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Th2 type inflammation promotes the gradual progression of HPV-infected cervical cells to cervical carcinoma

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Abstract

Objectives—To investigate the role of immunological parameters in tumorigenesis of cervical cancer in women infected with high risk human papillomavirus (hr-HPV), and determine whether key findings with human material can be recapitulated in the mouse TC1 carcinoma model which expresses hr-HPV epitopes.

Methods—Epithelial and lymphoid cells in cervical tissues were analyzed by immunohistochemistry and serum IL10 levels were determined by ELISA. Tumor draining lymph nodes were analyzed in the mouse TC1 model by flow cytometry.

Results—The mucosa was infiltrated by CD20+ and CD138+ cells already at cervical intraepithelial neoplasia 1 (CIN1) and infiltration increased in cervical intraepithelial neoplasia 3 (CIN3)/carcinoma in situ (CIS) and invasive cervical cancer (ICC), where it strongly correlated with infiltration by CD32B+ and FoxP3+ lymphocytes. GATA3+ and T-bet+ lymphoid cells were increased in ICC compared to normal, and expression in epithelial cells of the Th2 inflammation-promoting cytokine TSLP and of IDO1 was higher in CIN3/CIS and ICC. As a corollary, serum levels of IL10 were higher in women with CIN3/CIS or ICC than in normals. Finally we demonstrated in the mouse TC1 carcinoma, which expresses hr-HPV epitopes, an increase of cells expressing B cell or plasma cell markers or Fc receptors in tumor-draining than distal lymph nodes or spleen.

Conclusions—hr-HPV initiates a local Th2 inflammation at an early stage, involving antibody forming cells, and fosters an immunosuppressive microenvironment that aids tumor progression.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Introduction

Immunological mechanisms play a key role in the etiology and progression of many and perhaps all cancers [1-3], and recent studies support Virchow's postulate that inflammation promotes both carcinogenesis and tumor progression [4-6]. Invasive cervical cancer (ICC) provides an ideal system to investigate the relation of immunological mechanisms to tumor etiology and progression. The etiological agent, high risk human papillomavirus (hr-HPV), is known [7], its E6 and E7 genes encode tumor specific epitopes that can be recognized by immune T lymphocytes to cause cell destruction [8-11], and expression of these epitopes is intimately associated with the neoplastic transformation [12], a situation similar to that in rodent tumors caused by the polyoma virus, where neoplastic variants lacking the virus encoded cellular antigen could not be isolated by immunoselection [13]. Furthermore, the lesions preceding ICC are well defined, including cervical intraepithelial neoplasia (CIN) grades 1, 2, and CIN3/carcinoma *in situ* (CIS) [14-18], and archived cervical samples are available from women at different stages of the disease.

Chronic Th2 type inflammation is commonly seen during persistent infection with hr-HPV and promotes tumor progression [19], and a recent study suggests that immunological markers can be used to predict regression of CIN2-3 lesions [20]. We have applied immunohistochemistry (IHC) to archived cervical tissue samples as one approach to characterize the local immunological environment in cervical mucosa during the gradual progression from hr-HPV infected epithelial cells to ICC. Lymphoid cells were examined for the expression of FoxP3, a marker of regulatory T cells [21, 22]; CD20, a marker for B lymphocytes [23]; CD138 [24], a marker expressed on plasma cells but also on other cells including some epithelial cells; and CD32B [25], a marker which is expressed primarily by antigen presenting cells including dendritic cells (DC) and B cells and can downregulate an immune response after uptake of immune complexes [26]. Epithelial cells were examined for the expression of thymic stroma lymphopoietin (TSLP), an IL7-like cytokine, which instructs neighboring DC to promote Th2 inflammation [27-29], and indoleamine 2,3-dioxygenase 1 (IDO1), an enzyme which induces immunosuppression [30, 31]. We also studied the expression of two transcription factors, GATA3 and T-bet, which play key roles in determining Th cell differentiation [32, 33]. GATA3 promotes Th2 differentiation and induces Th2 cytokine production, while T-bet is essential for Th1 differentiation by upregulating Th1 cytokines and downregulating Th2 cytokines and can induce a shift from Th2 to Th1 dominance. It has been proposed that the relative expression of GATA3 and T-bet (G:T) determines the balance between Th1 and Th2 immune responses [32, 33].

