2008-02-07

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Shack, L. A.

Springer-Verlag

https://doi 10.1007/s00262-008-0460-2

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Article *in* Cancer Immunology and Immunotherapy · September 2008

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Cancer Immunol Immunother (2008) 57:1253–1262
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ORIGINAL ARTICLE

The neoplastically transformed (CD30^{hi}) Marek's disease lymphoma cell phenotype most closely resembles T-regulatory cells

L. A. Shack · J. J. Buza · S. C. Burgess

Received: 13 November 2007 / Accepted: 15 January 2008 / Published online: 7 February 2008

Abstract
Introduction Marek's disease (MD), a herpesvirus-induced lymphoma of chickens is a unique natural model of CD30-overexpressing (CD30^{hi}) lymphoma. We have previously proposed that the CD30^{hi} neoplastically transformed CD4+ T cells in MD lymphomas have a phenotype antagonistic to cell mediated immunity. Here were test the hypothesis that the CD30^{hi} neoplastically transformed MD lymphoma cells have a phenotype more closely resembling T-helper (Th)-2 or regulatory T (T-reg) cells.
Materials and methods We separated ex vivo-derived CD30^{hi}, from the CD30$^{lo/-}$ (non-transformed), MD lymphoma cells and then quantified the relative amounts of mRNA and proteins for cytokines and other genes that define CD4+ Th-1, Th-2 or T-reg phenotypes.
Results and discussion Gene Ontology-based modeling of our data shows that the CD30^{hi} MD lymphoma cells having a phenotype more similar to T-reg. Sequences that could be bound by the MD virus putative oncoprotein Meq in each of these genes' promoters suggests that the MD herpesvirus may play a direct role in maintaining this T-reg-like phenotype.

Keywords Regulatory T cell · Herpesvirus · Gene Ontology · Systems biology · Animal model

L. A. Shack and J. J. Buza equally contributed to this work.

L. A. Shack · J. J. Buza · S. C. Burgess (✉)
Department of Basic Sciences,
College of Veterinary Medicine, Wise Center,
Mississippi State University, Spring Street,
Box 6100, Mississippi State, MS 39762-6100, USA
e-mail: burgess@cvm.msstate.edu

J. J. Buza · S. C. Burgess
Institute for Digital Biology,
Mississippi State University, Mississippi State, USA

S. C. Burgess
Mississippi Agricultural and Forestry Experiment Station,
Mississippi State, USA

Introduction

Marek's disease (MD), a lymphomatous disease of chickens caused by the MD α-herpesvirus (MDV) is also a unique natural animal model for classical Hodgkin's, and non-Hodgkin's, human lymphomas. MD neoplastically transformed cells over-express tumor necrosis factor receptor superfamily member (TNFSFR) 8 [the "Hodgkin's disease antigen" (CD30)] [1]. Like human CD30^{hi} lymphomas [2, 3], the MD CD30^{hi} cells are rare [4] and surrounded by activated non-transformed lymphocytes. MDV latently infects CD30^{hi} MD lymphoma cells [5]. MDV's putative oncogenes are not acutely transforming in vitro [6–8], and survival and growth of MD CD30^{hi} cells depends on the local lymphoma environment [4]. MD lymphoma growth occurs despite specific immune responses to virus and host proteins [9]. Here we test our hypotheses that the CD30^{hi} MD lymphoma cells have a phenotype most resembling T-helper (Th)-2 [1, 4] or T-regulatory (T-reg) cells [10]; either of which could antagonize cytotoxic lymphocyte immunity and support tumorigenesis.

Th cells can be distinguished from T-reg cells based on gene expression. Th-1 cells express CD4 and produce high levels of interferon-gamma (IFN-γ) and interleukin (IL)-2, Th-2 cells (also CD4+) produce IL-4, -5, -10, and -13 [11]. In contrast, T-reg cells express CD4, major histocompatibility complex (MHC) class II (except in mouse), IFN-γ [12, 13], transforming growth factor beta (TGFβ), IL-10,

the transcription factor forkhead box (FOX) P3, IL-2 receptor α chain (CD25), high levels of cytotoxic T-lymphocyte associated molecule-4 (CTLA-4) [14–17], G protein-coupled receptor (GPR)-83 [18], and decreased SMAD-7 [19, 20]. Furthermore, T-reg function depends on CD30 expression and signaling [21–23]. The neoplastically transformed cells in MD lymphomas express the highest levels of MHC class II, CD25 and CD30 [4]. Furthermore, the MDV oncoprotein "meq" transactivates the CD30 promoter [1].

