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ORIGINAL PAPER

Genotype-Dependent Tumor Regression in Marek's Disease Mediated at the Level of Tumor Immunity

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Abstract Marek's disease (MD) of chickens is a unique natural model of Hodgkin's and Non Hodgkin's lymphomas in which the neoplastically-transformed cells over-express CD30 ($CD30^{hi}$) antigen. All chicken genotypes can be infected with MD virus and develop microscopic lymphomas. From 21 days post infection (dpi) microscopic lymphomas regress in resistant chickens but, in contrast, they progress to gross lymphomas in susceptible chickens. Here we test our hypothesis that in resistant chickens at 21 dpi the tissue microenvironment is pro T-helper (Th)-1 and compatible with cytotoxic T lymphocyte (CTL) immunity but in susceptible lines it is pro Th-2 or pro T-regulatory (T-reg) and antagonistic to CTL immunity. We used the B2, non-MHC-associated, MD resistance/susceptibility system (line $[L]6_1$/line $[L]7_2$) and quantified the levels of key mRNAs that can be used to define Th-1 (IL-2, IL-12, IL-18, IFNγ), Th-2 (IL-4, IL-10) and T-reg (TGFβ, GPR-83, CTLA-4, SMAD-7) lymphocyte phenotypes. We measured gene expression in both whole tissues (represents tissue microenvironment and tumor microenvironment) and in the lymphoma lesions (tumor microenvironment) themselves. Gene ontology-based modeling of our results shows that the dominant phenotype in whole tissue as well as in microscopic lymphoma lesions, is pro T-reg in both $L6_1$ and $L7_2$ but a minor pro Th-1 and anti Th-2 tissue microenvironment exists in $L6_1$ whereas there is an anti Th-1 and pro Th-2 tissue microenvironment in $L7_2$. The tumor microenvironment *per se* is pro T-reg, anti Th-1 and pro Th-2 in both $L6_1$ and $L7_2$. Together our data suggests that the neoplastic transformation is essentially the same in both $L6_1$ and $L7_2$ and that resistance/susceptibility is mediated at the level of tumor immunity in the tissues.

Keywords Animal model · Gene ontology · Herpesvirus · Lymphoma · Microenvironment · Regulatory T cell

Abbreviations

ADOL	Avian disease oncology laboratory
cHL	Classical Hodgkin's lymphoma
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T-lymphocyte associated antigen
C_t	Cycle threshold
CVM-MSU	College of Veterinary Medicine-Mississippi State University
DPI	Days post infection
FOX	Forkhead box protein
GO	Gene ontology
GPR	G protein-coupled receptor
HL	Hodgkin's lymphoma
IFN	Interferon
IL	Interleukin
L	Line
LCM	Laser capture microdissection
MD	Marek's Disease

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MDV	Marek's Disease virus
NHL	Non Hodgkin's lymphoma
NO	Nitric oxide
QPCR	Duplex reverse transcriptase real-time polymerase chain reaction
SEM	Standard error of mean
SMAD	Small mothers against decapentaplegic
SPF	Specific pathogen free
Th	T helper
T-reg	Regulatory T cell
TNSFR	Tumor necrosis factor receptor superfamily member
TRAF	Tumor necrosis factor receptor associated factor
USDA	United States Department of Agriculture

Introduction

Lymphomas are the 6th leading cause of death due to cancer, 4th greatest in economic impact and they account for 53% of the new cases of hematological malignancies in the USA [1]. It is imperative to understand the complex dynamics of host-tumor interactions within the tumor microenvironment for designing any anti-tumor strategy. To do so requires animal models, which fully mimic human tumor microenvironment and where both tumor and stromal cells can be studied within the natural tumor environment [2]. Marek's Disease (MD) is a lymphomatous disease of chickens caused by the MD α-herpesvirus (MDV) and is a unique natural model for human Hodgkin's (HL) and non-Hodgkin's lymphomas (NHL) which overexpress CD30 (CD30^{hi}; a.k.a. tumor necrosis receptor superfamily member [TNSFR-8] or the "Hodgkin's disease antigen") [3]. MD is a general model for CD30^{hi} T cell lymphomas which includes anaplastic large cell lymphoma, primary cutaneous anaplastic large cell lymphoma, adult T-cell leukemia/lymphoma, peripheral T-cell lymphoma, natural killer (NK)/T-cell lymphoma, nasal and enteropathy type T cell lymphoma [3, 4]. Like its human homologs, MD lymphomas are heterogeneous mixture of minority population of transformed cells (CD30^{hi}) surrounded by majority population of non transformed normal immune cells [5, 6]. However, MD transformed cells are not inherently immortal; they depend upon the local lymphoma environment for their survival and growth [5, 6].

