Role of the IL-23/IL-17 Axis in a Mouse Model of Psoriasiform Disease: An Immunological Approach

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SUMMARY:
Psoriasiform disorders, are a group of skin diseases characterized by thickened skin (acanthosis). Psoriasis and seborrheic dermatitis, prototypical examples of this disease class, affect up to 4% of the population (Johnson, 1978). The D2C T-cell receptor transgenic mouse model demonstrates severe lymphopenia and an immune reconstitution phase that models the immune dysregulation occuring in the setting of immune reconstitution syndromes. This includes patients with the acquired immunodeficiency syndrome (AIDS) undergoing HARRT therapy, a group of patients whom develop a high frequency of concommittant psoriasiform diseases (Reveille et al, 1990). D2C skin pathology closely mimics the clinicopatholgical features of human seborrheic dermatitis (Oble, 2006), a disease which is believed to have a multifactorial etiology but a poorly understood immune contribution. Previously it was shown that the pathophysiology in D2C mice was in part attributable to a lymphoproliferation of effector T-cells ($T_{effector}$) due to a lymphopenia of regulatory T-cells (T_{reg}) since the development of disease could be abrogated by the adoptive transfer of syngeneic $T_{reg.}$ It was hypothesized that this lymphoproliferation of $T_{effector}$ was accompannied by a greater polarization of T-cells to the T_H17 lineage and a greater liberation of IL-23/IL-17 axis cytokines. We demonstrated by enzyme-linked immunosorbent assay (ELISA) that there is in fact an increase in IL-23/IL-17 axis cytokines in the serum of D2C mice. Similalry, we demonstrated by RT-PCR analysis that there is an increased message level of these IL-23/IL-17 axis cytokines in lesional skin of D2C mice and that there is a vague correlation of these cytokine levels with disease stage. Given the intimate relationship between the T_H17 and T_{reg} lineages (antagonistic yet capable of reciprocal transmutation) and the previously ascertained role of T_{reg} in the model system, we also conducted pilot studies to examine in greater detail how T_{reg} subsets are altered by the process of transgenesis, and how these effects might effect disease pathogenesis. These preliminary results demonstrtaed that that both natural and induced T_{reg} are deficient in D2C mice however $CD25^+$ natural T_{reg} are particularly deficient in prediseased D2C animals and that disease progression and ultimately disease convalescence is associated with an expansion of both T_{reg} subsets, but with a preportionally greater expansion of the self-antigen specific natural $T_{reg,}$ perhaps reflecting the self-reactive nature of the 2C-TCR on the H-2^d expressing DBA/2 inbred strain, and the need to regulate self-reactive cells in this model system.

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Abstrac

The D2C T-cell transgenic mouse model of psoriasiform disease closely resembles human seborrheic dermatitis (Oble, 2006). Given the apparent importance of T_H17 cells in psoriasis, the activity of IL-23/IL-17 axis in this disease model was explored. Using an enzyme-linked immunosorbant assay (ELISA) approach, it was found that the IL-23/IL-17 axis cytokines (IL-6, IL-17, IL-22, and IL-23) are elevated in serum samples taken from mice at various points in disease progression. Similarly, reverse transcriptase PCR (RT-PCR) of RNA obtained from lesional D2C skin demonstrated that there is an increased message level of these same cytokines relative to the skin of wild-type control mice with a vague relationship between cytokine message levels and disease severity. Given the intimate relationship between T_{reg} and T_H17 cells, the status of T_{reg} was also explored in D2C mice. Pilot investigations demonstrated that relative to non-transgenic control mice, D2C mice are deficient in both natural and induced T_{reg} ; however, $CD25^+$ natural T_{reg} are particularly deficient in pre-diseased D2C animals and that disease progression and ultimately disease convalescence is associated with an expansion of both T_{reg} subsets, but with a proportionally greater expansion of the self-antigen specific natural $T_{reg,}$ perhaps reflecting the self-reactive nature of the 2C-TCR on the H-2^d expressing DBA/2 inbred strain, and the need to regulate self-reactive cells in this model system.