Materials and methods

Chickens and MDV

Lymphomas were produced in outbred SPF, MD-maternal antibody-free white leghorn chickens (Charles River Laboratories, SPAFAS Avian Products and Services, Wilmington, MA, USA), infected (11 days old) with MDV (GA/22, passage 18, 500 pfu, from the Avian Disease and Oncology Laboratory, East Lansing, MI, USA), housed in Petersime units in isolated rooms at Mississippi State University College of Veterinary Medicine (ad libitum food and water).

Lymphoma cell sorting

Lymphomas were removed from ten chickens (kidney, sciatic nerves, testis, bursa, spleen, mesentery, lung and liver) and immediately placed into ice cold phosphate buffered saline (PBS). The $CD30^{hi}$ were separated from the $CD30^{lo/-}$, lymphoma cells (Fig. 1a inset) by magnetic activated cell sorting and the $CD30^{hi}$ and $CD30^{lo/-}$ purity measured by flow cytometry (FACSCalibur, Becton Dickinson Biosciences) exactly as described [24].

RNA isolation and real-time PCR

We isolated RNA from three batches of 10^6 $CD30^{hi}$ and $CD30^{lo/-}$ cells using TRI reagent (Molecular Research Center, Inc.) and treated each with RNase-free DNAse 1 (Promega Corporation) exactly as described [24]. RNA concentrations were quantified (GeneSpec I spectrophotometer; MiraiBio, Alameda, CA, USA) and all RNA samples were adjusted to within a tenfold concentration of each other using RNase-free water. mRNA expression was measured for cytokines and other genes (shown in Fig. 1) to define CD4+ T cell phenotypes. We used a duplex real-time reverse transcriptase PCR (drtRT-PCR), with 28 S rRNA standard, exactly as described [25]: Platinum Quantitative RT-PCR ThermoScript One-Step System (Invitrogen, Carlsbad, CA, USA), 10 μM of each primer, 1 μM probe, an iCycler iQ Real-Time PCR Detection System [Bio-Rad Laboratories, Inc., Hercules, CA, USA; 50°C, 30 s; 95°C, 5 min + 45 × (95°C, 15 s; 60°C, 60 s)]. Most primer and probe sequences (Table 1) are previously published [26, 27], the novel primer/probe sets were designed using *Beacon Designer* (PREMIER Biosoft International, Palo Alto, CA, USA). All amplicons cross intron–exon boundaries. Each drtRT-PCR experiment, done in triplicate on 96-well plates, included no-template controls and a standard curve (10^{-1} – 10^{-6} total RNA made by mixing a 10 μl aliquot from all samples). All PCRs were normalized using the standard curves. The 28 S rRNA-specific mean threshold cycle (C_t) value for all target genes was calculated and used to normalize across PCR plates and between samples.

Proteomics

We isolated protein from three batches of 10^7 $CD30^{hi}$ and $CD30^{lo/-}$ cells by differential detergent fractionation (DDF) exactly as described to produce four fractions. Each DDF fraction predominantly contains proteins from different cellular locations (which directly relate to GO cell components) [28]. For each DDF, these were analyzed by two-dimensional liquid chromatography electrospray ionization tandem mass spectrometry (2D LC ESI MS^2) exactly as described [10] with one exception. Because we were searching only for the specific proteins described in Fig. 1, we first identified the predicted masses of tryptic peptides between 6 and 30 amino acids in length that could be derived from these peptides using the ExPASy PeptideCutter tool [29] and then did tandem mass spectrometry only on precursor ions with these masses (±1.5 Da, i.e., within the accuracy of the LCQ dex Xp plus mass spectrometer, ThermoElectron Corp., San Jose, CA, USA). We used decoy database searching exactly as described [10, 30] to calculate the probability that a tandem mass spectrometry match occurred by chance and, from these, the probability of the protein identification occurring by chance exactly as described [31, 32] (Table 2). We used isotope-free quantitative analysis based on spectral counting combined with the increased specificity given by including the quantitative aspects of the Sequest cross correlation (XCorr) [33].