After finding an increased number of Th2 type inflammation-associated cells and cytokines by IHC during the progression of hr-HPV induced cervical lesions, to further support our conclusion that Th2 type inflammation promotes their development, we evaluated the level of IL10 in sera from a similar group of subjects, including women from whom we had performed IHC. IL10 is one of the best studied Th2 type cytokines and has a general immunosuppressive function. It is expressed by cells of the innate and the adaptive immune system, including dendritic cells (DCs), macrophages, CD4 and CD8 T cells, and B cells [34], and increased level of IL10 has been observed in sera and cervical tissues from HPV-infected patients and associated with cervical high-grade lesions [35-37]. Finally, we analyzed by flow cytometry the involvement of B cells, plasma cells and cells expressing Fc receptors in the immune response to the mouse TC1 carcinoma, which expresses hr-HPV epitopes E6 and E7.

Materials and Methods

Samples

Both cervical tissue blocks and serum samples were selected from the HPV specimen repository at Dept. of Pathology, University of Washington. These samples were derived from three epidemiological studies conducted in Senegal, West Africa (Dr. Nancy Kiviat, principal investigator). Seventy nine Senegalese women were involved in this study, of which 21 had normal histology, 13 had CIN1, 23 had CIN3/CIS and 22 had ICC. Women with ICC (mean=46.6) were older than women with normal histology (mean=39.1), as were women with CIN1 (mean=47.3) and CIN3/CIS (mean=46.3). Seventy four women were positive for hr-HPV types. All women were HIV negative. Thirty-nine women contributed tissue block samples, 65 contributed serum samples and 25 women contributed both tissue block and serum samples. Both histological diagnoses and HPV genotyping data were available from the repository as well as demographic data, including age, HIV infection status. The studies were approved by the IRB at University of Washington.

Immunohistochemistry analysis (IHC)

Four-micron sections of formalin-fixed paraffin-embedded tissue were cut and placed on Superfrost Plus microscope slides. The sections were deparaffinized and rehydrated through graded alcohols. Antigen retrieval was carried out with Tris/EDTA pH 9.0 buffer in a tabletop autoclave. Endogenous peroxidase activity was blocked with 3% H₂O₂. In preliminary tests, each primary antibody was titrated across a range of dilutions to determine the optimal concentration that produced strong specific staining with no background. The slides were washed and ImmPRESS (Vector Laboratories; Burlingame, CA), an anti-mouse IgG polymerized reporter enzyme, was applied to each tissue. Color development was accomplished by incubation in diaminobenzidine (DAKO Corporation; Carpinteria, CA). The slides were counterstained in hematoxylin (DAKO Corporation), dehydrated through graded alcohols, cleared in xylene, and coverslipped with permanent mounting media. A slide without any primary antibody was used as a negative control. For each of the antibodies used in this study, these control slides demonstrated a complete absence of peroxidase staining.

The expression of each marker was evaluated semi-quantitatively by a highly experienced clinical technologist (J.M.). Specifically, epithelial expression of TSLP and IDO1 was scored using the semi-quantitative H-score method [38], taking into account both the staining intensity and the percentage of cells at that intensity. The staining intensity was scored as 0 (no staining), 1+ (weak staining), 2+ (moderate staining), or 3+ (intense staining). For each of the four staining intensity scores, the percentage of cells stained at the respective intensity was determined and multiplied by the intensity score to yield an intensity percentage score. The final staining scores were then calculated from the sum of the four intensity percentage scores; thus the staining score had a minimum value of 0 (no staining) and a maximum of 300 (100% of cells with 3+ staining intensity). When scoring expression of CD20, CD138, CD32B, or Foxp3 in the stroma, cells within 0.1 mm of the basement membrane of the normal cervical epithelium were counted and the number of cells/mm² was calculated, and the same region of the tissue was counted for all markers to maintain consistency. For CIN1 or CIN3 lesion tissues, cells in the stroma directly beneath the lesion were counted. For ICC tissues, the number of cells in 10 randomly selected high power fields (40X) of the stroma surrounding the tumor were counted, averaged and divided by the area of the stroma to give the number of cells/mm². For scoring expression of GATA3 and T-bet, serial sections were layered using Photoshop to ensure that identical areas were counted. The cells were counted within 0.1mm of the basement membrane to give number of cells/mm², after which the GATA3/T-bet ratio was calculated. As an

attempt to exclude investigator bias, the IHC data were independently evaluated and validated by a board-certified pathologist (M.Y.).

