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Temporal induction of immunoregulatory processes coincides with age-dependent resistance to viral-induced Type 1 Diabetes

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Abstract

The dilute plasma cytokine milieu associated with Type 1 diabetes (T1D), while difficult to measure directly, is sufficient to drive transcription in a bioassay that uses healthy leukocytes as reporters. Previously, we reported disease-associated, partially IL-1 dependent, transcriptional signatures in both T1D patients and the BioBreeding (BB) rat model. Here we examine temporal signatures in congenic BBDR.*lyp/lyp* rats that develop spontaneous T1D, and BBDR rats where T1D progresses only after immunological perturbation in young animals. After weaning, the BBDR temporal signature showed early coincident induction of transcription related to innate inflammation as well as IL-10- and TGF-β-mediated regulation. BBDR plasma cytokine levels mirrored the signatures showing early inflammation, followed by induction of a regulated state that correlated with failure of virus to induce T1D in older rats. In contrast, the BBDR.*lyp/lyp* temporal signature exhibited asynchronous dynamics, with delayed induction of inflammatory transcription and later, weaker induction of regulatory transcription, consistent with their deficiency in regulatory T cells. Through longitudinal analyses of plasma induced signatures in BB rats and a human T1D progressor, we have identified changes in immunoregulatory processes that attenuate a preexisting innate inflammatory state in BBDR rats, suggesting a mechanism underlying the decline in T1D susceptibility with age.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Keywords

Type 1 Diabetes; Inflammation; Gene Expression; Cytokine; Immune Regulation; Virus-induced diabetes

INTRODUCTION

Type 1 diabetes (T1D) is a T-cell mediated autoimmune disease that selectively targets pancreatic β-cells and results in life-long dependence on exogenous insulin for survival. T1D is thought to arise through the interaction of both genetic and environmental factors, with progressive loss of β-cells occurring over months to years in the presence of circulating islet autoantibodies (Ab); clinical diabetes occurs after ~80% of insulin secretory capacity is lost.

Several BioBreeding (BB) rat strains model human T1D with considerable fidelity[1]. Disease in these animals is characterized by insulitis, hyperglycemia, and selective β-cell destruction. Each expresses a high risk major histocompatibility complex (MHC) class II haplotype ($RT1B/D^u$)[2]. About 90% of BBDP rats develop T1D spontaneously but are lymphopenic. BBDR rats, derived from BBDP forbears, have a normal T cell phenotype, are never spontaneously diabetic, but can become diabetic after various immunological perturbations[1].

BBDR.*lyp/lyp* rats were developed by crossing the BBDP and BBDR strains, and 100% of rats of both sexes develop T1D spontaneously, typically around 9 weeks of age. BBDR.*lyp/lyp* rats, like BBDP, are lymphopenic (<15% normal T cell count) due to a mutation in *Gimap5*[3], a gene that encodes a GTP-binding protein necessary for post-thymic T cell survival[4, 5]. Polymorphism in this gene has been associated with human systemic lupus erythematosus as well as auto-Ab development in human T1D[6, 7]. The spontaneously diabetic phenotype of the BBDR.*lyp/lyp* rat is attributed to a deficiency in $CD4^+CD25^+$ ($Foxp3^+$) regulatory T cells (T_{reg}), as adoptive transfer of this population early in life prevents disease[8, 9].

Evidence suggests that T1D initiating events occur at the β-cell level. There, perturbants, possibly infection or oxidative stress, promote apoptosis and/or cytokine production, which activates/recruits tissue-resident antigen (Ag) presenting cells to the islet, and promote development of an adaptive immune response[10]. Consistent with this model, we have reported that by 40 days of age, β-cells of all BB sub-strains express the chemokine eotaxin, and their islets exhibit pro-inflammatory expression profiles[11–13]. This occurs before development of insulitis in BBDR.*lyp/lyp* rats, thereby providing a mechanism for recruitment of immune cells to the islet. Insulitis appears in BBDR.*lyp/lyp* rats after approximately 45 days of age[14] and the successful rescue of T1D requires adoptive transfer of T_{reg} prior to this time[8, 9]. T1D in BBDR.*lyp/lyp* rats is characterized by extensive β-cell destruction and infiltration of islets by T cells and other immunocytes[15, 16]. It has been proposed that all BB rats possess an underlying predisposition for T1D that progresses to overt diabetes with loss of immune regulation[11, 12] -hence the success of T_{reg} transfusion in prevention. WF rats share the high risk $RT1^u$ MHC haplotype but do not develop

spontaneous T1D. Their islets do not express eotaxin[11, 12] and T1D induction protocols only rarely induce disease.

A need exists for biomarkers of T1D that can sensitively and specifically detect disease-related immune activity prior to, and independent of, detection of auto-Abs towards islet cell antigens (Ag). Such markers should also report changes in the inflammatory state associated with disease progression or with response to therapeutic intervention. To address this need, we have applied a novel bioassay to both human and BB rat T1D, whereby the complex milieu of inflammatory mediators present in plasma can be indirectly detected through their ability to drive transcription in peripheral blood mononuclear cells (PBMC) drawn from healthy, unrelated donors[17–20]. Expressed genes are comprehensively measured with a microarray. We find that plasma of recent-onset T1D patients, compared to unrelated healthy controls, induces expression of a pro-inflammatory signature that consists, in part, of many IL-1 regulated genes related to immunological activation and immunocyte chemotaxis[17, 20]. This signature resolves in patients with longstanding (>10 years) T1D, thus associating it with active autoimmunity. Importantly, this disease-specific signature has been detected in plasma samples from persons at risk for T1D years before onset and prior to development of auto-Abs[17, 20].

Applying this approach to the BB rat, we have reported that plasma samples collected from both BBDR and BBDR.*lyp/lyp* animals at 60 days of age induce transcription of genes encoding cytokines, immune receptors, and signaling molecules consistent with shared susceptibility and immune activation[18]. The BBDR.*lyp/lyp* signature differed from that of the BBDR in that there was more robust induction of many IL-1–regulated genes. In addition, we observed in 60 day old BBDR rats the induction of a signature characterized by negative regulators of transcription and inflammation, consistent with the activity of T_{reg} cells. Building on these observations, we then showed that treatment of BBDR.*lyp/lyp* rats with IL-1 receptor antagonist (IL-1RA) delayed onset and, in part, normalized the signature[18].

As is the case for human T1D, the natural history of diabetogenesis in the BB rat remains incompletely defined. To address this, we report here the results of longitudinal studies of pathogenesis based on our assay. We examined the plasma-induced transcriptional signatures of BBDR.*lyp/lyp* and BBDR rats at four ages: 30 days (prior to islet eotaxin expression), 40 days (prior to insulitis in BBDR.*lyp/lyp* rats), 50 days (early insulitis in BBDR.*lyp/lyp* rats) and 60 days (prior to BBDR.*lyp/lyp* T1D onset). We observed distinct dynamics in the inflammatory and regulatory responses of the two BB sub-strains and characterized the ligands and T_{reg} populations associated with the temporal signatures. Importantly, we identified an age-dependent immunoregulatory process that supplants underlying innate inflammation in BBDR rats and correlates with the inability of Kilham's rat virus (KRV) to induce T1D in older BBDR rats.

RESULTS

Longitudinal analysis of the peripheral inflammatory milieu in BB rats

Plasma samples from separate cohorts of BBDR and BBDR.*lyp/lyp* rats were collected when the animals were 30, 40, 50 and 60 days old. This age range encompasses the interval between weaning until just prior to T1D onset in BBDR.*lyp/lyp* rats. The samples were co-cultured with PBMCs of healthy Brown Norway (BN) rats to induce transcription, which in turn was measured by microarray analysis. All rats were normoglycemic at the time of study, ensuring that induced gene expression was not a consequence of hyperglycemia. Control co-cultures were prepared using both autologous and allogeneic BN plasma (Figure 1A).

The BBDR.*lyp/lyp* and BBDR longitudinal data sets were analyzed with Short Time Series Expression Miner (S.T.E.M.), a software program that utilizes a non-parametric clustering algorithm for biological interpretation and comparison of temporal expression profiles[21]. S.T.E.M analysis of both the BBDR.*lyp/lyp* and BBDR data sets identified two significantly regulated profiles: Profile 7 (transcripts exhibiting increased temporal induction) and Profile 0 (transcripts exhibiting decreased temporal induction) (Figure 1B). When combined, profiles 7 and 0 of the BBDR.*lyp/lyp* signature possessed 513 regulated probe sets, whereas profiles 7 and 0 of the BBDR signature possessed contained 418 regulated probe sets. Consistent with the T1D susceptibility of both sub-strains, and the day 60 signatures reported previously[18], the longitudinal BBDR.*lyp/lyp* or BBDR signatures shared a significantly nonrandom ($p<10^{-6}$, $\chi 2$ test) intersection of 109 probe sets which represented 21.2% and 26.1% of the probe sets regulated by either plasma, respectively. One-way hierarchical clustering (probe sets only, Figure 1C) illustrates the overall expression levels of transcripts captured by the S.T.E.M. analysis across the experimental conditions. The BBDR.*lyp/lyp* and BBDR temporal signatures possessed a combined total of 822 regulated probe sets, a significant percentage of which ($259/822 = 31.5\%$, $p<10^{-6}$, $\chi 2$ test) were previously identified in comparisons of plasma induced signatures of day 60 BBDR.*lyp/lyp* and BBDR rats[18] (Figure 1D).

Selected regulated pathways identified by S.T.E.M. are presented in Table 1 and the regulated transcripts associated with these annotations are provided in Supplemental Table 1. Plasma from both BB sub strains induced immunological activation in the BN reporter PBMC as shown by identification of common Gene Ontology (GO) terms. These included positive regulation of immune response, positive regulation of cytokine production, and response to molecule of bacterial origin. A greater pro-inflammatory bias was present in the BBDR.*lyp/lyp* data set, documented by significant up-regulation of processes such as neutrophil chemotaxis, regulation of MAP kinase activity, and cytokine receptor binding. Conversely, the BBDR data set showed evidence of greater immunoregulatory activity as documented by significant up-regulation of processes that included regulation of leukocyte migration, regulation of inflammatory response, regulation of immune effector process, and negative regulation of cell adhesion.

