Molecular Immunology 59 (2014) 208–216

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Altered DNA methylation profile in Norwegian patients with Autoimmune Addison's Disease

Trine E. Bjanesoy[a], Bettina Kulle Andreassen[b,c,1], Eirik Bratland[d,1], Andrew Reiner[e], Shahinul Islam[a], Eystein S. Husebye[d,f], Marit Bakke[a,*]

[a] Department of Biomedicine, University of Bergen, Jonas Lies vei 91, 5009 Bergen, Norway
[b] Department of Molecular Biology, Institute of Clinical Medicine, University of Oslo, P.O Box 1171, 0318 Oslo, Norway
[c] Department of Biostatistics, Institute of Basic Medical Sciences, University of Oslo, P.O Box 1110, 0317 Oslo, Norway
[d] Department of Clinical Science, University of Bergen, P.O Box 7804, 5020 Bergen, Norway
[e] Stem Cell Epigenetics Laboratory, Institute of Basic Medical Sciences, Faculty of Medicine, University of Oslo, P.O Box 1110, 0317 Oslo, Norway
[f] Department of Medicine, Haukeland University Hospital, P.O Box 7804, 5020 Bergen, Norway

ARTICLE INFO

Article history:
Received 10 January 2014
Received in revised form 21 February 2014
Accepted 25 February 2014
Available online 22 March 2014

Keywords:
DNA methylation
Epigenetics
Autoimmunity
Autoimmune Addison's Disease
Adrenal gland

ABSTRACT

Autoimmune Addison's Disease (AAD) is an endocrine and immunological disease of uncertain pathogenesis resulting from the immune system's destruction of the hormone producing cells of the adrenal cortex. The underlying molecular mechanisms are largely unknown, but it is commonly accepted that a combination of genetic susceptibility and environmental impact is critical. In the present study, we identified multiple hypomethylated gene promoter regions in patients with isolated AAD using DNA isolated from CD4+ T cells. The identified differentially methylated regions were distributed evenly across the 10.5-kb-promoter regions covered by the array, and a substantial number localized to promoters of genes involved in immune regulation and autoimmunity. This study reveals a hypomethylated status in CD4+ T cells from AAD patients and indicates differential methylation of promoters of key genes involved in immune responses.

1. Introduction

Autoimmune disorders constitute a group of diseases that affect 5–10% of the population in western countries and around 3% worldwide (Shapira et al., 2010; Youinou et al., 2010). Apart from a few autoimmune diseases caused by mutations in single genes (Michels and Gottlieb, 2010), the etiology of most autoimmune diseases is still unclear. However, it is commonly accepted that a combination of genetic susceptibility and environmental factors are at play, causing breakdown of the natural tolerance mechanisms (Costenbader et al., 2012; Gonzalez et al., 2011; Wing and Sakaguchi, 2010). The relative importance of environmental factors is illustrated by the facts that monozygotic twins show moderate rates of concordance for autoimmune disorders and that drug-induced autoimmunity is observed after medication with demethylating agents (Javierre et al., 2011). As the environment directly impacts the epigenetic code, the surrounding milieu of an individual will directly affect gene regulatory mechanisms and disease development.

In autoimmune disorders, the most studied epigenetic mark is DNA methylation, which involves addition of a methyl group to cytosines by DNA methyl transferases (Dnmts). In differentiated mammalian cells >98% of all DNA methylation occurs on cytosines that are followed by guanines (i.e., a CpG site; Jin et al., 2011). The DNA methylation status of a genomic region will affect the expression of genes, but the outcome will depend on the location of the methylated cytosines. In general, whole genome sequencing studies suggest that hypermethylation of promoter regions is associated with gene silencing, whereas intragenic hypermethylation is linked to gene activation (Aran et al., 2011; Hon et al., 2012). However, many CpG-rich promoters (i.e., containing CpG islands) are kept demethylated regardless of the transcriptional activity of the corresponding gene (Hawkins et al., 2010). The DNA methylation signature is changed in many autoimmune diseases, as exemplified by global hypomethylation as well as hypo- or hypermethylation of specific loci (Lu, 2013; Selmi et al., 2012). Moreover, DNA methylation plays essential roles in normal transcriptional control of key

* Corresponding author. Tel.: +47 55 58 61 96.
E-mail address: Marit.Bakke@biomed.uib.no (M. Bakke).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.molimm.2014.02.018

factors in T cell differentiation and function, such as the interferon gamma locus (Sawalha, 2008), and the transcription factors FoxP3 (forkhead box P3 (Lal et al., 2009) and AIRE (Kont et al., 2011).

Primary adrenal insufficiency (Addison's disease) is a rare disease with a prevalence of around 100–140 per million inhabitants in European countries (Betterle et al., 2002; Laureti et al., 1999; Lovas and Husebye, 2002; Myhre et al., 2002). The major cause (80–90% of the cases) is an autoimmune reaction toward the adrenal gland, resulting from selective destruction of steroid hormone-secreting cells in the cortex. Autoimmune Addison's disease (AAD) may occur isolated, but in 50% of the cases another autoimmune diseases such as autoimmune thyroid disease and type 1 diabetes (T1D) is present (Eisenbarth and Gottlieb, 2004). The mechanisms underlying the initiation of the immune-mediated inflammation in AAD remain enigmatic, and as for other autoimmune diseases, it is believed that a combination of genetic and environmental factors affect disease development (Husebye and Lovas, 2009; Selmi et al., 2012). The vast majority of AAD patients (around 80–90% in different cohorts, (Betterle and Zanchetta, 2003; Erichsen et al., 2009)) display autoantibodies against the steroidogenic enzyme cytochrome P450 21-hydroxylase (CYP21), but the precise role of this immunoreaction in the pathogenesis of AAD is not well understood. It has been suggested that the destruction of the endocrine cells of the adrenal cortex is mediated by antigen specific CD4+T-helper cells, and executed by cytotoxic T-lymphocytes and macrophages (Bratland and Husebye, 2011). At the genetic level, several reports from different populations have confirmed a strong link to HLA-DRB*03 and HLA-DRB1*0404. The DRB1*0404 allele is particularly more frequent among AAD patients than other DRB1*04 alleles (Maclaren and Riley, 1986; Skinningsrud et al., 2011; Yu et al., 1999). As the differences between DRB1*0404 and the other DRB1*04 alleles influence the peptide binding capabilities of HLA molecules, a role of antigen-specific T cell responses in AAD pathogenesis is plausible (Gombos et al., 2007). The fact that homozygosity for the 5.1 allele of MIC-A gene (located in the HLA complex) increases the probability to develop AAD when present in combination with certain HLA genotypes (Triolo et al., 2009) supports a strong role of Human leukocyte antigen (HLA). Outside the HLA complex, polymorphisms in the genes encoding the immunomodulatory factors CTLA4 (cytotoxic T-lymphocyte antigen 4), CIITA (class II, major histocompatibility complex, transactivator), PTPN22 (protein tyrosine phosphatase, non-receptor type 22) and PD-L1 (programmed cell death 1 ligand 1) are commonly found in both AAD and other autoimmune diseases (Blomhoff et al., 2004; Ghaderi et al., 2006; Mitchell et al., 2009; Skinningsrud et al., 2008). In AAD patients, disease-associated polymorphisms are also frequently found in the genes encoding the vitamin D receptor and NLRP1 (NLR family, pyrin domain containing 1), important for cytokine secretion and inflammatory responses, respectively (Magitta et al., 2009; Pani et al., 2002). There is no published Genome-wide association study (GWAS) customized for AAD, and at present, we have very limited understanding of the genetic factors that contribute to AAD development at the individual level.

The aim of the present study was to investigate whether DNA methylation abnormalities were linked to AAD. Indeed, a number of differentially methylated regions (DMRs) were identified, the majority of which were hypomethylated in the patients. Moreover, the patients exhibited an overall hypomethylated status. Interestingly, a significant number of the DMRs localized to the promoters of genes encoding factors that are implicated in immune responses and autoimmunity. The results presented in this study might serve as a foundation for future experiments designed to unravel the molecular mechanisms underlying AAD, as well as to generate hypotheses regarding the importance of environment–genome interactions and autoimmunity.

2. Materials and methods

2.1. AAD patients and controls

Peripheral blood was collected in Ethylenediaminetetraacetic acid (EDTA) tubes from patients with isolated AAD and from healthy blood donors. All patients had previously been diagnosed with clinically evident primary adrenocortical insufficiency using standard biochemical measurements (Arlt and Allolio, 2003). All patients were positive for cytochrome CYP21-antibodies (CYP21-ab) and had been treated with cortisone combined with fludrocortisone since diagnosis (15 ± 14 years). All subjects were females from 20 to 60 year of age (mean 45 ± 13 years and 43 ± 8 years, for cases and controls, respectively). The patients were diagnosed in the age range of 13–54 years (mean age 30 years), and disease duration ranged from 4 to 37 (mean duration 15 years). All patients were born, raised and dwell in Western Norway (Hordaland and Rogaland Counties). All participants signed the informed consent form approved by the Norwegian Health Region West ethics committee (149/96–47.96).

