IL-4-induced p-STAT6. However, the reduced cytokine signaling in PD-1^hi FL TILs were not solely due to non-responsiveness of T_{FH} cells, but was also due to low responsiveness of CXCR5^ICOS^ non-T_{FH} cells. This subset also showed high expression of PD-1, and thus could represent truly exhausted T cells. PD-1, a member of the CD28 family, is an inhibitory receptor involved in maintenance of peripheral tolerance. Upon ligation, PD-1 blocks CD3/CD28-induced activation of PI3K and other signaling events downstream of the TCR by recruiting the protein tyrosine phosphatases SHP-1 and SHP-2. This culminates in the inhibition of T-cell proliferation, cytokine production and differentiation. How PD-1 suppresses cytokine-induced signaling in FL TILs remains to be determined, but this could also be mediated via recruitment of phosphatases SHP-1 and SHP-2. SHP-1 is a negative regulator of IL-4-induced signaling by suppressing IL-4 mediated phosphorylation of STAT6 and STAT6-mediated transcription of IL-4 responsive genes. Of note, we found that some FL patients TILs with highly impaired IL-4-, IL-10- and IL-21-induced signaling, showed normal levels of IL-2-, IL-7- and IL-15-induced p-STAT5. Furthermore, we observed that PD-1^hiCD4^+ T cell subsets with highly reduced IL-4-induced pSTAT6 responses were capable of mounting normal levels of IL-7-induced p-STAT5. IL-2, IL-7 and IL-15, but not IL-4 and IL-21 have been shown to rescue PD-1 inhibition, possibly mediated via STAT5 induced activation of Akt and thereby bypassing the PD-1-induced suppression. However, PD-1 needs to be ligated to exert its suppressive function. PD-1 has two known ligands, PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273). While PD-L1 expression can be induced in a variety of cell types in lymphoid and peripheral tissues, PD-L2 expression is restricted to myeloid cells. We found FL tumor cells to be negative for PD-L1 and PD-L2, in agreement with others studies which reported that FL tumor cells do
not express PD-L1. Instead, we found that histiocytes localized to T-cell rich areas between the malignant follicles expressed PD-L1, and some of these were colocalized with PD-1+ T cells. These PD-L1+ histiocytes likely deliver the negative message to PD-1hi FL TILs in vivo, making these T cells anergic to IL-4-, IL-10- and IL-21-induced cytokine signaling. We were not able to provide direct evidence for this hypothesis, but further support comes from the in vitro culture of PD-1hi TILs, which when cultured in the mix of other FL LN cells, but in the absence of PD-L1+ cells could regain their capacity to respond to cytokines, not seen in PD-1- cells. Furthermore, PD-1 is significantly upregulated in exhausted virus-specific CD4+ T cells during HIV and CMV infections, and blocking activation of PD-1 was shown to partially restore T cell function. Furthermore, a recent study by Yang et al further provides evidence for the existence of exhausted FL TIL subsets, induced by IL-12 via upregulation of TIM-3. The TIM-3+ cells were unresponsive to the cytokines IFN-γ, IL-6 and IL-12, and had intermediate expression of PD-1.

Further support comes from a recent study which showed the presence of PD-1hi infiltrating T cells in the bone marrow of FL patients with tumor infiltration, but not in lymphoma negative marrows, suggesting that FL cells alter the local immune microenvironment.

A role of PD-1 as a prognostic marker in FL is currently unclear. High numbers of PD-1+ lymphocytes were associated with improved survival in FL patients of whom the majority received combination chemotherapy, but with shorter survival in FL patients of whom the majority received Rituximab-containing regimens. However, our finding that the majority of FL TILs expressed PD-1, and that the signaling deficit observed in the PD-1hi subset was reversible upon disruption of the microenvironment and subsequent in vitro culture over time, has potential clinical significance. PD-1hi TILs in solid tumors has also been found to have dysfunctional TCR signaling, and disrupting the tumor microenvironment followed by in vitro culture over time was sufficient to partially restore TCR signaling. Also in Hodgkin’s lymphoma, the PD-1/PD-L1 axis contributes to an immune suppressive environment. Anti-PD-1 Ab therapy is emerging as one of the new promising ways to modulate the anti-tumor immune responses. Neutralizing anti-PD-1 Ab therapy was found to be safe and was well tolerated with evidence of single-agent clinical beneficial responses in 33% of patients with advanced hematologic malignancies. Phase II trials are currently ongoing for non-Hodgkin’s lymphoma, including FL.