Induced Genes Comprising Profiles 7 and 0

A total of 341 temporally up-regulated probe sets were assigned to profile 7, with the BBDR.*lyp/lyp* and BBDR signatures contributing 200 and 228 probe sets respectively (Figure 2A, Supplemental Table 2). Among the significantly regulated transcripts restricted to the BBDR.*lyp/lyp* profile 7 were the pro-inflammatory mediators *Cxcl2, Cxcl1*, and *Il1b*, which were increased at day 50 and peaked at day 60. Maximal induction of these transcripts by BBDR plasma occurred at age 40 days, when eotaxin expression is detectable in β-cells of both BB sub strains and before there is insulitis in BBDR.*lyp/lyp* rats[12]. Other genes related to proinflammatory activity exhibited similar temporal profiles, increasing over time in the BBDR.*lyp/lyp*. This contrasted with an early maximal response followed by attenuation in the BBDR time course study. These other gene transcripts included the immune receptor *Cd86; Sdc4* (syndecan-4), a transmembrane heparan sulfate proteoglycan that functions cooperatively with integrins as a receptor in intracellular signaling; the cytokine mediator *Hmgb1* (high-mobility group protein B1); as well as *Marcks* (alanine-rich C-kinase substrate) a protein necessary for neutrophil migration and adhesion[22]. Among the probe sets differentially up-regulated over time by BBDR.*lyp/lyp* plasma, some exhibited regulation opposite to that of BBDR plasma. This included *Srcap* (SNF2-related CBP-activating protein), a potentiator of Notch-dependent transcription[23] which has emerged as an important determinant in T cell differentiation[24] (Figure 2C).

The BBDR.*lyp/lyp* profile 7 and BBDR profile 7 share a significantly nonrandom intersection ($p<10^{-6}$, $\chi 2$ test; 87 probe sets that represent 43.5% and 38.2% of the profile regulated by either plasma, respectively, Figure2A and 2C). This intersection is indicative of underlying inflammatory processes present in both BB sub strains and included many transcripts associated with immunological activation. These included *Ccl2* (monocyte chemotactic protein-1); the T cell and NK cell recruiting chemokine *Cxcl16*; and *Tnfsf13* (TNF ligand superfamily member 13); immune receptors, including *Cd40, Csf1r* (colony stimulating factor 1 receptor), *Fcgr1a* (high affinity immunoglobulin gamma Fc receptor I), and the pattern recognition receptors *Cd14* and *Tlr7* (Figure2A and 2C).

As reflected by the significantly enriched GO terms (Table 1), many of the 228 temporally up- regulated probe sets belonging to BBDR profile 7 are known to be important in tempering immune responses and maintaining immune quiescence (Figure2A and 2C). They also show evidence of being regulated by IL-10 and/or TGF-β. Among these was *Klf4* (Kruppel-like factor 4) a zinc finger-containing transcription factor that regulates IL-10 expression[25]; *Hopx* (homeodomain-only protein), a transcription cofactor that is required by induced T_{reg} for mediating T cell unresponsiveness *in vivo*[26]; *Fcgr2b*, which encodes the inhibitory Fcγ receptor IIB[27]; and *Nr1h3*, that encodes the nuclear receptor LXR-α, signaling through which is important in regulating inflammation as LXR agonists have been reported to reduce LPS-induced IL-12 secretion, increase IL-10 secretion, and alter T cell activation[28].

A total of 485 probe sets were assigned to profile 0, with the BBDR.*lyp/lyp* and BBDR signatures contributing 313 and 190 probe sets, respectively (Figure 2B, Supplemental Table 2). The profile 0 signatures of the two BB sub-strains also share a significantly nonrandom

($p<10^{-6}$, $\chi 2$ test) intersection of 18 probe sets that represents 3.2% and 6.2% of the probe sets regulated by either plasma, respectively. Profile 0 of the BBDR.*lyp/lyp* rat revealed modest evidence of regulation as induction of certain transcripts related to positive regulation of inflammatory processes were less robust in samples collected closer to T1D onset. This included decreased temporal induction of *Il2*; the activation marker *Cd38*; *Sell*, the adhesion receptor L-selectin; the inflammatory regulator *Plcg2* (phospholipase C, gamma 2); the cell cycle regulator *Ccnd3* (cyclin D3), and *Unc13d* which is involved in the exocytosis of cytolytic granules from T cells and NK cells.

The biological activity associated with profile 0 of the BBDR signature mirrored the crescendo in immunoregulation detected in BBDR profile 7, showing down-regulation of genes important in mediating immune responses and cell adhesion. These included *Nfkb2*, the p100 precursor that is cleaved to produce the NF*k*B p52 protein; the receptors *Il4ra* and *Il1r2;* the cytokine *Lta* (lymphotoxin alpha); *Fosl2* (Fos-related Ag 2), a transcription factor of the activator protein-1 family important in the expression of cytokines; and *Nr4a3* (nuclear receptor subfamily 4, group A, member 3) a transcriptional mediator of inflammatory signals that is up regulated in macrophages upon LPS stimulation[29] (Figure 2D).

Overall the longitudinal signature analysis revealed evidence of immune activation and regulation in both BB sub strains; however the dynamics and magnitudes were very different. In the BBDR signature, induction of immunoregulatory gene expression, consistent with the presence of IL-10 and TGF-β, appears by day 40 and crescendos through day 60. This contrasts with the BBDR.*lyp/lyp* signature, which shows delayed and imbalanced induction of regulatory and proinflammatory gene expression. Specifically, robust proinflammatory gene expression is induced by day 50, with weak induction of immunoregulatory gene expression by day 60, consistent with their deficiency in T_{reg} cells. The plasma induced signature of BN rats exhibited neither of these activities (Figures 1 and 2).

Direct detection of inflammatory mediators

Cytokine levels in BBDR.*lyp/lyp*, BBDR, and BN plasma were measured by ELISA (Table 2, Figure 3). Consistent with its lymphopenia, levels of most mediators in plasma of the BBDR.*lyp/lyp* rat did not exceed detection thresholds at the time points analyzed. IL-13, which possesses both pro- and anti-inflammatory functions[30], was detected at increasing levels during the pre-onset period in BBDR.*lyp/lyp* plasma, consistent with previously reported eosinophilia and elevated IgE levels[12]. This result contrasted with the BBDR rat plasma in which more measurable mediators were detected. The pro-inflammatory cytokines (IL-2, IL-4, IL-18, CCL2, CXCL1) detected in the BBDR rat plasma mirrored the temporal transcriptional signature, showing higher levels indicative of immunological activation between ages 30 and 40 days followed by largely undetectable levels at older ages, consistent with induction of an immuno-regulated state.

qRT-PCR studies of the immunoregulatory state in BBDR rats

Previously, through the supplementation of co-cultures with IL-1RA, we demonstrated partial dependence of the BBDR.*lyp/lyp* signature on IL-1[18]. In support of the pathway analyses that suggested that the day 50 and day 60 BBDR signatures were in part dependent on increasing levels of IL-10 and/or TGF-β, day 30 BBDR plasma co-cultures were supplemented with one or both of these ligands and analyzed by qRT-PCR. Analysis of 4 induced transcripts related to immunoregulatory activity detected in the array studies (Figure 2C), and known to be regulated by IL-10 and/or TGF-β, showed significantly increased expression upon addition of the candidate ligands (Figure 4). These included *Mafb*, a transcription factor that antagonizes antiviral responses by blocking recruitment of co-activators to the transcription factor IRF3[31]; *Tgfbi* (TGF-β-induced), a secreted extracellular matrix adaptor protein that modulates cell adhesion[32] and is involved in T_{reg} induction[33, 34]; *Nqo1* (NAD(P)H:quinone oxidoreductase 1) which modulates inflammatory responsiveness of human monocytes[35]; and *Lrp1* (LDL receptor-related protein-1) a suppressor of inflammation capable of reducing expression of CCL2, TNF-α, and MMP-9 in macrophages, and down-regulating cell signaling through the IKK-NF-κB pathway[36].

Viral induction of T1D in BBDR rats

It is known that susceptibility to KRV-induced T1D in LEW.1WR1 rats declines with age[37, 38]. To determine if the changes in immunoregulatory plasma induced signature and the decline in plasma cytokine levels we observed in BBDR rats have a ‘clinical’ correlate, we studied KRV-induced diabetes in this strain. As shown in Table 3, using either virus alone or an optimized protocol using a priming dose of polyinosinic:polycytidylic acid (poly I:C) before infection, there was a clear decrease in the penetrance of diabetes with age ($p<0.001$). Forty percent of <30 day-old rats treated with KRV alone developed T1D, compared to 0% of >40 day-old rats. Among the rats infected after a priming dose of poly I:C, 100% of the <30 day-old rats developed T1D, compared to 0% of the >40 day-old rats. These data show that the age-dependent decline in T1D susceptibility following immunological perturbation in BBDR rats correlates with age-dependent acquisition of an immunoregulated state defined by emergence of the immunoregulatory plasma-induced signature and the decline in measurable proinflammatory cytokine levels.

Analysis of T_{reg} populations in BBDR.lyp/lyp and BBDR rats

The results described above indicate that immunoregulatory activity, possibly arising through the activation of T_{reg}, is induced early in life in BBDR rats. This activity arguably counteracts the underlying inflammatory processes, common to both BB sub-strains, to prevent spontaneous T1D in BBDR rats. In the pancreatic lymph nodes (PLN), we found that at day 25, day 40 and day 60, BBDR and BBDR.*lyp/lyp* rats had comparable numbers of total cells. However, in BBDR.*lyp/lyp* PLN, there was a 2–3 fold reduction in T cells ($CD3^+$) and a corresponding increase in B cell numbers. $CD4^+Foxp3^+$ T_{reg} comprised ~6% of the total cells in BBDR PLN compared to ~3% in BBDR.*lyp/lyp* PLN ($p<0.01$; data not shown). Furthermore, age-dependent, intra-strain changes in the percentages of $CD4^+Foxp3^+$ T_{reg} cells were not observed.

Because Helios expression was reported to differentiate thymic-derived natural (nT_{reg}) from induced regulatory T cells (iT_{reg}) in the periphery[39], we analyzed its expression in $Foxp3^+$ T_{reg} in spleens and PLN of both BB sub-strains. As shown in Figure 5, the expression levels of Helios in splenic and PLN T_{reg} were different in BBDR.*lyp/lyp* and BBDR rats. At all time points, BBDR rats showed higher proportions of T_{reg} expressing intermediate levels of Helios, and lower proportions of T_{reg} expressing negative/low levels of Helios (Figure 5). At age 60 days, the percentages of both $Helios^{intermediate}$ and $Helios^{neg/low}$ T_{reg} were significantly different in BBDR.*lyp/lyp* vs. BBDR rats whereas the frequency of the $Helios^{high}$ population was similar in both strains.