MD has advantage over murine models of lymphoma as it provides an opportunity to study the phenomenon of genotype dependent tumor regression as a model of spontaneous human lymphoma regression [7]. All chicken genotypes are susceptible to MDV infection, neoplastic transformation and microscopic lymphoma development. However, from 21 days post infection (dpi) these microscopic lesions regress in MD resistant genotypes but progress to gross lymphomas in MD susceptible genotypes [6, 8]. The fundamental genetic basis for the difference in lymphoma-regressing and progressing genotypes is poorly understood, though a very large body of work over almost 40 years has implicated several host immune factors, including innate cell-mediated immunity (CMI; including NK cells, monocytes); humoral, antigen-specific MHC class I-restricted cytotoxic T lymphocyte (CTL) immunity and cytokines (reviewed in [9]). At 21 dpi progressing lymphomas are CD4+ and CD4+ CD30^{hi} predominant with few CD8α+ T cells, whereas regressing lymphomas have many CD8α+ T cells, fewer CD4+ CD30^{hi} cells and the CD30 expression—though still above physiological levels in activated T cells [6]—is lower than in progressing lymphomas [8]. The neoplastically transformed MD lymphoma cells also have cytokine and other gene expression most similar to regulatory CD4+ T lymphocytes (T-reg) [5].

Here we test our hypothesis that, at the pivotal 21 dpi time point MD-resistant chicken genotypes have a tissue microenvironment congruent with CTL, where-as the tissue microenvironment in MD-susceptible genotypes is antagonistic to CTL. We tested this by quantifying the mRNAs for cytokines and other genes that determine T-helper (Th)-1; Th-2 and T-regulatory (T-reg) phenotypes: CD4+ Th-1 lymphocytes secrete high levels of interleukin (IL)-2 and interferon-gamma (IFNγ); CD4+ Th-2 lymphocytes secrete high levels of IL-4, IL-10 and IL-13 [10] and CD4+ T-reg lymphocytes express MHC II, transcription factor forkhead box protein (FOX) P3, G protein-coupled receptor (GPR)-83 [11], IL-2 receptor α chain (CD25), high levels of cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) [12–15] and decreased small mothers against decapentaplegic-7 (SMAD-7) [16, 17].

We used the B2; non-MHC-associated MD resistance/susceptibility (line [L]6_1/line [L]7_2) system [8]. We analyzed the gene expression profiles at whole tissue level (which represents both tissue microenvironment and tumor microenvironment) and subsequently at the level of microscopic lesions (tumor microenvironment) using Laser Capture Microdissection (LCM). Our Gene Ontology (GO)-based hypothesis testing demonstrates that: 1. a T-reg phenotype exists in both the tissue and tumor microenvironments in both resistant and susceptible genotypes; 2. a pro-inflammatory tissue microenvironment is present in both $L6_1$ and $L7_2$ tissues; 3. an anti-inflammatory and anti-CTL tumor microenvironment exists in microscopic lesions of both genotypes; 4. the susceptible genotype has an anti-CTL tissue microenvironment, whereas the resistant genotype has a pro-CTL tissue microenvironment. The fundamental differences between the genotypes exist at the level of the tissue

immune response and not at the level of the transformed cells.

Materials and Methods

Chickens, MDV and Tissue Sampling

Day old, specific pathogen free (SPF), MDV maternal antibody negative, $L6_1$ and $L7_2$ chickens were obtained from United States Department of Agriculture-Avian Disease Oncology Laboratory (USDA-ADOL, East Lansing, Michigan). These chickens were double wing-banded, housed in small groups in separate cages in an isolation facility at College of Veterinary Medicine-Mississippi State University, (CVM-MSU). Food and water was provided *ad libitum*. All chickens were infected on day 14 with MDV (GA/22 strain; passage 18; 500 pfu; intra-abdominally) obtained from USDA-ADOL (East Lansing, MI). On 21 dpi, five $L6_1$ and five $L7_2$ chickens were selected using the random number function in Microsoft excel using the list of wing band numbers, killed, kidney lymphomas harvested (kidney had the most visible gross lymphomas), snap frozen in liquid nitrogen, vacuum sealed in plastic bags and stored at −80°C until needed. All $L7_2$ birds that were not used for sampling developed gross lymphomas at later period and were euthanized. We confirmed that all chickens were MDV-infected by doing PCR on DNA isolated from the samples, using primers that amplify a fragment of the MDV Meq gene, exactly as described [8]. All animal practices and experiments were approved by the MSU-Institutional animal critical care and use committee.