Introduction

The D2C mouse is a spontaneous, 100% penetrant mouse model system of psoriasiform disease that shows high homology to the clinicopathological characteristics of human seborrheic dermatitis (Oble, 2006). The 2C TCR system forces (via the Lck promoter) the expression of T-cell receptor (TCR) transgenes, that generate a TCR which recognizes the ubiquitous, mitochondrial-derived, p2Ca peptide (LSPFPFDL) when presented by class I major histocompatibility proteins (MHC I). The intermediate affinity recognition of p2Ca by the 2C TCR when presented by K^b on the H-2^b genetic background (C57BL6) leads to the positive selection of transgenic TCR-expressing T-cells. However, the high affinity recognition of p2Ca by the 2C TCR when presented by L^d on the H-2^d background (BALB/c or DBA/2), leads to the negative selection of transgenic TCR-expressing T-cells, including the almost comprehensive deletion of double positive (DP) thymocytes. Since DP thymocytes are the precursors to SP (single positive) thymocytes which leave the thymus to become mature lymphocytes (CD4 and CD8), there is a dearth of these T-cell subsets in D2C mice. Furthermore, given that regulatory T-cells develop from CD4 SP thymocytes, T_{reg} are also severely lymphopenic in D2C animals. Although the Lck promoter drives 2C TCR transgene expression in all T-cells, this system is somewhat leaky since some cells delete the transgenes or rearrange endogenous TCR α chain genes (since these mice are not recombination activating gene -1/2 deficient) and form a new endogenous TCR-alpha chain (TCR α_E) that can pair with the transgenic β chain (TCR β_{Tg}) forming a TCR with new antigen specificity. This leads to a trickling of normal T cells that escape the negative selection process and populate the lymphopenic T-cell compartment. Subsequently, these infrequent $T_{effector}$ undergo a dysregulated expansion (homeostatic proliferation) that fills this void in the T-cell compartment created by the aforementioned deficient thymic output. This dysregulated $T_{effector}$ cell proliferation and associated cytokine storm is, in part, attributable to a lymphopenia of $T_{reg,}$ as the adoptive transfer of syngeneic T_{reg} to pre-diseased D2C mice can abrogate this lymphoproliferation and associated disease development (Oble, 2006). In non-manipulated D2C mice, the T-cell and T_{reg} compartments

stabilize with time (occurring between day 21 and day 80), leading to a more regulated immune environment and an associated disease convalescence (Oble, 2006).

It is hypothesized that this dysregulated immune expansion and resultant development of psoriasiform disease is accompanied / induced by a greater polarization of $T_{effector}$ to the T_H17 cell lineage and an upregulation of the IL-23/IL-17 cytokine axis. This prediction is based upon the recent discovery that the IL-23α chain (p19), which combines with the common p40 subunit, is upregulated in psoriasiform murine skin (Kopp et al., 2003, Lee et al., 2004) and is associated with an expansion of T_H17 cells and a greater liberation of IL-23/IL-17 axis cytokines (Moissec, 2009, Zaba et al., 2007). Interestingly, these revelations have significantly changed the understanding of psoriasis, which was thought to be induced by lymphocytes polarized to the T_H1 lineage and the resultant *in situ* generation interferon-gamma in lesional skin. This belief had been based largely upon the finding of elevated message levels of the common p40 subunit in psoriasiform skin. However, the aforementioned studies which demonstrated elevated IL-23α chain (p19) in psoriasiform tissue failed to demonstrate an increase in the IL-12 α chain (p35) in the same material, indicating that the elevated levels of the common p40 subunit in psoriasiform skin was contributed from an excess of tissue IL-23 (T_H17 axis) rather then IL-12 (T_H1 axis). To assess whether a similar pathophysiological cascade was operant in the D2C model, serum samples and rostral skin biopsies from variably diseased D2C mice and non-transgenic wild-type animals were assayed by ELISA and RT-PCR to look for elevations of IL-23/IL-17 axis cytokines and cytokine message respectively.

Given the intimate relationship between the T_H17 and T_{reg} lineage (antagonistic yet capable of bilateral lineage transmutation) and the previously ascertained role of T_{reg} in the model system (Oble, 2006), pilot flow cytometric immunophenotyping studies were also performed on lymph node cells to qualitatively and quantitatively evaluate differrent subsets of T_{reg}, and to ascertain whether they are differrential affected by transgenesis in the D2C system.

While other murine models of psoriasiform disease exist (Gudjonsson et al., 2007), none so closely replicate the clinicopathological features of human seborrheic dermatitis. Furthermore, the D2C system is an excellent model system of the immune reconstitution inflammatory syndrome (IRIS) which occurs in a variety of setting such as following chemotherapy or in AIDS patients following the initiation of highly active anti-retroviral therapy (HAART) (DeSimone et al., 2000). The spontaneous and 100% penetrant D2C system allows for a close analysis of these processes without the technical and ethical challenges associated with other model systems such as the xenotransplantation of human skin onto SCID mice (Gudjonsson et al., 2007). Furthermore, while the D2C and scurfy mouse model system appear to have some similar clinicopathological features (Clark et al., 1999), critical immunological differences exist between these model systems. For example, although scurfy mice are completely deficient in T_{reg} due to a point mutation in the FoxP3 gene, these animals also have a complete deficiency in T_H17 cells and thus are not as ideal as the D2C system to study T_H17 axis dysregulation.

Materials and Methods

All work was carried out under the supervision of Dr. Darryl Oble and Mariam Alkhawaja in the Department of Pathology, University of Manitoba. This work is ethically approved by the Bannatyne Campus Protocol Management and Review Committee. Fifty mice were used at various stages of disease to produce the sera, tissue and cells required for experiments.

