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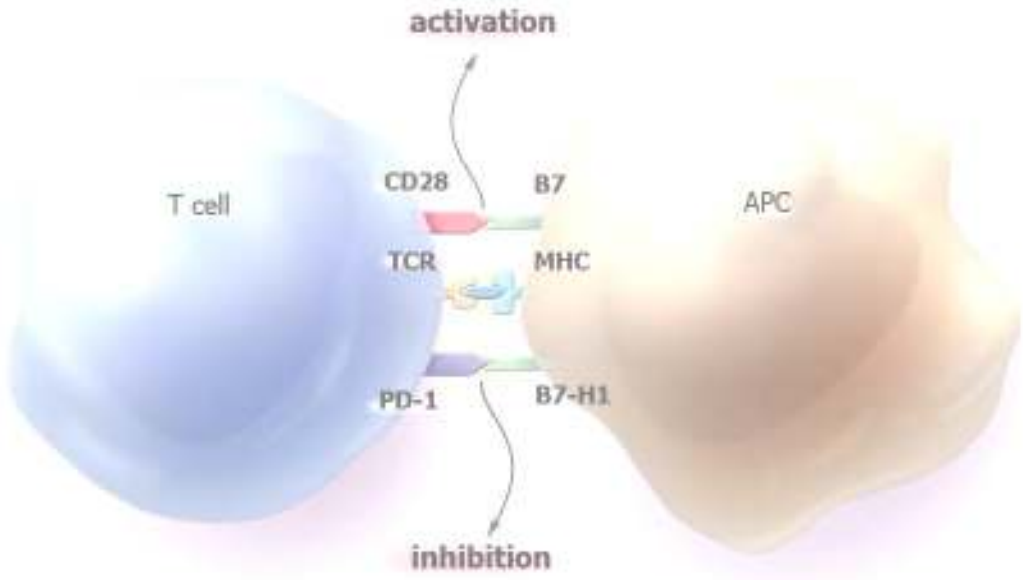
Expression of programmed death-1 (PD-1) and PD-1 ligands in human and murine tissue and their role in immunological tolerance



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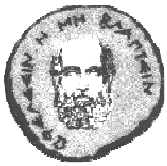
Expression of programmed death-1 (PD-1) and PD-1 ligands in human and murine tissue and their role in immunological tolerance

Genetic, immunologic, and immunohistochemical analysis of the PD-1/PD-L1 pathway in human systemic lupus erythematosus

George K. Bertsias, MD



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CONTENTS

1 ABSTRACT

The immune system has evolved to protect the body from foreign invading pathogens. To accomplish this critical role, T lymphocytes must discriminate between self and non-self; this property translates into immune recognition and elimination of infectious invaders while leaving host tissues intact. This highly selective process occurs through several complicated mechanisms. The first step is the elimination of self-reactive cells during T cell development in the thymus, aiming at a T cell repertoire that is 'self-tolerant'. However, this process is incomplete and autoreactive T cells that escape 'central' tolerance are controlled by peripheral mechanisms ('peripheral' tolerance). Autoimmunity results when either central or peripheral mechanisms fail.

Among the mechanisms involved in safeguarding of self-tolerance, B7/CD28 membrane receptors are crucial for fine-tuning of T cell function. Programmed death-1 (PD-1) is an inhibitory lymphocyte receptor that has recently emerged as a key player in induction and maintenance of tolerance. Compared to other B7 receptors, PD-1 has a broader role in regulation of immune responses considering its expression on activated T- and B-lymphocytes, the wide expression pattern of its ligands (PD-L1, PD-L2) on both lymphoid and non-lymphoid parenchymal cells, and its distinct mechanism of action. The role of PD-1 in tolerance is further highlighted by findings in PD-1-deficient mice which develop strain-specific autoimmune phenotypes, and in chronic viral infections which correlate with upregulation of PD-1 on virus-specific 'exhausted' T cells. In humans, a possible role for PD-1 in immune regulation is indicated by genetic-association studies which have shown certain polymorphisms of the PD-1 gene to confer increased risk for various autoimmune diseases. We sought to examine the role of PD-1 and PD-1 ligands in regulation of T cell function in systemic lupus erythematosus (SLE), the prototype of autoimmune disorder. SLE is characterized by hyper-active T cells and aberrant T cell-mediated autoantibody production and end-organ tissue injury.

We first performed PCR-based restriction fragment length analysis in 289 SLE patients and 256 matched healthy controls and confirmed the previously reported association with the PD1.3 (+7146G/A) single nucleotide polymorphism (SNP) (risk A allele associated with odds ratio 2.23 [95% confidence interval 1.55–3.38] for SLE). PD1.3 resides in an enhancer-like domain in intron 4 of the PD-1 gene, and the A allele has been shown to disrupt a RUNX1 binding site. To address the putative regulatory effects of PD1.3 SNP in PD-1 transcription, we performed transient transfection experiments in Jurkat T cells with reporter constructs expressing the firefly luciferase gene under the control of the SLE-associated A allele or the wild-type G allele of PD1.3. Over-expression of RUNX1 resulted in increased luciferase activity that was significantly higher with the G allele than with the A allele (by 36 ± 4%), indicating that PD1.3A may alter PD-1 expression. Indeed, two SLE patients homozygous for PD1.3 (A/A) had diminished PD-1 expression, assessed by flow cytometry, on peripheral blood $CD4^+$ $CD25^+$, $CD4^+$ $CD69^+$ and $CD4^+$ HLA-DR^+ T cell subsets compared to heterozygous (G/A) and wild-type (G/G) SLE patients. PD1.3 A/A patients also had defective induction of PD-1 on activated $CD4^+$ T cells following activation with suboptimal –but not optimal– concentration of anti-CD3/anti-CD28 antibodies, associated with impaired PD-1–mediated suppression of T cell proliferation and IFN-γ production under suboptimal concentrations of PD-L1.Fc.

To further characterize the role of PD-1 in regulation of immune responses in the context of SLE, we performed autologous mixed lymphocyte reaction (AMRL) experiments, which is an ex vivo model of autoreactivity against apoptotic self-antigens. During AMLR, regulatory circuits suppress effector T cells, and only a small degree of cell proliferation ensues. Compared to healthy controls, SLE patients had defective induction of PD-1 on AMLR $CD4^+$ $CD25^+$ and $CD4^+$ $CD69^+$ T cells; abnormalities were more pronounced in patients carrying the PD1.3A polymorphism. In contrast, PD-L1 expression on AMLR $CD14^+$ monocytes was comparable between SLE patients and healthy controls. In accordance, blockade of PD-1/PD-L1 interactions with monoclonal antibodies caused significant increase in AMLR T cell proliferation in healthy controls but not in SLE patients.

Since PD-1 is involved in regulation of effector T cells at the site of inflammation through interaction with PD-L1-expressing activated parenchymal cells, we examined the expression of PD-1/PD-L1 in renal biopsy samples from patients with lupus nephritis by immunohistochemistry. Weak-to-moderate PD-1 staining was detected in the glomeruli in 8 of 13 samples (62%) from patients with lupus nephritis compared with 0 of 9 (0%) control samples; PD-1 expression correlated with CD3 T cell staining. PD-L1 was detected in the renal tubules of both SLE patients (10 of 15, 67%) and controls (5 of 9, 56%), indicating a potential role for PD-1/PD-L1 in regulation of local immune inflammatory responses in SLE patients.

Despite its expression in the peripheral blood and inflamed tissues of SLE patients, the PD-1/PD-L1 pathway could be adversely affected by the lupus inflammatory microenvironment. To test this hypothesis, purified $CD4^+$ T cells from healthy controls were cultured in serum from non-homologous controls or active SLE patients and the effect of PD-1 crosslinking on T cell proliferation was assessed. At suboptimal PD-1 activation, incubation with SLE serum resulted in decreased suppression of T cell proliferation compared with normal serum (6.7 ± 3.6% versus 16.3 ± 2.7%); optimal PD-L1.Fc concentrations were able to overcome this effect and resulted in profound SLE T cell inhibition.

In summary, this study provides circumstantial evidence for an important role for the inhibitory PD-1/PD-L1 pathway in regulation of T cell function in human SLE. Importantly, our experiments indicate aberrant expression and/or function of PD-1 in lupus as a result of both direct (PD1.3 SNP, AMLR) and indirect (inflammatory microenvironment) effects. The expression of PD-1/PD-L1 in the affected tissues and during AMLR suggests a role of this pathway in maintenance of peripheral T cell tolerance and supports its rationale for its manipulation as a novel therapeutic option in SLE.

ΠΕΡΙΛΗΨΗ

Το ανοσολογικό σύστημα των θηλαστικών έχει αναπτυχθεί με στόχο την προστασία του οργανισμού έναντι ξένων παθογόνων μικροοργανισμών. Προϋπόθεση για τη λειτουργία αυτή είναι η δυνατότητα που έχουν τα Τ λεμφοκύτταρα να διακρίνουν τα ξένα αντιγόνα από τα αντιγόνα εαυτού, να αναγνωρίζουν δηλαδή και να αντιμετωπίζουν τους λοιμώδεις μικροοργανισμούς χωρίς να προξενούν βλάβη στον ίδιο τον οργανισμό. Η ιδιότητα αυτή των λεμφοκυττάρων εξασφαλίζεται μέσω σειράς περίπλοκων μηχανισμών που ξεκινούν με τον περιορισμό των αυτοδραστικών Τ λεμφοκυττάρων στο θύμο αδένα. Ωστόσο, ο μηχανισμός αυτός της «κεντρικής» ανοσολογικής ανοχής είναι ατελής με συνέπεια ορισμένοι κλώνοι αυτοδραστικών λεμφοκυττάρων να περνούν στην περιφέρεια όπου και ελέγχονται πλέον από μηχανισμούς «περιφερικής» ανοσολογικής ανοχής. Τυχόν διαταραχή της ανοσολογικής ανοχής (κεντρικής ή περιφερικής) οδηγεί σε αυτοανοσία.

Μεταξύ των μηχανισμών περιφερικής ανοσολογικής ανοχής, οι υποδοχείς της κυτταρικής μεμβράνης B7/CD28 είναι σημαντικοί για τον έλεγχο της ενεργοποίησης των Τ λεμφοκυττάρων. Ειδικότερα ο υποδοχέας programmed death-1 (PD-1) αναστέλλει τις ανοσολογικές αποκρίσεις των λεμφοκυττάρων και έχει ιδιαίτερο ρόλο στη ρύθμιση της ανοσολογικής ανοχής λόγω κάποιων χαρακτηριστικών που τον διαφοροποιούν από άλλους B7 υποδοχείς, όπως: η έκφρασή του στα ενεργοποιημένα Τ- αλλά και Β- λεμφοκύτταρα, το ευρύ πρότυπο έκφρασης των υποδοχέων του (PD-L1, PD-L2) σε κύτταρα του ανοσοποιητικού συστήματος αλλά και σε ενεργοποιημένα ιστικά παρεγχυματικά κύτταρα, και ο ειδικός μηχανισμός δράσης. Ο ανοσορρυθμιστικός ρόλος του PD-1 αναδείχθηκε σε μελέτες ποντικών με γενετική απαλοιφή (knock-out) του γονιδίου του PD-1, οι οποίοι αναπτύσσουν ποικίλες αυτοάνοσες διαταραχές, όπως επίσης σε χρόνιες ιογενείς λοιμώξεις όπου διαπιστώνεται ενισχυμένη έκφραση του PD-1 σε αντιγονο-ειδικά Τ λεμφοκύτταρα που βρίσκονται σε ανενεργή («ανοσο-ανεπαρκή») κατάσταση. Στους ανθρώπους, ο πιθανός ρόλος του PD-1 στον έλεγχο των ανοσολογικών αποκρίσεων προέκυψε από μελέτες συσχέτισης πολυμορφισμών του γονιδίου του PD-1 με διάφορα αυτοάνοσα νοσήματα. Με βάση αυτές τις παρατηρήσεις, εξετάσαμε το ρόλο του PD-1 και των συνδετών του (PD-1 ligands) στην ρύθμιση της λειτουργίας των Τ λεμφοκυττάρων στο Συστηματικό Ερυθηματώδη Λύκο (ΣΕΛ), το πρότυπο αυτοάνοσης διαταραχής στον άνθρωπο. Στο ΣΕΛ παρατηρείται υπέρμετρη ενεργοποίηση των Τ λεμφοκυττάρων που καθοδηγούν την παραγωγή αυτοαντισωμάτων από τα Β λεμφοκύτταρα και προκαλούν ιστική βλάβη σε όργανα-στόχους.