2.2. Isolation of CD4+ T cells and DNA

CD4+ T cells were isolated from 12 to 15 ml EDTA blood by the Dynabeads CD4 positive Isolation Kit (Life Technologies/Invitrogen) according to the manufacturer's protocol within 6 h of blood collection. Genomic DNA from CD4+ T cells was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol.

2.3. Global methylation analyses

The global DNA methylation status was determined on DNA isolated from CD4+ T cells from 16 AAD patients and 16 healthy controls by an ELISA assay (5-mC DNA ELISA kit, Zymo Research, Irvine, CA, USA). Five of these patients and four of the controls contributed to the MeDIP analysis. This kit is based on the recognition and quantification of the methylated DNA fraction using a 5-methylcytosine antibody and a standard curve generated by methylated/demethylated *E. coli* DNA. The output values were multiplied by the human CpG density/genome factor provided by the manufacturer (according to the manufacturer, the *E. coli* CpG density/genome length is 0.075 and human CpG density/genome length is 0.009 (28,700,086/3,137,161,264), and the fold difference between *E. coli* and human CpG density is 8.3).

2.4. MeDIP

Genomic DNA was isolated from nine AAD patients and five healthy control individuals. The DNA was sheared to fragments with an average of 400 bp. Methylated DNA was precipitated using the MagMeDIP kit (Diagnode, Belgium), which is based on magnetic beads and a 5-methylcytidine antibody. The precipitated DNA was purified and eluted from the beads by the IPure kit (Diagnode, Belgium) according to the manufacturer's protocol. To obtain sufficient amount of DNA for microarray hybridization, the immunoprecipitated DNA and input DNA were amplified using the WGA2 kit (Sigma, USA). Amplified products were controlled by qPCR using primer pairs specific for a methylated region (*TSH2B*) and unmethylated region (*GAPDH*) according to the manufacturer's protocol.

2.5. Array hybridization

The precipitated DNA was hybridized to the NimbleGen Human DNA Methylation 2.1 M Deluxe Promoter Array (NimbleGen-Roche, USA) by Roche NimbleGen Services (Iceland). This array design is

based on the HG18 genome build, and covers all transcript and microRNA promoters as well as all 28,226 annotated CpG islands with spacing throughout all tiled regions. Tiling of each transcript promoter begins 7.25 kb upstream of the transcription start site (TSS) and extends downstream 3.25 kb for a total of 10.5 kb of promoter coverage per transcript. Each miRNA promoter (475 total) is tiled from the mature miRNA sequence to 15 kb upstream.

2.6. Statistics

Preprocessing and quality control: The raw data included 2,129,040 annotated probes for each of the nine patients and five controls. 2.19% of the probe ids are matched to more than one probe sequence and were excluded from further analysis leading to 2,082,409 probe ids left. After removing probes whose positions have not been localized on the chromosome, the final number for the analysis became 2,000,078 probe ids. We used median-centered within-array normalization and quantile normalization for between array normalization.

Differentially methylated regions: We performed a *t* test for each of the 2,000,078 probes and defined the 1% percentile of the nominal *p* values as to be the threshold for an *interesting* probe. These interesting probes were then merged into DMR with neighboring probes if the distance to the next interesting probe was less than 500 bp. The region-wise test statistic was defined to be the sum of all (absolute values of the) *t* test statistics within the DMR. We ended up with 3373 DMRs (Supplementary Table S1_DMRs.xlsx).

2.7. Genomic annotation of DMRs

DMRs were associated with TSSs by identifying DMRs whose genomic coordinates intersected with the TSS coordinates. The 10.5 K gene promoter regions were divided into four subregions to further characterize the positions and properties of the identified DMRs (start:end pairs relative to the TSS: −7250/−4625, −4625/−2000, −2000/+625 and +625/+3250). The start coordinate is the first bp in the subregion, and the end coordinate is one past the final bp. Which DMRs overlap with which promoter subregions is presented in Supplementary Table S2_regions_overlap.xlsx. If a DMR overlaps with two or more subregions in a promoter region, it is associated with each.

2.8. CpG class assignment

Each promoter region or subregion was scanned by sliding a 500-bp window across the region, and calculating the CpG ratio and CG content at each position. CpG ratio was calculated as follows for each 500 bp window: Obs/Exp CpG = $N \times$ number of CpG/(number of C $\times$ number of G) *N*: total number of nucleotides. The region is high if there is a 500-bp window where the CpG ratio is > 0.75 and the C + G content is > 0.55. The region is low if there is no window with a CpG ratio > 0.48. The region is intermediate otherwise (Weber et al., 2007).

2.9. Gene ontology and pathway analysis

Gene ontology (GO) analysis was performed using the DAVID Bioinformatics Resource (Huang da et al., 2009) with a *p* value cut-off of 0.05. Pathway analysis and enrichment was done using the KEGG pathway component of the DAVID Bioinformatics Resource. Pathway enrichment was run using the default threshold = 2 and EASE score = 0.1.

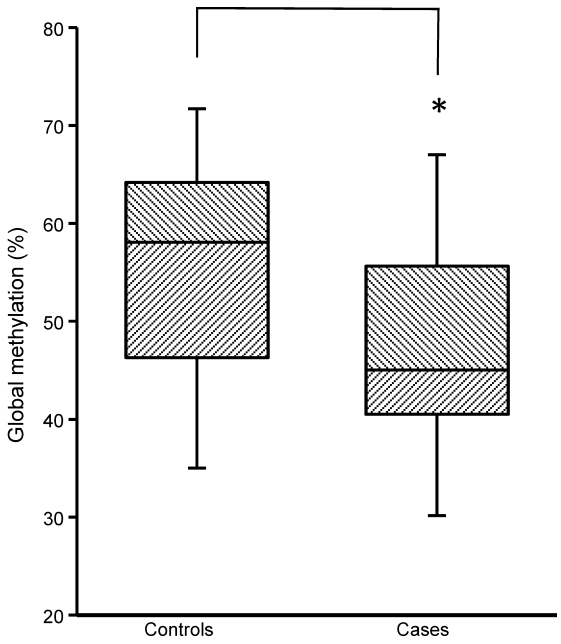

Fig. 1. Global DNA methylation levels (%) in CD4+ T cells from healthy controls ($n = 16$) and from AAD patients (cases) ($n = 16$) were determined by a 5-mC DNA ELISA kit. Students *t* test; $p = 0.0438$.

3. Results and discussion

3.1. Global and promoter-specific hypomethylation in patients with AAD

AAD is primarily associated with class II HLA alleles, suggesting that activation of CD4+ T helper cells is a necessity for the disease to develop. CD4+ T cells were therefore used as the source for DNA in further analyses on global DNA methylation and for the identification of potential genomic regions with altered methylation status in AAD patients. By MeDIP combined with array hybridization, we identified 3373 regions that were differentially methylated between AAD patients and controls (Supplementary Table S1). The majority of the DMRs were localized in promoter regions, and very few non-promoter CGIs were differentially methylated (i.e., <0.2%). Of the identified DMRs, 89% were hypomethylated in patients compared to controls (Supplementary Data, Table S1). Also, as determined by a 5-mC DNA ELISA assay, CD4+ T cell DNA from AAD patients was hypomethylated compared to controls (Fig. 1). The observed global methylation level in CD4+ T cell derived from controls is comparable to what has been previously reported (Ziller et al., 2013), and the relative decrease in methylation in AAD patients is analogous to what has been reported for other autoimmune diseases (Dong et al., 2011; Javierre et al., 2011; Lei et al., 2009). A recent comprehensive whole genome bisulfite dataset demonstrates that only around 20% of autosomal CpGs are dynamically regulated with regard to methylation. However, these CpG sites frequently localize to gene regulatory elements, explaining why relatively minor changes in global methylation can have major effects on cell function (Ziller et al., 2013). Cancer-derived cells are also generally hypomethylated, and whole genome DNA methylation analyses have revealed hypomethylated regions that cluster in the so-called partially methylated domains (PMDs; Hansen et al., 2011; Hon et al., 2012). PMDs are associated with gene expression hypervariability, presumably as a result of the methylation changes (Hansen et al., 2011; Hon et al., 2012). It remains to be determined whether a similar organization of hypomethylated regions exists in diseased cells from autoimmune patients.