Mice

Transgenic 2C mice on the B6 background were originally acquired from Herman Eisen at MIT and were bred back to the DBA/2 inbred strain at UBC (University of British Colombia, Vancouver, BC) before being adopted at the Genetic Model Centre at the University of Manitoba. Mice were genotyped for the presence of the 2C TCR transgenes by PCR analysis using primers against TCR chain genes: Vβ 8.2: 5'-AGA TAT CCC TGA TGG ATA CAA GGC – 3' and Jβ 2.5: 5'–CTA ACA CGA GGA GCC GAG TGC CTG – 3'. Alternatively, mice were genotyped for the presence of the 2C TCR transgenes by flow cytometry on lymph node cells using a monoclonal antibody directed against the 2C TCR (α-clonotypic mAb -1B2). Animals were bred and cared for at the Genetic Models Center at the University of Manitoba, and all experiments were performed in accordance with the standards of the Canadian Council for Animal Care. Mice used for the study included variably diseased D2C mice (Figure 1A), DBA/2 wild-type control mice, Scurfy mice and C57Bl6 wild-type control animals. C57Bl6 and DBA/2 control mice exhibited virtually identical immunological features in the assays performed and were sometimes used interchangeable as wild-type controls.

ELISA

Serum samples were collected from variably diseased D2C mice or wild-type control animals via intracardiac or saphenous vein puncture. ELISA Ready-Set-Go! kits (CAT# 88-7064-22, 88-7371-22, 88-7422-22, 88-7230-22) were purchased from eBioscience (San Diego, CA) and these serum ELISAs were performed as per the manufacturer's protocol. In brief, 96-well plates were coated with capture antibody overnight at 4ºC. After washing with PBS-Tween 20, the wells were blocked with assay diluent and incubated for an hour at room temperature (RT). Samples were subsequently incubated for 2 hours at RT. Captured cytokine was then detected during a 1 hour, RT incubation with the biotinylated detection antibody, followed by a 30-min RT incubation with Avidin-HRP (Horseradish peroxidase). The assay was developed during a 15-min RT incubation with the substrate solution followed by the addition of stop solution. The absorbance was measured at 450 nm on a SpectraMax plus plate reader (Molecular Devices, CA).

RT-PCR

Tissue for PCR was collected via dissection from mice and flash-frozen in Eppendorf tubes with liquid nitrogen. Frozen tissue was ground with mortar and pestle on liquid nitrogen and digested with an RNeasy Mini Kit (CAT# 74104, Qiagen, Toronto, ON). The mRNA was then transcribed into cDNA using random hexamer primers and SuperScript III Reverse Transcriptase (CAT# N8080127, 18080044 Invitrogen/Life technologies, Burlington, ON). cDNA was measured using a NanoDrop spectrophotometer (ND-1000 NanoDrop Products, Thermoscientific, DE). Samples were subsequently standardized using the ribosomal RNA house keeping gene, 18S. All PCR primers were purchased from Invitrogen and optimized to temperature and magnesium concentrations as indicated in the below table. Amplicons were electrophoresed on agarose gels

(1-2%) containing Sybr-safe green dye (CAT# S33102, Invitrogen) (Mullis et al., 1986). Photo and densitometry assessment was performed using a UV-transference readable camera and Alpha Innotech software (Protein Simple, Santa Clara, California).

PCR Optimization Details

Cytokine	Primers	Band size	Mg (mM)	Annealing Temperature (ºC)
IL-6	5`- TGT GCA ATG GCA ATT CTG AT -3` 5`- GGA AAT TGG GGT AGG AAG GA -3`	305bp	1	54
IL-17	5`-GCT CCA GAA GGC CCT CAG-3` 5`-CTT TCC CTC CGC ATT GAC A-3`	140bp	1	56
IL-22	5`- TTG AGG TGT CCA ACT TCC AGC A-3` 5`- AGC CGG ACG TCT GTG TTG TTA-3`	97bp	1	58
IL-23 p19	5`-AGC GGG ACA TAT GAA TCT ACT AAG AGA-3` 5`-CTC CTA GTA GGG AGG TGT GAA GTT G-3`	254bp	2.5	57.8
18S	5`-CTA CTA CCG ATTGGA TGG TTT AGT G-3` 5`-TAG ATA GTC AAG TTC GAC CGT CTT C-3`	140bp	1	53

Blood lymphocyte cell preparation

Blood from saphenous vein puncture was collected in a heparanized tube and subsequently centrifuged over a sucrose cushion (Isolymph, Gallard-Schlesinger Industries Inc., Planview, NY) at 1250 RPM for 5 minutes. The interface cells (buffy coat) were collected, washed, and resuspended in RMPI (Invitrogen).

Lymph Nodes and Spleen lymphocyte preparation

Lymph nodes or spleens were homogenized using scissors and expression through a metal sieve until a homogenous suspension was achieved. Macrophages and dead cells were removed with a Pasteur pipette or nylon mesh. When necessary, red blood cells were lysed in RBC lysis buffer (Biolegend, CA). The suspended cells were then washed and resuspended in RMPI.