Statistical analysis

Differences are always presented from the perspective of the neoplastically transformed $CD30^{hi}$ cells relative to the $CD30^{lo/-}$ cells. We express the results for the fold-difference in mRNA and the proteomics data for each gene as the mean percentage difference and mean difference ± 95% confidence interval (CI), respectively. The mean difference was used for the proteomics data because, on some occasions, the denominator (i.e., always the $CD30^{lo/-}$ value) was 0. Thus, for a given gene, if $CD30^{hi}$ cell

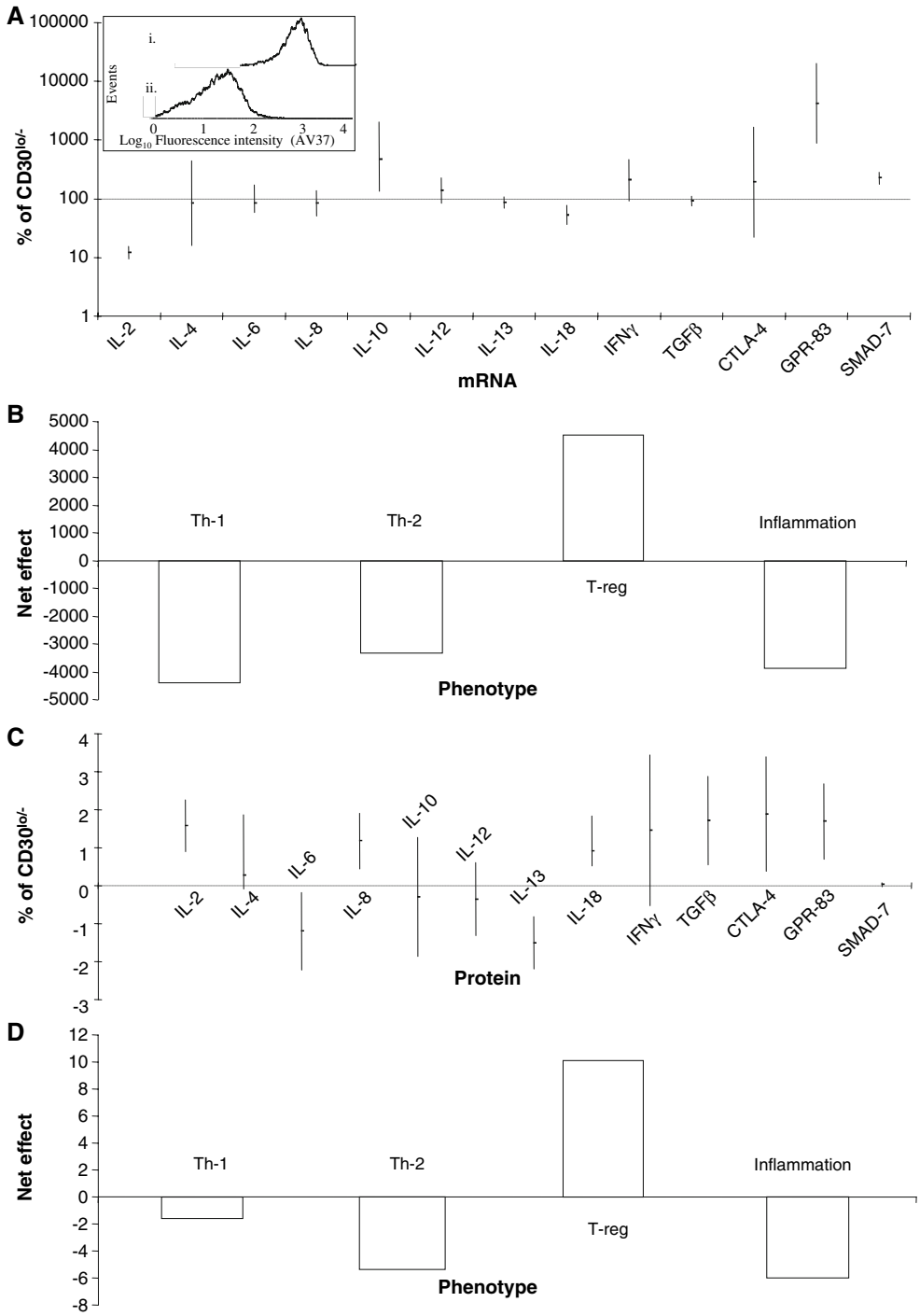

Fig. 1 Difference in cytokine and cell antigen mRNA (**a**) and protein (**c**) expression (measured by duplex real-time reverse transcriptase PCR and proteomics as described in M and M) of $CD30^{hi}$, relative to $CD30^{lo/-}$, MD lymphoma cells. The amount of $CD30^{lo/-}$ mRNA expression is set to 100% and protein expression is set to 0. The $CD30^{hi}$, were separated from the $CD30^{lo/-}$, lymphoma cells by magnetic activated cell sorting. Inset: the mean purity (±SEM) was 95.7 ± 3.3 and 93.9 ± 4.9%, for the (1) $CD30^{hi}$ and (2) $CD30^{lo/-}$ cells, respectively. GO-based hypothesis-driven quantitative modeling as described in M and M for the mRNA (**b**) and protein (**d**) shows that the $CD30^{hi}$ cells have a T-reg phenotype

mRNA expression was not different from the $CD30^{lo/-}$ cell mRNA expression then 100% would be included in the 95% CI Similarly, for the protein data, if the $CD30^{hi}$ cell protein expression was not different from the $CD30^{lo/-}$ cell mRNA expression then 0 would be included in the 95% CI.

Table 1 PCR probes and primers (fluorophore)

RNA target	Probe/primer	Sequence