ELISA

IL10 was measured in undiluted serum samples using the commercially available human IL10 immunoassay kit (R&D Systems), following the manufacturer's instruction. Each sample was measured twice.

Flow cytometry

We characterized the lymphocytes from spleens and TDLNs from C57BL mice bearing syngeneic TC1 tumors using flow cytometry analysis. Three 6-8 week old C57BL/6 mice were injected subcutaneously with 10^6 TC1 [39] cells in the right flank. When the tumors reached 8-10 mm in diameter, the mice were killed and spleen and tumor-draining lymph nodes were harvested immediately for flow cytometry analysis of following markers: CD45 (clone 30-F11), CD3 (clone 145-2C11), CD4 (clone GK1.5), CD19 (clone eBio1D3), CD138 (clone 281-2), CD32 (clone 93), and FoxP3 (clone FJK-16s; all from eBioscience). Spleen and lymph nodes from three age-matched naive mice were used as controls. Excised spleens and lymph nodes were mechanically minced by pressing with the plunger of a 3 mL syringe, cells were collected in 10 mL of ice-cold flow cytometry staining buffer (PBS with 1% FBS and 0.09% NaN₃), and filter through a 70 μ M cell strainer (BD Biosciences) to eliminate the clumps and debris. Red blood cells were lysed by incubation with 2 mL of red blood cell lysing buffer (Sigma) at room temperature for 10 min. The remaining lymphoid cells were washed with PBS and stained with various antibody combinations at 4°C for 30 min. Flow cytometry analysis was performed on a FACSCalibur instrument (Becton Dickinson, San Jose, CA). The data were analyzed using Flow Jo software (Tree Star, Ashland, OR).

Statistical Methods

Sections of cervical tissues of normal, CIN1, CIN3/CIS and ICC were semi-quantitatively investigated by IHC. Epithelial cells were analyzed for expression of TSLP and IDO1, and expression of the B cell marker CD20, the plasma cell marker CD138 and the inhibitory Fc receptor CD32B was measured in stromal infiltrating lymphoid cells. Pearson's Chi-Square Tests were used to compare baseline categorical variables and ANOVA was used to compare baseline continuous variables. Wilcoxon Rank Sum Tests were used to compare IHC marker expression and ELISA IL10 concentration between histologically normal tissue and CIN1, CIN3/CIS or ICC. Spearman correlation coefficients were used to determine correlation among IHC markers as well as between IHC markers and ELISA IL10 concentration. Student's *t* tests were used to analyze the data from flow cytometry. All *p*-values were adjusted for multiple comparisons using false discovery rates. A two-sided 0.05 test level determined statistical significance for all analyses. All analyses were conducted using SAS version 9.2 (SAS Institute Inc., Cary, NC).

RESULTS

Similar expression of immune response markers was observed in histologically normal cervical tissues regardless of hr-HPV infection status

We first analyzed 10 histologically normal cervical tissues, five from women positive for hr-HPV infection and five from women negative for hr-HPV infection. There were no differences for TSLP and IDO1 expression in epithelial cells or in the number of CD20, CD138, CD32B and FoxP3 positive lymphoid cells infiltrating the stroma. Although the expression of both GATA3 and T-bet transcription factors was higher in some hr-HPV+

histologically normal samples, there were no significant differences between histologically normal cervical samples from hr-HPV+ and hr-HPV- women. Therefore, we decided to combine these two groups as the negative control for subsequent analysis.

CIN3/CIS/ICC lesions were associated with Th2 inflammation and immunosuppression

To test the hypothesis that a Th2 type inflammation contributes to hr-HPV induced neoplasia, we quantitated the number of CD20+, CD138+, and CD32B+ lymphoid cells which infiltrated the cervical stroma in CIN1, CIN3/CIS and ICC as compared to negative controls (Fig. 1A-C). We found that CD20+ and CD138+ lymphoid cells were significantly increased already in CIN1 samples compared to negative controls (Figs. 1 and 2), and some CIN1 samples also had an increase of CD32B+ lymphoid cells (Fig. 2), although it was not statistically significant. The number of CD20+ lymphoid cells was comparable in CIN3/CIS and ICC, while CD32B+ and CD138+ lymphoid cells were significantly increased in ICC compared to CIN3/CIS. Expression of TSLP, a cytokine promoting a Th2 response, was significantly increased in epithelial cells from CIN3/CIS compared to negative controls (Fig. 3A). These data support the concept that HPV-induced cervical lesions are associated with a Th2 type inflammation involving B lymphocytes, plasma cells and CD32B+ lymphoid cells.