Signature comparison of BBDR, BBDR.lyp/lyp, and human patient plasma

To investigate possible parallels in pathogenesis between BB rat and human T1D, we next compared the temporal signature of the BBDR.*lyp/lyp* rat to that of a prospectively monitored person who developed T1D over several years; this person was the sibling of a T1D patient. This subject, who was also studied in our initial report[17], was diagnosed with T1D at the age of 21.7 years; plasma samples were collected at −5.3 years (auto-Ab negative), −3.3 years (+1 auto-Ab), −2.4 years (+3 auto-Abs), −1.5 years (+3 auto-Abs), −0.3 years (+3 auto-Abs), and +0.3 years (+4 auto-Abs) relative to onset.

As was done for the BB rat analyses, the longitudinal data was analyzed with S.T.E.M. and genes belonging to profiles 7 (temporally up-regulated) and 0 (temporally down-regulated) were identified as previously reported[20]. Among the >30,000 and >50,000 transcripts and variants interrogated by the Affymetrix RG230 2.0 rat and HG-U133 plus 2.0 human GeneChips, respectively, we identified probe sets for 10,903 orthologous genes present on both arrays. S.T.E.M. annotated 353 rat genes (513 total regulated probe sets) represented on the human array and 597 human genes (1,195 total regulated probe sets) present on the rat array. Among the total of 914 orthologues regulated to threshold levels in the two analyses, a significantly nonrandom ($p<0.001$, χ^2 test), intersection of 36 genes was identified.

To explore the relatedness of gene expression differences over time identified in the DR.*lyp/lyp* and human plasma samples, we employed ToppGene[40], a program that prioritizes genes based on their functional similarity. Including genes of the intersection, ToppGene identified significant functional identity ($p<0.05$) among 360 of the 914 orthologous genes (39.1%); specifically 205 genes regulated by BBDR.*lyp/lyp* plasma shared functional identity with the human signature and 191 genes regulated by human plasma shared functional identity with the BBDR.*lyp/lyp* signature (Figure 6A, B). Although the overall correlation is low 0.15 ($p=0.004$), shared patterns of induced gene expression were evident between the signatures, even among genes regulated to thresholds in a single species (Figure 6C, D). Pearson's correlation coefficients were calculated by comparing the last:first time point $\log_2$ ratios for genes exhibiting directional concordance. Among the 360 gene union, 229 (64%) genes exhibited directional concordance and were correlated (Pearson's correlation coefficient = 0.48, $p<10^{-6}$). Notably, 23/36 genes of the intersection exhibited directional concordance and high correlation in their induced gene expression (Pearson's correlation coefficient = 0.88, $p<10^{-6}$); these concordantly induced genes included *Il1b*, *Cxcl1*, *Cxcl2*, *Ier3* and others (Figure 6B).

Progression to T1D likely involves both increases in inflammation and decreases in regulation. We therefore hypothesized that the sequence of induced genes indicative of immunoregulation over time in the BBDR signature may not occur in the human longitudinal signature. We again identified orthologous content between the rat and human arrays for genes belonging to the BBDR profile 7 (n=159 genes) and profile 0 (n=139 genes). As shown in Figure 7, such patterns are evident. Importantly, up-regulation of transcripts associated with immunoregulation in the BBDR rat did not occur; instead we observed opposite temporal regulation during progression to T1D in the analysis of the human subject. Genes that were not up-regulated included *Hopx; Lgals1*, which encodes the immunoregulatory glycan-binding protein galectin-1 that in turn acts as a suppressive agent for T-cell responses[41]; the transcription factor *Mef2c* that promotes activation mediated cell death of macrophages[42]; *Cbl*, a negative regulator of immune receptor signal transduction; the inhibitory immunoreceptor *Pilra*[43] and others (Figure 7B). Conversely, among transcripts reduced over time in the BBDR profile 0, we observed an opposite pattern in the human T1D subject. The genes that did not down-regulate included the transcription elongation factor *Ell2* that directs immunoglobulin secretion in plasma cells[44]; *Carm1* (coactivator-associated arginine methyltransferase 1) that enhances NF-κB mediated transcription[45] and others.

DISCUSSION

The present studies extend our previous report that BBDR.*lyp/lyp* and BBDR plasma collected at 60 days of age can induce transcription in healthy BN PBMCs[18]. Plasma of both BB sub-strains induced a signature indicative of innate immune activation. However, as we reported to occur in human T1D relative to unrelated healthy controls[17, 20], the signature associated with disease progression in BBDR.*lyp/lyp* rats included more robust induction of genes regulated by IL-1 and differential regulation of NF-κB signaling. A key feature distinguishing BBDR plasma was induction of many genes involved in negative regulation of transcription and inflammation, consistent with the presence of T_{reg} cell activity.

The present longitudinal study shows the dynamics and balance of the inflammatory and regulatory responses in the two BB sub-strains to be highly distinct. BBDR rats exhibited concurrent induction of transcription consistent with both pro-inflammatory and immunoregulatory processes by age 40 days; this is the age at which eotaxin expression by pancreatic β-cells is histologically detected in both BB sub-strains[11, 12]. In contrast, longitudinal samples collected from the BBDR.*lyp/lyp* rat, showed very different responses. Compared to day 40 BBDR plasma, only a modest pro-inflammatory signature is induced by day 40 BBDR.*lyp/lyp* plasma. That pro-inflammatory signature becomes increasingly more robust at days 50 and 60. At the latter time points, BBDR.*lyp/lyp*, but not BBDR rats, already show histological evidence of low grade insulitis[14] that subsequently progresses, leads to β-cell destruction, and eventually T1D onset at approximately 60 days of age[11]. Evidence of immunoregulatory activity in the BBDR.*lyp/lyp* signature (e.g. induction of IL-10 dependent transcripts like *Fcgr2b*, *Klf4*, *Lrp1*, and *Hopx*), was delayed, becoming detectable only at 60 days.

Previous studies of plasma cytokine levels in 60 old rats found only IL-13 to be elevated in BBDR.*lyp/lyp* as compared to BBDR rats[18]. The longitudinal studies here reveal a much more complex, dynamic, and informative process. For example, IL-2 levels, important in the activation of CD8+ T cells by CD4+ T cells, were highest at 40 days of age in both BB sub-strains. Plasma levels of IL-10, produced by FoxP3+CD4+ T_{reg} and important for the suppression of auto-reactive T cells, were not significantly different between the strains over time. However, the highest IL-10 level was observed at day 40 in BBDR rats, compared to day 60 in BBDR.*lyp/lyp* rats, indicating asynchronous induction of regulatory components in the two BB sub-strains.

Consistent with the robust inflammatory gene expression induced by day 40 BBDR plasma, generally higher cytokine levels were observed at days 30 and 40 in BBDR rats. This, however, was followed by lower levels at 50 and 60 days of age, which we interpret as induction of an immune quiescent state (Figure 3). The general failure to detect elevated peripheral cytokine levels by ELISA in the BBDR.*lyp/lyp* rat, despite spontaneous disease progression, is most likely due to its lymphopenia.

Compared with the BBDR, BBDR.*lyp/lyp* PLN possessed approximately half the number of $CD4^{+}Foxp3^{+}$ T_{reg}. We also found T_{reg} that express intermediate levels of Helios accumulate in BBDR rats, whereas BBDR.*lyp/lyp* rats preferentially harbored a $Helios^{neg/low}$ population. Recent reports suggest that Helios expression is not restricted to nT_{reg}, but is also observed in stimulated effector T-cells and iT_{reg}[46–48] where expression levels decline in the absence of continuous Ag stimulation[46, 47]. Furthermore, when compared to $Helios^{-}$ T_{reg}, $Helios^{+}$ T_{reg} appear to exhibit increased immunosuppressive activity[49]. While the differing temporal signatures induced by plasma of the two BB sub-strains are associated with differing abundances of T_{reg}, the increased proportion of $Helios^{intermediate}$ T_{reg} in BBDR compared to BBDR.*lyp/lyp* rats suggests that these immunoregulators are more active in the BBDR rat.

BB rats possess T1D susceptibility beyond that conveyed by the MHC (in BBDR and BBDR.*lyp/lyp* rats) and *Gimap5* deficiency (in BBDR.*lyp/lyp* rats). This is demonstrated in the BBDR rat by β-cell eotaxin expression and pro-inflammatory islet expression profiles in the absence of insulitis[12, 13], as well as the ability of induction protocols, such as inoculation with KRV, to trigger T1D in BBDR rats, but not MHC-identical WF rats. A key unanswered question is why both BBDR and BBDR.*lyp/lyp* rats have a basal inflammatory signature at all. One possibility is an intrinsic immunoregulatory defect. Speculatively, another source of inflammation could be intestinal hyper-permeability[50], which could lead to the translocation of bacteria and/or LPS and a heightened systemic innate inflammatory state. Since TLR4 and IL-1R1 signaling are mediated through the adaptor protein MyD88 to activate NF-κB, and either or both could contribute to the intersecting portions of the BBDR.*lyp/lyp* and BBDR signatures, we previously investigated LPS levels and found them significantly higher in BBDR.*lyp/lyp* and BBDR rat serum compared with that of BN rats[18]. Consistent with this overall hypothesis, is the report of Valladares et al., where administration of *Lactobacillus johnsonii* N6.2. to diabetic prone BB rats resulted in higher expression levels of the tight junction protein claudin, potentially ameliorating intestinal barrier dysfunction, and significant delay of T1D onset[51]. A similar underlying pathomechanism, involving

interactions between inherited susceptibility, gut microbiota, and environmental triggers (e.g. viruses) may also exist in the LEW1.WR1 rat, as antibiotic treatment has been determined to protect rats from KRV-induced insulitis and T1D[52]. Inflammatory states influenced by bacterial flora and viral infections may not only promote breaking of tolerance by elevating innate inflammation but may also promote β-cell dysfunction, including endoplasmic reticulum (ER) stress, contributing to their susceptibility as targets for T-cell mediated destruction[13, 53, 54]

The present study reveals the early induction of active homeostatic mechanisms that may reflect attenuation of an underlying inflammatory and disease progression in the BBDR rat. The “fertile field hypothesis” proposes that viral infection alone might not be sufficient to induce T1D in the absence of other inflammatory factors[55]. Consistent with this hypothesis we found that the decline in susceptibility to KRV-induced T1D with age in BBDR rats correlates with induction of a more regulated state. KRV is well known to alter the immunoregulatory environment[56]. KRV infects T and B lymphocytes (but not β-cells), while not causing the T lymphopenia observed in the spontaneously diabetic BBDR.*lyp/lyp* rat. KRV does, however, diminish proliferative and cytolytic responses[57], as well as the number of splenic CD4+CD25+ Tregs[58]. Overall our data suggest that KRV infection disrupts immune regulation, transforming a "regulated predisposition" into disease progression, which are the focus of future studies that are aimed at determining how KRV infection alters temporal dynamics of inflammatory and regulatory processes in KRV-infected BBDR rats.