Cryosectioning and Laser Capture Microdissection (LCM)

Tissue samples were transferred from −80°C to a cryostat (Leica Microsystems Inc., Bannockburn, IL) on dry ice, and warmed to −20°C before sectioning; 8 μm cryosections were cut and placed directly into 1 ml of TRI reagent (Molecular Research Center, Cincinnati, OH) or onto Histogene LCM slides (Molecular Devices Sunnyvale, CA). Cryosections were stored (for no more than a week) at −80°C until LCM. Cryosections were stained with Histogene Frozen Section Staining solution (Molecular Devices Sunnyvale, CA) following the manufacturer's protocol. Briefly, cryosections were ethanol fixed (75%) for 30 s, rehydrated in nuclease free water for 30 s, stained with Histogene Staining solution (100 μL per slide for 20 s), washed in nuclease free water for 30 s and dehydrated in 75%, 95% and 100% ethanol for 30 s each followed by final dehydration step in xylene for 5 min and allowed to air dry for 5 min. Air dried stained slides were placed in slide box with fresh desiccant and were used for LCM the same day. LCM was done using the PixCell IIe Laser Capture Microdissection system (Molecular Devices Sunnyvale, CA) and CapSure Macro LCM caps (Molecular Devices Sunnyvale, CA). MD microscopic lesions (Fig. 1a, b) were located and excised (laser power: 45–55 mw for 3–5 ms). A new cap was used for each sample.

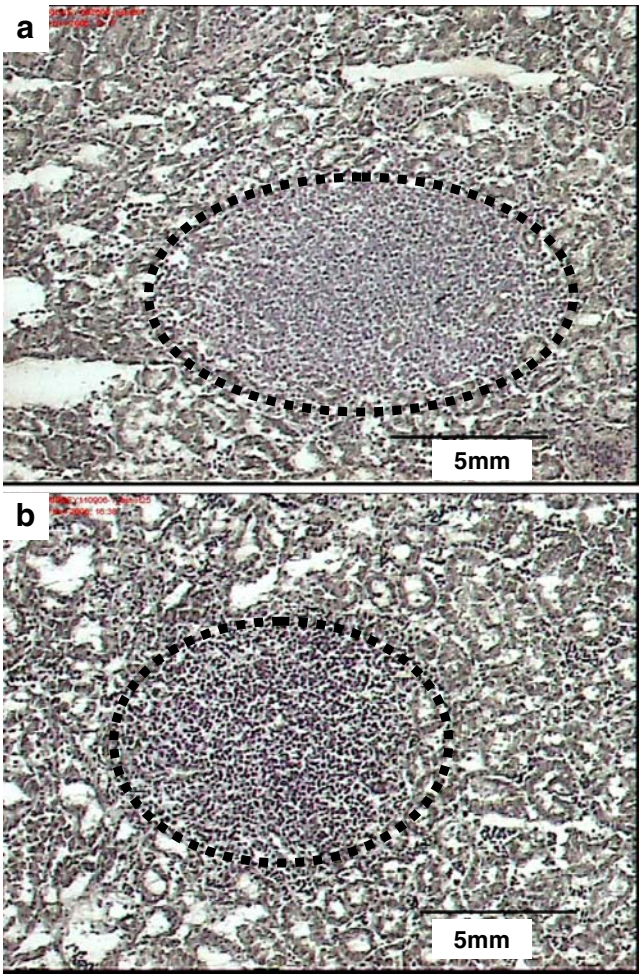

Fig. 1 Photomicrographs of kidneys at 21 dpi with MDV (see M&M), stained with "Histogene LCM frozen section staining kit" showing similarity in size of microscopic lymphoma lesions (*circled*) between $L6_1$ (**a**) and $L7_2$ (**b**)