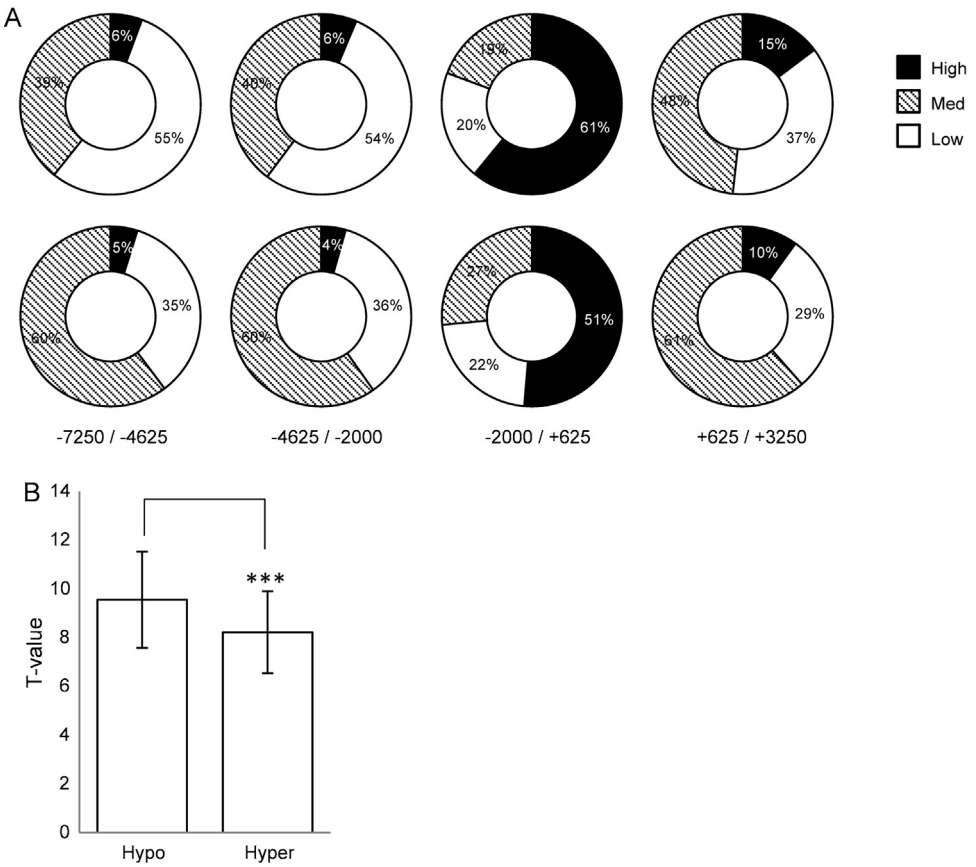

Fig. 2. (**a**) The distribution of high, intermediate (med), and low CpG density regions across all tiled promoters covered by the array is shown in the upper panel. The distribution across promoter regions of high, intermediate (med), and low CpG density DMRs identified in this study is shown in the lower panel. Different distribution of the various CpG categories between the identified DMRs and all tiled promoter regions was determined with Pearson's chi-squared test of association: For promoter region +625/+3250: $\chi^2 = 46.5$, ds = 2, $p = 8.0 \times 10^{-11}$; for promoter region −2000/+625: $\chi^2 = 46.5$, ds = 2, $p = 1.8^{-6}$; for promoter region −4625/−2000: $\chi^2 = 109.5$, ds = 2, $p < 2.2 \times 10^{-16}$; for promoter region −7250/−462: $\chi^2 = 109.5$, ds = 2, $p < 2.2 \times 10^{-16}$. (**b**) Relative levels of differential methylation levels in hypo- and hypermethylated DMRs calculated as the absolute *t* statistic value. Students *t* test, $p = 2.7 \times 10^{-27}$ ($n = 2280$ for hypomethylated DMRs, $n = 275$ for hypermethylated DMRs). The data are presented as mean values ±SE.

As described in Section 2.7, the promoter regions covered by the array were divided into four subregions to further characterize the positions and properties of the identified DMRs. The identified DMRs were distributed uniformly across the four promoter subregions (Supplementary Table S2). We categorized the DMRs into high, intermediate, and low CpG content, according to the criteria posted by (Weber et al., 2007; see Section 2.8). As expected, based on the documented enrichment of CGIs around TSSs (Deaton and Bird, 2011), the largest proportion of the DMRs classified as "high" was found in the −2000/+625 promoter regions (Fig. 2a). The largest proportion of DMRs in all other promoter regions was those DMRs with intermediate CpG content, which might reflect the fact that medium CpG-containing genomic regions are more frequently subjected to differential methylation (Irizarry et al., 2009). The relative level of differential methylation (calculated as the absolute *t* statistic value, Supplementary Data, Table S1) was slightly higher for hypomethylated than for hypermethylated DMRs (Fig. 2b). The relative differences in methylation did not differ between the different promoter regions, or between regions classified as high, intermediate, or low with regard to CpG content (Supplementary Table S2).

3.2. DMRs localize to promoters of genes with essential roles in immunity

A considerable number of the identified DMRs localized to promoters of genes with immunological functions, some of which have already been associated with AAD. The majority of the genes discussed here were differentially methylated in the proximal promoter region (−2000/+625). However, several potentially interesting genes carried abnormal methylation marks in other promoter subregions, demonstrating the importance of examining the whole tiled promoter (Supplementary Table S2). As we detected relatively mild differences in MeDIP profiles between patients and controls, these methylation changes could not be validated by bisulfite sequencing (data not shown). In the remaining of Section 3.2, "hypomethylated" and "hypermethylated" refer to that the DMR was hypo or hypermethylated in patients compared to controls.

Hypomethylated DMRs localized to several interferon regulated genes. Among 201 genes recently shown to be upregulated by type I interferons in PBMCs of healthy humans (Waddell et al., 2010), 13% were differentially methylated in AAD patients compared to controls in our dataset. These included well-described target genes of interferons such as *IRF2* (interferon regulatory factor 2), *IRF7* (interferon regulatory factor 7), *EIF2AK2* (eukaryotic translation initiation factor 2-alpha kinase 2), *USP18* (ubiquitin specific peptidase 18), *OAS2* (2'-5'-oligoadenylate synthetase 2), and *CXCL10* (C-X-C motif chemokine 10/interferon gamma-induced protein 10). A number of these genes are also hypomethylated in naïve CD4+ T cells of patients with SLE, a systemic autoimmune disorder with a strong interferon signature (Coit et al., 2013). While the type I interferon pathway is considered to play a dominant role in systemic autoimmune disorders, limited data are available on its role in organ-specific autoimmune disorders such as AAD. Yet, IFNα is

elevated in serum of patients with autoimmune endocrinopathies, including children with newly diagnosed T1D (Chehadeh et al., 2000; Mavragani et al., 2013). Furthermore, IFNα has been suggested as a possible endogenous predisposing factor for adrenal inflammation, as treatment of chronic hepatitis C infection with recombinant IFNα induces the level of CYP21-Ab (Wesche et al., 2001), causes transient primary adrenal insufficiency (Krysiak et al., 2011; Tran et al., 2008), and worsen clinical features of already existing AAD (Oshimoto et al., 1994). With regard to CXCL10, it is of interest that several independent studies demonstrate that this chemokine is elevated in sera of AAD patients (Bratland et al., 2013; Ekman et al., 2013; Rotondi et al., 2005). Considering the identification of DMRs in these gene promoters, it might be hypothesized that the type I interferon pathway is involved in the development of AAD. Whether this represents an epigenetic "poising" of the type I interferon system (as has been described for SLE (Coit et al., 2013)), or merely a reflection of the ongoing adrenalitis in AAD, remains to be established.

Genes encoding members of the NLRP family of proteins (nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing; also called NALP) also contained DMRs in their promoter regions. Specifically, *NLRP*-1, -5, -7, -8, -12, and -13 were found to contain hypomethylated DMRs in their promoter regions in our group of patients. The NLRPs are members of the nucleotide oligomerization domain (NOD)-like receptors (NLRs) that play critical roles in innate immune function (Lupfer and Kanneganti, 2013). Such functions have been ascribed NLRPs 1 and 12, and the identification of NLRP1 in our dataset is of particular interest since SNPs in this gene have been associated with AAD (Magitta et al., 2009; Zurawek et al., 2010). However, the DMRs identified here do not overlap with the described SNPs. Upon recognition of microbial ligands or danger-associated molecular patterns NLRP1 forms part of the inflammasome, which cleaves the zymogen pro-caspase 1 into enzymatically active and highly proinflammatory caspase 1 (Franchi et al., 2012). Intriguingly, another central component of the inflammasome, *PYCARD*, was also found to be hypomethylated in AAD patients. PYCARD (PYD and CARD domain containing, also known as ASC; apoptosis-associated speck-like protein containing a CARD) is a key adaptor protein that mediates the inflammatory and pro-apoptotic effects of the NLRP1. Once the inflammasome containing NLRP1, PYCARD, and pro-caspase 1 has been established, the proinflammatory cytokines IL1β and IL18 are produced from inactive precursors by the activated caspase 1. Interestingly, the genes encoding the receptors for IL1β, IL1R1 and IL1R2, were also found to contain hypomethylated DMRs. The activated inflammasome has widespread effects, including increased T cell-dependent antibody responses, increased IFN-γ production by T_H1 cells, and increased rate of differentiation of naïve T cells into T_H17 effector cells (Shaw et al., 2011). These findings might indicate that NLRP1 and the inflammasome are implicated in the onset or perpetuation of AAD. A similar role for the inflammasome has been suggested for other autoimmune diseases, including RA, vitiligo and T1D (Jin et al., 2007; Kastbom et al., 2008).