28 S	Probe	5′-(HEX)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3′
	F	5′-GGCGAAGCCAGAGGAAACT-3′
	R	5′-GACGACCGATTTGCACGTC-3′
IL-2	Probe	5′-(FAM)-ACTGAGACCCAGGAGTGCACCCAGC-(TAMRA)-3′
	F	5′-TTGGAAAATATCAAGAACAAGATTCATC-3′
	R	5′-TCCCAGGTAACACTGCAGAGTTT-3′
IL-4	Probe	5′-(FAM)-AGCAGCACCTCCCTCAAGGCACC-(TAMRA)-3′
	F	5′-AACATGCGTCAGCTCCTGAAT-3′
	R	5′-TCTGCTAGGAACTTCTCCATTGAA-3′
IL-6	Probe	5′-(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)-3′
	F	5′-GCTCGCCGGCTTCGA-3′
	R	5′-GGTAGGTCTGAAAGGCGAACAG-3′
IL-8	Probe	5′-(FAM)-TCTTTACCAGCGTCCTACCTTGCGACA-(TAMRA)-3′
	F	5′-GCCCTCCTCCTGGTTTCAG-3′
	R	5′-TGGCACCGCAGCTCATT-3′
IL-10	Probe	5′-(FAM)-CGACGATGCGGCGCTGTCA-(TAMRA)-3′
	F	5′-CATGCTGCTGGGCCTGAA-3′
	R	5′-CGTCTCCTTGATCTGCTTGATG-3′
IL-12β	Probe	5′-(FAM)-CTGAAAAGCTATAAAGAGCCAAGCAAGACGTTCT-(TAMRA)-3′
	F	5′-TGGGCAAATGATACGGTCAA-3′
	R	5′-CAGAGTAGTTCTTTGCCTCACATTTT-3′
IL-13[a]	Probe	5′-(FAM)-CTGCCACAGTGCTGGACAACATGACCG-(TAMRA)-3′
	F	5′-CAAGGATCGGAAGCTGTCAGAG-3′
	R	5′-GGCGGGCAGTTCGTCATG-3′
IL-18	Probe	5′-(FAM)-CCGCGCCTTCAGCAGGGATG-(TAMRA)-3′
	F	5′-AGGTGAAATCTGGCAGTGGAAT-3′
	R	5′-ACCTGGACGCTGAATGCAA-3′
CTLA-4[a]	Probe	5′-(FAM)-TTGTCTTCTCTGAATCGCTTTGCCCACG-(TAMRA)-3′
	F	5′-CAGCATCATCATCTCAGCCATTG-3′
	R	5′-GCATTTTCACATAGACCCCAGTAG-3′
GPR83[a]	Probe	5′-(FAM)-TCCGCCACCAGCCTGTTCATCGTCA-(TAMRA)-3′
	F	5′-CGTCATCATCAAGAGCAAACGC-3′
	R	5′-ACAAAACGAGCCAGTGTAAAAGG-3′
IFN-γ	Probe	5′-(FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA)-3′
	F	5′- GTGAAGAAGGTGAAAGATATCATGGA-3′
	R	5′-GCTTTGCGCTGGATTCTCA-3′
SMAD7[a]	Probe	5′-(FAM)-TCCCAGTAAGCCACCACGCACCAGT-(TAMRA)-3′
	F	5′-GCTCTCAGATTCTCAAGTTATTCAGG-3′
	R	5′-CCGACCCACACGCATCTTC-3′
TGFβ	Probe	5′-(FAM)-ACCCAAAGGTTATATGGCCAACTTCTGCAT-(TAMRA)-3′
	F	5′-AGGATCTGCAGTGGAAGTGGAT-3′
	R	5′-CCCCGGGTTGTGTGTTGGT-3′