A better understanding of how the FL tumor microenvironment affects tumor infiltrating T cells can facilitate the design of improved immunotherapy of FL. Although the high PD-1 expression and the corresponding reduced cytokine signaling observed in CD4+ FL TILs seemed to be physiologic as this
phenomenon also was seen in tonsil T cells, this process might negatively influence FL patients’ therapeutic response to immunotherapy. To overcome this, combination therapies which also take into consideration to neutralizing negative regulators of T cell responses, including PD-1, might offer therapeutic benefit. Our data suggests that anti-PD-1 Ab in combination with immunotherapy might be particularly effective in FL due to the high percentage of PD-1+ TILs in this subtype of non-Hodgkin’s lymphoma, as compared to DLBCL and MCL, and warrants further investigation.
Acknowledgments

J.H.M. was supported by the Norwegian Cancer Society, the Research Council of Norway and South East-Regional Health Authorities. J.M.I. was supported as a Leukemia & Lymphoma Society Fellow and by the National Institutes of Health (K99 CA 143231-01). R.L. is a Clinical Research Professor of the American Cancer Society. This work has been supported by the National Institutes of Health (CA 34233 and CA 33399), the Leukemia and Lymphoma Society, and the Integrative Cancer Biology Program (U56 CA112973).

Authorship

Contribution: J.H.M. and J.M.I. designed the research, performed experiments and wrote the paper. J.B. designed the research and provided patient samples. D.K.C., R.H., J.T. and J.S. performed experiments. H.E.K performed experiments and provided tonsil specimens. J.D. provided patient samples and revised histopathological analysis of patient samples. A.K. provided patient samples and clinical data. A.A.A. provided patient samples, clinical data and performed data analysis. R.L. designed the research, supervised the study, provided patient samples and wrote the paper. All authors approved the final manuscript.

Conflict-of-interest disclosure:

All authors declare no competing financial interests.
References


30. Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 Associate with Immunoreceptor Tyrosine-Based Switch Motif of Programmed Death 1 upon Primary Human T Cell


Figure legends

Figure 1. Tumor infiltrating T cells in FL biopsies have suppressed cytokine signaling.
Tumor specimens from patients with DLBCL, MCL, FL, CLL or peripheral blood mononuclear cells (PBMC) from healthy donors were stimulated with cytokines for 15 minutes. Signaling was then stopped by fixation, followed by permeabilization and detection of cytokine-induced phosphorylation of STATs by phospho-flow cytometry. (A) Representative FACS plots of Tumor infiltrating T cells (TILs) or normal PBMC T cells were identified based on their co-expression of CD3 and CD5, and lack of CD20 expression, and histograms of IL-4-induced p-STAT6 and IL-21-induced p-STAT3 in TILs from a FL tumor sample (FL-J117) and a healthy donor is shown as median fold change (FC), relative to unstimulated cells. On the archsinh scale, a FC of 1.75 corresponds to a difference of 1 log_{10}. Scatter plots of cytokine-induced phosphorylation in TILs from DLBCL, MCL, FL, and in PBMC T cells from healthy donors. (B) IL-4-induced p-STAT6, (C) IL-10 induced p-STAT3 and (D) IL-21 induced p-STAT3 and (E) IL-7 induced p-STAT5. Each dot represents one patient sample: DLBCL: n=12, MCL: n=19, FL: n=14, CLL: n=14, healthy donor PBMCs (normal): n=6. Significance between groups was determined by unpaired Mann-Whitney U test.

Figure 2. T cell composition is skewed towards CD4^+CD45RO^+ T cells in FL LN biopsies.
Immunophenotypic analysis of the lymphoma patient specimens. Shown is scatter plot of percentage positive cells out of total CD3^+ T cells in the tumor sample of (A) CD4^+ T cells, (B) CD4^+CD45RO^+ memory T cells and (C) FoxP3^+CD25^+CD4^+ T regulatory cells. DLBCL: n=12 (n=4 for FoxP3), MCL: n=19, FL: n=14, CLL: n=14, tonsil: n=4. Significance between groups was determined by unpaired Mann-Whitney U test.

Figure 3. CD4^+CD62L^−CD45RO^+ FL TILs, but not the corresponding autologous PBMC T cells have reduced cytokine signaling capacity
IL-4-induced phosphorylation of p-STAT6 was analyzed in malignant LN and in autologous PBMC samples from FL patients by combining CD3, CD5, CD4, CD8, CD62L and CD45RO specific Abs with p-STAT6 Ab in the phospho-flow cytometry assay. (A) Gating strategy of live cells to identify CD3^+CD4^+ T cells and then subsequent gating based on expression of CD62L and CD45RO to identify CD62L^+CD45RO^+, CD62L^−CD45RO^+, CD62L^+CD45RO^−, and the corresponding histograms of IL-4-induced p-STAT6 in the various CD4^+ T cell subsets. (A) Representative FACS data from one FL patient is shown, (B) mean FC of IL-4-induced p-STAT6^+ cells in CD4^+ T subsets from FL LN and autologous PBMC. Mean ± SEM, n=5. (C) Mean
FC of IL-4-induced p-STAT6, relative to unstimulated cells in CD4^+ T subsets from tonsil and autologous blood samples from healthy tonsil donors. Mean ± SEM, n=3. Significance between groups was determined by unpaired two-tailed student t-test.