Another striking observation was the high number of hypomethylated DMRs in genes that are often mutated in lymphoid malignancies. These DMRs were located to promoters of genes encoding both chromatin modifiers and histone regulators such as *BRCA1* (encoding breast cancer type 1 susceptibility protein), *KAT6A/MYST3* (K (Lysine) Acetyltransferase 6A), *WHSC1* (probable histone-lysine N-methyltransferase NSD2), *DNMT3A* (DNA (cytosine-5)-methyltransferase 3A) and *BRD3* (bromodomain-containing protein 3), but also critical components of activating and anti-apoptotic signaling pathways such as *CARD11* (caspase recruitment domain-containing protein 11), *BIRC5* (baculoviral inhibitor of apoptosis repeat-containing 5/surviving), and *MCL1* (induced myeloid leukemia cell differentiation protein; Knoechel and Lohr, 2013). These findings underline possible converging pathways and mechanisms of autoimmunity and lymphoid neoplasms, both at genetic and epigenetic levels.

Hypomethylated DMRs also mapped to a strikingly high number of genes encoding enzymes of the mitogen-activated protein kinase (MAPK) family. In particular, we identified members of the p38 MAPK pathway, a signaling cascade involved in many autoimmune diseases (Cuenda and Rousseau, 2007). Also, this pathway is implicated in many processes in CD4+ T cells (Dodeller et al., 2005), including integration of extracellular signals, T cell activation and differentiation and production of proinflammatory cytokines (Mavropoulos et al., 2013). Specifically, we found DMRs in the promoter region of the gene encoding MAPK13 (mitogen-activated protein kinase 13, also called p38δ), and in genes up- and downstream of MAPK13 in the p38 MAPK signaling cascade: MAP3K4 (encoding MAPK kinase kinase 4), MAP2K6 (encoding MAPK kinase 6), LAT (linker for activation of T cells), and the p38 MAPK substrate ATF2 (activating transcription factor 2). The JNK pathway, another MAPK signaling pathway converging with p38 at ATF2, was also represented among the hypomethylated DMRs. Similar to the p38 MAPK pathway, the JNK signaling cascade is activated by proinflammatory stimuli, but also in response to TcR stimulation (Dong et al., 2002). Both p38 and JNK pathways are therefore involved in the activation and differentiation of antigen-specific T cells, predominantly of the T_H1 type. It is presumable, therefore, that these pathways are involved in the development of disease-specific IFNγ producing T cells recognizing CYP21 that have previously been demonstrated in AAD patients (Bratland et al., 2009; Rottembourg et al., 2010). Hypomethylated DMRs were identified in a considerable number of genes encoding members of the tumor necrosis factor (TNF) superfamily. Among these was *TNFRSF1A* encoding TNF-R1, the receptor of TNFα. TNFα is a classical proinflammatory cytokine with a demonstrated role in a range of autoimmune diseases such as RA, SLE, multiple sclerosis (MS), and T1D, and also a target for immunotherapeutic intervention in those diseases (Aggarwal, 2003). Furthermore, we found hypomethylated DMRs in the gene *TNFSF14* (encoding lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells, or LIGHT). LIGHT is a powerful stimulatory factor for T cells that may contribute to the breach of immunological tolerance in autoimmune diseases (Wang et al., 2001). Based on the pivotal role played by members of the TNF superfamily in a wide array of autoimmune disorders, we suggest that the findings reported herein indicate a role for the TNF superfamily also in the pathogenesis of AAD.

It is also worth noting that a considerable proportion of the DMRs demonstrating the quantitatively largest methylation difference between patients and controls and the highest number of probe hits, mapped promoters of genes with no previously known roles in the immune response or in autoimmunity; for example, *GRIK5* (encoding glutamate receptor, ionotropic, kainite 5) and *FBLN1* (encoding fibulin 1). The potential roles for these factors in AAD, if any, are still to be described. Moreover, of interest is also that the promoter region of the gene encoding StAR (steroidogenic acute regulatory protein) was hypomethylated in AAD patients. StAR is essential in steroid hormone biosynthesis as the transporter of cholesterol to the inner mitochondrial membrane (Papadopoulos and Miller, 2012). Another gene expressed in adrenocortical cells found to be hypomethylated in AAD patients is MC2R (melanocortin receptor 2), the receptor for adrenocorticotropic hormone (ACTH). Whether these findings represent an effect from long-term steroid supplementation in the AAD patients, or rather an intrinsic dysfunction of the adrenal cortex that predisposes for autoimmune adrenalitis remain an open question. CYP21, against which antibodies is the prime known marker for AAD, was not found to be differentially methylated in AAD patients.

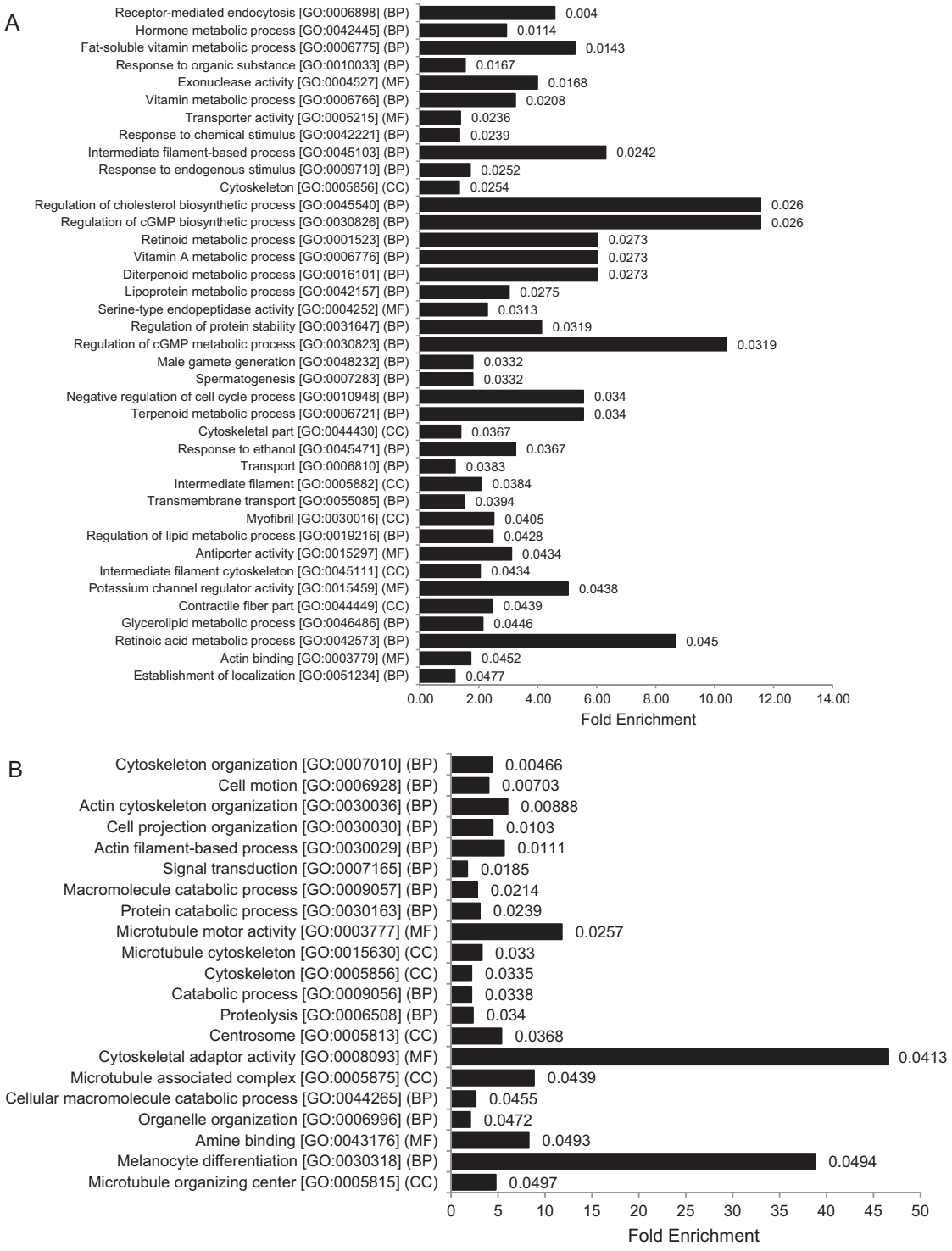

Fig. 3. (**a**) GO analyses of genes with hypomethylated DMRs in the proximal promoter region (−2000/+625). (**b**) GO analyses of genes with hypermethylated DMRs in the proximal promoter region (−2000/+625). GO categories: BP, biological processes; MF, molecular function; and CC, cellular compartments. The different GO terms are given to the left followed by the ID in brackets. The *p* values are given behind each bar.