RNA Isolation and Real-Time PCR

Total RNA was isolated from ~100 μg of tissue sections using TRI reagent (Molecular Research Center, Cincinnati, OH) exactly following manufacturer's protocol. Total RNA from each microdissected sample was isolated using the Pico Pure RNA isolation kit (Molecular Devices Sunnyvale, CA) exactly following the manufacturer's protocol. RNA concentrations were quantified (ND-1000

spectrophotometer; NanoDrop Technologies, Wilmington, DE) and adjusted to within 10-fold concentration of each other using RNAase free water. For comparing mRNA expression, we used a duplex reverse transcriptase real-time PCR (QPCR), with 28S rRNA as a positive control for each PCR exactly as described [5]; iCycler iQ Real-Time PCR Detection System [Bio-Rad Laboratories Inc., Hercules, CA]; Platinum Quantitative RT-PCR Thermo-Script One-Step System [Invitrogen, Carlsbad, CA]; 100 pM of each primer [except 28S which was 1 pM]; 1 pM of all probes; 2.5 µl template RNA and RNAse free water; cycle conditions: 50°C, 30 min; 95°C, 5 min+45× [95°C, 15 s; 60°C, 60 s]). All primer and probe sequences (Table 1) are previously published and all amplicons (except 28S) cross intron-exon boundaries [5, 18–21]; although 28S has no introns in it, it is routinely used as an internal control and its RNA template far exceeds its DNA template. Each QPCR experiment was done in triplicate and included no-template controls. Differences in the mean QPCR results were compared using one way analysis of variance.

Gene Ontology (GO) Based Quantitative Modeling

We tested our hypotheses using GO-based modeling of our QPCR data exactly as described [5]. Briefly, we used the computational tool *GOmodeler* [22], which scores the effects of each gene product on a process as either "pro" (+1), "anti" (−1), "no effect" (0) or "no data" (blank cell), then multiplies these score by the QPCR data for each gene. The net effect of each phenotype is the sum of scores of each gene for that phenotype and the net overall phenotype is the sum of scores of each phenotype.

Table 1 PCR probes and primers (fluorophore)

RNA target	Probe/Primer	Sequence	Accession
28S	Probe	5′-(HEX)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3′	X59733
	F	5′-GGCGAAGCCAGAGGAAACT-3′	
	R	5′-GACGACCGATTTGCACGTC-3′	
IL-2	Probe	5′-(FAM)-ACTGAGACCCAGGAGTGCACCCAGC-(TAMRA)-3′	AF221080
	F	5′-TTGGAAAATATCAAGAACAAGATTCATC-3′	
	R	5′-TCCCAGGTAACACTGCAGAGTTT-3′	
IL-4	Probe	5′-(FAM)-AGCAGCACCTCCCTCAAGGCACC-(TAMRA)-3′	NM_001007079
	F	5′-AACATGCGTCAGCTCCTGAAT-3′	
	R	5′-TCTGCTAGGAACTTCTCCATTGAA-3′	
IL-10	Probe	5′-(FAM)-CGACGATGCGGCGCTGTCA-(TAMRA)-3′	AJ621614
	F	5′-CATGCTGCTGGGCCTGAA-3′	
	R	5′-CGTCTCCTTGATCTGCTTGATG-3′	
IL-12β	Probe	5′-(FAM)-CTGAAAAGCTATAAAGAGCCAAGCAAGACGTTCT-(TAMRA)-3′	AJ564201
		5′-TGGGCAAATGATACGGTCAA-3′	
	F	5′-CAGAGTAGTTCTTTGCCTCACATTTT-3′	
IL-18	R	5′-(FAM)-CCGCGCCTTCAGCAGGGATG-(TAMRA)-3′	AJ276026
	F	5′-AGGTGAAATCTGGCAGTGGAAT-3′	
	R	5′-ACCTGGACGCTGAATGCAA-3′	
CTLA-4	Probe	5′-(FAM)-TTGTCTTCTCTGAATCGCTTTGCCCACG-(TAMRA)-3	AM236874
	F	5′-CAGCATCATCATCTCAGCCATTG-3′	
	R	5′-GCATTTTCACATAGACCCCAGTAG-3′	
GPR-83	Probe	5′-(FAM)-TCCGCCACCAGCCTGTTCATCGTCA-(TAMRA)-3′	XM_425651
	F	5′-CGTCATCATCAAGAGCAAACGC-3′	
	R	5′-ACAAAACGAGCCAGTGTAAAAGG-3′	
IFNγ	Probe	5′-(FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA)-3′	Y07922
	F	5′- GTGAAGAAGGTGAAAGATATCATGGA-3′	
	R	5′-GCTTTGCGCTGGATTCTCA-3′	
SMAD-7	Probe	5′-(FAM)-TCCCAGTAAGCCACCACGCACCAGT-(TAMRA)-3′	XM_427238
	F	5′-GCTCTCAGATTCTCAAGTTATTCAGG-3′	
	R	5′-CCGACCCACACGCATCTTC-3′	
TGFβ	Probe	5′-(FAM)-ACCCAAAGGTTATATGGCCAACTTCTGCAT-(TAMRA)-3′	M31160
	F	5′-AGGATCTGCAGTGGAAGTGGAT-3′M31160	
	R	5′-CCCCGGGTTGTGTGTTGGT-3′	

F: forward; R: reverse.