Εξετάστηκαν 289 ασθενείς με ΣΕΛ και 256 υγιείς εθελοντές για την παρουσία του PD1.3 (+7146G/A) μονονουκλεοτιδικού πολυμορφισμού με τεχνική PCR και χρήση ειδικής νουκλεάσης περιορισμού (PCR-based restriction fragment length polymorphism analysis). Διαπιστώθηκε αυξημένη συχνότητα του πολυμορφικού αλληλίου Α στους ασθενείς με ΣΕΛ σε σχέση με τους υγιείς (σχετικός κίνδυνος 2.23, 95% διάστημα εμπιστοσύνης 1.55-3.38). Ο PD1.3 πολυμορφισμός εντοπίζεται σε μια πιθανή ρυθμιστική περιοχή στο intron 4 του γονιδίου PD-1 και το Α αλλήλιο καταργεί μια θέση πρόσδεσης του μεταγραφικού παράγοντα RUNX1 με πιθανή επίπτωση στη μεταγραφή του γονιδίου. Το ενδεχόμενο αυτό εξετάστηκε με πειράματα μόλυνσης (transfection) Jurkat Τ κυττάρων με πλασμιδιακές κατασκευές (constructs) που εκφράζουν το γονίδιο luciferase υπό τον έλεγχο του πολυμορφικού Α ή του άγριου τύπου G αλληλίου. Υπερέκφραση του RUNX1 προκάλεσε έκφραση του γονιδίου luciferase η οποία ήταν σημαντικά αυξημένη παρουσία του G σε σχέση με το πολυμορφικό Α αλλήλιο κατά 36 ± 4%, υποδεικνύοντας πιθανή τροποποίηση της έκφρασης του PD-1 παρουσία του PD1.3A πολυμορφισμού. Πειράματα κυτταρομετρίας ροής ανέδειξαν μηδαμινή έκφραση του PD-1 στην επιφάνεια ενεργοποιημένων $CD4^+$ $CD25^+$, $CD4^+$ $CD69^+$ και $CD4^+$ HLA-DR^+ Τ λεμφοκυττάρων σε 2 ασθενείς ΣΕΛ ομοζυγώτες για το Α αλλήλιο του PD1.3 (A/A), σε σχέση με ετεροζυγώτες (G/A) και άγριου τύπου (G/G) ασθενείς. Επιπλέον, οι PD1.3 A/A ασθενείς παρουσίαζαν μειωμένη έκφραση PD-1 σε $CD4^+$ Τ λεμφοκύτταρα μετά από ενεργοποίηση με χαμηλές –αλλά όχι αυξημένες– συγκεντρώσεις anti-CD3/anti-CD28 αντισωμάτων, και αντιστοίχως μειωμένη δράση του PD-1 ως προς την αναστολή του κυτταρικού πολλαπλασιασμού και της έκκρισης IFN-γ.

Για να μελετηθεί περαιτέρω ο ρόλος του PD-1 στις ανοσολογικές αποκρίσεις στο λύκο, πραγματοποιήθηκαν καλλιέργειες Τ λεμφοκυττάρων με αυτόλογα μη-Τ κύτταρα τα οποία είχαν ακτινοβοληθεί ώστε να προκληθεί μικρού βαθμού απόπτωση (autologous mixed lymphocyte rections, AMLR). Οι καλλιέργειες αυτές αποτελούν πειραματικό πρότυπο για τη μελέτη της αυτοδραστικότητας έναντι ιδίων αποπτωτικών αντιγόνων, και κινητοποιούν ρυθμιστικούς μηχανισμούς καταστολής της δράσης των Τ λεμφοκυττάρων. Διαπιστώθηκε μειωμένη έκφραση του PD-1 στα $CD4^+$ $CD25^+$ και $CD4^+$ $CD69^+$ Τ κύτταρα των αυτόλογων καλλιεργειών στους ασθενείς με ΣΕΛ σε σχέση με τους εθελοντές. Επιπλέον, ασθενείς με τον PD1.3A πολυμορφισμό είχαν σημαντικά μειωμένη έκφραση του PD-1. Αντιθέτως, η έκφραση του PD-L1 στα $CD14^+$ μονοκύτταρα/μακροφάγα δε διέφερε μεταξύ ασθενών και εθελοντών. Σε συμφωνία με τα αποτελέσματα αυτά, αποκλεισμός του PD-1/PD-L1 συστήματος με ειδικά μονοκλωνικά αντισώματα ενίσχυσε τον Τ κυτταρικό πολλαπλασιασμό στις καλλιέργειες από υγιείς εθελοντές αλλά όχι από ασθενείς με ΣΕΛ.

Επειδή το PD-1 συμμετέχει στη ρύθμιση των Τ λεμφοκυττάρων στο σημείο της φλεγμονής μέσω αλληλεπίδρασης με ενεργοποιημένα παρεγχυματικά κύτταρα που εκφράζουν PD-L1, θελήσαμε να μελετήσουμε με ανοσοϊστοχημεία την έκφραση του PD-1/PD-L1 σε βιοψίες νεφρού από ασθενείς με νεφρίτιδα του ΣΕΛ. Διαπιστώθηκε σπειραματική έκφραση του PD-1 σε 8 από τα 13 (62%) δείγματα νεφρίτιδας ΣΕΛ, σε σχέση με κανένα από τα 9 (0%) δείγματα υγιούς νεφρικού ιστού. Η έκφραση του PD-1 συσχετίστηκε με αυτή του Τ κυτταρικού δείκτη CD3. Έκφραση του PD-L1 ανιχνεύθηκε στα νεφρικά σωληνάρια ασθενών (10 από 15, 67%) και υγιών (5 από 9, 56%), υποδεικνύοντας συμμετοχή του PD-1/PD-L1 στη ρύθμιση των φλεγμονωδών Τ κυτταρικών αποκρίσεων σε ιστικό επίπεδο.

Παρά την έκφραση του PD-1 στο περιφερικό αίμα και τους προσβεβλημένους ιστούς (νεφροί) των ασθενών, η λειτουργία του ανοσορρυθμιστικού συστήματος PD-1/PD-L1 ενδεχομένως να τροποποιείται στο φλεγμονώδες περιβάλλον του λύκου. Το ενδεχόμενο αυτό εξετάστηκε με καλλιέργειες φυσιολογικών $CD4^+$ Τ λεμφοκυττάρων παρουσία ορού από υγιείς εθελοντές ή από ασθενείς με ενεργό ΣΕΛ. Η δράση του PD-1 μετά από ενεργοποίηση με την πρωτεΐνη PD-L1.Fc, εκτιμήθηκε μέσω του βαθμού αναστολής του Τ κυτταρικού πολλαπλασιασμού. Σε συνθήκες μερικής ενεργοποίησης, διαπιστώθηκε μειωμένη ανασταλτική δράση του PD-1 παρουσία ορού από ασθενείς με ΣΕΛ σε σχέση με υγιείς εθελοντές (μείωση πολλαπλασιασμού κατά 6.7 ± 3.6% έναντι 16.3 ± 2.7%). Ωστόσο, ενεργοποίηση του PD-1 με αυξημένες συγκεντρώσεις PD-L1.Fc προκάλεσε σημαντικού βαθμού αναστολή του Τ κυτταρικού πολλαπλασιασμού ακόμα και παρουσία ορού από ΣΕΛ.

Συμπερασματικά, τα ευρήματα της μελέτης αυτής υποστηρίζουν ένα σημαντικό ρόλο του ανασταλτικού συστήματος PD-1/PD-L1 στη ρύθμιση των Τ λεμφοκυττάρων στο ΣΕΛ. Τεκμηριώνεται διαταραχή της έκφρασης και λειτουργίας του PD-1 ως συνέπεια «άμεσων» (PD1.3 πολυμορφισμός, καλλιέργειες με αυτόλογα αποπτωτικά κύτταρα) και «έμμεσων» (φλεγμονώδες περιβάλλον) επιδράσεων στο λύκο. Η έκφραση του PD-1/PD-L1 σε πειραματικά πρότυπα αυτοδραστικότητας και σε προσβεβλημένους ανθρώπινους ιστούς υποδεικνύουν συμμετοχή του ανοσορρυθμιστικού αυτού συστήματος στην Τ ανοσολογική ανοχή με δυνατότητα παρέμβασης για θεραπευτικούς σκοπούς.

2 ABBREVIATIONS

AIRE	Autoimmunr regulator
AMLR	Autologous mixed lymphocyte reaction
APC	Antigen-presenting cell
APECED	Autoimmune polyendocrinopathy, candidiasis, ectodermal dystrophy
BAFF	B cell activator of the TNF family
BLyS	B-lymphocyte stimulator
BTLA	B and T lymphocyte attenuator
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
FasL	Fas ligand
FoxP3	Forkhead box P3
GWA	Genome-wide association
HAT	Histone acetyl-transferase
HDAC	Histone deacetylase
HRV	Herpes rhinovirus
HSV	Herpes simplex virus
HVEM	Herpes entry mediator
ICOS	Inducible costimulator
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked inheritance
IRF	IFN regulatory factor
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
mTEC	Medullary thymic epithelial cell
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor –κB
NOD	Non-obese diabetic (mouse)
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PD-L1/2	Programmed death-1 ligand ½
PI3K	Phosphoinositide-3 kinase
PKC	Protein kinase C
RA	Rheumatoid arthritis
RAG	Recombination activating gene
RTEC	Renal tubular epithelial cell
RUNX	Runt-related transcription factor
SHP	SH-2 domain containing tyrosine phosphatase
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
TCR	T cell receptor
TLR	Toll-like receptor
TRAIL	TNF-related apoptosis-inducing ligand
T_{REG}	Regulatory T cell

3 INTRODUCTION

3.1. Pathways for immunological self-tolerance. Central tolerance

The adaptive immune system mounts highly specific responses against pathologic agents which are encountered during the lifetime of a vertebrate. This is made possible by populations of T and B lymphocytes that express unique receptors generated by random rearrangements or mutations in the genes encoding them. The randomness of this process is dictated by the fact that the immune system cannot predict the exact pathogens that will affect the host. As a corollary, however, a large majority (20-50%) of receptors generated in this way will actually target determinants derived from the host's own genome; the activation and differentiation of lymphocytes bearing such receptors can result in autoimmune disease and therefore, must be avoided. This constitutes the issue of **immunological self-tolerance**, which is an essential feature of the immune system that works to protect tissue antigens from becoming targets of damaging immune responses.

The first step for maintenance of self-tolerance is the elimination of self-reactive cells during T-cell development in the thymus. The goal of **central tolerance** is to achieve a T-cell repertoire that is self-tolerant; this is done by purging any newly generated receptor that is capable of strongly engaging antigen in its environment (reviewed in [1-3]). At initial stage, T cell receptors (TCRs) are selected to recognize a composite ligand comprising antigen peptide fragments bound to major histocompatibility complex (MHC) molecules. These self-peptide/MHC composites are displayed on the surface of cortical thymic epithelial cells and $CD4^+CD8^+$ double positive thymocytes expressing TCRs that weakly bind these ligands are stimulated to survive (**positive selection**), whereas the majority of thymocytes whose receptors do not recognize self-MHC are permitted to die. A small proportion of self-reactive TCRs may also trigger an editing process during which the offending TCR α-chain is replaced or diluted with a second, less self-reactive α-chain.