3.3. Gene ontology and pathway analyses

We next performed a GO analysis on DMRs localized to the proximal promoter region (2000/+625) as described in Section 2.9. Analyses were carried out for all DMRs (supplementary figure S1), and separately for hypo- (Fig. 3a, see Supplementary Data, Table S3 for list of genes under each GO term) and hypermethylated DMRs (Fig. 3b, see Supplementary Data, Table S4 for list of genes under each GO term). Note that few hypermethylated DMRs were found in the 2000/+625 region (12%; 75/644).

The GO analysis revealed significant enrichment of DMRs within the categories *biological processes* (BP), *molecular function* (MF), and *cellular compartments* (CC). The enrichment of hypomethylated DMRs in the BP-category terms receptor-mediated endocytosis, transport activity, response to stimulus by organic, chemical and endogenous substances and hormone metabolic processes, as well

as in the MF-category term transporter activity (Fig. 3a), probably reflect the combination of steroid hormone supplemental therapy and the lack of endogenous steroid hormone production in the AAD patients. Central genes in several of these BP are present in our dataset. These include *APOE* (apolipoprotein E), *SCAP* (sterol regulatory element-binding protein cleavage-activating protein), and *ABCG1* (TP-binding cassette subfamily G member 1) that are crucial for regulation of cellular uptake and transmembrane traffic of cholesterol, the precursor of all steroid hormones (Mahley, 1988; Nakajima et al., 1999; Schmitz et al., 2001). Moreover, genes encoding proteins that are directly interacting with the glucocorticoid receptor and known to modulate the effects of glucocorticoid hormones (e.g., *PPARA*, encoding peroxisome proliferator-activated receptor alpha and *STAT5B*, encoding signal transducer and activator of transcription 5B; Bougarne et al., 2009; Wyszomierski et al., 1999) were found to be hypomethylated. It is well established that excessive amounts of endogenous or exogenous glucocorticoids may leave a transient and also stable epigenetic imprints (Khulan and Drake, 2012). As the AAD patients in this study have taken exogenous glucocorticoids on a daily basis for periods of 4–40 years, an impact on DNA methylation levels was somewhat expected. In fact, several hypomethylated DMRs identified in AAD patients in the present study, localize to genes previously described to be hypomethylated in chronic obstructive pulmonary disease patients treated with systemic steroids (Wan et al., 2012). These hits include genes encoding proteins with diverse biological functions (e.g., *SLC22A18* (solute carrier family 22 member 18), *SCNN1A* (amiloride-sensitive sodium channel subunit alpha), *DYRK4* (dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 4), *IRF7* (interferon regulatory factor 7), and *AIF1* (Allograft inflammatory factor 1, an interferon-γ inducible Ca^{2+} binding EF hand protein). Five related metabolic process terms within the BP-category were significantly enriched for hypomethylated DMRs (Fig. 3a). These terms, the terpenoid-, diterpenoid-, retinoid-, vitamin A-, and retinoic acid metabolic processes all consisted of the same four genes, namely *ALDH1A2* (retinaldehyde dehydrogenase 2), *ALDH8A1* (Aldehyde dehydrogenase 8 family, member A1), *CYP26C1* (cytochrome P450, family 26, subfamily c, polypeptide 1), and *RPE65* (retinal pigment epithelium-specific 65 kDa protein). This is an interesting observation in light of recent discoveries regarding the proinflammatory and immunoregulatory roles of retinoic acid and vitamin A (Hall et al., 2011).

Within the MF-category, we found enrichment of hypomethylated DMRs in the term exonuclease activity, including genes like *APTX* (aprataxin) and *RAD9* (RAD9), which are both involved in DNA damage control (Niida and Nakanishi, 2006; Sperka et al., 2012; Fig. 3a). This could be related to the increased extent of genomic DNA damage and killing of lymphocytes by reactive oxygen intermediates in many autoimmune diseases (Bashir et al., 1993). A second MF-term that showed enrichment of hypomethylated DMRs was serine-type endopeptidase activity, including genes like *FURIN* (FURIN) and *KLK6* (Kallikrein-6; Fig. 3a). These factors are involved in cleavage of viral proteins and cytokine precursors, and promotion of lymphocyte survival, respectively (Scarisbrick et al., 2011; Tay et al., 2012). The CC-category revealed significant enrichment of both hypo- and hypermethylated DMRs in genes connected to the cytoskeleton (Fig. 3a and b), and related terms such as microtubule cytoskeleton and intermediate filaments (Fig. 3b). We propose that this might reflect the activation and polarization of T cells and lymphocytes that take place in a T cell mediated autoimmune disease such as AAD (Filbert et al., 2012).

To determine the potential enrichment of pathways in our dataset, we used the KEGG functional annotation tool in DAVID (as described in Section 2.9). Although a substantial number of the genes containing hypomethylated DMRs in their proximal promoter regions are involved in immunological pathways, none of these pathways were enriched. The only significantly enriched pathways were found for hypermethylated DMRs and included melanogenesis and axon guidance (Table 1). Since the number of genes with hypermethylated DMRs in each pathway was only three, and since the *p* values approached the borderline of significance, this finding should be interpreted with caution. However, the enrichment of hits within the melanogenesis pathway is noteworthy since one of the cardinal signs of AAD is hyperpigmentation of the skin due to excessive production of ACTH (Bratland and Husebye, 2011).

In the present study, we utilized DNA isolated from CD4+ T cells as the starting material to identify DMRs. This pool consists of different subpopulations of cells, and in the body, the relative abundance of each subtype will depend on various signals present in their milieu. In fact, the distribution of CD4+ T cell subtypes is frequently altered in autoimmune diseases (Buckner, 2010). In AAD, an increased percentage of activated T cells is observed in patients with recent onset of disease, and moreover, a decreased percentage of suppressive T_{REGS} is evident (Coles et al., 2005; Rabinowe et al., 1984). This characteristic of autoimmune diseases, together with the fact that the DNA methylome differs between CD4+ T subtypes (Lee et al., 2009) can potentially confound our results. For example, in our dataset, DMRs were localized to the promoter regions of *LIF* (leukemia inhibitory factor), *PIAS3* (E3 SUMO-protein ligase PIAS3), and *RORC* (retinoic acid receptor (RAR)-related orphan receptor C). It has previously been demonstrated that these genes can be regulated by methylation, and moreover these genes are differentially expressed in CD4+ T cell populations (Bixler et al., 2013; Cao et al., 2011; Kluge et al., 2011; Mycko et al., 2012; Santarlasci et al., 2012; Shin et al., 2011; Thomas et al., 2012). Although the limited number of cells hampered experiments on individual CD4+ T cell subpopulations at this time, such experiments should be possible in the coming years as the DNA methylation techniques advance and allow satisfactory analyses on few cells. It is already possible to separate most known subpopulations based on cell-specific markers (Bendall et al., 2011). Another limitation is that the assay does not provide information about whether the identified DMRs are causes or consequences of the disease. Moreover, as indicated by the GO-analyses (discussed in Section 3.3), it is possible that the abnormal methylation pattern in AAD patients has developed partly as a result of medication. However, such changes are also valuable to monitor as they may hint to genomic regions and cellular pathways that are important to take into account for upcoming proposals for improved patient treatment and care. Future studies should be designed to determine when and where differences in methylation status arise. Important tools for such analyses will be cohorts of individuals that have not yet developed AAD, but have CYP21-Ab, and animal models that allow cell tracking in combination with DNA methylation analyses.

Table 1
Pathway analyses (by the KEGG functional annotation tool in DAVID) on genes with hypermethylated DMRs in the proximal promoter region (−2000/+625).

Pathway ID (KEGG)	Pathway term	Genes	Fold enrichment	*p* value
hsa04916	Melanogenesis	EDNRB, MITF, KRAS	7.337662338	0.05679425
hsa04360	Axon guidance	NCK2, KRAS, PLXNC1	5.631229236	0.09016057

4. Conclusion

Dysregulation of epigenetic processes is an essential contributing factor in the development of autoimmune diseases, underscoring the importance in identifying and mapping epigenetic alterations in patients. Our results suggest that a number of genes involved in immune regulation and autoimmunity carry abnormal DNA methylation signatures in a cohort of AAD patients. It remains to be determined whether these epigenetic changes has arisen as a consequence of the disease and/or medical treatment, or whether they are contributing to disease development. Nevertheless, this report can serve as a foundation to reveal the molecular mechanisms underlying AAD.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2014.02.018.