Regulated inflammatory responses may also underlie T1D progression in humans. Elevated cytokine levels have been reported in auto-Ab negative healthy first degree relatives of T1D patients[59]. Other studies suggest that mechanisms similar to those observed in the BBDR rat may exist in humans to control autoimmune responses towards pancreatic β-cells. For example, Petrich de Marquesini et al.[60] examined islet-Ag-specific T-cell responses and observed that auto-Ab-negative first-degree relatives of T1D patients had a balance of inflammatory T cells and T_{reg} intermediate between that of recent onset T1D patients and unrelated healthy controls. Tree et al.,[61] have isolated naturally occurring IL-10-secreting islet auto-Ag-specific regulatory $CD4^+$ T_{reg} from unrelated healthy controls (with no family history of T1D) that suppress β cell-specific T-cell responses. Finally, the existence of islet auto-Ab-positive family members of T1D patients that never progress to overt diabetes further supports the existence of active mechanisms that control progression of autoimmunity.

How faithfully animals model human T1D is an important ongoing subject of debate. The present study bears on this question, but with limitations. We demonstrate parallels and divergences in pro-inflammatory activities related to T1D susceptibility and progression in the BBDR.*lyp/lyp*, BBDR, and human longitudinal signatures. In all three there is a basal pro-inflammatory signature. In both the BBDR and BBDR.*lyp/lyp* there is also evidence of an immunoregulatory signature over time, but in the latter it is “too little, too late.” In the single human case we studied there are increases in inflammation and decreases in regulation with time, which we speculate may be the consequence of measurements made after destabilization of a regulated state by an “environmental trigger”. There are, however,

limitations to these correlational observations. Although many of the correlations were statistically significant, their magnitude was small. In addition, the human subject in this cross-species comparison was diagnosed with T1D fairly late, at 21 years of age, and lacked the usual HLA-DR3 or DR4 high risk haplotype. It remains to be determined if our observations are representative of a general pattern of at-risk humans who eventually develop T1D. It is possible that patterns of regulatory and inflammatory activity may differ between patients diagnosed very early in childhood vs. adolescence or early adult. For all these reasons, the parallels must be considered suggestive and not definitive.

This report further validates the plasma-induced transcription assay as a tool for understanding, and a biomarker of, the pathogenesis of T1D. With it we have confirmed the existence of an underlying innate inflammatory state in two rat strains with genetic susceptibility to T1D. More importantly, we have discovered the emergence of an immune-regulated state that changes with age in BBDR rats, which do not become spontaneously diabetic. This study provides a foundation for future human longitudinal analyses aimed at assessing the inflammatory state associated with diabetes susceptibility and disease progression. Such studies will address whether human T1D arises through a failure to establish a regulatory state as observed in the BBDR.*lyp/lyp* rat or alternatively through the destabilization of a regulated inflammatory state as in the BBDR rat induced to become diabetic by infection.

MATERIALS AND METHODS

Animals

BN and BBDR.*lyp/lyp* rats were bred at the Medical College of Wisconsin (MCW). BBDR rats, which are homozygous for wild-type *Gimap5*, were obtained either from the colony at MCW or from BRM, Inc. (Worcester, MA); the latter were housed at the University of Massachusetts Medical School. BBDR rats are not lymphopenic and do not develop insulitis or spontaneous T1D. They are, however, susceptible to T1D induced by depletion of T_{reg} cells or by infection with KRV[38, 56, 62]. All rats at both institutions were housed under specific pathogen-free conditions with standard light/dark cycles, and were fed a regular chow diet and water *ad libitum*. All federal guidelines (http://grants1.nih.gov/grants/olaw/references/phspol.htm) for use and care of laboratory animals were followed and all protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the respective institutions.

PBMC cultures

Blood was collected from BB rats that were fasted for 16 hours prior to euthanasia and only those whose blood glucose concentration was <130 mg/dl (Bayer Ascensia Elite XL glucometer, Bayer HealthCare, Tarrytown, NY, USA) were used. Rats were anesthetized with isofluorane and blood was collected by heart puncture into an EDTA vacuum tube (Becton-Dickinson, Franklin Lakes, NJ USA). Plasma was separated by centrifugation then stored at −80°C until use.

Fresh PBMCs from healthy male BN rats ~180 days old (to avoid any variability introduced by estrous or pubertal status) were isolated by density gradient centrifugation. As described[18], transcription was induced by culturing PBMCs for 6 h at 37°C in 5% CO_2 with 20% autologous BN (self-baseline control), allogeneic BN (healthy-unrelated control), BBDR.*lyp/lyp*, or BBDR plasma. RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Certain cultures were supplemented with 20 ng/ml TGF-β (R&D Systems, Minneapolis, MN; #240-B) and/or 50 ng/ml IL-10 (R&D Systems; #1064-IL). RNA from each culture was independently analyzed by either array analysis or quantitative RT-PCR (qRT-PCR).

Gene Chip Analysis

Purified RNA (~50 ng) was amplified (Affymetrix two-cycle cDNA synthesis kit, Affymetrix, Santa Clara, CA, USA) and labeled targets were hybridized to the RG230 2.0 array according to manufacturer's instructions. Image data were quantified with Affymetrix Expression Console Software and normalized with Robust Multichip Analysis (www.bioconductor.org) to determine signal log ratios[63]. Time series data were analyzed using S.T.E.M.[21]. With S.T.E.M., genes were assigned to predefined model profiles based upon their temporal expression changes (utilizing these input parameters: absolute difference between the values of any two time points = $|\log_2 \text{ratio}| > 0.5$; maximum number of units a model profile may change between time points = 1). As is an option with the software, the replicate data for each time series were considered from the perspectives of both the mean, as well as the median. The details and relationship between these analyses are provided in Supplemental Tables I and II. Significant differences in induced gene expression between conditions (time points or strain) were further assessed by a non-parametric rank product test[64]; these analyses are provided in Supplemental Table 2. Hierarchical clustering and generation of heat maps was accomplished with Genesis[65].

Orthologous rat and human probe sets were mapped using the Affymetrix support document: HG-U133_Plus_2.na32.ortholog.csv.zip. Functional relatedness of the plasma induced signatures associated with progression of BBDR.*lyp/lyp* rat versus human T1D relied on ToppGene (Transcriptome Ontology Pathway PubMed based prioritization of Genes,[40]), a software that prioritizes genes based on their functional similarity. Gene expression data have been deposited in NCBI's Gene Expression Omnibus[66] and are accessible through GEO SuperSeries GSE40498 (rat:GSE40497; human:GSE40496).

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from cultured PBMCs using TRIzol reagent, and qRT-PCR for *Tgfbi*, *Mafb*, and *Lrp1* was performed[17] and analyzed[67] as described. Each biological replicate (n=7) was analyzed in duplicate and the correct size of the amplified product was confirmed by gel electrophoresis on 1.5% agarose gels. Primer designs and reaction performance parameters are provided in Supplemental Table 3.

Direct Detection of Inflammatory Mediators in Plasma

Plasma of day 60 BB rats and day 180 BN rats were assayed with the BeadLyte cytokine assay kit (Millipore, Billerica, MA) per the manufacturer's protocol and a Bio-Plex Luminex

100 XYP instrument. Concentrations were calculated with the Bio-Plex Manager 4.1 software and a 5 parameter curve fitting algorithm applied for standard curve calculations. TGF-β levels were measured with Quantikine ELISAs (R&D Systems, Minneapolis, MN).

Viral Induction of Diabetes in BBDR Rats

Poly I:C (Sigma, St. Louis, MO) was dissolved in Dulbecco's PBS, filter sterilized, and stored at −20°C. KRV (UMass strain) was obtained from stocks maintained in our laboratories as described[56]. Cohorts of male and female BBDR rats 25–30 days old or 40–45 days old, were injected intraperitoneally with either KRV alone (10^7 plaque forming units (PFU)), or poly I:C (1.0 μg/g body wt on 3 consecutive days) followed by inoculation with KRV (10^7 PFU). Animals were screened twice weekly for glycosuria (CliniStix, Bayer HealthCare, Elkhart, IN) beginning 1 week after the start of each protocol. Diabetes in glycosuric rats was diagnosed as a capillary glucose concentration >250 mg/dl (Accu-Chek; Roche Diagnostics, Indianapolis, IN). Rats were screened until diabetes onset or day 40 after KRV injection.