The selected thymocytes move from the cortex toward the medulla and they further test their TCRs for self-reactivity. This occurs on medullary thymic epithelial cells (mTECs) and dendritic cells (DCs) which express T-cell costimulatory moleculres such as CD80 (B7.1) and CD86 (B7.2), the ligands for CD28 expressed on T cells. At this point, TCRs that bind strongly to self-peptide/MHC composites trigger the death of thymocytes (**negative selection**) [4]. The key function of mTECs in this process is due to expression of numerous tissue-specific antigens that are apparently not required for their direct function and are normally present only in specialized peripheral organs. To this end, expression of many tissue-specific antigens is directed by the autoimmune regulator (AIRE) protein, which possesses partially understood function of ubiquitin ligase and transcription factor [5, 6]. Patients with mutations in the AIRE gene develop autoimmune disorders in a variety of peripheral organs, collectively called APECED (autoimmune polyendocrinopathy, candidiasis, ectodermal dystrophy) syndrome, presumably due to inability of the thymus to delete organ-specific autoreactive T cells.

However, the aforementioned processes are unlikely to purge all possible self-reactive receptors generated during lymphocyte development. The **'incompleteness' of central tolerance** might be explained by the fact that some tissue-specific antigens and anticipating genes are expressed only later in life. Second, it may be difficult for the immune system to settle an affinity threshold of a TCR towards self antigens that might allow it to be functionally autoreactive in the mature state. Moreover, lymphocytes may change their sensitivity to an antigen at various stages during their mature life, with the possibility that a TCR with too low affinity to be negatively selected might still trigger auto-immunity in the periphery. Indeed, healthy individuals have been shown to harbor self-reactive T cells in the periphery and therefore, the immune system has developed mechanisms that deal with tolerance in the peripheral lymphoid organs (**peripheral tolerance**), providing the safety net to prevent autoimmunity.

3.2. Mechanisms of peripheral immune tolerance

Tolerance induction among mature T cells in the lymphoid periphery operates through many mechanisms and failure of these mechanisms may result to autoimmunity. First, autoreactive T cells may be prevented from encountering antigen in a context that could lead to cell activation (**immune ignorance**) (**FIGURE 3.1**). Organs such as the brain, gonads, and eye, are considered immune-privileged sites because foreign antigens that are placed within them do not provoke an immune response and often, tolerance is induced instead. These organs maintain stronger barriers to routine entry of lymphocytes than other non-lymphoid tissues [7]. One example is the blood-brain barrier where tight junctions between endothelial cells of the brain vasculature prevent the access of lymphocytes to the central nervous system; its importance in tolerance has been demonstrated in experimental autoimmune encephalomyelitis (EAE).

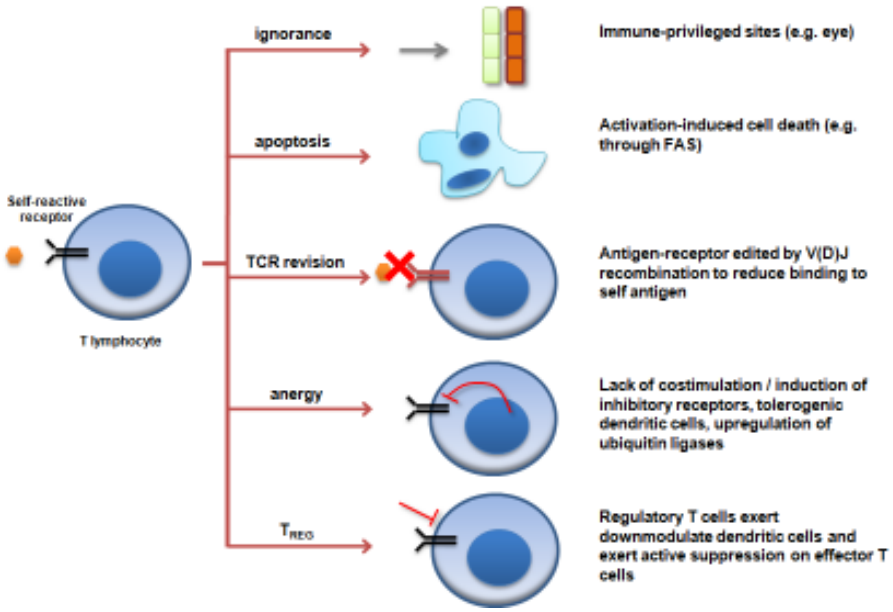


Figure 3.1. Mechanisms of peripheral T cell tolerance

A second mechanism to safeguard tolerance is though deletion of mature autoreactive T cells, which is achieved to a large extent though apoptotic cell death. After an initial phase of activation and differentiation to effector cells, most activated peripheral T cells die upon further encounter with antigen (**activation-induced cell death**) [3, 8, 9]. Apoptosis is prominently regulated by interactions between the Fas death receptor (CD95) and it ligand (FasL), as demonstrated by the lymphoproliferative and autoimmune disorders in Fas- or FasL-deficient mice. Surface expression of FasL is induced on activated T cells which may as well secrete soluble FasL in an auto/paracrine fashion. As a result, activated T cells have the potential of killing each other, a property that enables T cells to negatively regulate their own responses after initial waves of expansion. Apart from the Fas/FasL interactions, TNF- and TRAIL-mediated apoptosis may also operate in elimination of autoreactive T cells.

Another strategy to regulate self-reactive T cells is through revision of the TCR into one that is self-tolerant. Although TCR α- and β-gene rearrangements occur during T lymphocyte development in the thymus, it is now recognized that weak tolerogens and superantigens can drive peripheral T cells to re-express the proteins that mediate DNA recombination (RAG1 and RAG2) and to re-arrange and express diverse novel antigen receptor genes encoding TCRs that no longer recognize the tolerogen (**antigen receptor revision**) [10, 11]. This observation was first described in TCR Vβ5 transgenic mice but it may also occur in non-manipulated immune systems. However, TCR revision appears to be a risky proposition since re-expression of the recombinase machinery in mature lymphocytes offers the potential for aberrant juxtra-position of cellular oncogenes and lymphocyte-specific promoters. Whether such genomic instability increases the risk for cellular transformation and lymphomas is not known. A further danger in TCR revision is that the individual exposes itself to the

possibility of continued auto-aggression. Thus, it is not yet clear whether the revision process results to stringent selection against overt self-reactivity, as in the thymus during T cell maturation, or subsequent selection events are initiated to eliminate newly generated autoreactive T cells.

Optimal activation of T cells requires antigen-presenting cells (APCs) such as DCs, which have been activated to express costimulatory molecules that act in concert with MHC/peptide-TCR interactions. The most crucial set of costimulatory ligands are CD80/CD86, which engage the CD28 receptor expressed by T cells to augment cytokine gene expression, glucose uptake and utilization, cell survival, and responsiveness on subsequent re-stimulation (see SECTION 3.3 for details). TCR engagement in the absence of costimulation results in a hyporesponsive state termed anergy. **T-cell anergy** is thought to represent one mechanism of peripheral tolerance that maintains T cells in an 'off' state in response to self-antigens [1, 4]. Particularly relevant in this process is the maturation/activation status of DCs which determines the T-cell fate [12, 13]. Thus, maturation of DCs in the presence of pro-inflammatory cytokines and/or microbial 'patterns' to engage the innate immunity toll-like receptors (TLRs) results to enhanced expression of costimulatory molecules that promote robust T cell activation and immune response. In contrast, immature DCs activated in the absence of TLR signaling or in presence of IL-10 or TGF-β, acquire a 'tolerogenic' phenotype with low levels of expression of costimulatory molecules and suppress peripheral T cell responses or induce antigen-specific tolerance.

The **molecular pathways involved in induction of anergy** rather than full activation of T cells have been recently explored and provide insight for the better understanding of immune regulation mechanisms [5, 14, 15]. Specifically, anergic T cells are incapable of activating the Lck and ZAP-70 kinases, resulting in decreased phosphorylation of TCR-ε/ζ chains and impaired activation of Ras, ERK, and JNK MAP kinases. Instead, these cells have increased Fyn kinase activity, increased levels of free intracellular Ca^{+2}, PLC-γ1 phosphorylation, and phosphatidyl-inositol 1,4,5 triphosphate (**FIGURE 3.2**). Moreover, anergic T cells show enhanced activation of Rap1, another small GTP-binding protein of the Ras family, which inhibits IL-2 gene transcription. Ca^{+2} signaling is critical for anergy induction and this is mediated primarily by NFAT, a transcription factor regulated by the protein phosphatase calcineurin. During full T cell activation, NFAT proteins are de-phosphorylated and translocate to the nucleus where they cooperate with AP-1 to induce transcription of genes associated with T cell activation. In T cell unresponsiveness, a new set of NFAT-dependent genes (independent of NFAT-AP1 complexes) are induced, which function as negative regulators of TCR signaling and TCR-induced transcription. The anergy-associated genes include at least three E3 ubiquitin ligases, namely Itch, Cbl-b, and GRAIL, which interfere with TCR/CD28 and cytokine receptor signaling by promoting their degradation. Deletion or mutation of Itch and Cbl-b in mice is associated with disseminated autoimmune disease.

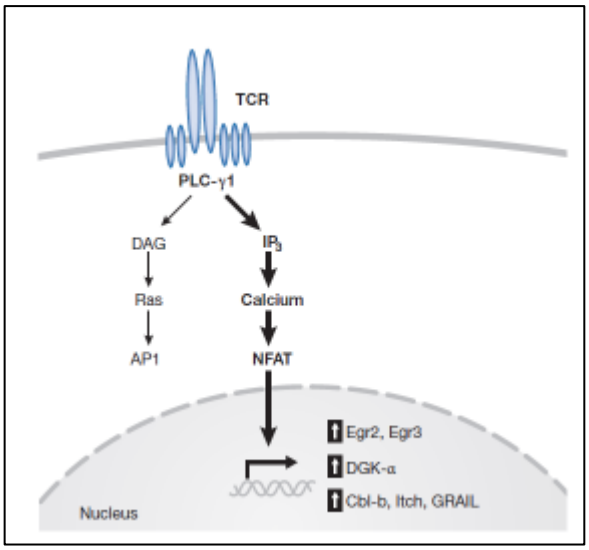


Figure 3.2. In the state of T cell anergy, Ca^{+2}-dependent activation NFAT pathways result in transcription of E3 ubiquitin ligases, namely Itch, Cbl-b, and GRAIL, which interfere with TCR/CD28 signaling.

A significant development in the field of tolerance was the reemergence of the concept that T cell reactivity is controlled by a distinct subset of T cells with regulatory function, named **regulatory T cells** (T_{REG}) [1, 16, 17]. The majority of these cells constitutively express CD25, the α chain of IL-2 receptor, and constitute 5-10% of $CD4^+$ T cells in rodents and 1-2% of $CD4^+$ T cells in humans. T_{REG} are produced in the thymus as a functionally mature subpopulation of T cells but can also be induced from naïve T cell in the periphery. Thymus-derived T_{REG} (also known as 'naturally-occurring' or 'natural' T_{REG}) may possess TCRs with high affinity for thymic MHC/self-peptide ligands and this could result from positive selection of highly self-reactive T cells to the T_{REG} lineage, or resistance of differentiated T cells to negative selection. In addition, the

intensity of the interaction between T cell accessory molecules and their ligands on thymic stromal cells contributes to the generation of T_{REG}. A cardinal feature of $CD4^+$ $CD25^+$ T_{REG} is the expression of the transcription factor FoxP3 (forkhead box P3), a member of the forkhead/winged-helix family of transcription factors. FoxP3 is a master regulator of T_{REG} development and function in humans and mice; forced expression of FoxP3 can convert naïve T cells to T_{REG} both phenotypically and functionally. Mutations within the FoxP3 gene cause a severe autoimmune syndrome known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance), which is a rare disease in male children characterized by autoimmune (type 1) diabetes, severe allergy, and inflammatory bowel disease. Studies have proposed a model in which FoxP3 overrides (or hijacks) the transcription machinery for effector T cells, thereby functionally converting them to T_{REG}. However, it is currently unknown the exact mechanism by which the FoxP3 complex containing the transcriptional factors NFAT and AML1/Runx1 (together with HATs and HDACs) controls the genes that mediate suppression.