References

Aggarwal, B.B., 2003. Signalling pathways of the TNF superfamily: a double-edged sword. Nat. Rev. Immunol. 3, 745–756.

Aran, D., Toperoff, G., Rosenberg, M., Hellman, A., 2011. Replication timing-related and gene body-specific methylation of active human genes. Hum. Mol. Genet. 20, 670–680.

Arlt, W., Allolio, B., 2003. Adrenal insufficiency. Lancet 361, 1881–1893.

Bashir, S., Harris, G., Denman, M.A., Blake, D.R., Winyard, P.G., 1993. Oxidative DNA damage and cellular sensitivity to oxidative stress in human autoimmune diseases. Ann. Rheum. Dis. 52, 659–666.

Bendall, S.C., Simonds, E.F., Qiu, P., Amir el, A.D., Krutzik, P.O., Finck, R., Bruggner, R.V., Melamed, R., Trejo, A., Ornatsky, O.I., Balderas, R.S., Plevritis, S.K., Sachs, K., Pe'er, D., Tanner, S.D., Nolan, G.P., 2011. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. Science 332, 687–696.

Betterle, C., Zanchetta, R., 2003. Update on autoimmune polyendocrine syndromes (APS). Acta Biomed. 74, 9–33.

Betterle, C., Dal Pra, C., Mantero, F., Zanchetta, R., 2002. Autoimmune adrenal insufficiency and autoimmune polyendocrine syndromes: autoantibodies, autoantigens, and their applicability in diagnosis and disease prediction. Endocr. Rev. 23, 327–364.

Bixler, S.L., Sandler, N.G., Douek, D.C., Mattapallil, J.J., 2013. Suppressed Th17 levels correlate with elevated PIAS3, SHP2, and SOCS3 expression in CD4 T cells during acute simian immunodeficiency virus infection. J. Virol. 87, 7093–7101.

Blomhoff, A., Lie, B.A., Myhre, A.G., Kemp, E.H., Weetman, A.P., Akselsen, H.E., Huseby, E.S., Undlien, D.E., 2004. Polymorphisms in the cytotoxic T lymphocyte antigen-4 gene region confer susceptibility to Addison's disease. J. Clin. Endocrinol. Metab. 89, 3474–3476.

Bougarne, N., Paumelle, R., Caron, S., Hennuyer, N., Mansouri, R., Gervois, P., Staels, B., Haegeman, G., De Bosscher, K., 2009. PPARalpha blocks glucocorticoid receptor alpha-mediated transactivation but cooperates with the activated glucocorticoid receptor alpha for transrepression on NF-kappaB. Proc. Natl. Acad. Sci. USA 106, 7397–7402.

Bratland, E., Husebye, E.S., 2011. Cellular immunity and immunopathology in autoimmune Addison's disease. Mol. Cell Endocrinol. 336, 180–190.

Bratland, E., Skinningsrud, B., Undlien, D.E., Mozes, E., Husebye, E.S., 2009. T cell responses to steroid cytochrome P450 21-hydroxylase in patients with autoimmune primary adrenal insufficiency. J. Clin. Endocrinol. Metab. 94, 5117–5124.

Bratland, E., Hellesen, A., Husebye, E.S., 2013. Induction of CXCL10 chemokine in adrenocortical cells by stimulation through toll-like receptor 3. Mol. Cell Endocrinol. 365, 75–83.

Buckner, J.H., 2010. Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases. Nat. Rev. Immunol. 10, 849–859.

Cao, W., Yang, Y., Wang, Z., Liu, A., Fang, L., Wu, F., Hong, J., Shi, Y., Leung, S., Dong, C., Zhang, J.Z., 2011. Leukemia inhibitory factor inhibits T helper 17 cell differentiation and confers treatment effects of neural progenitor cell therapy in autoimmune disease. Immunity 35, 273–284.

Chehadeh, W., Weill, J., Vantyghem, M.C., Alm, G., Lefebvre, J., Wattre, P., Hober, D., 2000. Increased level of interferon-alpha in blood of patients with insulin-dependent diabetes mellitus: relationship with coxsackievirus B infection. J. Infect. Dis. 181, 1929–1939.

Coit, P., Jeffries, M., Altorok, N., Dozmorov, M.G., Koelsch, K.A., Wren, J.D., Merrill, J.T., McCune, W.J., Sawalha, A.H., 2013. Genome-wide DNA methylation study suggests epigenetic accessibility and transcriptional poising of interferon-regulated genes in naive CD4+ T cells from lupus patients. J. Autoimmun. 43, 78–84.

Coles, A.J., Thompson, S., Cox, A.L., Curran, S., Gurnell, E.M., Chatterjee, V.K., 2005. Dehydroepiandrosterone replacement in patients with Addison's disease has a bimodal effect on regulatory (CD4 + CD25hi and CD4 + FoxP3 +) T cells. Eur. J. Immunol. 35, 3694–3703.

Costenbader, K.H., Gay, S., Alarcon-Riquelme, M.E., Iaccarino, L., Doria, A., 2012. Genes, epigenetic regulation and environmental factors: which is the most relevant in developing autoimmune diseases? Autoimmun. Rev. 11, 604–609.

Cuenda, A., Rousseau, S., 2007. p38 MAP-kinases pathway regulation, function and role in human diseases. Biochim. Biophys. Acta 1773, 1358–1375.

Deaton, A.M., Bird, A., 2011. CpG islands and the regulation of transcription. Genes Dev. 25, 1010–1022.

Dodeller, F., Skapenko, A., Kalden, J.R., Lipsky, P.E., Schulze-Koops, H., 2005. The p38 mitogen-activated protein kinase regulates effector functions of primary human CD4 T cells. Eur. J. Immunol. 35, 3631–3642.

Dong, C., Davis, R.J., Flavell, R.A., 2002. MAP kinases in the immune response. Annu. Rev. Immunol. 20, 55–72.

Dong, R., Zhao, R., Zheng, S., 2011. Changes in epigenetic regulation of CD4+ T lymphocytesin biliary atresia. Pediatr. Res. 70, 555–559.

Eisenbarth, G.S., Gottlieb, P.A., 2004. Autoimmune polyendocrine syndromes. N. Engl. J. Med. 350, 2068–2079.

Ekman, B., Alstrand, N., Bachrach-Lindstrom, M., Jenmalm, M.C., Wahlberg, J., 2013. Altered chemokine Th1/Th2 balance in Addison's disease: Relationship with hydrocortisone dosing and quality of life. Horm. Metab. Res..

Erichsen, M.M., Lovas, K., Skinningsrud, B., Wolff, A.B., Undlien, D.E., Svartberg, J., Fougner, K.J., Berg, T.J., Bollerslev, J., Mella, B., Carlson, J.A., Erlich, H., Husebye, E.S., 2009. Clinical, immunological, and genetic features of autoimmune primary adrenal insufficiency: observations from a Norwegian registry. J. Clin. Endocrinol. Metab. 94, 4882–4890.

Filbert, E.L., Le Borgne, M., Lin, J., Heuser, J.E., Shaw, A.S., 2012. Stathmin regulates microtubule dynamics and microtubule organizing center polarization in activated T cells. J. Immunol. 188, 5421–5427.

Franchi, L., Munoz-Planillo, R., Nunez, G., 2012. Sensing and reacting to microbes through the inflammasomes. Nat. Immunol. 13, 325–332.

Ghaderi, M., Gambelunghe, G., Tortoioli, C., Brozzetti, A., Jatta, K., Gharizadeh, B., De Bellis, A., Pecori Giraldi, F., Terzolo, M., Betterle, C., Falorni, A., 2006. MHC2TA single nucleotide polymorphism and genetic risk for autoimmune adrenal insufficiency. J. Clin. Endocrinol. Metab. 91, 4107–4111.

Gombos, Z., Hermann, R., Kiviniemi, M., Nejentsev, S., Reimand, K., Fadeyev, V., Peterson, P., Uibo, R., Ilonen, J., 2007. Analysis of extended human leukocyte antigen haplotype association with Addison's disease in three populations. Eur. J. Endocrinol. 157, 757–761.

Gonzalez, S., Aguilera, S., Urzua, U., Quest, A.F., Molina, C., Alliende, C., Hermoso, M., Gonzalez, M.J., 2011. Mechanotransduction and epigenetic control in autoimmune diseases. Autoimmun. Rev. 10, 175–179.