Flow cytometry

Single cell lymphocyte preparations were counted and resuspended in Iscoves media (Invitrogen). 10^5-10^6cells/well were seeded in round bottom 96 well plates then stained in FACS buffer which consisted of PBS containing 2% BSA (Bovine serum albumin). Staining with relevant monoclonal antibodies (mAb) and fluorochrome labeled strepavidin (SA) took place for 20 min at 4ºC followed by a 20 min fixation step in paraformaldehyde (1%). For intercellular flow cytometry staining, additional steps were taken following surface antigen labeling as directed by the FoxP3 intercellular staining kit / protocol (eBioscience, San Diego, CA). In general, fluorochrome dilutions were as follows: Fitc labeled mAb/SA (1:100); PE labeled mAb/SA (1:300); PerCP/PE-Cy5/PE-Cy7 labeled mAb/SA (1:200); APC labeled mAb/SA (1:200); and Biotinylated mAb (1:200). See the below table for mAb used in the study. Flow cytometric data was collected within the Department of Immunology on both the FACS CantoII and the Facscalibur flow cytometers (BD Biosciences, Mississauga, ON). Data was collected with FACSDiva or CellQuest Pro software (BD Biosciences) and analyzed with FloJo (Tree Star Inc., Ashland, OR) software.

Antigen	Clone	Vender
CD3	145-2C11	Abcam (Cambridge, MA)
CD4	GK1.5	eBioscience
FoxP3	FJK-16S	eBioscience
Lag3	eBio C9B7W	eBioscience
GITR	DTA-1	eBioscience
CD39	24DMS1	eBioscience
CD73	eBioTy/11.8	eBioscience
CD69	H12F3	eBioscience
CD25	PC61.5	eBioscience

Data analysis

Statistical analysis was done comparing the means of study groups using a one-tailed Student's T-test with the help of GraphPrism software (GraphPad Software Inc., La Jolla, CA).

Results and Discussion

Serum from variable diseased D2C animals (Fig. 1a) and non-transgenic wild-type control mice was assayed for the presence of T_H17 axis cytokines by ELISA (Fig 1B-1E). As previously described (Oble, 2006), D2C disease is arbitrarily divided into 4 stages corresponding to the extent of rostral skin changes: S0 (no apparent disease which could be representative of either pre-diseased or convalescent animals); S1 (minimal periocular erythema / swelling); S2 (extensive periocular disease +/- lid fusion); S3 (extensive periocular disease with spread to contiguous tissues).

The ELISA data showed significantly higher levels of all T_H17 axis cytokines in the serum of severely affected D2C mice (stages S2-S3) relative to non-transgenic wild-type control animals which was found to be statistically significant using Students T-test ($p < 0.05$). For all of the cytokines, there was a vague association between disease activity and serum concentration of these factors as convalescent S0 animals possessed values close to wild-type control mice. Given this association, lumping all D2C mice together, regardless of disease stage, did not result in statistically significant differences relative to control animals.

IL-6 works with IL-23 to polarize naive $CD4^+$ cells to the T_H17 lineage (Streek et al., 2008), whereas IL-17 and IL-22 are downstream cytokines in the T_H17 inflammatory cascade that are believed to be responsible for the thickened skin phenotype (Martin et al., 2012) (Ma et al., 2008). While a biphasic pattern of cytokine expression was expected, with peak levels of IL-6 and IL-23 (T_H17 axis initiator cytokines) present in earlier disease stages, followed by peak levels of IL-22 and IL-17 (T_H17 axis effector cytokines) occurring at later stages of disease, this pattern was not clearly observed and considerable variability was observed between disease stages. In fact, higher levels of IL-6 were expressed in advanced staged disease relative to peak levels of IL-23 observed in early disease. A similar discrepancy was seen between IL-17 and IL-22 with IL-17 levels peaking in early disease while IL-22 peak concentrations being seen in S2-S3 D2C mice. These observations are not completely unexpected given the known difficulties in detecting T_H17 axis cytokines in the circulation (personal communication from Dr. Jude Ozonna). However, future studies will involve the serial collection of serum from the same

animals (collecting serum at each stage of disease) to reduce the variability of this approach as each data point in the current analysis reflects a different animal. Nevertheless, to exclude this variability and the effects of metabolism of these factors etc. in the circulation, it was next sought to determine whether there were similar elevations of T_H17 axis cytokines in the lesional tissue of D2C mice. An RT-PCR approach (performed in collaboration with Oble lab M.Sc. student Mariam Alkhawaja) to detect message levels of T_H17 axis cytokines was utilized (Fig. 2-3) after first normalizing cDNA samples with the rRNA housekeeping gene 18s (Fig. 3A). An increased production of T_H17 axis cytokines within D2C lesional skin was highly suggested for all of the factors examined. Message levels for all of these factors were undetectable in control skin or in the skin from Scurfy mice, which is in keeping with the absence of T_H17 cells in these animals (in addition to the absence of T_{reg}). Although the difference between diseased D2C mice and control mouse skin was only statistically significant for IL-23 (Fig. 2A) and IL-17 (Fig. 2B) it is anticipated the differences between diseased D2C mice and control mouse skin will be significant for the other factors when a more precise real-time PCR approach is used, as the semiquantitative band densitometry method was insensitive to faint bands generated from D2C skin despite a complete absence of amplicon from the control animals. Nevertheless, relative to the serum ELISA results, the RT-PCR data demonstrated a better correlation with disease activity and conformed better to the hypothesized biphasic relationship between initiator and effector T_H17 axis cytokines. For both IL-6 (Fig. 3B) and IL-23 (Fig 2A), particularly with IL-23, there was a definite tendency of early stage disease (S0/S1) to have a greater amount of message relative to late disease stages (S2/S3) whereas the converse was true for the T_H17 effector cytokines IL-17 (Fig 2B) and IL-22 (Fig. 3C). Although a definite trend in this correlation between disease stage and cytokine concentration can be appreciated from this RT-PCR data, additional animals will be studied to better solidify this relationship. Furthermore message levels will be correlated with protein levels using a semiquantitative immunohistochemical approach to confirm that the increased T_H17 axis cytokine message level translates into a greater production of T_H17 axis cytokines in D2C lesional skin.