We also determined the expression of the highly immunosuppressive molecule IDO1 by epithelial cells and the frequency of stroma-infiltrating FoxP3+ lymphoid cells. Both were significantly increased in CIN3/CIS and ICC compared to the negative control (Fig. 3B-C).

There was a highly significant correlation between the number of CD20+, CD138+ and CD32B+ cells in the stroma ($\rho=0.64$, 0.68 , and 0.64 respectively, $p<.0001$). The number of stromal CD32B+ cells was significantly correlated with epithelial IDO1 expression ($\rho=0.56$, $p=0.0007$) and with the number of stromal Foxp3+ cells ($\rho=0.79$, $p<.0001$) (Fig. 4 A-B). There were also significant correlations between the number of CD20+ or CD138+ cells and IDO1 expression ($\rho=0.41$, $p=0.03$), between the number of CD20+ or CD138+ cells and the number of FoxP3+ cells ($\rho=0.72$, $p<.0001$ and $\rho=0.63$, $p<.0001$, respectively), and between stromal FoxP3+ cells and epithelial IDO1 expression ($\rho=0.48$, $p=0.007$) (Fig. 4C). There was a similar significant correlation between TSLP expression and IDO1 expression ($\rho=0.62$, $p<.0001$, Fig. 4D). These observations support the hypothesis that Th2 inflammation is closely associated with local immunosuppression.

Expression of GATA3 and T-bet was significantly increased in ICC

We next determined the expression in stroma infiltrating lymphoid cells, of two transcription factors, GATA3 and T-bet, which are important for the development of Th2 and Th1 immune responses. The expression of both factors was increased in CIN1 and the difference between the normal control and ICC was significant ($p=0.01$); however, there was a great variation between samples. The GATA3/T-bet ratio, an indicator of a Th1 to Th2 shift, increased in CIN1 and CIN3/CIS/ICC from normal controls but the difference was not statistically significant (supplemental Fig.).

Serum levels of IL10 were significantly increased in CIN3/CIS and ICC

We analyzed 65 serum samples for the level of IL10. There was no significant difference in serum samples from women with histologically normal cervix mucosa whether they were hr-HPV+ or hr-HPV-. In contrast, IL10 levels in sera were significantly higher in women with CIN3/CIS or ICC compared to women with normal histology (Fig. 5A). Among 25 women who contributed both tissue and blood samples, we found significant correlation between IL10 level and the number of infiltrating CD32B+ cells ($\rho=0.52$, $p=0.02$, Fig. 5B).

Th2 type lymphoid cells were more frequent in tumor draining lymph nodes than in distal lymph nodes or spleens from TC1 tumor-bearing mice or lymph nodes from naïve mice

To confirm and extend our observations on archived cervical mucosa blocks from human subjects, we performed flow cytometry studies in mice bearing a transplanted TC1 carcinoma which expresses the E6 and E7 epitopes of hr-HPV and is frequently used as a preclinical model for assessing immunity to HPV induced tumors [39, 40]. We sampled material from tumor-draining lymph nodes (TDLN) from mice with subcutaneously growing TC1 tumors (>5 mm mean diameter) and from distal lymph nodes (DLN) and spleen, as well as lymph nodes (NLN) and spleens from age matched naïve syngeneic mice.

As shown in Fig.6, the most striking difference was a significant ($p<0.01$) increase of CD19+ (B cells), CD138+ (plasma cells) and CD32+ (Fc receptor positive) cells in TDLN as compared to DLN or NLN (Fig. 6A). There was a statistically significant ($p<0.05$) increase of CD19+, CD138+, and CD32+ cells in spleens from TC1 tumor-bearing versus naïve mice, but the differences for CD19 and CD32 cells were substantially less than for the tumor-draining nodes. There was also a significantly increased percentage of FoxP3+ cells among CD4+ cells in tumor-draining lymph nodes and spleens from the TC1-bearing mice (Fig. 6A). In contrast, the frequency of CD3+, CD4+ and CD8+ cells was significantly reduced in TDLN from TC1 bearing mice as compared to NLN or DLN; similar differences were seen when comparing spleens from TC1-bearing versus naïve mice (Fig. 6A). Fig. 6B illustrates data from a typical experiment.