Hall, J.A., Grainger, J.R., Spencer, S.P., Belkaid, Y., 2011. The role of retinoic acid in tolerance and immunity. Immunity 35, 13–22.

Hansen, K.D., Timp, W., Bravo, H.C., Sabunciyan, S., Langmead, B., McDonald, O.G., Wen, B., Wu, H., Liu, Y., Diep, D., Briem, E., Zhang, K., Irizarry, R.A., Feinberg, A.P., 2011. Increased methylation variation in epigenetic domains across cancer types. Nat. Genet. 43, 768–775.

Hawkins, R.D., Hon, G.C., Lee, L.K., Ngo, Q., Lister, R., Pelizzola, M., Edsall, L.E., Kuan, S., Luu, Y., Klugman, S., Antosiewicz-Bourget, J., Ye, Z., Espinoza, C., Agarwahl, S., Shen, L., Ruotti, V., Wang, W., Stewart, R., Thomson, J.A., Ecker, J.R., Ren, B., 2010. Distinct epigenomic landscapes of pluripotent and lineage-committed human cells. Cell. Stem. Cell 6, 479–491.

Hon, G.C., Hawkins, R.D., Caballero, O.L., Lo, C., Lister, R., Pelizzola, M., Valsesia, A., Ye, Z., Kuan, S., Edsall, L.E., Camargo, A.A., Stevenson, B.J., Ecker, J.R., Bafna, V., Strausberg, R.L., Simpson, A.J., Ren, B., 2012. Global DNA hypomethylation coupled to repressive chromatin domain formation and gene silencing in breast cancer. Genome Res. 22, 246–258.

Huang da, W., Sherman, B.T., Lempicki, R.A., 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57.

Husebye, E., Lovas, K., 2009. Pathogenesis of primary adrenal insufficiency. Best Pract. Res. Clin. Endocrinol. Metab. 23, 147–157.

Irizarry, R.A., Ladd-Acosta, C., Wen, B., Wu, Z., Montano, C., Onyango, P., Cui, H., Gabo, K., Rongione, M., Webster, M., Ji, H., Potash, J.B., Sabunciyan, S., Feinberg, A.P., 2009. The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat. Genet. 41, 178–186.

Javierre, B.M., Hernando, H., Ballestar, E., 2011. Environmental triggers and epigenetic deregulation in autoimmune disease. Discov. Med. 12, 535–545.

Jin, Y., Mailloux, C.M., Gowan, K., Riccardi, S.L., LaBerge, G., Bennett, D.C., Fain, P.R., Spritz, R.A., 2007. NALP1 in vitiligo-associated multiple autoimmune disease. N. Engl. J. Med. 356, 1216–1225.

Jin, B., Li, Y., Robertson, K.D., 2011. DNA methylation: superior or subordinate in the epigenetic hierarchy? Genes Cancer 2, 607–617.

Kastbom, A., Verma, D., Eriksson, P., Skogh, T., Wingren, G., Soderkvist, P., 2008. Genetic variation in proteins of the cryopyrin inflammasome influences susceptibility and severity of rheumatoid arthritis (the Swedish TIRA project). Rheumatology (Oxford) 47, 415–417.

Khulan, B., Drake, A.J., 2012. Glucocorticoids as mediators of developmental programming effects. Best Pract. Res. Clin. Endocrinol. Metab. 26, 689–700.

Kluge, A., Dabir, S., Vlassenbroeck, I., Eisenberg, R., Dowlati, A., 2011. Protein inhibitor of activated STAT3 expression in lung cancer. Mol. Oncol. 5, 256–264.

Knoechel, B., Lohr, J.G., 2013. Genomics of lymphoid malignancies reveal major activation pathways in lymphocytes. J. Autoimmun. 45, 15–23.