Previously, the adoptive transfer of T_{reg} was shown to be sufficient to abrogate disease development in pre-diseased D2C mice, indicating a role for T_{reg} lymphopenia in the mechanism of disease pathogenesis. This data is entirely consistent with the apparent role of T_H17 cells in inducing D2C disease as T_{reg} and T_H17 have a known antagonistic relationship, with a deficiency of regulatory cells likely resulting in an imbalance towards the T_H17 axis. However, it has recently been shown that T_{reg} are not a homogenous population, but rather represent a heterogeneous group of cells possessing a variety of regulatory properties and thus it was attempted to better clarify which T_{reg} subsets were present within D2C mice and how the process of transgenesis affected their ontogeny. As seen in Fig 4a, D2C mice possess $FoxP3^+$ cells belonging both to the classically described, self-Ag specific, $CD4^+CD25^+FoxP3^+$ natural T_{reg} (nT_{reg}) that are produced during normal thymic development as well as $CD4^+CD25^-FoxP3^+$ induced T_{reg} (iT_{reg}) that are peripherally derived after expansion by their exogenous Ag. Given the overlapping immunophenotype of different T_{reg} subsets and the fact that some T_{reg} are $CD25^-$, while some activated $T_{effector}$ express intermediate levels of CD25 and can purportedly upregulate FoxP3, additional markers were explored to better isolate different T-cell subsets. The overlapping histograms in Fig. 4B showing expression of various markers on $CD4^+CD25^{int\text{-}hi}$ T_{reg} and $CD4^+CD25^{lo}$ $T_{effector}$ demonstrate that only GITR can successfully discriminate between these populations in actively diseased D2C mice. Although CD39 and CD73 are preferentially

expressed by T_{reg}, as can be appreciated in the histograms from the non-D2C wild-type control mice, this differential expression is lost in actively diseased D2C mice mostly likely secondary to the activation of $T_{effector}$ in D2C mice, as both CD39 and CD73 are known to be "activation" markers. This activation of $T_{effector}$ in the D2C model system, which accompanies the disorganized CD4 expansion, can be appreciated by the CD4 vs. CD69 dot plots in Fig 4B. While only 10.5% of $CD4^+$ live lymphocytes express the acute activation marker CD69 in wild-type control mice, 56% of $CD4^+$ live lymphocytes are $CD69^+$ in the representative dot plot from a S2 D2C mouse.

Therefore a $FoxP3^+$ $GITR^+$ live lymphocyte gating strategy was used to isolate T_{reg} from $T_{effector}$ in D2C mice followed by the examination of T_{reg} for expression of CD25 to discriminate between nT_{reg} and iT_{reg}. As seen from the average values of these subsets (derived from a few animals in a pilot experiment), pre-diseased D2C mice have a marked deficiency of both nT_{reg} (0.08% of the wild-type control value) and iT_{reg} (0.9% of the wild-type control value). This more selective deficiency of nT_{reg} in pre-diseased D2C mice is better illustrated in the CD25 histograms of Fig. 4C (gated upon $FoxP3^+$, $GITR^+$ live lymphocytes), where the S0 D2C mice are seen to possess a clear population of $CD25^-$ iT_{reg}, but only a rudimentary population of $CD25^+$ $nT_{reg.}$ Once D2C animals develop significant cutaneous pathology, there is an associated expansion of both T_{reg} subsets as appreciated by the numbers in Fig. 4a (23% of the wild-type control value for nT_{reg} and 31.5% of the wild-type control value for iT_{reg}) and the CD25 histogram of Fig. 4C for the S2 D2C mouse. This expansion (288-fold for nT_{reg} and 35-fold for iT_{reg}) ultimately leads to disease convalescence and it is tempting to speculate that the preferential expansion of nT_{reg} is critical for this establishment of disease remission as the transgenic 2C TCR is directed against a self-Ag. Furthermore, the previous T_{reg} purification strategy from syngeneic DBA/2 mice, employed for the adoptive transfer of T_{reg} to pre-diseased D2C animals, involved a positive selection approach that utilized a magnetic bead-labeled αCD25 mAb that preferentially captured the nT_{reg} population (Oble, 2006). Future work will involve the adoptive transfer of unfractionated T_{reg} as well as nT_{reg} and iT_{reg} alone to pre-diseased D2C animals to ascertain whether either subset is more paramount in the prevention of psoriasiform skin changes in the D2C model system.