F forward, *R* reverse

[a] Designed during this work

GO-based quantitative modeling

Our GO-based modeling was based on specific hypotheses, framed in GO biological process (GOBP) terms, defining the phenotype of CD4+ T cells and inflammation. To derive quantitative data, we combined GO-annotation with our quantitative mRNA or protein expression data using the computaional tools at *AgBase* [34]. We had to overcome

Table 2 Proteins and probability (*P*) that the protein identification could have occurred by chance (based on decoy database searching as described in M and M)

Protein	*P*
gi|3087785|emb|CAA12025.1| interleukin-2	$2.0e^{-67}$
gi|27803086|emb|CAC15566.2| interleukin-6	$3.4e^{-63}$
gi|1729918|sp|P09531|TGFB1_CHICK Transforming growth factor β-1	$7.0e^{-52}$
gi|47087195|ref|NP_998736.1| interleukin 12B	$1.7e^{-48}$
gi|8919963|emb|CAB96214.1| interleukin-18	$2.0e^{-47}$
gi|54792251|emb|CAF18428.1| interleukin-13	$4.9e^{-43}$
gi|50745619|ref|XP_426254.1| Predicted: similar to G protein-coupled receptor 83	$2.8e^{-36}$
gi|90968221|emb|CAJ86460.1| cytotoxic T-lymphocyte-associated protein 4	$4.9e^{-23}$
gi|51173886|emb|CAF18432.1| interleukin-10	$4.9e^{-21}$
gi|119318|sp|P08317|IL8_CHICK Interleukin-8	$8.2e^{-21}$
gi|1708496|sp|P49708|IFNG_CHICK Interferon gamma	$4.9e^{-13}$
gi|54792249|emb|CAF18427.1| interleukin-4	$4.9e^{-13}$
gi|50774673|ref|XP_427238.1| Predicted: similar to SMAD7	$7.0e^{-04}$

Table 3 *GOmodeler* table after scoring the regulatory effects of each gene product examined on Th-1, Th-2, T-reg cell differentiation and inflammation

Gene product	Th-1	Th-2	T-reg	Inflammation
IL-2	1		1	–1
IL-4	–1	1	1	
IL-6		1	–1	1
IL-8			1	1
IL-10	–1	1	1	0
IL-12	1	–1		
IL-13	–1	1		
IL-18	1	1	1	1
IFNγ	1	–1	1	1
TGFβ	–1	0	1	–1
CTLA-4	–1	–1	1	–1
GPR-83	–1	–1	1	–1
SMAD-7	1	1	–1	1

Pro +1, *Anti* –1 and *No effect* 0. When no data is present in the published literature the cell is left blank. Annotation followed the GO Consortium guidelines (http://www.geneontology.org/GO.annotation.shtml)

the limitation of the GO that most literature for any species is not yet curated and so functional annotations from this literature are not yet present in the GO databases. Although the GOBP terms exist for gene products controlling T-reg development and regulation (GO:0045066, GO:0045590, GO:0045591 and GO:00455890), and there is a literature on the genes involved, the literature and GO databases are unconnected [10, 34]. Thus, we first annotated the chicken genes, using orthology to the human and mouse genes, to GO:0045066, GO:0045590, GO:0045591 and GO:00455890. To retrieve existing GO annotations, and add futher computational- and literature-based annotations, the GO annotation processes we use are decribed elsewhere [35, 36]. The computational tool *GOmodeler* [35], first scores the effects of each gene product, a process as either "pro" (+1), "anti" (–1), "no effect" (0) or "no data" (blank cell) (Table 3). Then the quantitative drtRT-PCR or proteomics data are used to calculate a quantitative effect for each gene (i.e., to give a quantitative value in each cell). Finally, net effects are calculated and both the mRNA (Fig. 1b) and the protein data (Fig. 1d).