Peripheral generation of T_{REG} from naïve T cells is driven when antigen stimulation occurs in the presence of TGF-β, IL-2 and IL-10 [16]. Not all types of peripheral T_{REG} express FoxP3 and it remains to be determined whether they are functionally stable in vivo. There are **multiple modes of T_{REG}-mediated suppression** that take place in a synergistic and sequential manner. Initially, antigen-activated T_{REG} are recruited to APCs and outcompete antigen-specific naïve T cells regarding interactions, especially with DCs. Then, T_{REG} modulate DC function (for instance, they promote the downregulation of DC costimulatory molecules), thereby hindering the activation of other T cells that are recruited to DCs. T_{REG} may then further differentiate to secrete granzyme/perforin, IL-10, and other immunosuppressive cytokines against other effector T cells. The fate of T cells that are suppressed by T_{REG} is unclear; that is, whether they remain inactivated, undergo apoptosis, or become anergic.

3.3. B7/CD28 family receptors in regulation of T cell function

Optimal T cell activation requires the engagement of TCR with a cognate MHC/peptide ligand (signal 1) and a second costimulatory signal (signal 2) mediated by other cell surface molecules. The most well characterized costimulatory-accessory molecules are those of the B7/CD28 family of membrane receptors, but others belonging to the TNF/TNF-receptor family have also been described. However, recently it has become apparent that these pathways not only provide critical 'positive' second signals that promote and sustain T cell responses, but they also contribute 'negative' signals that downregulate T cell function (reviewed in [18-23]). Moreover, the designation of these pathways as 'costimulatory' versus 'co-inhibitory' may not always be valid, as they may exert differential effects under certain circumstances in vitro and in vivo. Overall, the ultimate fate of T cells and in turn immune responses is determined by the interplay between positive and negative T cell costimulatory pathways (**FIGURE 3.3**).

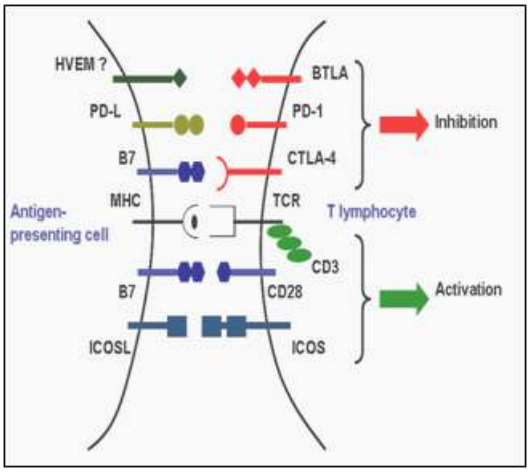


Figure 3.3. Regulation of T cell responses by positive (CD28, ICOS) and negative (PD-1, CTLA-4, BTLA) costimulatory receptors.

The prototype costimulatory pathway is the **CD28/CD80/CD86** pathway, where CD80 (B7.1) and CD86 (B7.2) expressed on APCs bind to CD28 receptor on T cells and promote enhanced T cell proliferation and secretion of cytokines, such as IL-2, TNF, and IFN-γ. CD86 is basally expressed on DCs, macrophages, and B cells and is further upregulated upon activation; CD80 is expressed only in activated APCs. The CD28 signaling pathway primary involves a small subset of molecules that are also implicated in TCR signal transmission, in particular the phosphoinositide-3 kinase (PI-3K) pathway with downstream activation of AKT,

and activation of TEC protein kinases and of IL-2-inducible T-cell kinases. AKT cooperates with the TCR-induced activation of protein kinase C (PKC)-θ to activate the NF-κB pathway. Activation of TEC is important for the regulation of PLC-γ1, contributing to intracellular Ca^{+2} signals and PKC activation. Therefore, CD28 functions to lower the TCR threshold to antigen-specific stimuli but also have a selective imprint on the transcriptional program induced by TCR signaling. In addition to activation of transcription factors, such as NF-κB, AP1, and NFAT, CD28 costimulation has a major impact on DNA demethylation and chromatin remodeling [24, 25]. Consisted with these findings, blocking the CD28/B7 pathway inhibits T cell-mediated immune responses and ameliorates autoimmune disease in several murine models.

However, CD80/CD86 can also attenuate T cell responses by binding to the **inhibitory receptor CTLA-4**. In contrast to CD28 which is constitutively expressed on resting T cells, CTLA-4 is rapidly upregulated following T cell activation and binds B7 molecules with a higher affinity than does CD28, therefore leading to the termination of the immune response. Apart from competing with the CD28/B7 pathway, CTLA-4 crosslinking mediates inhibitory signals through interaction with the serine/threonine phosphatase PP2A, which is a negative regulator for the MAPK/ERK/JNK pathway [26, 27]. CTLA-4 can also be found in membrane lipid rafts and is able to suppress raft aggregation mediated by TCR and CD28. The importance of CTLA-4 as a negative regulatory T cell molecule in maintenance of peripheral tolerance is highlighted by the observation that CTLA-4-deficient mice develop massive lymphoproliferation and early death. Also, in several animal models blockade of CTLA-4 signaling results in exacerbation of autoimmune disease. The programmed death-1 (PD-1)/B7-H1/B7-DC pathway is another immunoregulatory pathway that critically affects peripheral T cell functions as described below (see SECTION 3.4).

Recent findings suggest that upregulation of B7 on T cells is also important for in vivo T cell homeostasis. Specifically, CD80/CD86 may be important for transmission of inhibitory signals by $CD4^+$ $CD25^+$ T_{REG}, and B7 may not only engage CTLA-4 on T cells but also deliver signals into the T cell.

The fact that many of the T cell immune responses persisted in the absence of CD28/B7-mediated costimulation suggested that other costimulatory pathways existed. **Inducible costimulator (ICOS)** is a CD28 homolog expressed on activated T cells and interacts with ICOS-ligand (ICOS-L, B7h), which is expressed in lymphoid but also non-lymphoid tissues, such as kidney, liver, lung, and testes. ICOS stimulates PI3-K activity, augments T cell differentiation and cytokine production, and also provides critical signals for immunoglobulin production. In vivo studies have demonstrated that ICOS can stimulate production of both Th1 and Th2 cytokines during priming and effector T cell responses; however, it does not regulate IL-2 secretion by naïve T cells. In accordance with this, ICOS/B7h blockade ameliorates collagen-induced arthritis and ICOS-deficient NZB/NZW and MRL-lpr lupus-prone mice show decreased autoimmune manifestations. However, there is also evidence to support an immunoregulatory function of ICOS/B7h under certain conditions in vivo. For example, in EAE, ICOS-deficient mice and early ICOS blockade at the time of immunization exacerbates disease, presumably due to Th1 polarization. In allergy and transplantation studies, ICOS has been shown to be important for T cell regulatory function. More recent studies have shown that the ICOS/B7h pathway is critical for induction of tolerance through interactions with tolerogenic DCs [28-30].

B and T lymphocyte attenuator (BTLA) is a novel CD28 family member with immunoregulatory properties that shares functional similarities with the inhibitory receptor PD-1 (see SECTION 3.4). BTLA is induced on T cells during activation (especially Th1 cells) but it is also expressed on B lymphocytes, and interacts with HVEM (herpes entry mediator) expressed on activated lymphoid cells. Cross-linking BTLA with antigen receptor stimulates with tyrosine phosphorylation which recruits SHP-1 and SHP-2 and inhibits activation of the TCR ζ-chain complex [31-35]. BTLA-deficient T cells show increased response to TCR stimulation in vitro and BTLA-deficient mice have enhanced susceptibility to EAE. However, the exact role and the relative contribution of BTLA compared to other inhibitory receptors have not been determined yet.

The continuously expanding family of B7/CD28 costimulatory molecules also includes B7-H3 and B7-H4 [20, 22]. **B7-H3** (B7RP-2) is induced on T cells co-cultured with DCs and following stimulation with phorbol esters. Its receptor has not yet been identified but is distinct from known CD28 family members. Although initial studies

showed that B7-H3 stimulated IFN-γ production by T cells, there is evidence from in vitro and in vivo studies to support a role as negative regulator. Under Th1 polarizing conditions, B7-H3-deficient mice develop more severe airway inflammation associated with enhanced T cell responses. Moreover, B7-H3-deficient mice develop EAE two days earlier than wild-type littermates. **B7-H4** (B7x/B7S1) has also been described as negative regulator of T cell function. Human and mouse B7-H4 mRNA is expressed broadly in both lymphoid and non-lymphoid tissue; protein B7-H4 is induced on lymphocytes and DCs/monocytes following activation. B7-H4.Ig binds to a receptor on activated but not naïve T cells, which is not yet identified. Functional studies have shown B7-H4 to deliver a signal that inhibits TCR-mediated T cell proliferation and IL-2 production. In vivo administration of anti-B7-H4 blocking mAb enhances T cell priming and markedly accelerates the onset and severity of EAE. Altogether, B7-H4 may serve to downregulate immune responses in peripheral tissues and play a role regulation of T cell tolerance.

3.4. The emerging role of PD-1/PD-1 ligands in tolerance and immune homeostasis

3.4.1. Expression of PD-1/PD-1 ligands

Programmed death-1 (PD-1, CD279) is a 288 amino acid (50-55 kDa) type I transmembrane protein composed of one immunoglobulin (Ig) domain and an intracellular domain containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) [20, 36-40]. The extracellular domain shares 21-33% sequence identity with other CD28/B7 family members, namely CTLA-4, CD28, and ICOS. Structural and biochemical analyses have shown that PD-1 is monomeric in solution as well as on cell surface. PD-1 is encoded by the *pdcd1* gene on chromosome 1 in mice and chromosome 2 in humans. It consists of 5 exons; exon 2 encodes the Ig domain, exon 3 the transmembrane domain. Splice variants of PD-1 have been isolated, which are expressed at levels similar to full-length PD-1 in resting human peripheral blood mononuclear cells. Of note, the PD-1Δex3 variant, which lacks exon 3, encodes an mRNA that lacks the transmembrane domain but its significance in immune regulation remains unknown [41, 42]. PD-1 is expressed during thymic development primarily on $CD4^-$ $CD8^-$ (double negative) cells and double negative *γδ* thymocytes, and is induced on peripheral $CD4^+$ and $CD8^+$ T cells, B cells, and monocytes upon activation. PD-1 is also expressed at low levels on NK-T cells and activated myeloid $CD11c^+$ DCs but its function in these cell subsets is not known. Intracellular localization of PD-1 has been reported in $CD4^+$ $CD25^+$ T_{REG} [43].

PD-1 is minimally expressed on resting lymphocytes but is induced after activation with TCR/BCR crosslinking or other polyclonal stimuli. Surface expression is detected at 24 hours after stimulation; its peaks at 48-72 hours and remains detectable for up to 7 days. The transcriptional **regulation of PD-1 expression** is currently unknown but NFATc1 is implicated in activation-induced expression in T cells [44]. In murine B cells, certain 'danger' signals abrogate anti-IgM-induced PD-1 expression, including CpG oligodeoxynucleotides (via TLR-9/MyD88) and LPS (via TLR-4) [45]. Cytokines, such as IL-12, IL-18, and IL-4 (via STAT6) also negatively regulate PD-1 expression, whereas others (TNF, IL-1) do not.

Two ligands for PD-1 have been identified, namely **PD-1 ligand 1 (PD-L1, B7-H1, CD274)** and PD-1 ligand 2 (PD-L2, B7-DC, CD273). They are both type I transmembrane glycoproteins composed of Ig extracellular domains and share 20% amino acid identities with CD80/CD86. PD-L1 is a 290 amino acid protein encoded by the *Cd274* gene on mouse chromosome 19 and human chromosome 9, which comprises seven exons. There is one reported splice variant of PD-L1 in humans consisting of a sequence lacking the IgV-like domain encoded in exon 2 [46]. The mutant should not be able to bind PD-1 but its functional significance has not been studied. The IgV-like domain of PD-L1 is essential for interaction with PD-1 but certain mutants of PD-L1 unable to bind PD-1 can costimulate T cell proliferation providing evidence for another receptor. Interestingly, surface plasmon resonance studies have recently demonstrated specific interaction between PD-L1 and B7.1 (CD80) with an affinity intermediate between those of B7.1/CD28 and B7.1/CTLA-4. The interaction between

PD-L1 and B7.1 is mediated through their IgV-like domains and its functional significance is discussed below (SECTION 3.4.2).