Another T_{reg} subset which has a purported role in the maintenance of B-cell homeostasis, and which is predicted to be similarly deficient in D2C mice, given the polyclonal hypergammaglobulinemia and associated autoantibody formation in the model system (Oble, 2006), is the $CD4^+CD25^-FoxP3^-Lag3^+$ T_{reg} subset. Due to technical difficulties, these $Lag3^+$ T_{reg} were not identified (even in control mice) probably due to the attempt to stain this extracellular antigen via a surface staining approach, since it is know to be quickly cleaved by the ADAM10 and ADAM17 metalloproteases. Future studies will utilize an intercellular staining protocol to quantitate this antigen in D2C mice from various disease stages, and the adoptive transfer of these $Lag3^+$ T_{reg} will be attempted to try to decouple the T and B-cell autoimmunity present in D2C mice.

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Figure 1

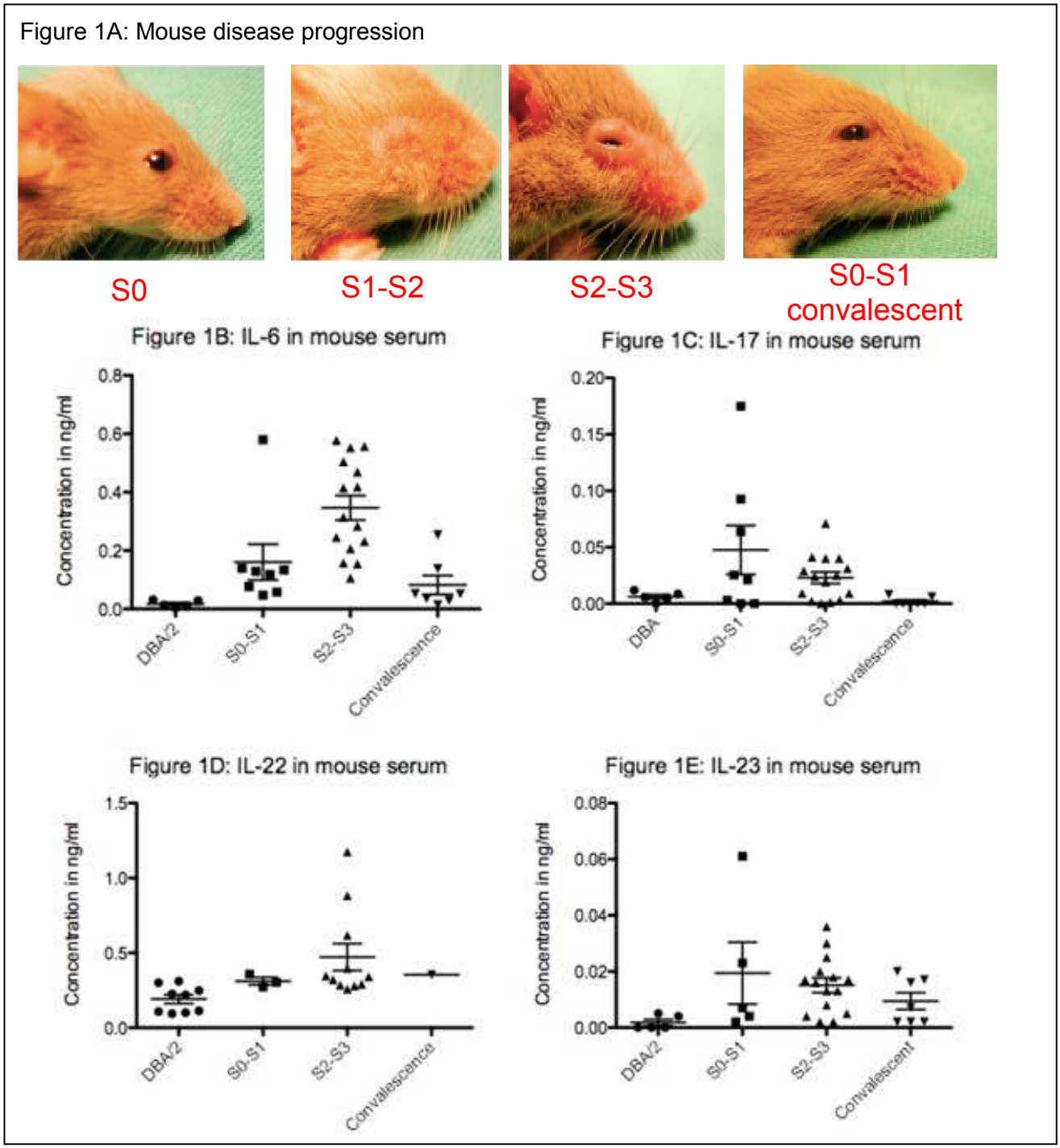

Figure 1: A. Representative photos of mice during disease course. Typical progression from pre-disease, S0-S1, S2-S3, and finally into convalescence. Note perioccular fusion and swelling. B-E: Serum cytokine levels measured via ELISA in DBA/2 wild-type control animals, D2C early disease (S0-S1), D2C late disease (S2-S3), and convalescent D2C animals.