Discussion

The microenvironment of most cancers is highly immunosuppressive, both in humans and in mouse models [41], a problem that needs to be better understood and overcome to make systemic immunotherapy for cancer more efficacious.

There is complete peripheral tolerance to E6 and E7 epitopes expressed by hr-HPV induced cervical cancers (35, 36). It is associated with tumor-infiltrating lymphocytes which express the FoxP3 marker of Treg cells [42, 43], and agents counteracting Treg cells can partially counteract the growth of mouse TC1 tumors which express E6 and E7 [44-47]. We have applied IHC to analyze archived cervical tissue samples representing several stages during the progression from hr-HPV infected cervical epithelial cells to ICC, and have focused on cellular markers that have been associated with a Th2 type inflammation, hypothesizing that such inflammation contributes to the local, tumor-related immunosuppression. To complement our work, we assayed sera for the IL10 and we applied flow cytometry to measure the expression of several of the markers in the TC1 mouse model, focusing on markers for B cells, plasma cells and cells expressing the Fc receptor.

We are aware that our approach has several limitations. Functional studies cannot be performed on archived material and there are problems relating the expression of surface markers to cellular functions and few of the markers, with the possible exception of CD20, are entirely specific for a given cell type. Furthermore, our sample size is small, and the mean age of women with normal histology was about 7 years lower than for women with cervical lesions, although the mean age of women with CIN1 was comparable to that of women with CIN3/CIS and ICC. Taking these limitations into account, our findings still give strong support to the hypothesis that a Th2 type inflammation and local immunosuppression facilitate the progression of hr-HPV infected cervical epithelium to ICC.

We observed a statistically significant increased stromal infiltration of lymphocytes expressing CD20 or CD138 markers already at CIN1, as well as the infiltration of some

CIN1 samples by lymphoid cells expressing CD32B. This indicates that a Th2 lymphoid cell response is involved already when there is the first histological evidence of (pre)neoplastic transformation caused by the hr-HPV infection. Stromal lymphocytes expressing FoxP3, which is primarily although not exclusively expressed by Treg cells, were detected in CIN3/CIS and ICC and correlated significantly ($p<0001$) with an increase of lymphoid cells expressing CD20, CD138 or CD32B. Our finding on increased proportion of CD138+ cells in early hr-HPV induced lesions confirms and extends observations by Ovestad et al [20]. There was an analogous increase of cells expressing B (CD19) and plasma (CD138) cell markers and of cells expressing Fc receptors (CD32) in tumor-draining lymph nodes and, to a less extent, in spleens and distal lymph nodes from mice with established TC1 tumors which express the HPV 16 E6 and E7 proteins.

Our findings that the stroma of hr-HPV induced cervical lesions is infiltrated by B cells as well as by lymphoid cells expressing CD138 and CD32B are reminiscent of early reports that implicated antibodies in the evasion of tumors from control by immunological mechanisms [48-52] and, importantly, with recent findings from studies in a HPV16 transgenic mouse model where there is complete tolerance to E6 and E7 epitopes [53, 54]. It is noteworthy that most patients with ICC have antibodies to hr-HPV E6 and E7 proteins and that a reverse correlation has been described for the presence of such antibodies and prognosis [55]. In addition, antibodies can be detected at the precancerous lesions, though less frequently [56]. The antibody level is higher in cervical washings than in serum [57], stressing the importance of the local tumor microenvironment.

Although findings relating antibodies to a downregulated anti-tumor response have been controversial, a role of antibodies and antigen-antibody complexes in the regulation of tumor immunity lends credence from the discovery of different Fc receptors on antigen presenting cells [58-60]. By expression of either a stimulatory (CD32A/C) or an inhibitory Fc receptor (CD32B), antigen-antibody complexes play a role in the regulation of immune responses [61], and manipulation of antigen presentation via Fc receptors can have therapeutic benefit [54, 60].

TSLP facilitates the induction of a Th2 type response by conditioning myeloid DC to express OX40 ligand (OX40L) [29]. Recent studies in breast [62] and pancreatic [63] carcinoma indicate that inflammatory Th2 cells which promote tumor development are driven by TSLP made by cancer cells and cancer-associated fibroblasts. We observed elevated TSLP expression in cervical epithelial cells from women with CIN3/CIS ($p=0.01$), extending these findings to cervical cancer and indicating that TSLP may be a potential therapeutic target whose role in promoting tumor growth needs to be further investigated.