The numbers of putative Meq binding sites in promoters

There is more Meq in the $CD30^{hi}$, than the $CD30^{lo/-}$, MD lymphoma cells [1] and Meq activates or represses gene transcription [1, 6, 37–39]. It is possible that differences in amounts of mRNA between $CD30^{hi}$ and $CD30^{lo/-}$ MD lymphoma cells may be caused by Meq directly. We identified the genomic location of each cytokine (by BLASTN searches against the chicken genome sequence) and extended each sequence by 2,500 bp 5′ of the annotated ORF start. These sequences were then searched using *Alibaba2* and *MatInspector* (core and matrix similarity values of 1.0 and $\geq$0.9, respectively), for activator protein-1 (AP-1), MERE I and II sequences [39] that could potentially bind Meq, exactly as described [1].

Results

Cell sorting and differential mRNA and protein expression

The $CD30^{hi}$ and $CD30^{lo/-}$ purity was 95.7 ± 3.3 and $93.9 \pm 4.9\%$ (mean $\pm$ SEM), respectively (Fig. 1a inset). For statistical confidence that $CD30^{hi}$ lymphoma cells expressed a different amount of mRNA or protein compared to the $CD30^{lo/-}$ cells (at $\alpha = 0.05$), then 100% or 0 cannot be included in the 95% CI of the mean percentage

Table 4 Change in mRNA expression and numbers of AP1, MERE I and II in promoters of each gene

	Δ mRNA (%)	AP1	MERE I/II	Sum
IL-2	10	18	5	23
IL-4	0	12	5	17
IL-6	0	11	4	15
IL-8	0	21	7	28
IL-10	524	23	11	34
IL-12	0	9	4	13
IL-13	0	13	7	20
IL-18	53	13	3	16
CTLA-4	0	7	3	10
GPR-83	4,154	8	5	13
IFNγ	0	18	4	22
SMAD-7	224	6	3	9
TGFβ	0	5	1	6

difference and mean difference for mRNA and protein, respectively. The $CD30^{hi}$ lymphoma cells express less IL-2 and IL-18 mRNA, but more IL-10, GPR-83 and SMAD-7 mRNA, than $CD30^{lo/-}$ lymphoma cells (Fig. 1a). $CD30^{hi}$ lymphoma cells express less IL-6 and IL-13 proteins, but more IL-2, IL-8, IL-18, TGFβ, CTLA-4 and GPR-83 proteins, than $CD30^{lo/-}$ lymphoma cells (Fig. 1a). Because we used the DDF method to isolate the proteins, we can identify the cellular component that the given protein was primarily isolated from [28], these are shown in the Table.

GO-based quantitative modeling

Even though there is no 100% agreement between differentially expressed mRNAs and proteins, GO-based model is pro T-reg, anti Th-1, -2 and inflammatory for both mRNA (Fig. 1b) and protein (Fig. 1d).

Meq binding sites in promoters of mRNAs examined

The numbers of putative Meq binding sites in promoters with relative rank order of mRNA expression for each cytokine. The predicted numbers of putative Meq binding AP1 and MERE I and II binding sequences are shown in Table 4.

Discussion

The suggestion that the MDV Meq protein's presence in cells is necessary, but not alone sufficient, for lymphomagenesis, is clear from the range of genetic resistance and susceptibility of chickens to MD, i.e., whether a given MDV causes gross lymphomas depends on the chicken genotype. Furthermore, although MD resistance is mediated to some degree at the level of virus load [27, 40], we have demonstrated that MDV lymphomagenesis is a continuum and lymphomagenesis is also mediated at the level of numbers of transformed cells [4] and lesion development [41]. Here we did not examine the factors that may mediate genetic MD resistance and susceptibility. Instead we defined one aspect of the MD lymphoma immune environment and how the neoplastically transformed cells differ from the reactive cells in MD lymphomas. This is important because the outcome of any oncogenic herpesvirus infection depends not only on intracellular host virus gene interactions but also on interactions between the neoplastically transformed cells and the immune system. We show for the first time a herpesvirus transformed cell that has a phenotype most closely resembling T-reg.