Figure 2: IL-23 / IL-17 Message in Rostral Skin

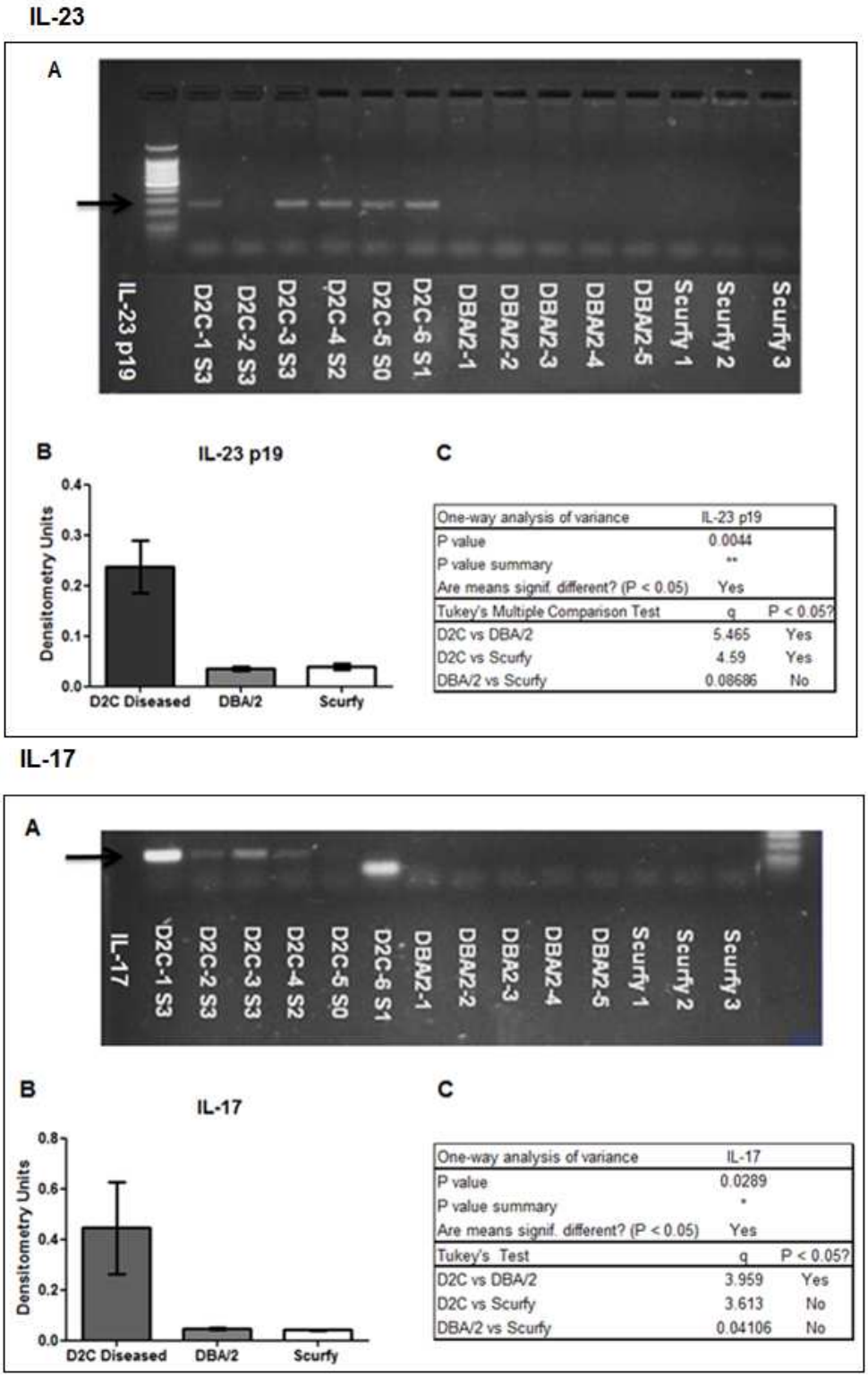

Figure 2: Agarose gels and quantification of cytokine message by band densiometry showing a statistically significant difference between diseased D2C mice and wild-type controls for both IL-23 and IL-17.Scurfy mice data is also included. The IL-23 p19 subunit is a 254bp band while IL-17 is a 140bp band. Arrows indicate specific bands.