In contrast to other B7 family members, **PD-L1 displays a wide pattern of expression** in both lymphoid and non-lymphoid tissues. Specifically, PD-L1 is expressed on resting and upregulated on activated T, B, myeloid, and dendritic cells. PD-L1 transcripts have been detected in various tissues, with high levels of expression in placenta, heart, lung and liver, and lower expression levels in spleen, lymph nodes, and thymus. IFN-γ-stimulated epithelial and endothelial cells readily upregulate PD-L1. Various stimuli induce PD-L1 expression on APCs, such as TCR/BCR croslinking, LPS, anti-CD40, type I and II IFN, TLRs, IL-4, IL-12, and GM-CSF. Analyses of the human PD-L1 promoter indicate that both constitutive and inducible PD-L1 expression depends on two IFN regulatory factor-1 (IRF-1) binding sites [47]. PD-L1 expression in cell lines is also positively regulated by MyD88, TRAF6, MEK, JAK2, and negatively by PTEN [48-50]. Moreover, a recent study has shown that the micro-RNA miR-513 targets the 3'-untranslated region of PD-L1 mRNA and represses IFN-γ-induced PD-L1 translation [51].

PD-L2 is encoded by the *Pdcd1lg2* gene which is adjacent to *Cd274*. The gene comprises six exons in mice and seven in humans. Three splice variants have been isolated from activated human mononuclear cells; one lacks the IgV-like exon and presumably loses the capacity to bind PD-1, another form drops out the IgC-like domain, and the third variant loses both the IgC-like and the transmembrane domains and might represent a soluble form of PD-L2 [52, 53]. PD-L2 has a much more restricted expression than PD-L1 and is induced on macrophages and DCs but also on airway tract and renal tubular epithelial cells. Less is known about transcriptional regulation of PD-L2. Its induction by IFN-γ is mediated by NF-κB. PD-L2 is also induced by GM-CSF, IL-4, IL-13, and IFN-γ [54-56].

3.4.2. Signaling through PD-1/PD-1 ligands and regulation of T cell function

PD-1 functions as negative regulator of T cell responses in terms of cell proliferation/survival, cytokine production and differentiation. Upon ligand engagement PD-1 is phosphorylated on its intracellular tyrosine residues (ITIM and ITSM) and recruits two phosphatases, SH-2 domain containing tyrosine phosphatase 1 (SHP-1) and SHP-2. Mutation studies have shown that the ITSM is essential for the inhibitory function of PD-1. Also, the association between SHP-1 and PD-1 is weaker compared to that between SHP-2 and PD-1, and PD-1 exerts its function by getting into proximity with TCR and bringing SHP-2 into the synapse [57-59]. PD-1 ligation inhibits PI3-K activity and downstream activation of Akt, as well as TCR-induced activation of CD3ζ, ZAP-70, and PKC-θ [57, 60, 61]. Erk activation is also inhibited by PD-1 but this effect is overcome through cytokine receptor signaling [61, 62]. PD-1 downregulates the expression of Bcl-2 and Bcl-xL, and also of the transcription factors GATA-3, Tbet, and Eomes [63] (**FIGURE 3.4**). The results of these effects include inhibition of T cell proliferation, reduced expression of surface activation markers (CD25, CD69, CD40-ligand), and reduced cytokine production (IL-2, IL-4, IL-5, IL-10, IFN-γ, IL-17).

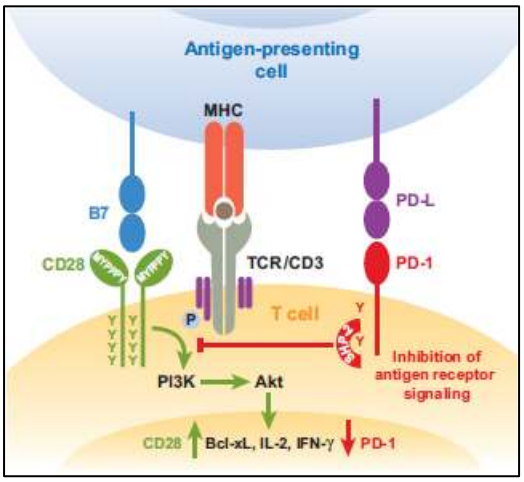


Figure 3.4. Activation of PD-1 results in inhibition of PI-3-kinase–induced activation of Akt. The Erk pathway is also suppressed resulting in decreased Bcl-xL, IL-2 and IFN-γ gene expression.

The **outcome of PD-1 signaling is affected by the level of antigen stimulation and CD28 costimulation**; excess TCR/CD28 costimulation or exogenous IL-2 may overcome the inhibitory effects of PD-1. In line with this, $CD4^+$ T cells are more 'resistant' to PD-1 activation compared to $CD8^+$ T cells. PD-1

activation suppresses the effects of ICOS, IL-4, and IL-21 on T cells but may be blocked by IL-7 and IL-15 in a STAT5-dependent manner [53, 62].

The effects of PD-1 are mediated through engagement with its specific ligands, PD-L1 and PD-L2. Thus, several in vitro and in vivo studies have shown that both PD-L1/PD-1 and PD-L2/PD-1 interactions inhibit T cell responses [53, 61, 64-66]. However, there is also evidence to suggest that PD-1 ligands can exert co-stimulatory – rather than co-inhibitory – effects on T cells. For example, resting T cells stimulated with low levels of anti-CD3 mAb (to crosslink TCR) and immobilized PD-L1.Ig or PD-L2.Ig had increased proliferation and IFN-γ secretion [67-73]. These effects seem to be both PD-1-dependent and independent and could be due to a second, yet unidentified, receptor. This is further supported by the results of a recent study showing that B7-H1 (PD-L1) on naïve T cells is required to condition immature DCs to undergo efficient maturation when they encounter microbial infection [74]. Since immature DCs do not express PD-1, T cell PD-L1 should signal through another receptor.

Interestingly, **PD-L1 and PD-L2 may also act as receptors** to deliver signals into PD-L1/PD-L2-expressing cells ('reverse signaling'). For example, soluble PD-1 inhibits DC activation and increases IL-10 production presumably via PD-L1 or PD-L2. PD-L1-deficient T cells show enhanced activation following TCR crosslinking, with potential implications for T:T interactions [75, 76]. In contrast, active RA patients have circulating anti-PDL1 mAbs which are capable of costimulating T cells and inducing their apoptotic cell death [77]. Similarly, B7-DC (PD-L2) crosslinking has been shown to augment DC maturation and antigen-presenting capacity, as well as T cell responses [78-81]. Recently, B7.1:PD-L1 bi-directional interactions have been described; ligation of PD-L1 on $CD4^+$ T cells by B7.1, or ligation of B7.1 on $CD4^+$ T cells by PD-L1, delivers an inhibitory signal [82, 83]. Taken together, these results emphasize the **complexity of interactions between B7 family members** and that the designation of a molecule as 'receptor' versus 'ligand' is arbitrary, since both properties may co-exist. Also, the mode of action and activation outcome of a receptor (or ligand) depends on both the stage of immune response (priming, effector) and the expression level of other accessory molecules. Importantly, specific stimulatory or inhibitory antibodies may preferentially affect specific interactions (e.g. PD-L1:PD-1), while leaving others unaffected (e.g., PD-L1:B7.1). This might explain discrepancies in the results of functional studies and should be considered in potential therapeutic trials.

3.4.3. The role of PD-1/PD-1 ligands in immune tolerance and autoimmunity

3.4.3.1. PD-1 deficient mice

The first indication that PD-1 plays a critical role in regulation of tolerance and autoimmunity came from observations in PD-1-deficient (*PD-1*$^{-/-}$, *pdcd1*$^{-/-}$) mice. Aged (14 months) *PD-1*$^{-/-}$ **C57Bl/6 mice** spontaneously develop mild glomerulonephritis and arthritis at a low frequency (50%) [84]. The severity of these phenotypes is accelerated by the introduction of the *lpr* (lymphoproliferative) mutation. On the **BALB/c** background, PD-1 deficiency causes fatal dilated cardiomyopathy with production of antibodies against cardiac troponin I (cTnI). Specifically, BALB/c *PD-1*$^{-/-}$ mice start dying from 5 weeks and approximately 50% of these mice die by 5 months due to severe congestive heart failure [85, 86]. The non-obese diabetic (**NOD**) mouse is a model of type-1 (autoimmune) diabetes and its incidence is affected by both environmental and genetic factors; 40-80% of the female NOD mice and 10-40% of the male NOD mice develop diabetes by 30 weeks. Introduction of PD-1 deficiency accelerates the onset and incidence of diabetes and all female and male NOD *PD-1*$^{-/-}$ mice develop diabetes by 10 weeks with severe insulitis [87, 88]. Notably, PD-L1 is highly expressed on pancreatic beta (β) cells in pancreatic islets with insulitis, suggesting that PD-L1 may serve as a barrier to suppress effector $PD\text{-}1^+$ diabetogenic T cells. Since T cells are more strongly activated in islets than in draining lymph nodes, PD-1/PD-L1 interaction seems to inhibit the *in situ* priming and activation of T cells. Indeed, mechanistic studies have shown that PD-L1 on β cells rather than on lymphoid cells is critical for maintenance of peripheral tolerance and induction of diabetes [89]. In line with this observation, PD-L1-deficient

NOD mice also develop severe diabetes, similar to PD-1-deficient littermates. Of interest, approximately one-third of BALB/c mice with combined deficiency in the inhibitory receptors PD-1 and FcgRIIb develop autoimmune hydronephrosis, which is not observed in either BALB/c-*Fcgr2b*$^{-/-}$ or BALB/c-*pdcd1*$^{-/-}$ mice [90]. Hydronephrotic mice produced autoantibodies against urothelial antigens, including uroplakin IIIa, and these antibodies were deposited on the urothelial cells of the urinary bladder. These observations suggest cross talk between PD-1 and other immunoinhibitory receptors (FcgRIIb) on the regulation of autoimmune diseases.

3.4.3.2. Functional in vivo studies

PD-1 and PD-1 ligands provide inhibitory signals that regulate both central and peripheral tolerance. PD-1 is expressed on maturing thymocytes, PD-L1 is expressed on the thymic cortex and thymocytes themselves, and PD-L2 expression is limited to the thymic medulla. PD-1/PD-L1 interaction inhibits positive selection during transformation of double negative ($CD4^-$ $CD8^-$) to double positive ($CD4^+$ $CD8^+$) thymocytes [91, 92]. Thus, absence of PD-1 increases double positive thymocyte numbers but there is also evidence that the PD-1/PD-L1 pathway might affect negative selection of autoreactive T lymphocytes [93, 94]. Moreover, PD-1 has been identified as candidate gene in a microarray analysis of aberrant central tolerance in NOD mice [95]. However, the contribution of central tolerance disturbances in pathogenesis of autoimmunity in PD-1 deficient mice remains unclear.