Flow Cytometry Studies

Single cell suspensions were prepared from spleens and pancreatic lymph nodes of days 25, 40, and60 BB rats. Cell surface staining was carried out by incubating cells with anti-CD4 (clone W3/25) at 4C for 30 min, followed by intracellular staining with anti-Foxp3 (clone FJK-16s) and anti-Helios (clone 22F6) using the Foxp3 staining kit from eBioscience (San Diego, CA). Abs were obtained from eBioscience or BioLegend (San Diego, CA). Stained cells were analyzed on a FACSAria flow cytometer and sorter (BD Bioscience, San Jose, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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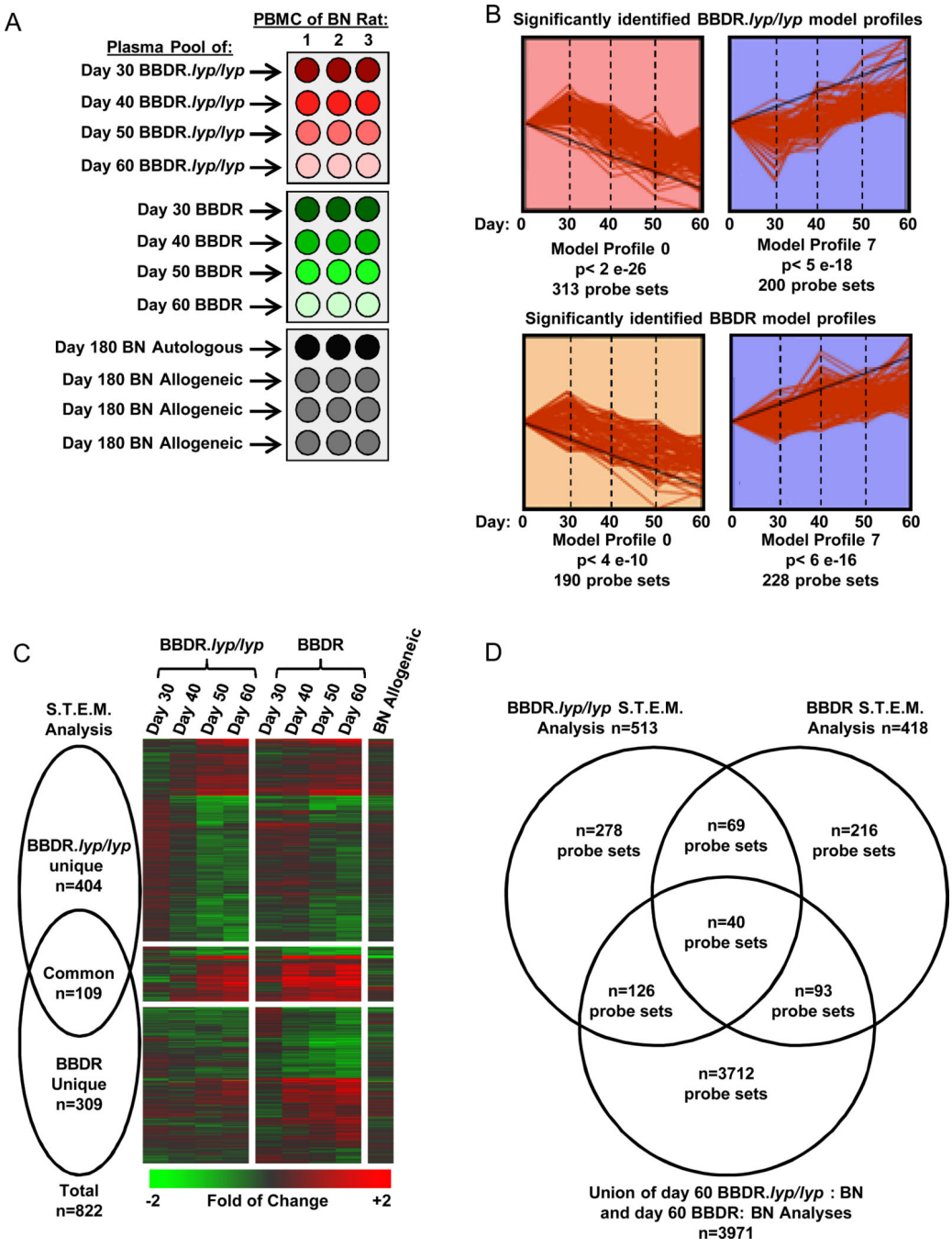

Figure 1.

Transcriptional signatures induced by plasma longitudinally collected from BBDR and BBDR.*lyp/lyp* rats. A. Study design: responder PBMCs of three BN rats were each cultured under 10 different conditions: autologous serum (n = 3 cultures); allogeneic BN serum (n = 9 cultures); day 30, day 40, day 50 and day 60 BBDR.*lyp/lyp* serum pools (n = 3 cultures each), day 30, day 40, day 50 and day 60 BBDR serum pools (n = 3 cultures each). The serum pools were each created from an equal contribution of six individual, normoglycemic rats. Gene expression was measured independently in each culture, and all data were

normalized with that of the autologous induction. B. Significantly regulated model profiles identified by the S.T.E.M. software. C. A Venn diagram and one-way hierarchical clustering (probe sets only) illustrating the relationship between the probe sets temporally regulated by BBDR.*lyp/lyp* and BBDR plasma (profiles 7 and 0 combined). Expression levels illustrated in the heat maps are normalized against the mean of day 30 BBDR and day 30 BBDR.*lyp/lyp*. D. Relationship between the regulated probe sets identified by S.T.E.M. analysis of BBDR.*lyp/lyp* and BBDR longitudinal plasma induced profiles compared to those previously identified when directly comparing samples collected from day 60 BBDR and day 60 BBDR.*lyp/lyp* rats[18].

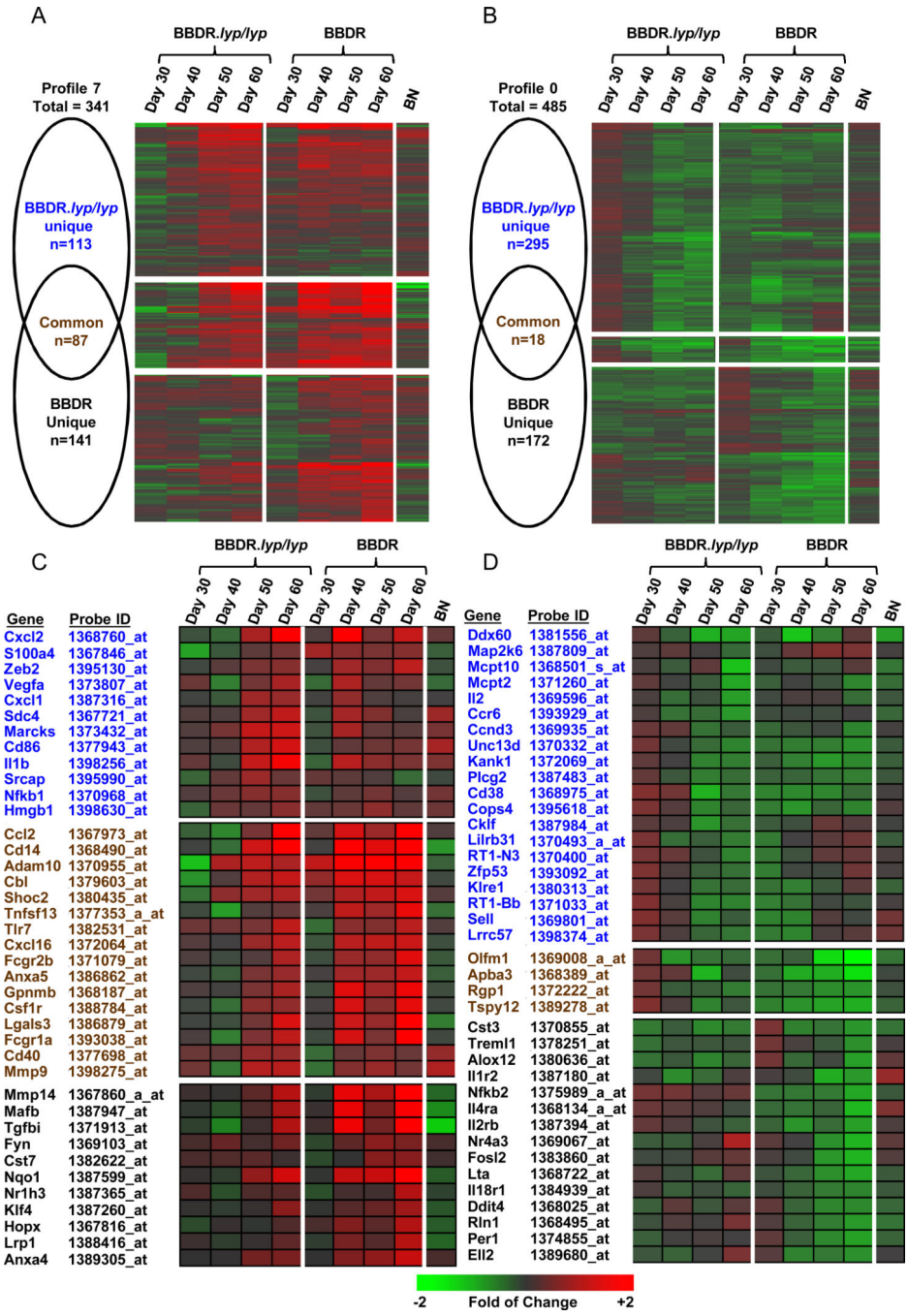

Figure 2.

Analysis of BBDR.*lyp/lyp* and BBDR S.T.E.M. Profiles 7 and 0. Panels A and B: Venn diagrams and one-way hierarchical clustering (probe sets only) illustrating the relationship between the probe sets temporally regulated by BBDR.*lyp/lyp* and BBDR plasma belonging to S.T.E.M. profiles 7 and profiles 0, respectively. Panels C and D. Expression levels of well annotated probe sets temporally regulated by BBDR.*lyp/lyp* and BBDR plasma belonging to S.T.E.M. profiles 7 and profiles 0, respectively. In panels A–D heat maps (and font colors) correspond to the three subsets defined by each Venn diagram. Expression levels illustrated

in the heat maps are normalized against the mean of day 30 BBDR and day 30 BBDR.*lyp/lyp*.