Results

MD Lesions at 21 dpi

The developing MD lymphoma lesions at 21 dpi from resistant and susceptible genotypes are indistinguishable (Fig. 1a, b).

QPCR Based Gene Expression in Whole Tissue and Microscopic Lymphoma Lesions

mRNA expression is presented as 40-mean cycle threshold (C_t) values (± standard error of mean [SEM]) (Fig. 2a, b). In whole tissues $L6_1$ expressed significantly more IL-18, IFNγ, and GPR-83, but less IL-10 and SMAD-7 mRNA than $L7_2$ (Fig. 2a). In microscopic lesions $L6_1$ produced more IL-4 and less **TGF β**, GPR 83, SMAD-7 and CTLA-4 mRNA (Fig. 2b). IL-2 was below the detectable level of the assay in both whole tissues and in microscopic lesions, and IL-10 and IFNγ mRNA were below detectable limits in microscopic lesions of $L6_1$ and $L7_2$.

GO-Based Modeling

In the whole tissue samples, our GO-based modelling showed that the tissue microenvironment in both $L6_1$ and $L7_2$ is similarly pro T-reg and pro-inflammatory (Fig. 3a). However, $L6_1$ is also pro Th-1 and anti Th-2, whereas $L7_2$ is anti Th-1 and pro Th-2. At the level of developing microscopic MD-lesions (tumor microenvironment), both