There is ample evidence to suggest that **PD-1/PD-1 ligands interactions are critical for the functional restriction of peripheral self-reactive T cells** that escape negative selection. Initial studies in 2C-TCR transgenic mice which recognize H-$2L^d$-bearing cells, indicated that PD-1 is important for peripheral tolerance. Thus, in the autoimmune background (H-$2^{b/d}$), $2C^+$ autoreactive T cells are eliminated in the thymus but a few cells migrate to the periphery. In *PD-1*$^{-/-}$ mice, the number of mature $2C^+$ T cells in the thymus is unchanged but autoreactive T cells in the periphery are not inhibited and mice die of a graft-versus-host-like disease [84]. The role of PD-1/PD-L1 was studied using an *in vivo* system of T cell anergy whereby naïve $CD8^+$ OT-1 TCR tg cells are transferred into C57Bl/6 mice [96]. OT-1 T cells specifically recognize a peptide epitope of chicken ovalbumin (OVA), and virtually all transferred cells are engaged by antigen after intravenous administration of soluble OVA as a tolerogen. As a result, there is an initial expansion of OT-1 T cells which then undergo apoptosis and surviving cells become anergic to OVA restimulation. The authors showed that, before their exit to the periphery, T cells in lymphoid organs rapidly upregulated PD-1 upon tolerogen recognition. Blockade of PD-1/PD-L1 interaction while T cells were still in lymphoid organs prevented anergy, and could render anergic T cells responsive to antigen. Similarly, $CD8^+$ T cells specific for influenza hemagglutinin expressed as a self-antigen become functionally tolerized and express high levels of surface PD-1 by the time of their first division. Blockade of PD-1 or PD-L1 (but not PD-L2) at the time of self-antigen encounter abrogated tolerance induction and promoted T cell differentiation into effector cytolytic $CD8^+$ T cells [97].

Probst *et al.* [98] examined the role of PD-1 in peripheral tolerance using DC-specific inducible expression of T cell epitopes by recombination (DIETER) mice, in which two dominant T cell epitopes of lymphocytic choriomeningitis virus (LCMV), GP(33–41) and NP(396–404), can be inducibly presented on $CD11c^+$ cells by tamoxifen treatment. Induced presentation of these antigens on resting DCs prior to LCMV infection efficiently induces tolerance of antigen-specific $CD8^+$ T cells. In mixed bone marrow chimeras of RAG-deficient DIETER, B6-thy-1.1 and B6-thy-1.2-*pdcd1*$^{-/-}$ mice, tamoxifen pre-treatment prior to LCMV infection efficiently induces tolerance on thy-1.1^+ PD-1-sufficient $CD8^+$ T cells but not on thy-1.2^+ PD-1-deficient T cells. Because the $CD8^+$ T cell response against another immunodominant epitope of LCMV, NP(276–286), is unchanged between PD-1-sufficient and PD-1-deficient cells by tamoxifen pre-treatment, the loss of tolerance is not due to a non-specific activation of PD-1-deficient T cells. In this model, injection of anti-CTLA-4 blocking antibody further increased the number of antigen-specific PD-$1^{-/-}$ $CD8^+$ T cells, suggesting that CTLA-4 and PD-1 play non-redundant roles in the induction of T cell tolerance by resting DCs.

3.4.3.3. Experimental disease models

PD-1/PD-1 ligand interactions are important not only in the initial phase of activation and expansion of self-reactive T cells but also influence self-reactive T cell effector function upon antigen encounter.

Administration of anti-PD-1 or anti-PD-L1 mAb to 1- to 10-week-old prediabetic **NOD** female mice and to male NOD mice (usually resistant to diabetes) led to the rapid onset of diabetes, and it was associated with increased IFN-γ producing GAD-reactive splenocytes [87]. The broad temporal range of the effects of anti-PD-1/PD-L1 contrasts with the diabetes-provoking effects of anti-CTLA-4 mAb only in neonatal female NOD mice, implying that CTLA-4 regulates the initial phase of disease, whereas PD-1/PD-L1 interactions regulate both the initiation and progression of autoimmune diabetes in NOD mice. Islet expression of PD-L1 correlates with the severity of insulitis and is critical for regulation of self-reactive T cells [89]. More recently, in a model of antigen-specific therapy in which administration of antigen-coupled fixed splenocytes induces tolerance and reverses diabetes in NOD mice, PD-1/PD-L1 interactions were required for both the induction and maintenance of $CD4^+$ T cell tolerance [99]. Blockade of PD-1 or PD-L1 reversed anergy in islet-antigen-specific T cells, whereas CTLA-4 blockade did not break tolerance, indicating a unique function for PD-1/PD-L1 interactions in maintaining T cell anergy.

PD-1 and PD-1 ligands also regulate self-reactive T cells on the **EAE** model of human multiple sclerosis [100]. PD-1, PD-L1, and PD-L2 are all expressed on cellular infiltrates within the meninges during active EAE disease in C57BL/6 mice. PD-L1 is expressed in the CNS on inflammatory cells as well as on astrocytes and vascular endothelial cells. PD-L1 is specifically induced on $CD11b^+$ APCs by IL-12 and on microglial cells by IFN-γ. Anti-PD-1 or anti-PD-L2 mAb administration during the induction of EAE accelerated disease onset and severity, increased CNS inflammatory infiltrates, and led to increased myelin oligodendrocyte glycoprotein (MOG)-reactive T cells and antibodies. Subsequent studies using blocking antibodies in different mouse strains, such as Balb/c, or gene-deficient animals suggested that PD-1 and PD-L1, but not PD-L2, are responsible for regulating the severity of disease in most mouse strains [101, 102]. Cytokine production is important in the pathogenesis of EAE, and *pdcd1*$^{-/-}$ and *cd274*$^{-/-}$ T cells secrete increased amounts of inflammatory cytokines, including IL-17 and IFN-γ, in recall responses to MOG. Adoptive transfer studies underscored the critical role of PD-L1 in limiting MOG-reactive effector T cells and showed that PD-L1 both on the transferred T cell and in the recipient restrains encephalitogenic T cell responses [75].

3.4.4. PD-1 and PD-1 ligands in human autoimmune diseases

3.4.4.1. Expression of PD-1/PD-L1 in human autoimmune diseases

Several groups have examined the expression of PD-1 and PD-1 ligands in patients with autoimmune disease. In **Sjögren's syndrome**, PD-1 is expressed by salivary T cells and PD-L1 by salivary epithelial cells indicating active PD-1/PD-L1 interactions [103, 104]. Mataki *et al.* detected PD-1 and PD-L1/PD-L2 expression by immunohistochemistry in liver tissue of patients with **autoimmune hepatitis** and **primary biliary cirrhosis** [105]. Affected muscle from patients with idiopathic **inflammatory myopathies** expressed PD-L1 [106], and patients with **myasthenia gravis**, an auto-antibody–mediated neuromuscular disorder, had increased percentage of circulating $PD\text{-}1^+$ T cells and $PD\text{-}L1^+$ monocytes [107]. Enhanced expression of PD-1 on T cells and of PD-L1 on T, B, and macrophage/dendritic cells is seen in inflamed colon from **inflammatory bowel disease** patients [108]. Compared to healthy controls, patients with **type-1 diabetes mellitus** had decreased PD-1 mRNA in purified peripheral blood $CD4^+$ T cells, whereas there was no defect in ICOS, CD28, CTLA-4, and BTLA expression [109]. Other researchers have reported a correlation between circulating $CD4^+$ $CD45R0^+$ $CD26^{high}$ memory T cells and **multiple sclerosis** disease severity [110]. This subset of T cells expressed high levels of Th1 activation markers and low levels of surface PD-1.

More extensive studies have been performed in patients with **rheumatoid arthritis** (RA). Synovial fluid from active RA patients is enriched in PD-1^+ $CD4^+$ T cells, which also express CTLA-4 and produce IL-10 [111]. Similarly, Wan *et al.* [42] reported increased expression of PD-1 and PD-L1 by sunovial fluid T cells and macrophages derived from RA patients as opposed to osteoarthritis controls. The expression of PD-L1 on monocytes could be induced *in vitro* by inflammatory cytokines (IFNγ, TNF) that were produced abundantly in RA-derived synovial fluid. Furthermore, the authors were able to characterize a soluble form of PD-1 that corresponded to an alternative splice variant (PD-1Δex3) of PD-1 transcript. Soluble PD-1 correlated with RF titers and could functionally block the regulatory effect of PD-1 on T cells [42]. Other investigators have found autoantibodies against PD-L1 in sera of 29% of RA patients compared to 4% in healthy controls, correlating with RA activity; immobilized anti-PDL1 autoantibodies stimulated $CD4^+$ T cells proliferation [77].

Taken together, these data – although circumstantial – indicate aberrant expression of PD-1 and PD-1 ligands in patients with autoimmune disorders and suggest that PD-1/PD-1 ligand interactions participate in regulation of T cell responses in the context of human autoimmunity.

3.4.4.2. Genetic association studies

A role for PD-1/PD-1 ligands in humans is strongly suggested by polymorphisms in the PD-1 gene (PDCD1) that have been associated with human autoimmune diseases, such as systemic lupus erythematosus (SLE), RA, ankylosing spondylitis, type-1 diabetes mellitus, Grave's disease, multiple sclerosis, and atopy [112]. To date, >30 **single nucleotide polymorphisms** (SNPs) have been identified, most of which are found in conserved regions in intronic sequences of PDCD1. Of note, the PD1.3 SNP (G+7146A) has been associated with development of SLE (in Europeans and Mexicans), type-1 diabetes, and multiple sclerosis in most but not all ethnic groups. A recent meta-analysis utilized data from 9 studies involving a total of 3,366 SLE patients and 4,317 unrelated controls and found a significant association between PD1.3 SNP and SLE in Latin Americans (adjusted odds ratio [OR] 3.1, 95% confidence interval [95% CI] 1.4–6.5, p=0.003, for the risk A allele), but not in patients of European and African descent [113]. The PD1.3A allele was a risk factor for lupus nephritis in European descendants (OR 2.2, 95% CI 1.5–3.5, p<0.001). Another polymorphism, PD1.5C was also a risk factor for SLE in Europeans (OR 1.3, 95% CI 1.0–1.6, p=0.031). PDCD1 genetic variation may influence the risk and expression of SLE, and effects of PD-1 polymorphisms may vary according to the ethnic background, similar to the effects of mouse PD-1 deficiency in different genetic backgrounds [114].

Importantly, the **PD1.3 SNP is a putative regulatory polymorphism** located in an enhancer-like domain in intron 4 of PDCD1 [115]. Electrophoretic mobility shift assays have shown the risk A allele to disrupt a binding site for the runt-related transcription factor 1 (RUNX1, also known as AML1), with potential impact on PD-1 transcription. To this end, German patients with multiple sclerosis who carried the PD1.3A allele had reduced PD-1–mediated inhibition of IFN-γ production [116]. In accordance, **RUNX1** regulates the expression other autoimmune-related genes. Helms *et al.* [117] reported that a SNP on putative RUNX1-binding site, which locates between SLC9A3R1 and NAT9, is associated with the development of psoriasis. Tokuhiro *et al.* [118] reported that a SNP on the RUNX1-binding site of SLC22A4, which encodes an organic cation transporter, is associated with the development of RA. They further identified that a SNP in the intron 6 of RUNX1 gene itself is associated with the development of RA.

Polymorphisms in the PD-L1 gene have been associated with Grave's disease [119] but not with SLE [120, 121]. PD-L2 gene polymorphisms have been associated with Taiwan SLE patients [120].

3.4.5. The role of PD-1/PD-1 ligands in infections and host defense

PD-1 and its ligands have important roles in regulating immune defenses against pathogens causing acute and chronic infections. PD-1/PD-1 ligands determine the outcome of infection by regulating the delicate balance between effective anti-microbial immune defenses and immune-mediated tissue damage. Mice

deficient in PD-1 clear an adenovirus infection more rapidly but develop more severe hepatocellular injury than wild-type mice [122]. In a mouse model of herpes stromal keratitis, PD-L1 blockade exacerbated keratitis, increasing HSV-1-specific effector $CD4^+$ T cell expansion and IFN-γ production and survival [123]. Infection by human rhinovirus (HRV) is one the most frequent causes of the common cold and is associated with weak pathogen-specific immune responses which render patients susceptible to secondary bacterial infection. Kirchberger *et al.* [124] found that HRV-treated DCs fail to induce potent T cell responses, and this is – at least in part – due to upregulation of PD-L1 on DCs. Addition of blocking anti-PD-L1 antibodies restored the stimulatory capacity of DCs and therefore, PD-1/PD-L1 interactions appear to play an important role in HRV-induced T cell anergy. These studies suggest that the PD-1/PD-1 ligands pathway limits the potentially detrimental consequences of vigorous anti-pathogen effector T cells but may also result to immune paresis and defective pathogen elimination.