**Figure 4. Differential expression of PD-1 in CD4^+ FL TILs and autologous PBMC CD4^+ T cells and negative association with IL-4-induced p-STAT6.**

Immunophenotypic analysis of inhibitory receptors CTLA-4, BTLA and PD-1 was performed in FL LN and autologous blood samples. (A) Representative FACS plot of TILs and peripheral blood T cells from a representative FL patient. (B) Mean percentage of PD-1^+ cells out of CD4^+ T cells. FL: n=3, healthy donors: n=3. (C) Mean percentage of BTLA^+ cells out of CD4^+ T cells. FL: n=3, normal: n=3. (D) Scatter plot of IL-4-induced p-STAT6 in CD4^+ T cells vs. percent PD-1^+ cells out of CD4^+ T cells in LN and PBMC from FL patients and in tonsil and PBMC from healthy donors. (E) Scatter plot of IL-4-induced p-STAT6 in CD4^+ T cells vs. percentage of BTLA^+ cells out of CD4^+ T cells in LN and PBMC from FL patients and in tonsil and PBMC from healthy donors. Statistical difference between groups was determined by paired two-tailed student t-test.

**Figure 5. The cytokine signaling deficit in FL TILs is restricted to PD-1^hi CD4^+ T cells, and includes T_{FH} cells and non-T_{FH} cells.**

FL LN specimens were cultured with or without IL-4 for 15 minutes and then assayed for IL-4-induced phosphorylation of p-STAT6 by combining CD3, CD5, CD4, PD-1 specific Abs with p-STAT6 ab in the phospho-flow cytometry assay. (A) IL-4-induced p-STAT6 was determined in CD4^+ TILs gated on different expression levels of PD-1. (B) Data is shown as mean FC ± SEM, FL: n=7. (C) IL-4-induced p-STAT6 was determined in CD4^+ FL TILs, based on expression of ICOS and CXCR5. Shown is one representative case, and (D) Mean FC ± SEM, FL: n=3. (E) IL-4-induced p-STAT6 was determined in CD4^+ tonsil T cells, based on expression of ICOS and CXCR5. Mean FC ± SEM, tonsil: n=3. Statistical difference between groups was determined by paired two-tailed student t-test.

**Figure 6. In vitro culture of FL LN specimens over time restores IL-4-induced p-STAT6 in PD-1^hi CD4^+ T cells, but does not require neutralization of PD-1.**

FL LN specimens were cultured over time as specified in materials and methods with anti-PD-1 neutralizing Ab or with an isotype control Ab, washed twice with PBS and then restimulated with or
without IL-4 for 15 min. IL-4-induced p-STAT6 and the expression of PD-1 were measured by phospho-flow cytometry. (A) Representative histograms of IL-4-induced p-STAT6 in FL LN TILs pre-cultured with control ab or anti-PD-1 ab for 30 min or 48 h before the cells were washed and restimulated with IL-4 for 15 min. (B). Time course of IL-4-induced p-STAT6 is shown in CD4+ or CD8+ T cell subsets. Results are shown as FC of IL-4-induced p-STAT6, relative to unstimulated cells for 2 different FL donors. (C) FL LN were pre-cultured in the presence of an isotype control ab for 30 min or 48 hours before washing and restimulated with or without IL-4. IL-4-induced p-STAT6 was then determined in PD-1−, PD-1int or PD-1hi CD4+ TILs. Shown is Mean ± SEM, FL: n=5. (D) Same experiment as described in C), using tonsils. Shown is Mean ± SEM, tonsil: n=3. p<0.021 as determined by paired two-tailed t-test.

**Figure 7. Histiocytes in the T-cell rich zones express PD-L1**

Representative follicular lymphoma paraffin sections stained for PD-L1 (5H1) (A,C) and CD3 (B,D). A and C show PD-L1 staining cells predominantly in the T-cell rich zones between the neoplastic follicles. Inset figure C is a high power photomicrograph illustrating the dendritic process of the 5H1 staining histiocytes. B and D stained for CD3 reveal staining predominantly between the neoplastic follicles in the same distribution as the PD-L1 staining cells. E and F show the interfollicular zone stained for PD-L1 (brown) and PD-1 (red). F, follicle; IF, interfollicular zone. (Hematoxylin counterstain, original magnification x50: A, B; x200: C, D; Inset in C: x400; E: x250, F: x400).
Figure 1.
Figure 2.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
High PD-1 expression and suppressed cytokine signaling distinguish T cells infiltrating follicular lymphoma tumors from peripheral T cells


Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include digital object identifier (DOIs) and date of initial publication.