Analyzing T cells by phenotype analysis alone is not trivial because, with the exception of the T and the B cell receptors, proteins are not definitive markers of cell phenotype per se. CD25, though considered a phenotypic marker of human, mouse and rat T-reg cells, is expressed by non-suppressive, activated CD4+ T cells [42]. In addition to the antigens that we used to define T cell phenotype, tumor necrosis factor receptor superfamily members (TNFRSF)-4 (a.k.a. CD134 and OX40), TNFRSF9 (a.k.a. CD137 and 4-1BB) and TNFRSF18 (a.k.a. GITR) are also suggested to be markers of T-reg phenotype and could provide additional information. However, all are non-specific as T-reg markers [43–47]. TNFRSF-4 has also recently been demonstrated to negatively regulate T-reg development [48] and enhances the numbers of tumor antigen-reactive CD4 T cells [49]. Regardless, the GO annotation for all three is very poor and thus these genes would not significantly contribute to our GO modeling. In contrast, FOXP3 is considered a "master gene required for the development and function of T-reg" [50]. We would have liked to use FOXP3 in this work but no FOXP3 ortholog has (yet) been identified in the chicken and we were unable to identify any ESTs with high-enough sequence identity in the EST databases to identify an ortholog. However, although FOXP3 is currently considered as one of the most specific markers for naturally occurring CD4(+)CD25(+) T-regulatory cells, expression of FOXP3 is a normal consequence of CD4(+) T cell activation and is not an exclusive marker of human T-reg [51, 52]. FOXP3 expression occurs after in vitro stimulation of human CD4(+)CD25(–) cells and although FOXP3 expression is strongly associated with hyporesponsiveness of activated T cells, it is not directly correlated with T-reg suppressive capabilities and in humans, expression of endogenous FOXP3 is not sufficient to induce regulatory T cell activity or to identify T-reg cells [52]. The lack of FOXP3 in the chicken was the reason that we used GPR-83 and SMAD7. GPR-83 is up-regulated in human

CD4(+)CD25(+) T-regulatory cells, is directly linked to FOXP3 expression in human cells and in mouse it is "critically involved in the peripheral conversion of FOXP3-negative to FOXP3-expressing regulatory T cells in vivo" [18]. SMAD7 negatively regulates FOXP3 and thus T-reg cannot have high SMAD7 expression [19].

Apart from surface phenotype, T cells are described functionally and because of this, we used cytokine production profile to define T-reg in addition to cellular antigens. Generally, the cytokine profiles that are used to distinguish (the various subtypes of) T-reg cells from T effector cells are increased levels of IL-10, TGFβ and IFNγ, and decreased IL-2. Although our modeling overall suggests that the CD30^{hi} MD lymphoma cells are more similar to a T-reg, then Th1 or Th2 cells, our data are not completely consistent with this classical T-reg phenotype at a gene-by-gene level. This is perhaps not surprising given that these CD30^{hi} MD lymphoma cells are not physiological T-reg cells, but are virus transformed CD4+ T cells. Our protein and mRNA data also do not correlate perfectly but this is expected to some degree because there is a generally low correlation between amounts of an mRNA and amounts of protein in a cell [53, 54]. IL-2 and IL-18 are of most concern, as these have the greatest conflict and decreased IL-2 expression is one critical determinant of a T-reg phenotype. However, because each DDF fraction contains proteins predominantly from different cellular locations [28], we can quantitatively define the differences in cellular distribution of protein between the CD30$^{lo/-}$ and CD30^{hi} MD lymphoma cells (Fig. 2). In the CD30^{hi} MD lymphoma cells both IL-2 and IL-18 are preferentially distributed in the differential detergent fractions that represent the least superficial areas of the cell [28]. This suggests that IL-18 is

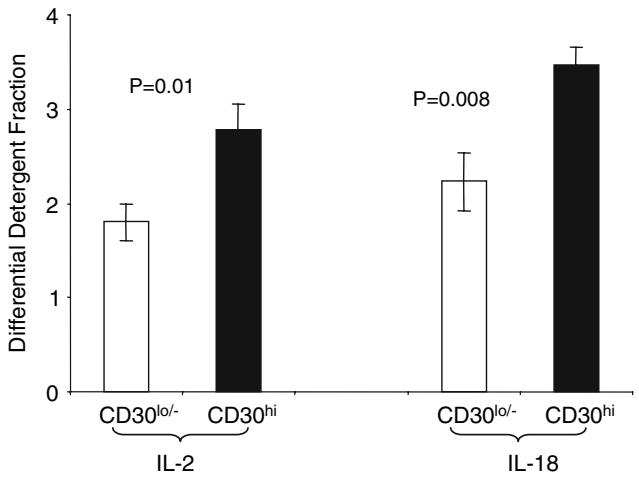

Fig. 2 Difference in IL-2 and IL-18 protein distribution in CD30$^{lo/-}$ and CD30^{hi} lymphoma cells mean (±SEM). Both IL-2 and IL-18 protein are preferentially distributed in the differential detergent fractions representing the least superficial areas of the cell and the less soluble proteins