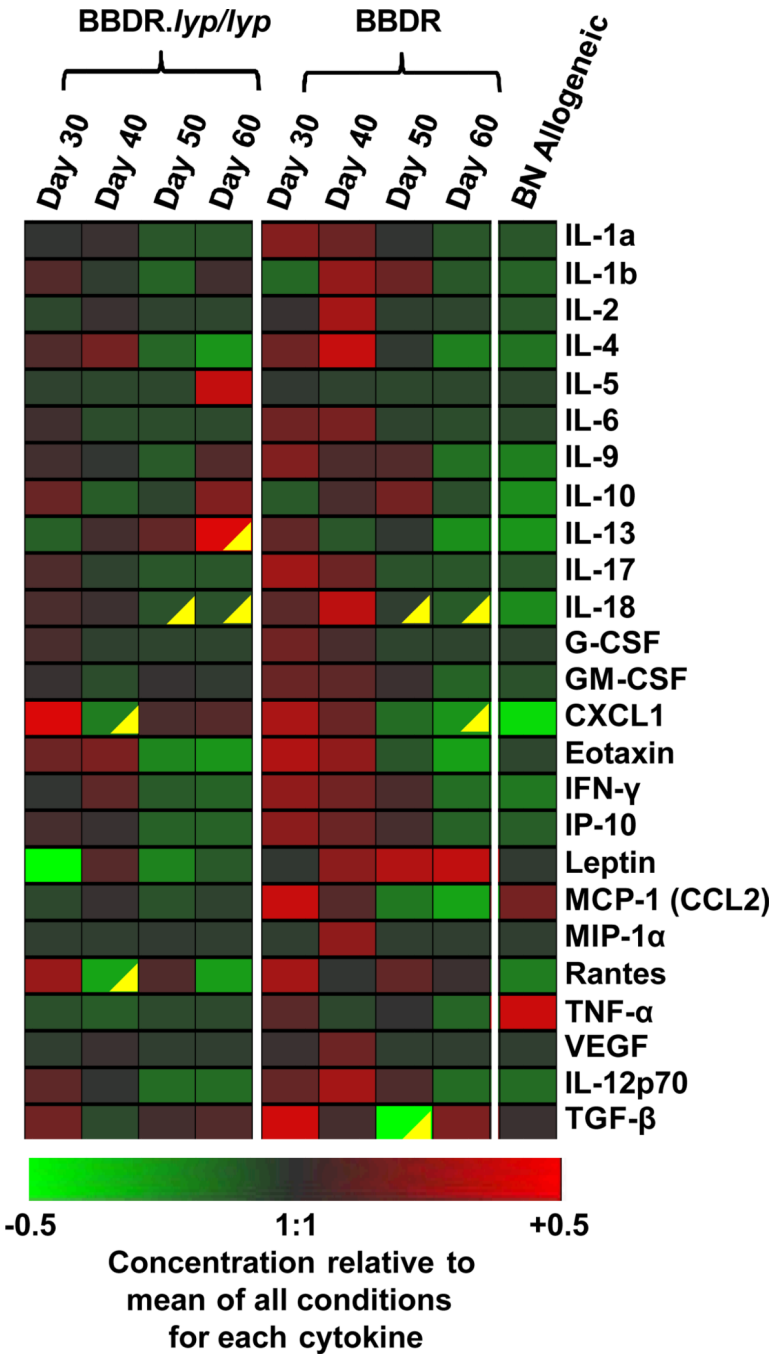

Figure 3.

Heat map representing mean concentrations of individual cytokines in longitudinally collected plasmas of BBDR.*lyp/lyp* and BBDR rats. Color representation (green to red) for each individual cytokine reflects the normalized ratio between the concentration measured at given strain/time-point relative to the mean concentration of all strains/time-points. Yellow triangles denote $p < 0.05$ Wilcoxon rank-sum test BBDR.*lyp/lyp* or BBDR versus intra-strain day 30.

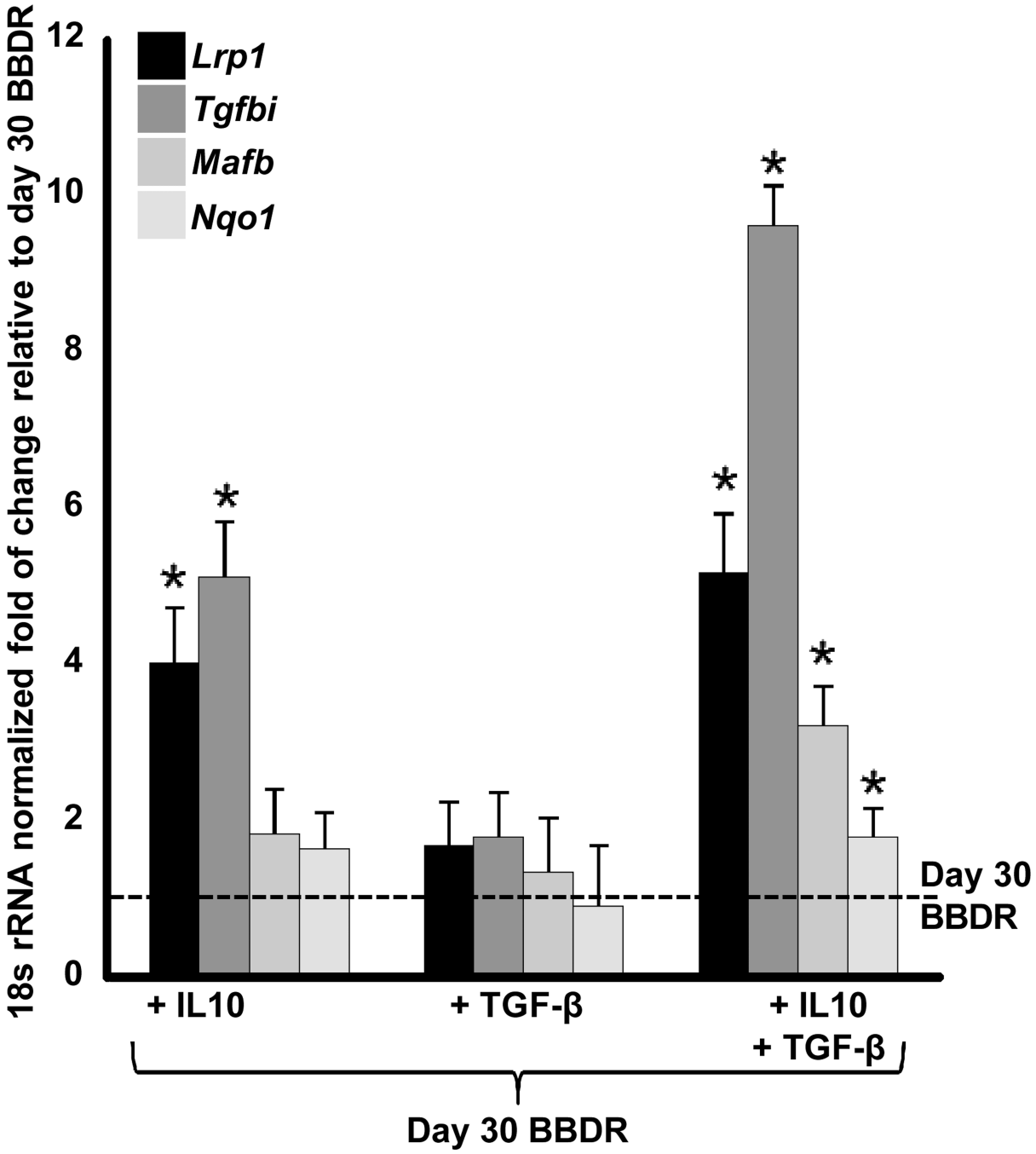

Figure 4.

Investigation of IL-10 and TGF-β as ligands associated with the BBDR temporal signature. PBMCs of seven BN rats each were cultured under four different conditions: *1*) day 30 BBDR plasma pool (*n* = 7 cultures); *2*) day 30 BBDR plasma supplemented with 20 ng/ml TGF-β (*n* = 7 cultures); *3*) day 30 BBDR plasma supplemented with 50 ng/ml IL-10 (*n* = 7 cultures); *4*) day 30 BBDR plasma supplemented with 50 ng/ml IL-10 and 20 ng/ml TGF-β (*n* = 7 cultures). The plasma pools were created from an equal contribution of six normoglycemic rats independent of those used in the microarray-based signature studies.

Gene expression was measured independently, in duplicate, for each culture by qRT-PCR and normalized against the abundance of 18s rRNA. Data is expressed relative to day 30 expression levels for each gene. *$p<0.05$, ANOVA, relative to day 30.

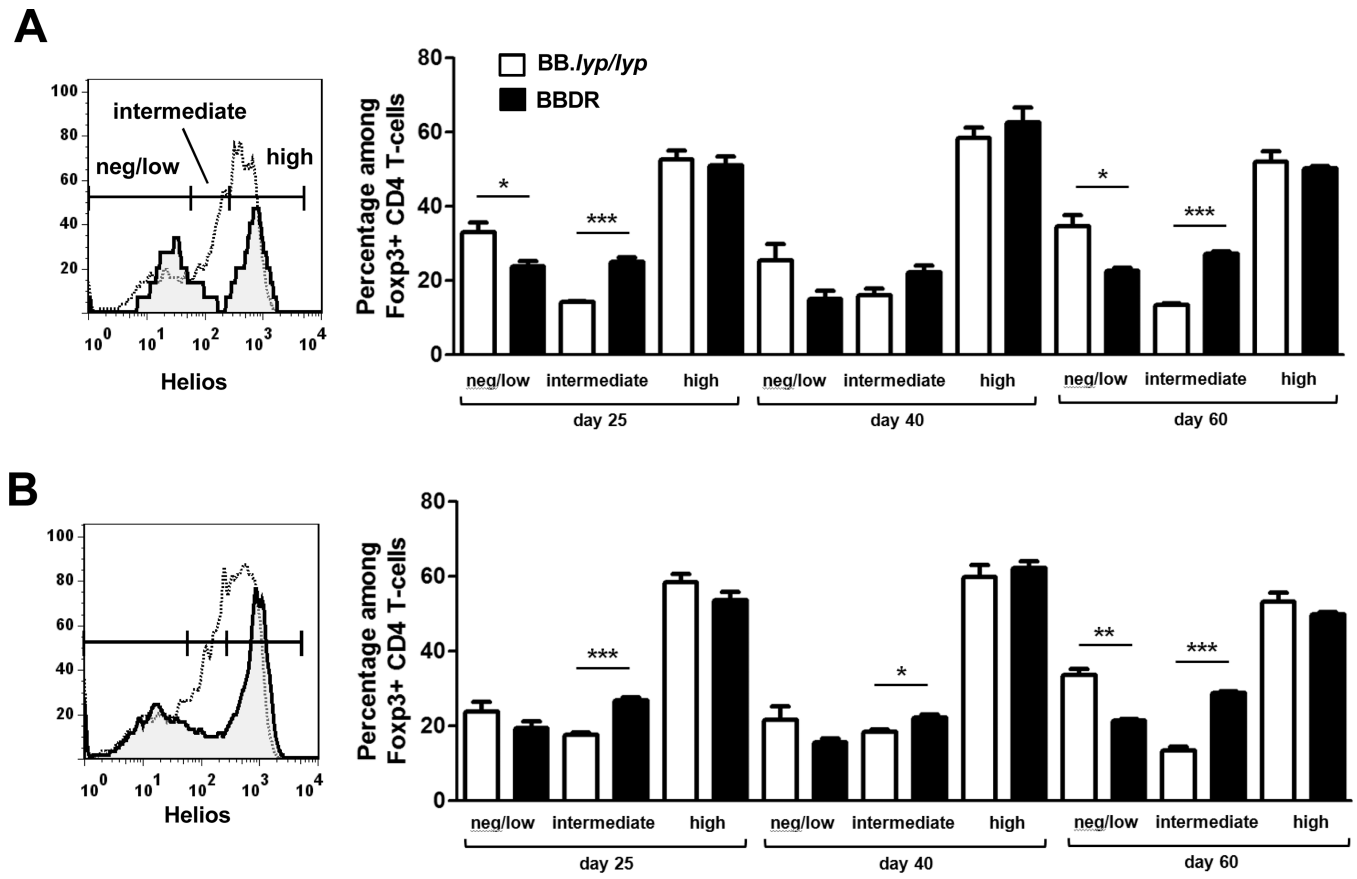

Figure 5.