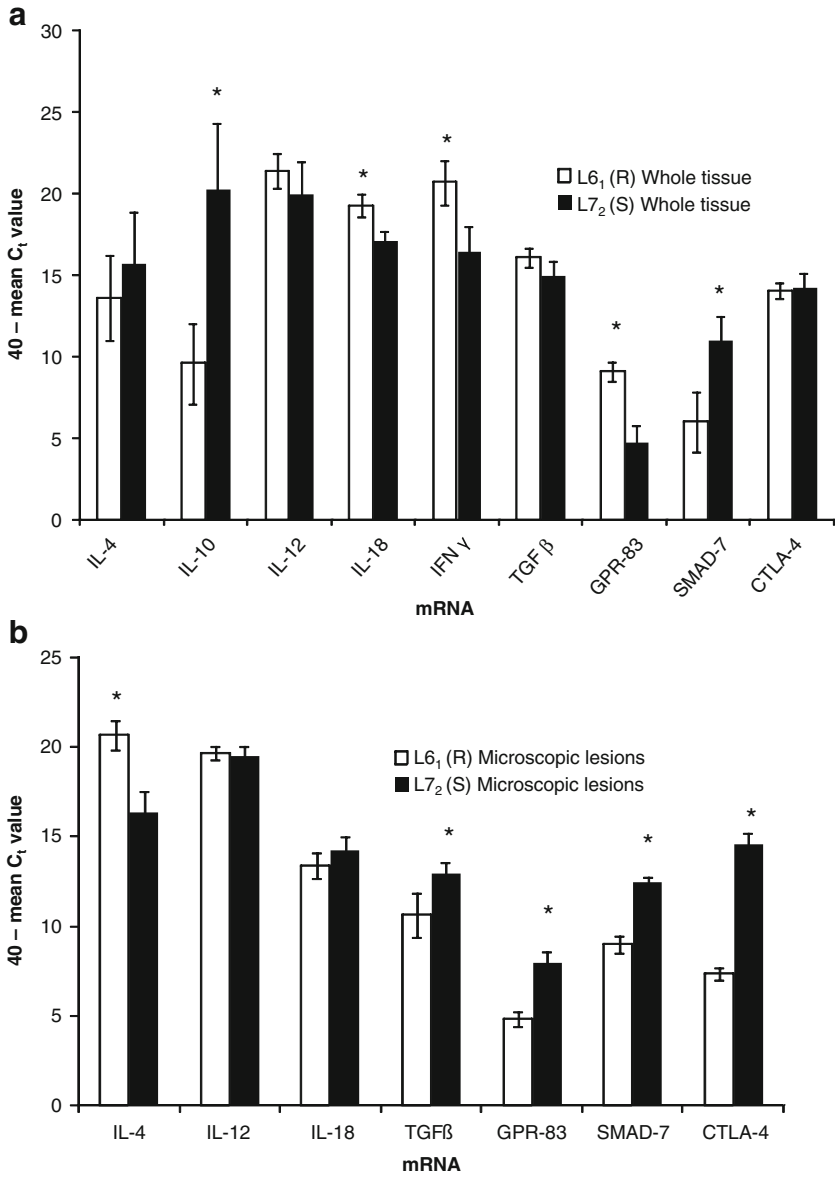

Fig. 2 mRNA expression profiles from kidney from MDV-infected chickens (see M&M) at 21 dpi for line 6_1 (resistant to gross lymphomas) and 7_2 (susceptible to gross lymphomas) presented as 40-mean Ct (±SEM, *=$P<0.05$) at the level whole tissue (**a**) and microscopic lesions (**b**)

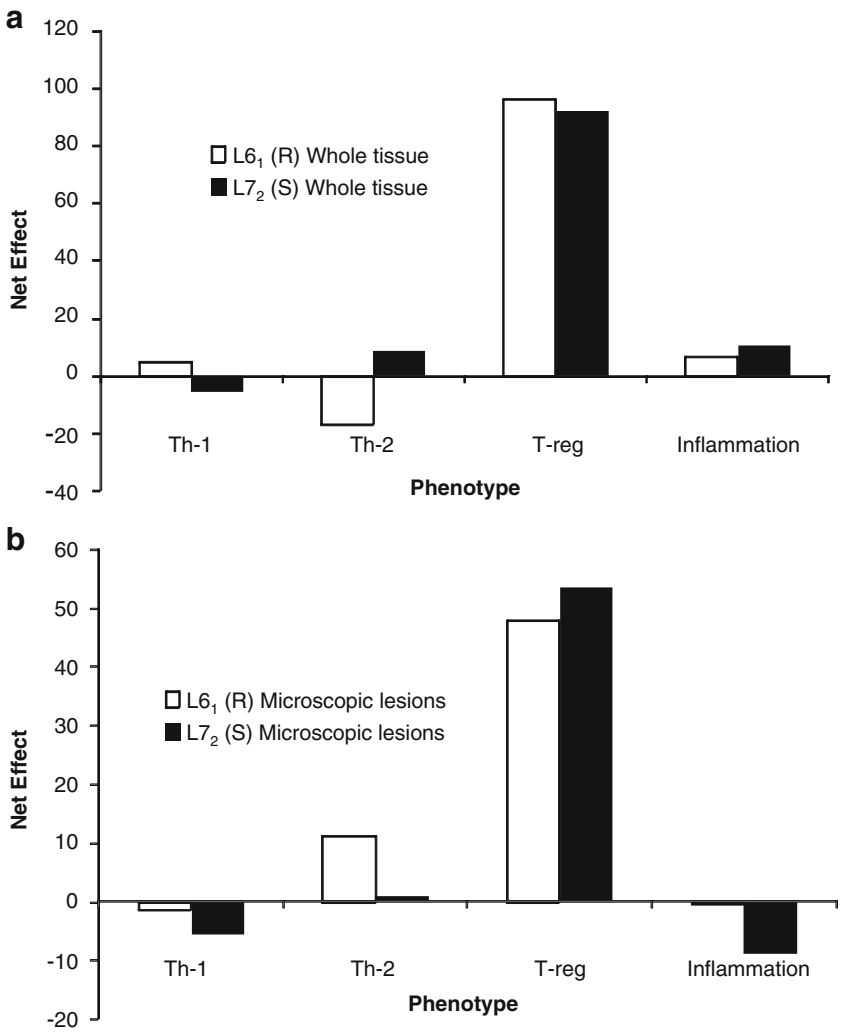

Fig. 3 Gene ontology (GO)-based quantitative modeling shows that at the whole tissue level both the resistant $L6_1$and the susceptible $L7_2$ genotype have a pro T-reg microenvironment but also $L6_1$ has a pro Th-1 and anti Th-2 microenvironment while susceptible genotypes have the opposite (**a**). Microscopic lesions in both $L6_1$ and $L7_2$ have a common phenotype which is pro T-reg, pro Th-2 and anti Th-1 which is antagonistic to cytotoxic T cell mediated immunity (**b**)

$L6_1$ and $L7_2$ are similarly high pro T-reg and in contrast to the whole tissues, both $L6_1$ and $L7_2$ are anti-Th-1, pro Th-2 and anti-inflammatory (Fig. 3b).