Indeed, several microorganisms that cause **chronic infection** appear to have exploited PD-1/PD-1 ligands to evade the immune responses and establish persistent infection. Viruses that cause chronic infections can render virus-specific T cells nonfunctional and thereby silence the antiviral T cell response. PD-1 is upregulated upon activation, and a functionally significant high level of expression is maintained by **'exhausted' $CD8^+$ T cells** in mice chronically infected with LCMV [125]. In vivo administration of antibodies that blocked PD-1/PD-L1 interactions enhanced T cell responses and importantly, led to a substantial reduction in viral burden. Subsequently, three groups simultaneously reported that PD-1 is highly expressed on HIV-specific $CD8^+$ T cells in **HIV-infected individuals** [126-128]. PD-1 expression correlated with viral load, declining CD4 counts, and decreased capacity of $CD8^+$ T cells to proliferate in response to HIV antigen *in vitro*. There was also a direct correlation between PD-1 expression on HIV-specific $CD4^+$ T cells and viral load. Long-term non-progressors had functional HIV-specific memory $CD8^+$ T cells with markedly lower PD-1 expression, in contrast to typical progressors who expressed significantly upregulated PD-1, which correlated with elevated plasma viral load [129]. Importantly, blocking PD-1/PD-L1 interactions *in vitro* reversed the exhaustion of HIV-specific $CD8^+$ and $CD4^+$ T cells and restored proliferation and cytokine production. Other investigators extended this work to other chronic viral infections and have demonstrated PD-1 upregulation on HBV- and HCV-specific T cells, associated with impaired antiviral T cell responses [130, 131]. In addition, PD-L1 is also upregulated on peripheral blood $CD14^+$ monocytes and myeloid DCs in patients with chronic HBV infection, and on $CD14^+$ and T cells in HIV patients.

The PD-1/PD-1 ligands pathway may also be important in the chronicity of bacterial infections, as in the case of ***Helicobacter pylori*** chronic gastritis and gastroduodenal ulcers. Following exposure to *H. pylori*, PD-L1 is induced on human gastric epithelial cells contributing to defective T cell responses that are insufficient to clear infection [132]. Anti-PD-L1 blocking antibodies enhance T cell proliferation and IL-2 production in co-cultures of gastric epithelial cells exposed to *H. pylori* and $CD4^+$ T cells, suggesting that PD-L1 may play an important role in regulating T cell responses during *H. pylori* infection. In mice, **parasitic infections** by *Taenia crassiceps* and *Schistosoma mansoni* cause PD-L1 and PD-L2 upregulation on macrophages, correlating with T cell anergy [133]. Furthermore, PD-L1 and PD-L2 have distinct roles in the immune response to the protozoan parasite ***Leishmania mexicana***. Mice deficient in PD-L1 are resistant to *L. mexicana* infection, whereas mice deficient in PD-L2 developed exacerbated disease with increased parasite burdens [134]. Notably, PD-L1 knock-out mice exhibited a diminished Th2 response, which may explain their increased resistance.

The key roles of PD-1 and PD-1 ligands in soothing T cell responses during chronic infections drive the development of strategies to manipulate PD-1/PD-1 ligands interactions to restore T cell responses during chronic infections. This pathway may have evolved to limit aberrant immune-mediated damage to the host during infection. It is important that we better understand the precise immunoregulatory role of this pathway to determine how to modulate it so as to activate effective pathogen-specific T cells while minimizing the risk of autoimmunity and immunopathology.

3.4.6. The role of PD-1/PD-1 ligands in transplantation

Several lines of evidence suggest that PD-1/PD-L1 interactions control engraftment of solid organs and graft-versus-host disease (GVHD). Redundancy among negative costimulatory pathways is important for controlling alloreactive T cells, and studies have demonstrated unique roles for the PD-1/PD-1 ligands and CTLA-4/B7 pathways. PD-1 and PD-L1 are significantly upregulated on alloreactive T cells in transplant recipients [135]. In heart transplant models PD-L1 is upregulated on allografts as early as one day after heart transplant [136], whereas PD-1 and PD-L2 are induced later, presumably because they are expressed primarily by infiltrating cells. In heart, corneal, and skin transplant models, administration of anti-PD-L1, but not anti-PD-L2, blocking antibodies accelerated transplant rejection [137-139]. PD-1 upregulation occurs after the onset of GVHD, and PD-L1 is extensively expressed on most cells in GVHD target organs.

To examine the role of PD-L1 on the donor *versus* the recipient in the acquired tolerance model of CTLA-4-Ig, PD-L1 knock-out mice were used as graft donors or recipients of fully MHC mismatched cardiac allografts and given CTLA-4-Ig treatment [140]. PD-L1$^{-/-}$ recipients of wild-type cardiac allografts had accelerated graft rejection compared with wild-type recipients given CTLA-4-Ig therapy. PD-L1$^{-/-}$ cardiac allografts were accepted by wild-type recipients treated with CTLA-4-Ig, yet histologic examination showed evidence of severe chronic rejection and vasculopathy. These data indicate that **PD-L1 in the graft protects from local pathology and chronic rejection**, whereas PD-L1 expression in the recipient immune system is required for induction and/or maintenance of transplantation tolerance after CTLA-4-Ig therapy [141]. Therefore, PD-1 agonists may be useful for promoting transplant tolerance and preventing local inflammation that leads to chronic graft arterial disease and rejection.

To this end, PD-L1-Ig, but not PD-L2-Ig, plus cyclosporine A significantly enhanced cardiac allograft survival over that of cyclosporine A or PD-L1-Ig alone and led to decreased intragraft expression of IFN-γ and CCR5/CXCR3mRNA. PD-L1-Ig also had synergistic effects when given with rapamycin and led to permanent survival of fully MHC mismatched cardiac allografts. PD-L1-Ig promoted long-term graft survival in CD28$^{-/-}$ recipients and reduced cardiac transplant arteriosclerosis when given in conjunction with anti-CD154 mAb [136, 142]. These findings suggest that **PD-1 targeting**, when used together with agents in current clinical use or in clinical trials, may improve the survival of solid organ transplantation. The mechanisms involved in induction of allograft tolerance through PD-1/PD-1 ligands are not completely understood but seem to include apoptosis of alloreactive T cells and control of regulatory versus effector T cell generation [137, 143].

3.4.7. The role of PD-1/PD-1 ligands in tumor immunology

Although tumors express antigens that can be recognized by host T cells, immunologic clearance of tumors is rare. PD-L1 expression on many tumors is a mechanism, by which tumors escape from immunosurveillance, and may act in concert with other immunosuppressive signals [144]. PD-L1 expression has been shown in situ on a variety of **solid tumors** including breast, lung, colon, ovarian, melanoma, bladder, liver, salivary, stomach, gliomas, thyroid, thymic epithelial, head, and neck. PD-1 expression is upregulated on tumor infiltrating lymphocytes, and this may also contribute to tumor immunosuppression. Importantly, studies relating PD-L1 expression on tumors to disease outcome show that PD-L1 correlates with unfavorable prognosis in kidney, ovarian, bladder, breast, gastric, and pancreatic cancer but not small cell lung cancer. In addition, higher levels of PD-L1 on tumors may facilitate advancement of tumor stage and invasion into deeper tissue structures [145-148].

The PD-1 pathway may also play a role in **hematologic malignancies** [149-154]. Thus, PD-L1 is expressed on multiple myeloma cells but not on normal plasma cells, and also some primary T cell lymphomas, particularly anaplastic large cell T lymphomas. PD-1 is highly expressed on the T cells of angioimmunoblastic lymphomas, and PD-L1 on the associated follicular dendritic cell network. In nodular lymphocyte-predominant Hodgkin lymphoma, the T cells associated with lymphocytic and/or histiocytic cells express PD-1.

In accordance with these findings, manipulation of the PD-1/PD-L1 pathway may be used to reverse the immunocompromised status of tumor-bearing host and activate the immune system to eradicate tumours. Administration of anti-PD-L1 antibodies has been shown to augment antitumor responses, as measured by cytotoxicity and cytokine production. **Treatment with anti-PD-L1 mAb** *in vivo* delays, but does not halt, tumorigenesis of PDL1-expressing mouse myeloma cell lines, and PD-L1 blockade can also improve the outcome of immunotherapy [155-158]. Currently, a fully human anti-PD-1 mAb has been developed and is in Phase I clinical trials for cancer.

3.5. Systemic lupus erythematosus (SLE): the prototype of autoimmune diseases

3.5.1. Epidemiology and clinical manifestations of SLE

SLE is a multisystem, autoimmune, connective tissue disease with a broad range of clinical presentations. There is a peak age of onset in young women between 18 and 40 years and a women-to-men ratio of 9:1. Ethnic groups, such as those with African or Asian ancestry, are at greatest risk of developing SLE, which can be more severe than in white patients. SLE is a chronic illness that can be life threatening when major organs are affected, but more commonly results in chronic debilitating ill health. The prevalence of lupus ranges from approximately 40 cases per 100,000 persons among Northern Europeans to more than 200 per 100,000 persons among blacks. Incidence rates also vary widely across different ethnic groups but typically range 3–5 cases per 100,000 persons per year [159].

The widely recognized presentation of a young woman with inflammatory arthritis and a typical 'butterfly' facial rash is not common. Rather, non-specific symptoms of fatigue, malaise, oral ulcers, arthralgia, photosensitivity, lymphadenopathy, sicca (dry eyes and mouth), headache, raynaud's phenomenon, and mild hair loss are more likely presentations. Therefore, there is often a considerable delay before the diagnosis is considered in patients with low-grade disease. Renal involvement develops insidiously and if not detected early, carries high risk of progression to renal impairment. Neuropsychiatric manifestations are common in SLE and may present in the context of active disease, or as an isolated event. There is a wide spectrum of clinical manifestations that may be grouped into neurologic (including the CNS, cranial, and peripheral nerves) and psychiatric (including psychosis and depression). SLE may also cause serositis (pleurisy, pericarditis, or peritonitis), gastrointestinal manifestations (abdominal pain, anorexia, nausea, vomiting, mesenteric vasculitis, lupus hepatitis), lung involvement (pneumonitis, pulmonary hemorrhage, pulmonary hypertension, pulmonary embolism), and cardiac manifestations (pericarditis, myocarditis, endocarditis, valve disease, coronary artery disease).

The diagnosis of lupus is based on careful and thorough clinical evaluation and recognition of multisystem involvement, corroborated by abnormalities in basic laboratory investigations such as complete blood count (often showing anemia or thrombocytopenia or lymphopenia), renal and liver function, and acute phase reactants (usually high erythrocyte sedimentation rate [ESR] with normal C-reactive protein [CRP]). Serum complement levels (C3, C4) are depressed in patients with active SLE and are often used as a surrogate marker to monitor disease activity. Some autoantibodies are also useful in diagnosis of SLE. These include antibodies to nuclear antigens (antinuclear antibodies [ANA], anti-dsDNA antibodies) and antibodies to the extractable nuclear antigens (ENA) such as Ro (SS-A), La (SS-B), ribonucleoprotein (RNP), and Sm. For a disease with such protean manifestations and variable course as SLE, the American College of Rheumatology (ACR) has established classification criteria (**TABLE 3.1**) to allow comparisons of patients from different centers.

Prognosis in lupus patients has improved from an approximate 4-year survival rate of 50% in the 1950s to a 15-year survival rate of 80% today. Even so, a patient in whom lupus is diagnosed at 20 years of age still has a 1 in 6 chance of dying by 35 years of age, most often from lupus itself or infection. Later, myocardial

infarction and stroke become important causes of death. Generally, major organ involvement (nephritis, neuropsychiatric, cardio-respiratory) or multiple manifestations are associated with worse outcome in SLE.