Differential expression of Helios in T_{reg} of BBDR.*lyp/lyp* and BBDR rats. Expression of Helios was analyzed in $CD4^+Foxp3^+$ Tregs from spleens (A) and pancreatic lymph nodes (B) of sex-matched 25, 40 or 60-day-old rats. Representative histograms of Helios expression (gated on $CD4^+Foxp3^+$ cells) of 60-day-old DP (solid line with shaded area) and DR (dotted line) rats are shown on the left. The right panels summarize the results from 3–4 rats per strain at each time point (mean±sem). *$p < 0.05$, ** $p < 0.005$, ***$p < 0.0005$, significantly different between BBDR.*lyp/lyp* and BBDR rats.

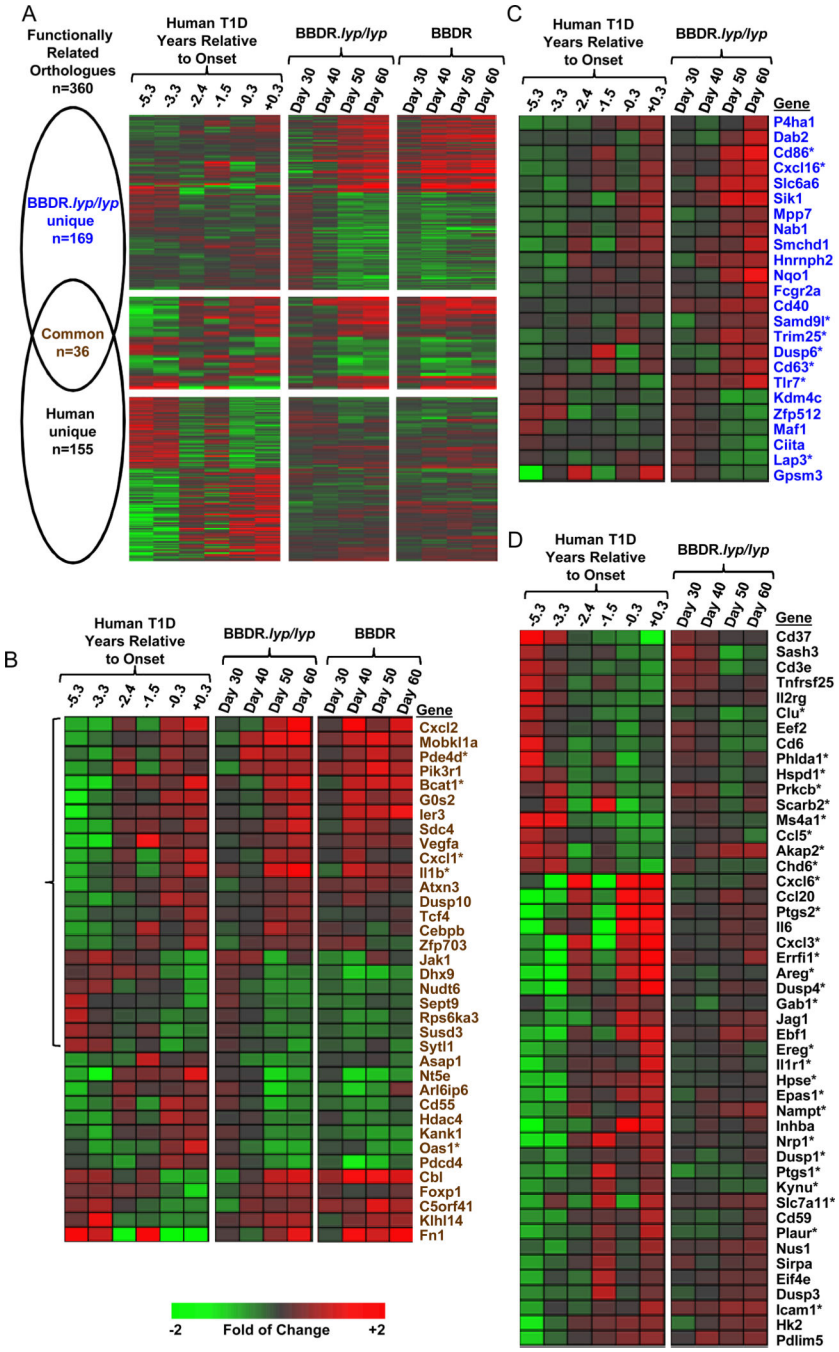

Figure 6.

Comparison of the BBDR.*lyp/lyp* longitudinal plasma-induced signature to that associated with human T1D progression. A. Venn diagram and one-way hierarchical clustering (genes only) for each component of the Venn diagram illustrating the relationship between the 360/914 functionally-related, direct orthologues induced plasma collected during human or BBDR.*lyp/lyp* rat T1D progression. Gene expression induced by longitudinally collected BBDR plasma is shown for comparison. B. One-way hierarchical clustering (genes only) of the 36 genes of the commonly regulated intersection. The 23 directionally concordant

orthologues are bracketed. C. One-way hierarchical clustering (genes only) of selected genes among the 169 orthologous genes uniquely regulated by BBDR.*lyp/lyp* plasma. D. One-way hierarchical clustering (genes only) of selected genes among the 155 genes uniquely regulated by human longitudinal plasma. Genes significantly regulated by human recent onset T1D patients described in our seminal report[17] are indicated by *. Expression levels illustrated in the BB rat heat maps are normalized against the mean of day 30 BBDR and day 30 BBDR.*lyp/lyp*; expression levels illustrated in the human heat maps are normalized against the mean of all time points.

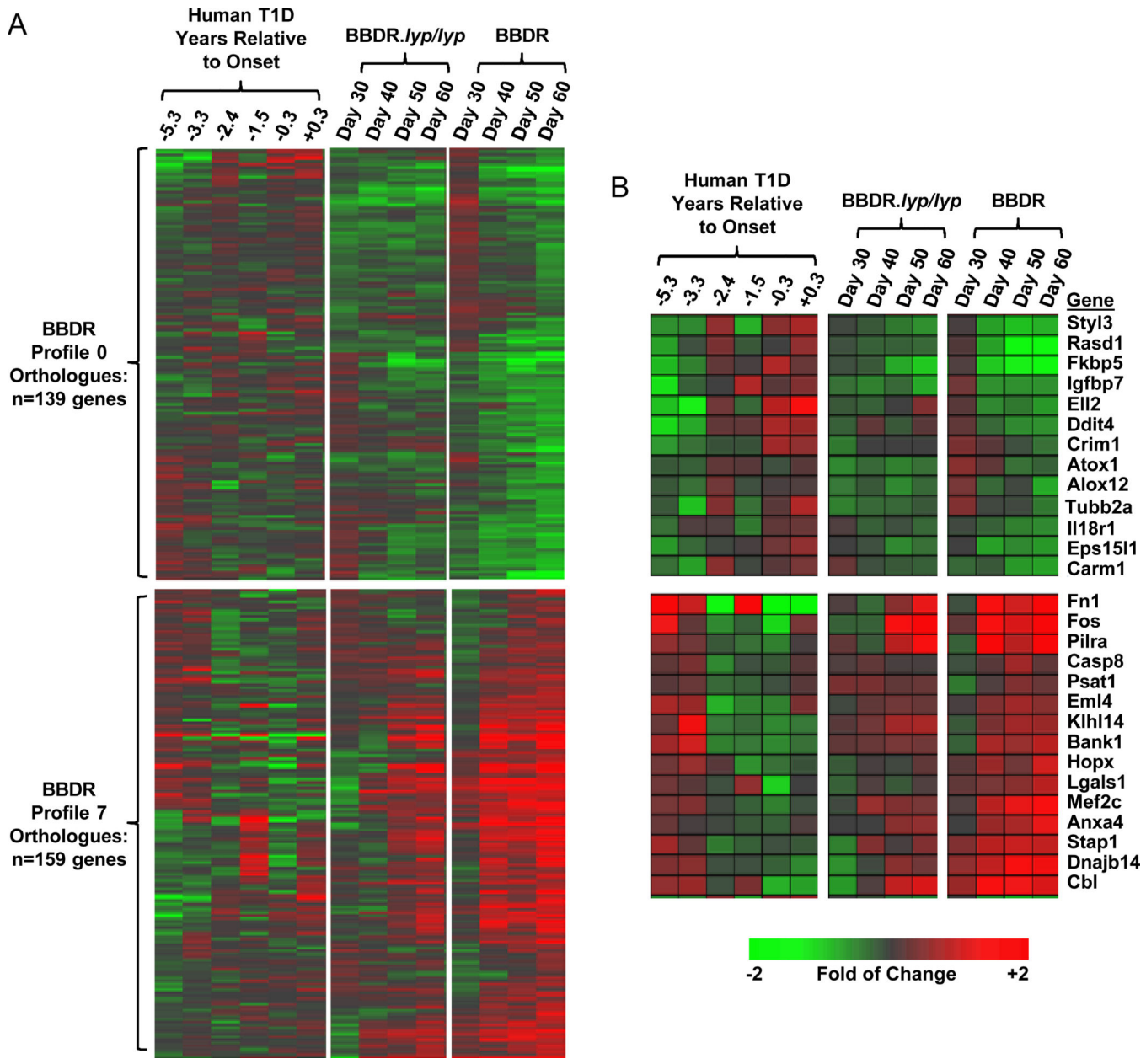

Figure 7.