Discussion

Here we have identified the micro-environments of MD tumors at both the whole tissue and microscopic lesion level at the seminal time-point of lymphoma regression and progression in a natural animal model of CD30-overexpressing lymphoma. We used mRNA expression data from a panel of defining genes, to perform GO based quantitative hypothesis testing to validate our hypothesis that the tissue micro-environment is compatible with the genotype in which lymphoma regression occurs and not in the genotype with lymphoma progression.

In the MD system the role of cytokines has previously been focused on the virological (rather than neoplastic transformational) stages [20, 23–28]. Xing and Schat [25] proposed that IFNγ and nitric oxide (NO) may affect MDV pathogenesis. Kaiser et al. [20], like us, leveraged the power of MD-resistant and -susceptible chicken genotypes to compare cytokine expression in splenocytes and proposed that IL-6 and IL-18 may play an important role in immune the response that could lead to lymphoma progression in susceptible genotypes and what they referred to as the maintenance of latency in resistant genotypes. More recently, Heidari et al. [28] suggested a Th-2 cytokine profile (upregulated IL-4, IL-10, IL-13) in chicken splenocytes in the cytolytic phase of MD. Though splenocytes are one model for studying the immunity and MDV pathogenesis, they may not mimic the MD tissue and tumor microenvironment in non-lymphoid tissues. Regardless, none of the preceding work took the descriptive quantitative genetics to functional modeling.

The increase in IL-18 mRNA in $L6_1$ that we measured contrasts with Kaiser's data [20] in which there was no increase in IL-18 mRNA in resistant genotypes when compared to age matched uninfected controls. We did not

detect IL-2 mRNA in either whole tissue or microscopic lesions in both $L6_1$ and $L7_2$. IL-2 is a crucial immune-modulator cytokine for T cell proliferation and is required for maintenance of T-reg cells *in vivo* [29]. However, the MDV "Meq" oncogene binds the IL-2 promoter and represses IL-2 transcription [30] and there are high levels of Meq in MD lymphoma cells [6]; furthermore our previous work has demonstrated less IL-2 mRNA from *ex vivo*-derived purified $CD30^{hi}$ MD lymphoma cells compared to $CD30^{lo}$ cells [5]. Our IL-2 data again contrasts with that of Kaiser et al. [20] who identified more IL-2 mRNA in L7 splenocytes at 21 dpi compared to uninfected controls, but the IL-2 mRNA in the spleen is probably derived from activated, rather than transformed, T cells. Also, the high levels of IL-4 in both $L6_1$and $L7_2$ would be predicted to directly suppress IL-2 transcription [28].GPR-83 is selectively upregulated in T-reg cells of both humans and mice and is critically involved in mediating T-reg functions as well as in development of induced T-reg cells [11]. However, recently Lu et al. [31] suggested that GPR-83 is dispensable for T-reg functions. Though the role of GPR-83 in T-reg biology is questioned in one publication, it is still generally accepted to be a selective marker for T-reg cells and so we included it our work here. SMAD 7 is the member of the inhibitory type of SMADs which acts in a negative feedback for TGFβ signaling. Since the expression of inhibitory SMADs is induced by TGFβ [32] increased SMAD 7 expression suggests an increase in the TGFβ expression which triggers this negative feedback loop [33]. This is in accordance with our data, which show an increase in TGFβ and SMAD 7 mRNA expression in $L7_2$ tumor microenvironment.

Our GO-based modeling demonstrates that a T-reg phenotype predominates in both $L6_1$ and $L7_2$ at both whole tissue and microscopic lesion levels (Fig. 3a and b). The whole tissue consists of a heterogeneous mixture of large numbers of transformed cells which are transcriptionally very active and normal immune and non immune kidney cells. We propose that the T-reg phenotype is contributed by the transformed cells and the relatively weaker Th-1 phenotype in $L6_1$ and Th-2 phenotype in $L7_2$ are indicative of host immune responses from non transformed cells in the tissues. When the mRNA from the surrounding tissue (tissue microenvironment) is removed both, $L6_1$ and $L7_2$ have a similar phenotype (i.e. pro-T-reg, anti Th-1, pro-Th-2 and anti-inflammatory) i.e. antagonistic to CTL. Our result is consistent with the cellular profiles previously identified in MD lymphomas by immunohistochemistry [8] and flow cytometry [6], as well as evidence of specific CTL anti-tumor immunity [3, 9], and together; support our hypothesis that in $L6_1$ the tissue microenvironment is congruent with CTL mediated immunity leading to lymphoma regression while a T-reg/Th-2 phenotype is dominant in $L7_2$ which is consistent with continued lymphomagenesis.