The tolerogenic molecule IDO1 is expressed in most invasive cancers as well as in tolerogenic dendritic cells and sometimes in macrophages and endothelial cells [30]. We found a significant increase of IDO1 in cervical epithelial cells from women with CIN3/CIS and ICC, indicating that IDO1 plays a role in establishing peripheral tolerance to hr-HPV transformed cells and that agents counteracting IDO1 may promote the immunological destruction of transformed cells.

Independent evidence that progression from histologically normal cervical epithelium to ICC is related to a Th2 inflammation comes from the demonstration of a statistically significant increase of the level in serum of IL10, a Th2 type cytokine, in CIN2-3/CIS ($p=0.01$) and ICC ($p=0.0009$), as compared to the histologically negative control, and in ICC as compared to CIN1 ($p=0.01$). It remains unclear whether a Th2 type inflammation promotes tumor growth via immunostimulation of the neoplastic cells [64] in addition to decreasing the impact of potentially tumor-destructive mechanisms.

Further and more extensive investigations of antibody forming cells, antibodies and immune complexes, TSLP and IDO1, as well as of IL10 and other Th2 type cytokines in hr-HPV infected women may aid prognosis. They may also guide the development of more effective ways to treat hr-HPV induced lesions, which, if left untreated, often progress to ICC, one of the most common female cancers world-wide. The findings may be relevant also for other tumors caused by hr-HPV, such as some carcinomas of the head and neck or anus, and probably also for cancers of other causations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research highlights

- The hr-HPV infected cervical mucosa is infiltrated by CD20+ and CD138+ cells already at CIN1
- Infiltration by Th2 lymphoid cells increases with severity of lesions and TSLP and IDO1 are associated with progression to ICC
- Serum IL10 levels are increased in cervical high grade lesions and ICC.

Figure 1. Th2 type lymphoid cells are significantly associated with CIN3/CIS and ICC

The presence of stromal infiltrating CD20+ (A), CD138+ (B) and CD32B+ (C) lymphoid cells was determined on cervical tissue blocks by immunohistochemistry analysis. Wilcoxon Rank Sum Tests were used to compare IHC marker expression in cervical tissues with various histological diagnosis.

Figure 2. Stromal infiltrating CD20+, CD138+ and CD32B+ positive lymphoid cells are increased in CIN3/CIS and ICC

Figure 3. Cervical lesions are associated with a local immunosuppressive environment

The epithelial expression of Th2 type cytokine TSLP (A) and IDO1 (B), as well as stromal infiltrating FoxP3+ cells were determined on cervical tissue blocks by immunohistochemistry analysis. Wilcoxon Rank Sum Tests were used to compare IHC marker expression in cervical tissues with various histological diagnoses.

Figure 4. Correlation between Th2 inflammation and immunosuppression in cervical epithelium
Spearman correlation coefficients were used to determine correlation between stromal CD32B+ cells and FoxP3+ cells (A), between stromal CD32B+ cells and epithelial IDO1 expression (B), between stromal infiltrating Foxp3+ cells and epithelial IDO1 expression (C), and between epithelial TSLP and IDO1 expression (D).

Figure 5. IL10 was elevated in serum samples collected from women with CIN3/CIS and ICC
 Serum level IL10 was determined by the ELISA assay (A), and significantly correlated with epithelial CD32B+ cells (B) by Spearman correlation coefficients test.

Figure 6. Tumor draining lymph nodes from TC1 mice have increased CD19+, CD138+, CD32+ lymphocytes

Lymphocytes from spleens of TC1 bearing mice and naïve mice were analyzed for CD19+, CD138+, CD32+ cells by flow cytometry analysis. Similar analysis was performed on lymphocytes collected from tumor-draining lymph nodes (TDLNs) and distal lymph nodes (DLNs) from TC1 bearing mice as well as normal lymph nodes (NLNs) from naïve mice (3 mice/group). Each type of cells was presented as the percentage of CD45+ leukocytes. Data are presented as mean±SEM (A). Representative dotspots show CD19+, CD138+ and CD32+ cells in NLNs, DLNs and TDLNs (B).