Comparison of BBDR longitudinal plasma-induced signature to that associated to human T1D progression. A. One-way hierarchical clustering (genes only) for orthologous content between the rat and human arrays for genes belonging the BBDR profile 0 (n=139 genes, top panel) and profile 7 (n=159 genes, bottom panel). Gene expression induced by longitudinally collected BBDR.*lyp/lyp* plasma for these probe sets is shown for comparison. B. Expression levels of selected genes of the BBDR temporal signature. Among the 159 genes assigned to BBDR profile 7, 4 were ascribed to human profile 0: *Fn1, Cbl, Klhl14, Bank1*. Among the 139 genes assigned to BBDR profile 0, 11 were ascribed to human profile 7: *Igfbp7, Fkbp5, Rasd1, Sytl3, F5, Ell2, Crim1, Ddit4, Tubb2a, Carm1, Rab5c*. Expression levels illustrated in the BB rat heat maps are normalized against the mean of day

30 BBDR and day 30 BBDR.*lyp/lyp*; expression levels illustrated in the human heat maps are normalized against the mean of all time points.

Table 1

Significantly regulated pathways identified by STEM analysis

		BBDR.*lyp/lyp* Profile 7		**BBDR Profile 7**		**BBDR.*lyp/lyp* Profile 0**		**BBDR Profile 0**	
Gene Ontology Identifier	**Category Name**	**Genes n=**	**p-value**	**Genes n=**	**p-value**	**Genes n=**	**p-value**	**Genes n=**	**p-value**
GO:0001664	G-protein coupled receptor binding	5	8.9E-03		n.s*		n.s		n.s
GO:0001819	positive regulation of cytokine production	8	4.9E-04	6	2.6E-03		n.s		n.s
GO:0002237	response to molecule of bacterial origin	14	5.0E-04	15	5.8E-03		n.s		n.s
GO:0002366	leukocyte activation involved in immune response	5	6.5E-04		n.s		n.s		n.s
GO:0002685	regulation of leukocyte migration		n.s	5	4.8E-04		n.s		n.s
GO:0002697	regulation of immune effector process		n.s	5	1.0E-02		n.s		n.s
GO:0002764	immune response-regulating signaling pathway		n.s	7	1.4E-03	5	6.7E-03		n.s
GO:0005125	cytokine activity	6	1.8E-04		n.s		n.s		n.s
GO:0005126	cytokine receptor binding	7	1.1E-04		n.s		n.s		n.s
GO:0006954	inflammatory response	14	3.6E-06	13	2.0E-05		n.s		n.s
GO:0007155	cell adhesion	14	1.0E-02	14	1.6E-03		n.s	9	1.0E-02
GO:0007162	negative regulation of cell adhesion		n.s	5	2.1E-04		n.s		n.s
GO:0030593	neutrophil chemotaxis	5	4.3E-06		n.s		n.s		n.s
GO:0043405	regulation of MAP kinase activity	7	2.1E-04		n.s		n.s		n.s
GO:0045087	innate immune response	10	2.2E-03	15	4.7E-03	7	7.1E-03		n.s
GO:0045321	leukocyte activation	8	5.3E-03	15	2.9E-04		n.s		n.s
GO:0050727	regulation of inflammatory response		n.s	6	2.5E-03		n.s		n.s
GO:0050776	regulation of immune response	10	1.0E-02	12	8.1E-05	12	1.2E-03		n.s
GO:0050778	positive regulation of immune response	5	1.0E-02	10	2.1E-05	9	6.7E-03		n.s
GO:0080134	regulation of response to stress		n.s	11	4.5E-03		n.s		n.s

*Not Significant

Table 2

Cytokine/chemokine levels (pg/ml) in BBDR.*lyp/lyp*, BBDR, and BN rats

Cytokine	Day 30 BBDR.*lyp/lyp*	Day 40 BBDR.*lyp/lyp*	Day 50 BBDR.*lyp/lyp*	Day 60 BBDR.*lyp/lyp*	Day 30 BBDR	Day 40 BBDR	Day 50 BBDR	Day 60 BBDR	Day 180 BN	Lower Detection Limit
IL-1α	7.2±6.1	9.3±7.0	0	0	53.7±41.9	31.6±24.2	6.9±5.5	0	0	>27
IL-1β	17.4±4.1	12.6±2.6	9.5±3.9	15.6±4.4	9.7±5.2	30.5±13.4	21.4±6.6	11.7±6.0	11.8±8.1	>27
IL-2	20.2±7.4	73.1±37.9	26.8±8.9	20.7±6.9	69.1±40.9	**2292.6±1817.5**‡	29.7±9.4	23.8±9.7	3.9±3.9	>75
IL-4	22.3±4.5	**29.5±3.3***	8.0±2.1	1.9±1.9	**30.8±9.0**‡	**78.4±36.9**‡	16.9±6.5	5.1±3.9	6.7±3.3	>27
IL-5	0.3±0.3	0	0	19.6±10.8	0.9±0.9	0.3±0.3	0	0	0	>10
IL-6	32.6±23.4	0	0	1.6±1.3	107.2±95.0	115.2±93.2	6.3±6.3	0	1.8±1.8	>250
IL-9	206.7±53.2	192.9±74.3	149.0±64.5	224.1±43.9	373.6±173.2	238.0±85.7	214.2±10.0	105.0±28.5	102.9±55.8	>250
IL-10	85.4±17.7	47.7±12.2	62.2±22.4	107.2±29.9	48.4±13.9	111.4±60.4	101.6±30.1	58.2±19.4	42.7±24.2	>27
IL-13	28.2±13.0	**36.0±6.8**‡	**43.9±8.6**‡	**90.4±21.1***†≠	65.3±35.5	31.0±14.6	30.3±5.0	14.1±2.2	14.1±4.4	>27
IL-17	5.2±3.4	1.1±0.5	0	0	21.4±13.9	7.8±3.7	0.3±0.2	0	0	>10
IL-18	**32.4±4.0***	**36.0±15.2***	**14.9±4.0**‡≠	**14.6±3.0**‡≠	**39.4±2.6***	**614.5±400.4***	**19.6±3.3***≠	**12.4±3.3**‡≠	3.2±1.6	>10
G-CSF[1]	3.4±3.4	0.2±0.2	0	0	10.7±10.2	3.3±3.3	0	0	0	>10
GM-CSF[2]	9.5±6.3	3.8±3.8	7.9±3.8	6.9±5.2	33.9±29.1	17.7±8.5	8.1±1.6	0	2.9±2.9	>27
CXCL1[3]	**395.3±53.8**‡	**235.1±22.7**≠	303.5±57.4	302.5±45.0	**361.0±52.8**‡	315.3±45.9	245.6±25.4	**227.1±29.9**≠	199.8±34.7	>27
Eotaxin	16.9±2.7	18.8±2.8	2.2±1.4	0.9±0.9	**30.5±12.5**‡	23.0±6.7	7.7±4.4	0	8.4±2.8	>27
IFN-γ	20.2±14.1	33.4±14.3	5.6±2.7	3.9±2.3	98.7±67.5	63.6±43.7	**27.1±15.1***	1.6±1.0	0	>27
IP-10	5.2±3.4	3.4±1.9	0.1±0.1	0	17.0±11.9	12.0±9.1	4.7±2.7	0	0.2±0.2	>10
Leptin	1000.3±384.4	1588.8±189.9	1470.6±421.0	1727.4±607.1	1626.5±374.2	2104.9±504.1	2461.3±578.4	2478.8±602.3	1438.4±203.9	>10
CCL2[4]	69.3±22.1	70.8±13.9	70.4±27.4	80.0±33.0	124.9±27.2	79.8±14.5	54.8±18.8	45.8±21.1	87.6±7.3	>75
MIP-1α[5]	0	0	0.1±0.1	0	0	10.6±8.1	0	0	0	>10
Rantes	**8553.0±3551.9**‡	**1331.8±341.5**≠	5649.2±2984.1	1496.9±416.0	9946.4±3710.4	3926.1±2134.3	9449.8±7031.8	6757.0±4493.9	1530.2±176.7	>27
TNF-α	1.2±0.8	0.5±0.5	1.5±0.8	1.6±0.9	13.5±12.7	2.1±1.9	3.5±2.2	0	16.9±3.1	>10
VEGF[6]	0	0.1±0.1	0	0	0.1±0.1	0.6±0.6	0	0	0	>27
IL-12p70	11.4±6.5	5.8±4.5	0	0	12.9±7.8	23.8±12.5	10.5±8.1	0	0	>27
TGF-β	6788.2±838.9	5351.0±538.5	6073.4±769.2	7416.1±2728.6	9406.9±2079.4	6283.0±1246.6	**2475.8±308.5**≠	6959.1±899.8	6506.1±3122.2	>1.9

[1] G-CSF, Granulocyte colony-stimulating factor.

[2] GM-CSF, Granulocyte/macrophage colony-stimulating factor.

[3] CXCL1, Growth-regulated oncogene alpha, chemokine.

[4] CCL2, Monocyte chemoattractant protein.

[5] MIP-1α, Macrophage inflammatory protein-1α.

[6] VEGF, Vascular endothelial growth factor.

Data are means ± SE of six rats per group (pg/ml). Each sample was tested in duplicate using the Millipore BeadLyte cytokine assay kit, except for TGF-β. Significant differences for mean cytokine levels meeting detection thresholds are indicated:

[*] P <0.01 Wilcoxon rank-sum test versus BN;

[‡] P <0.05 Wilcoxon rank-sum test versus BN;

[†] P<0.01 Wilcoxon rank-sum test BBDR.*lyp/lyp* versus BBDR at same time point;

[≠] P< 0.05 Wilcoxon rank-sum test BBDR.*lyp/lyp* or BBDR versus intra-strain Day 30.

Table 3

Penetrance of T1D in BBDR rats treated with Poly I:C or viral infection at different ages

KRV (10^7 PFU)		Poly I:C x3, followed by KRV	
Age <30 days	Age >40 days	Age <30 days	Age >40 days
40% (4/10)	0% (0/6)*	100% (6/6)	0% (0/6)*

Penetrance of T1D in BBDR rats treated with poly I:C alone (1 μg/gm on 3 consecutive days) or Kilham rat virus infection (10^7 PFU) alone, or KRV infection (10^7 PFU) given the day after poly I:C priming (1 μg/gm daily on 3 consecutive days) at different ages. Rats were followed until diabetes onset or day 40 after the start of treatment.

*overall $p<0.001$ vs. <30 days group, Fisher exact statistic.