Both $L6_1$ and $L7_2$ have a pro inflammatory phenotype in whole tissues, inflammation is causative factor in carcinogenesis in general [34] and inflammation is linked to various types of lymphomas [34, 35]. The inducible transcription factor NF-kappaB is a pivotal regulator of genes involved in immune-inflammatory pathways, cell cycle progression and inhibition of apoptosis promoting carcinogenesis in mice and humans [36]. The MDV Meq protein binds the CD30 promoter and enhances CD30 transcription [3], which in turn can activate the NF-kappaB transcription factor via the CD30-tumor necrosis factor receptor associated factor (TRAF) (1,2,3)-NF-kappaB signaling pathway [37]. The high amounts of Meq protein, over-expression of CD30 in transformed cells in all genotypes (regardless of MD-susceptibility or -resistance) in the first week after MDV infection [6] and the pro-inflammatory profile in both $L6_1$ and $L7_2$ in our current work together suggest that the genetic pathways of inflammation are also common to MD.

The tumor microenvironment is critical in development and maintenance of lymphoma generally [38] and this is also true for MD [6]. A complex network of cytokines and cell-to-cell contact mediated interactions between the transformed cells and surrounding reactive infiltrate can lead to further proliferation of neoplastic cells [38]. In classical Hodgkin's lymphoma (cHL), cytokine production by the transformed cells and the surrounding reactive infiltrating cells acts in autocrine and paracrine ways to result in the survival and proliferation of transformed cells and the maintenance of immunosuppressive microenvironment [39]. Aberrant activation of the STAT pathway is a postulated mechanism employed by neoplastic cells in HL derived cell lines to escape cell death [40] and the reactive infiltrate in HL is primarily comprised of Th-2 type of cells enriched in T-reg cells, though not always with a classical Th-2 type cytokine profile [38, 41]. These reactive cells express CTLA-4 and are anergic (which may be due to increased TGFβ and IL-10 expression). In human Epstein-Barr virus (EBV) positive tumors, genetically engineered TGFβ resistant CTLs had better antitumor activity than unmodified CTLs, suggesting the inhibitory role of TGFβ [42]. Also, EBV-infected HL transformed cells express the Epstein-Barr nuclear antigen-1 (EBNA-1) gene which upregulates the expression of chemokine (C-C motif) ligand (CCL20) binding, which is a strong chemoattractant of T-regs to the tumor microenvironment [43]. Alvaro et al. [44, 45] used the cellular composition of HL tumor microenvironment as a prognostic marker and suggested that a low number of cytotoxic T cells in reactive infiltrate correlate with increase in anti-apoptotic mechanisms in neoplastic cells. Wahlin et al. [46] proposed that the presence of more

of CD8+ T cells is a positive prognostic marker in human follicular lymphoma. Overall our results here and previously [5] suggest that the initial latently transformed minority cells which are CD4+CD30^{hi} are of T-reg phenotype and these cells induce the infiltrating CD4+T cells to the T-reg phenotype in both $L6_1$ and $L7_2$. In $L6_1$ a Th-1 tissue microenvironment would support CD8+ T cell-mediated immunity and CD8+ T cells have been observed in these lesions previously (8). Furthermore, IFNγ-secreting CD4+ T cells could recruit macrophages (also previously described in resistant MD lines [8]), induce MHC class I up regulation on the target transformed T-cells and promote restricted CD8+ CTL cells [47]. The mechanisms by which such a Th-1 could "over-ride" the T-reg type response within the neoplastic lesions themselves is unclear, but the Th-1 bias we observed is a clear distinction between the resistant and the susceptible MHC congenic lines. The strength of the MD system for understanding how the tissue and tumor microenvironment effects genetically-determined lymphoma regression or progression, and which we took advantage of, is that it is a natural system in the context of a non-manipulated immune environment with predictable pathogenesis.

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