Figure 3

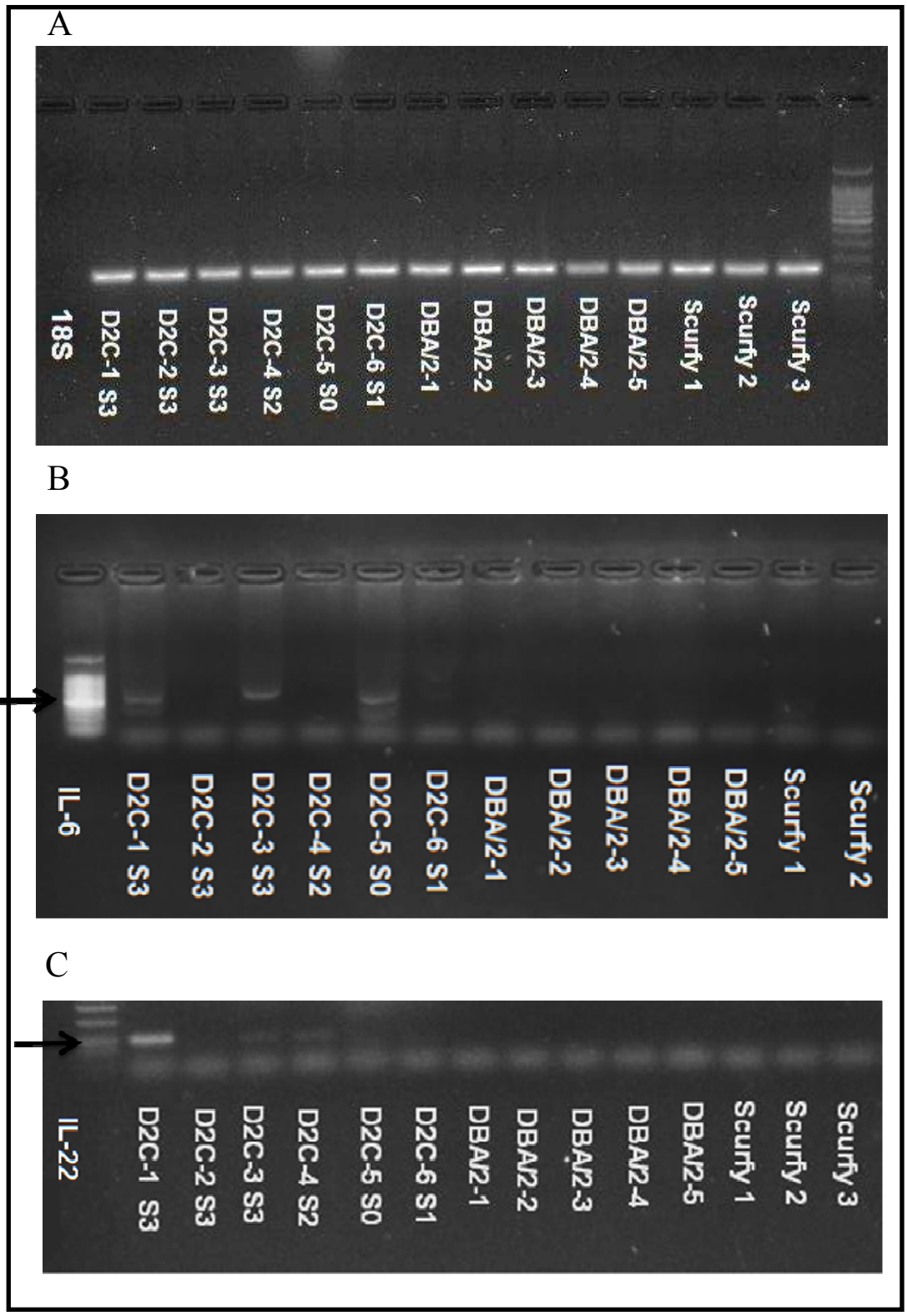

Figure 3: Agarose gels demonstrating expression of A: the 18S rRNA housekeeping gene which has been normalized accross samples, B: IL-6 is a 305 bp band, C: IL-22 is a 97 bp band. Arrows indicate specific bands.

Figure 4

4A

Mouse	Diseased D2C	Pre-Diseased D2C	Non-Transgenic Wild-Type
Lymphocytes	$6.89x10^6$	$7.72x10^5$	$1.13x10^7$
FoxP3 (%)	2.00%	0.32%	4.17%
FoxP3+	$1.29x10^5$	$2.48x10^3$	$4.71x10^5$
CD25+ (%)	57%	11%	69%
CD25+	$7.68x10^4$	$2.83x10^2$	$3.25x10^5$
CD25- (%)	27%	42%	24%
CD25-	$3.53x10^4$	$1.05x10^3$	$1.12x10^5$

Figure 4: 4A. Enumeration of lymphocytes and T_{reg} populations in actively diseased D2C mice, Pre-diseasesd D2C mice, and non-transgenic wild-type controls (data represents averages of similarly affected animals). B. Histograms illustrating expression of CD39, CD73, and GITR on $CD4^+CD25^+$ T_{reg} and $CD4^+CD25^-$ $T_{effector}$ from the indicated animals. CD4 vs. CD69 dot plots are gated upon live lymphocytes. C. Enumeration of nT_{reg} and iT_{reg} by examining CD25 expression on live $FoxP3^+GITR^+$ cells