retained in the cell in its pre-pro and/or pro-protein isoforms, which fits with the known physiology of IL-18 secretion. IL-2 may be actively or passively retained in the cell; regardless, the IL-2 DDF profile in the CD30^{hi} MD lymphoma cells suggests that IL-2 secretion will be lower in the CD30^{hi} MD lymphoma cells and this is consistent with a T-reg phenotype and with low IL-2 mRNA expression.

Although we have circumstantial evidence suggesting that the MD lymphoma environment is antagonistic to T cell mediated immunity [1, 4, 9, 41], after cytokine expression the next logical functional evidence to define a T-reg-like phenotype would be to demonstrate T-reg-associated suppressor activity in an in vitro assay [e.g. contact-dependent CD30^{hi} MD lymphoma cell inhibition of proliferation and cytokine secretion induced by TCR cross-linking of CD4(+)CD25(–) responder T cells]. However, such experiments are challenging in MD. The CD30^{hi} MD lymphoma cells are absolutely dependent on the lymphoma environment for their survival [4]. Even when MD cell lines can be produced from lymphomas, such cell lines are highly in vitro-adapted and may not be good functional models of the MD lymphoma cells in vivo [4, 9]. Finally, there is persistent MDV reactivation with concomitant cell death, in both ex vivo CD30^{hi} MD lymphoma cells and MD cell lines.

Virus survival critically depends on immune evasion and virus activation of T-reg is a known immune evasion strategy, e.g. herpes simplex virus [55] and the tumorigenic Epstein-Barr [56], hepatitis C [57] and murine leukemia [58] viruses. Tumor growth also depends on immune evasion. The T-reg-like phenotype of the CD30^{hi} MD cells could help to explain the lack of CD8+ T cells in developing MD lesions in susceptible, compared with resistant, chicken genotypes [41]. In support, the T-reg-like response induced by persistent infection of mice by Friend retrovirus causes the loss of tumor transplant rejection ability [59].

Anti-tumor immunity not targeting virus- or mutated host-antigens is fundamentally "autoimmunity". T-reg are essential for controlling autoimmunity [60, 61] and CD30 signaling very early in disease pathogenesis is critical to T-reg function [23]. Such CD30 signaling is likely in MD [4]. T-reg requires activation with specific antigens to develop suppressive activity but subsequent immune suppression is antigen- and MHC-independent [62]. This property is consistent with a hypothesized mechanism of CD4+: CD4+ T cell co-antigen presentation via MHC class II in MD lymphomas [4].

MDV appears to be similar to other transforming viruses that have usurped T-reg to perturb tumor immunity. Meq is a transcriptional regulator of the CD30 gene [1], and meq transcriptionally regulates the expression of many other mRNAs [37]. Because the CD30^{hi} lymphoma cells have the most meq protein [1], we speculated that the differences in

cytokine mRNA expression may be due to greater numbers of meq binding sequences in the promoters of these genes. However, there was a low correlation coefficient (–0.11) between the numbers of predicted AP1, MERE I and II binding sites, and the difference in mRNA expression. Despite a poor overall correlation, T cell lymphomas generally have AP-1 activation [63, 64] and one of the target genes is IL-2 and IL-2 is a critical T cell proliferation regulator [65–67]. Notably our ex vivo data do agree with previous in vitro data that Meq (presumably homodimers) represses IL-2 [38]. TGFβ, which has the lowest number of predicted AP1, MERE I and II binding sites, is particularly interesting because previous in vitro work has demonstrated that Meq increases TGFβ expression [37]. Though we would have predicted differential expression of TGFβ mRNA, we identified higher expression by the CD30^{hi} cells at the protein level only. Regardless, and although the mechanistic details need further experimental clarification, our work suggests that the MD herpesvirus may play a direct role in maintaining a T-reg-like phenotype via the meq protein.

Acknowledgments This work was supported by a USDA NRI 2004-35204-14829. We acknowledge Tibor Pechan for running the mass spectrometer and the extremely valuable input of two anonymous reviewers.

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