Kont, V., Murumagi, A., Tykocinski, L.O., Kinkel, S.A., Webster, K.E., Kisand, K., Tserel, L., Pihlap, M., Strobel, P., Scott, H.S., Marx, A., Kyewski, B., Peterson, P., 2011. DNA methylation signatures of the AIRE promoter in thymic epithelial cells, thymomas and normal tissues. Mol. Immunol. 49, 518–526.
Krysiak, R., Boldys, A., Okopien, B., 2011. Autoimmune polyglandular syndrome type 2 induced by treatment with interferon alpha. Am. J. Med. Sci. 341, 504–507.
Lal, G., Zhang, N., van der Touw, W., Ding, Y., Ju, W., Bottinger, E.P., Reid, S.P., Levy, D.E., Bromberg, J.S., 2009. Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. J. Immunol. 182, 259–273.
Laureti, S., Vecchi, L., Santeusanio, F., Falorni, A., 1999. Is the prevalence of Addison's disease underestimated? J. Clin. Endocrinol. Metab. 84, 1762.
Lee, C.G., Sahoo, A., Im, S.H., 2009. Epigenetic regulation of cytokine gene expression in T lymphocytes. Yonsei. Med. J. 50, 322–330.
Lei, W., Luo, Y., Lei, W., Luo, Y., Yan, K., Zhao, S., Li, Y., Qiu, X., Zhou, Y., Long, H., Zhao, M., Liang, Y., Su, Y., Lu, Q., 2009. Abnormal DNA methylation in CD4+ T cells from patients with systemic lupus erythematosus, systemic sclerosis, and dermatomyositis. Scand. J. Rheumatol. 38, 369–374.
Lovas, K., Husebye, E.S., 2002. High prevalence and increasing incidence of Addison's disease in western Norway. Clin. Endocrinol. (Oxf) 56, 787–791.
Lu, Q., 2013. The critical importance of epigenetics in autoimmunity. J. Autoimmun. 41, 1–5.
Lupfer, C., Kanneganti, T.D., 2013. Unsolved Mysteries in NLR Biology. Front Immunol. 4, 285.
Maclaren, N.K., Riley, W.J., 1986. Inherited susceptibility to autoimmune Addison's disease is linked to human leukocyte antigens-DR3 and/or DR4, except when associated with type I autoimmune polyglandular syndrome. J. Clin. Endocrinol. Metab. 62, 455–459.
Magitta, N.F., Boe Wolff, A.S., Johansson, S., Skinningsrud, B., Lie, B.A., Myhr, K.M., Undlien, D.E., Joner, G., Njolstad, P.R., Kvien, T.K., Forre, O., Knappskog, P.M., Husebye, E.S., 2009. A coding polymorphism in NALP1 confers risk for autoimmune Addison's disease and type 1 diabetes. Genes Immun. 10, 120–124.
Mahley, R.W., 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. Science 240, 622–630.
Mavragani, C.P., Niewold, T.B., Chatzigeorgiou, A., Danielides, S., Thomas, D., Kirou, K.A., Kamper, E., Kaltsas, G., Crow, M.K., 2013. Increased serum type I interferon activity in organ-specific autoimmune disorders: clinical, imaging, and serological associations. Front Immunol. 4, 238.
Mavropoulos, A., Orfanidou, T., Liaskos, C., Smyk, D.S., Billinis, C., Blank, M., Rigopoulou, E.I., Bogdanos, D.P., 2013. p38 mitogen-activated protein kinase (p38 MAPK)-mediated autoimmunity: lessons to learn from ANCA vasculitis and pemphigus vulgaris. Autoimmun. Rev. 12, 580–590.
Michels, A.W., Gottlieb, P.A., 2010. Autoimmune polyglandular syndromes. Nat. Rev. Endocrinol. 6, 270–277.
Mitchell, A.L., Cordell, H.J., Soemedi, R., Owen, K., Skinningsrud, B., Wolff, A.B., Ericksen, M., Undlien, D., Husebye, E., Pearce, S.H., 2009. Programmed death ligand 1 (PD-L1) gene variants contribute to autoimmune Addison's disease and Graves' disease susceptibility. J. Clin. Endocrinol. Metab. 94, 5139–5145.
Mycko, M.P., Cichalewska, M., Machlanska, A., Cwiklinska, H., Mariasiewicz, M., Selmaj, K.W., 2012. MicroRNA-301a regulation of a T-helper 17 immune response controls autoimmune demyelination. Proc. Natl. Acad. Sci. USA 109, E1248–E1257.
Myhre, A.G., Undlien, D.E., Lovas, K., Uhlving, S., Nedrebo, B.G., Fougner, K.J., Trovik, T., Sorheim, J.I., Husebye, E.S., 2002. Autoimmune adrenocortical failure in Norway autoantibodies and human leukocyte antigen class II associations related to clinical features. J. Clin. Endocrinol. Metab. 87, 618–623.
Nakajima, T., Ota, N., Kodama, T., Emi, M., 1999. Isolation and radiation hybrid mapping of a highly polymorphic CA repeat sequence at the SREBP cleavage-activating protein (SCAP) locus. J. Hum. Genet. 44, 350–351.
Niida, H., Nakanishi, M., 2006. DNA damage checkpoints in mammals. Mutagenesis 21, 3–9.
Oshimoto, K., Shimizu, H., Sato, N., Mori, M., 1994. A case of Addison's disease which became worse during interferon therapy: insulin secretion under hyposmolarity. Nihon. Naibunpi. Gakkai. Zasshi. 70, 511–516.
Pani, M.A., Seissler, J., Usadel, K.H., Badenhoop, K., 2002. Vitamin D receptor genotype is associated with Addison's disease. Eur. J. Endocrinol. 147, 635–640.
Papadopoulos, V., Miller, W.L., 2012. Role of mitochondria in steroidogenesis. Best Pract. Res. Clin. Endocrinol. Metab. 26, 771–790.
Rabinowe, S.L., Jackson, R.A., Dluhy, R.G., Williams, G.H., 1984. Ia-positive T lymphocytes in recently diagnosed idiopathic Addison's disease. Am. J. Med. 77, 597–601.
Rotondi, M., Falorni, A., De Bellis, A., Laureti, S., Ferruzzi, P., Romagnani, P., Buonamano, A., Lazzeri, E., Crescioli, C., Mannelli, M., Santeusanio, F., Bellastella, A., Serio, M., 2005. Elevated serum interferon-gamma-inducible chemokine-10/CXC chemokine ligand-10 in autoimmune primary adrenal insufficiency and in vitro expression in human adrenal cells primary cultures after stimulation with proinflammatory cytokines. J. Clin. Endocrinol. Metab. 90, 2357–2363.
Rottembourg, D., Deal, C., Lambert, M., Mallone, R., Carel, J.C., Lacroix, A., Caillat-Zucman, S., le Deist, F., 2010. 21-Hydroxylase epitopes are targeted by CD8 T cells in autoimmune Addison's disease. J. Autoimmun. 35, 309–315.
Santarlasci, V., Maggi, L., Capone, M., Querci, V., Beltrame, L., Cavalieri, D., D'Aiuto, E., Cimaz, R., Nebbioso, A., Liotta, F., De Palma, R., Maggi, E., Cosmi, L., Romagnani, S., Annunziato, F., 2012. Rarity of human T helper 17 cells is due to retinoic acid orphan receptor-dependent mechanisms that limit their expansion. Immunity 36, 201–214.
Sawalha, A.H., 2008. Epigenetics and T-cell immunity. Autoimmunity 41, 245–252.
Scarisbrick, I.A., Epstein, B., Cloud, B.A., Yoon, H., Wu, J., Renner, D.N., Blaber, S.I., Blaber, M., Vandell, A.G., Bryson, A.L., 2011. Functional role of kallikrein 6 in regulating immune cell survival. PLoS One 6, e18376.
Schmitz, G., Langmann, T., Heimerl, S., 2001. Role of ABCG1 and other ABCG family members in lipid metabolism. J. Lipid Res. 42, 1513–1520.
Selmi, C., Lu, Q., Humble, M.C., 2012. Heritability versus the role of the environment in autoimmunity. J. Autoimmun. 39, 249–252.
Shapira, Y., Agmon-Levin, N., Shoenfeld, Y., 2010. Defining and analyzing geoepidemiology and human autoimmunity. J. Autoimmun. 34, J168–J177.
Shaw, M.H., Kamada, N., Warner, N., Kim, Y.G., Nunez, G., 2011. The ever-expanding function of NOD2: autophagy, viral recognition, and T cell activation. Trends Immunol. 32, 73–79.
Shin, J.E., Park, S.H., Jang, Y.K., 2011. Epigenetic up-regulation of leukemia inhibitory factor (LIF) gene during the progression to breast cancer. Mol. Cells 31, 181–189.
Skinningsrud, B., Husebye, E.S., Pearce, S.H., McDonald, D.O., Brandal, K., Wolff, A.B., Lovas, K., Egeland, T., Undlien, D.E., 2008. Polymorphisms in CLEC16A and CIITA at 16p13 are associated with primary adrenal insufficiency. J. Clin. Endocrinol. Metab. 93, 3310–3317.
Skinningsrud, B., Lie, B.A., Lavant, E., Carlson, J.A., Erlich, H., Akselsen, H.E., Gervin, K., Wolff, A.B., Erichsen, M.M., Lovas, K., Husebye, E.S., Undlien, D.E., 2011. Multiple loci in the HLA complex are associated with Addison's disease. J. Clin. Endocrinol. Metab. 96, E1703–E1708.
Sperka, T., Wang, J., Rudolph, K.L., 2012. DNA damage checkpoints in stem cells, ageing and cancer. Nat. Rev. Mol. Cell Biol. 13, 579–590.
Tay, F.P., Huang, M., Wang, L., Yamada, Y., Liu, D.X., 2012. Characterization of cellular furin content as a potential factor determining the susceptibility of cultured human and animal cells to coronavirus infectious bronchitis virus infection. Virology 433, 421–430.
Thomas, R.M., Sai, H., Wells, A.D., 2012. Conserved intergenic elements and DNA methylation cooperate to regulate transcription at the il17 locus. J. Biol. Chem. 287, 25049–25059.
Tran, H.A., Song, S., Lojewski, R.J., Reeves, G.E., 2008. Exacerbation of hepatitis C induced subclinical hypoadrenalism by Interferon-alpha2beta: A case report. Cases J. 1, 157.
Triolo, T.M., Baschal, E.E., Armstrong, T.K., Toews, C.S., Fain, P.R., Rewers, M.J., Yu, L., Miao, D., Eisenbarth, G.S., Gottlieb, P.A., Barker, J.M., 2009. Homozygosity of the polymorphism MICA5.1 identifies extreme risk of progression to overt adrenal insufficiency among 21-hydroxylase antibody-positive patients with type 1 diabetes. J. Clin. Endocrinol. Metab. 94, 4517–4523.
Waddell, S.J., Popper, S.J., Rubins, K.H., Griffiths, M.J., Brown, P.O., Levin, M., Relman, D.A., 2010. Dissecting interferon-induced transcriptional programs in human peripheral blood cells. PLoS One 5, e9753.
Wan, E.S., Qiu, W., Baccarelli, A., Carey, V.J., Bacherman, H., Rennard, S.I., Agusti, A., Anderson, W.H., Lomas, D.A., DeMeo, D.L., 2012. Systemic steroid exposure is associated with differential methylation in chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care. Med. 186, 1248–1255.
Wang, J., Lo, J.C., Foster, A., Yu, P., Chen, H.M., Wang, Y., Tamada, K., Chen, L., Fu, Y.X., 2001. The regulation of T cell homeostasis and autoimmunity by T cell-derived LIGHT. J. Clin. Invest. 108, 1771–1780.
Weber, M., Hellmann, I., Stadler, M.B., Ramos, L., Paabo, S., Rebhan, M., Schubeler, D., 2007. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat. Genet. 39, 457–466.
Wesche, B., Jaeckel, E., Trautwein, C., Wedemeyer, H., Falorni, A., Frank, H., von zur Muhlen, A., Manns, M.P., Brabant, G., 2001. Induction of autoantibodies to the adrenal cortex and pancreatic islet cells by interferon alpha therapy for chronic hepatitis C. Gut 48, 378–383.
Wing, K., Sakaguchi, S., 2010. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nat. Immunol. 11, 7–13.
Wyszomierski, S.L., Yeh, J., Rosen, J.M., 1999. Glucocorticoid receptor/signal transducer and activator of transcription 5 (STAT5) interactions enhance STAT5 activation by prolonging STAT5 DNA binding and tyrosine phosphorylation. Mol. Endocrinol. 13, 330–343.
Youinou, P., Pers, J.O., Gershwin, M.E., Shoenfeld, Y., 2010. Geo-epidemiology and autoimmunity. J. Autoimmun. 34, J163–J167.
Yu, L., Brewer, K.W., Gates, S., Wu, A., Wang, T., Babu, S.R., Gottlieb, P.A., Freed, B.M., Noble, J., Erlich, H.A., Rewers, M.J., Eisenbarth, G.S., 1999. DRB1*04 and DQ alleles: expression of 21-hydroxylase autoantibodies and risk of progression to Addison's disease. J. Clin. Endocrinol. Metab. 84, 328–335.
Ziller, M.J., Gu, H., Muller, F., Donaghey, J., Tsai, L.T., Kohlbacher, O., De Jager, P.L., Rosen, E.D., Bennett, D.A., Bernstein, B.E., Gnirke, A., Meissner, A., 2013. Charting a dynamic DNA methylation landscape of the human genome. Nature 500, 477–481.
Zurawek, M., Fichna, M., Januszkiewicz-Lewandowska, D., Gryczynska, M., Fichna, P., Nowak, J., 2010. A coding variant in NLRP1 is associated with autoimmune Addison's disease. Hum. Immunol. 71, 530–534.