	Criterion	Definition
1	Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
2	Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3	Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
4	Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by physician
5	Arthritis	Nonerosive arthritis involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion
6	Serositis	a) Pleuritis--convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion, OR b) Pericarditis--documented by ECG or rub or evidence of pericardial effusion
7	Renal disorder	a) Persistent proteinuria greater than 0.5 grams per day or grater than 3+ if quantitation not performed, OR b) Cellular casts--may be red cell, hemoglobin, granular, tubular, or mixed
8	Neurologic disorder	a) Seizures--in the absence of offending drugs or known metabolic derangements; OR b) Psychosis--in the absence of offending drugs or known metabolic derangements
9	Hematologic disorder	a) Hemolytic anemia--with reticulocytosis, OR b) Leukopenia--less than 4,000/mm^3 total on 2 or more occasions, OR c) Lyphopenia--less than 1,500/mm^3 on 2 or more occasions, OR d) Thrombocytopenia--less than 100,000/mm^3 in the absence of offending drugs
10	Immunologic disorder	a) Positive LE cell preparation, OR b) Anti-DNA: antibody to native DNA in abnormal titer, OR c) Anti-Sm: presence of antibody to Sm nuclear antigen, OR d) False positive serologic test for syphilis known to be positive for ≥6 months and confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test
11	Antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug-induced lupus" syndrome

Table 3.1. The 1982 Revised Criteria for Classification of Systemic Lupus Erythematosus. The proposed classification is based on 11 criteria. For the purpose of identifying patients in clinical studies, a person shall be said to have systemic lupus erythematosus if any 4 or more of the 11 criteria are present, serially or simultaneously, during any interval of observation. Modified from Tan EM, *et al.* Arthritis Rheum 1982;25:1271-7.

3.5.2. Pathogenesis of SLE

3.5.2.1. Overview

SLE is a complex, multisystem autoimmune disease caused by several defects in innate and adaptive immunity. The autoimmune nature of SLE became apparent from studies that demonstrated presence of inflammation and deposition of antibodies and complement components in affected organs, such as the kidneys and the skin. Kidneys from patients with lupus nephritis were shown to contain antibodies that bound native, double-stranded DNA; these were '**auto-antibodies**', since they bound a normal constituent of the patients' cells and tissues. Anti-dsDNA antibodies are specific to SLE and are found in sera of 70% of SLE patients as compared to 0.5% of healthy individuals or patients with other autoimmune diseases. Numerous other autoantibodies (with specificity against nucleosomes, Ro/La, Sm, NMDA receptor, phospholipids, actinin, C1q and other antigens) are detected in 10–90% of SLE patients and contribute to pathogenesis of specific manifestations. This is evidenced by: a) clinical studies demonstrating associations between autoantibodies and lupus manifestations, b) studies of tissues from SLE patients, and c) studies in animal models of lupus [160].

The obvious source of nucleosomes and other antigens in SLE is the cellular debris released as a result of **apoptosis**. Defects in the clearance of apoptotic cells have been described in SLE and these defects could lead to aberrant uptake by macrophages, which then present the previously intracellular antigens to T and B lymphocytes, thus driving the auto immune process. Studies have examined possible defects in the clearance of apoptotic bodies, including complement (C1q, C2, C4) deficiencies, defects in macrophage handling, and presentation of these antigens to the immune system.

Cytokine patterns might also be important in the pathogenesis of lupus. Investigations have drawn attention to the overexpression of the type I interferon pathway in patients. In accordance, a common IRF5 haplotype, driving enhanced expression of multiple unique isoforms of IRF5, has been described as an important genetic risk factor for the disease. Furthermore, serum levels of interleukin-10 (IL-10) and IL-21 are high in SLE patients and correlate with disease activity. Both cytokines exert multiple biologic effects including stimulation of B lymphocytes to differentiate into antibody-producing plasma cells. Also, the B-lymphocyte stimulator (BLyS) is a member of the TNF-ligand superfamily that promotes the proliferation and survival of B lymphocytes. Circulating levels of BLyS are elevated in lupus as well as in RA and Sjögren's syndrome. Levels of BLyS correlate with serum anti–dsDNA titers and may thus be associated with the increased activity of lupus in some patients.

Environmental factors have also been implicated in pathogenesis of SLE. Since 90% of patients with lupus are female, an important role for female hormones seems likely but a protective role for male hormones or an effect of genes on the X chromosome is also possible. Sunlight is another factor that can exacerbate lupus. Moreover, an antecedent viral-like illness may occur at the onset of lupus or immediately before a flare. A temporal association between Epstein-Barr virus (EBV) infection and SLE onset has been described and a case-control study in young adults has detected significantly higher levels of anti-EBV antibodies and EBV DNA in lupus patients. However, the fact that 90% of the adult population is infected with EBV while the prevalence of SLE remains low emphasizes the multifactorial nature of the pathogenesis of this disorder.

3.5.2.2. Defects in immunological tolerance in lupus

The autoimmune process in SLE is a "T cell-driven response", which involves autoreactive T cells and elevated titers of high-affinity anti-DNA IgG that has undergone somatic hypermutation. Several mechanisms of peripheral immune tolerance are abnormal in **SLE T cells**, including: a) resistance to the induction of anergy, b) reduced apoptosis and impaired clonal deletion of autoreactive T cells, c) increased spontaneous signalling and decreased threshold for the activation of T cells, and d) reduced number and/or function of regulatory T cells (in addition to indirect factors such as an abnormal cytokine production that contribute to immune deviation) [161, 162]. Specific molecular and signaling abnormalities identified include exaggerated intracellular Ca^{+2} responses, increased intracellular phosphorylation, decreased TCR/CD3ζ chain expression and replacement by FcR γ-chain, syk recruitment to the TCR complex, increased translocation of CaMKIV from the cytoplasm to the nucleus, which results in increased binding of CREM to the IL-2 and c-fos promoters, and decreased availability of pCREB because of increased PP2A activity in SLE T cells, both resulting to impaired IL-2 production, CD40 ligand overexpression, and upregulation of a COX-2 dependent pathway resulting in resistance to apoptosis [163]. Moreover, SLE T cells have pre-clustered lipid rafts which harbor a different array of molecules than the lipid rafts from normal T cells, including the hyaluronic acid receptor CD44 that guides T cells to inflamed tissues. Mitogen-activated protein (MAP) kinase responses are also hampered in SLE, which is associated with decreased DNA methylation and distorted transcription factor activation. Taken together, T cells from SLE patients exhibit several abnormalities that favor their activation/effector functions but also trigger an altered gene transcription program resulting to a non-functional cell.

T cells in SLE provide excessive 'help' to B cells to differentiate into autoantibody-producing plasma cells and this is mediated via CD40-ligand/CD40 and ICOS/ICOS-ligand interactions. In addition, recent work has demonstrated **disturbed B cell tolerance** in human SLE patients. Thus, an important tolerance checkpoint

that operates in healthy subjects to censor autoreactive (9G4) B cells in the mature naïve compartment, thereby preventing the expansion of these cells into the memory compartment, has been shown to be faulty in SLE [164]. Moreover, in some SLE patients, peripheral B cell tolerance checkpoints were found to be defective, with a further increase in autoreactivity within the mature naïve compartment [165]. Other abnormalities include naïve B cell lymphopenia, increased transitional B cells, and an expansion of peripheral activated memory B cells and plasmablasts. The frequency and absolute number of plasmablasts in patients with SLE correlates with overall disease activity and autoantibody titers [166]. Signaling abnormalities have also been implicated in the B cell hyperactivity in SLE patients. Memory B cells in SLE have decreased surface expression of the inhibitory FcgRIIB receptor, associated with diminished suppression of BCR-induced Ca^{+2} responses [167]. Then, SLE patients have increased serum BAFF (B cell activator of the TNF family) levels, a cytokine that promotes (autoreactive) B cell survival [168]. Finally, aberrant expression and/or signaling through TLRs in lupus B cells contributes to differentiation into plasma cells, even in the absence of T cells [169, 170].

Dendritic cells are crucial in determining the fate of immune response and whether T cells will acquire a tolerogenic *versus* effector phenotype. Evidence suggests that DCs are involved in the pathogenesis of SLE; their maturation process is abnormal and their cytokine secretion and T cell stimulation is biased. These abnormalities might be both DC-intrinsic and the result of lupus serum which contains several factors capable of inducing phenotypic and functional changes in DCs [171].

3.5.2.3. The genetic basis of SLE

The strong genetic component of SLE has been known for at least 30 years based on observations of disease concordance of 2-5% in dizygotic compared to 24-57% in monozygotic twins and a sibling risk of 20-39. Progress in identifying these genetic factors was initially slow and it was based on candidate gene association studies and genetic linkage analysis in multiplex families. In the last two years, **genome-wide association (GWA) scans** have dominated efforts in gene mapping for autoimmune diseases [172]. In GWA scanning, no particular hypothesis is being addressed. Rather, hundreds of thousands of hypotheses are being addressed simultaneously, without regard to biologic plausibility. In SLE, two high-density case-control GWA analyses utilizing 300,000–500,000 SNPs have been published to date [173, 174]. Despite the vast number of simultaneously tested SNPs, the genomic 'densities' achieved by the used chips were not high enough to achieve definite genome-wide coverage. Also, the cohorts employed in these studies were of white, northern European ancestry and thus, represent only a proportion of the human population.

Nonetheless, important **lupus susceptibility genes** have been identified based on the results of GWA scans and meta-analyses of fine-mapping studies [175, 176]. Genes that are most strongly associated with SLE (*p* values ranging 10^{-9} to 10^{-52}) are: the *MHC* (major histocompatibility complex) locus (HLA alleles, C4A complement null allele), the *ITGAM* (integrin α_M protein) influencing leucocyte trafficking and/or the uptake of apoptotic cells/immune complexes, the *IRF5* (IFN regulatory factor 5) regulator of type I IFN responses, the *BLK* (B-lymphoid tyrosine kinase) involved in BCR-mediated B cell activation, and the *STAT4* mediator of cytokine signaling. Strong associations (*p* values ranging 10^{-5} to 10^{-7}) have been identified for *PTPN22* (protein tyrosine phosphatase non-receptor 22), a negative regulator of lymphocytes, and *FCGR2A* (Fcγ-receptor 2A) involved in IgG-mediated phagocytosis. Several other genes have been identified in only one of the two GWA analyses or in fine-mapping studies and are involved in the control of apoptosis, B cell signaling, methylation and ubiquitination, and costimulation [176]. By considering how these genes fall into clusters with shared function we understand how dysregulation at key immunological steps may predispose to the development of SLE.

4 Research questions and aim of the study

SLE is characterized by disturbed T- and B-cell tolerance; there is increased proportion of activated $CD4^+$ T cells with enhanced effector function that offer aberrant help to B cells to produce pathogenic autoantibodies. The activated phenotype of lupus T cells may result from an imbalance between costimulatory ('positive') and coinhibitory ('negative') signals that originate from membrane receptors. Such abnormalities could contribute to: a) break of tolerance of self-reactive T cells, b) increased effector function and tissue injury, and c) perpetuation of aberrant immune responses in lupus. PD-1/PD-1 ligands is a novel coinhibitory pathway that is implicated in regulation of tolerance and pathogenesis of autoimmunity based on evidence from experimental models and genetic association studies. However, the role of PD-1/PD-1 ligands in T cell activation in the context of human autoimmunity has not been defined. To this end, the following biological questions were set:

a) Does the PD-1/PD-1 ligands pathway regulate T cell responses in healthy individuals and in patients with SLE?
b) Is there a role for PD-1/PD-1 ligands in the immunopathogenesis of SLE?
c) Could the manipulation of the PD-1/PD-1 ligands pathway be used to reverse lupus T cell abnormalities?

In specific, the following **research questions** were addressed:

1. Is PD-1 a susceptibility gene in a cohort of Cretan (Greek) SLE patients? What are the functional consequences of PD-1 gene polymorphisms in SLE patients?
2. What is the expression and function of PD-1/PD-1 ligands in lupus T and B lymphocytes as compared to healthy individuals and patients with other autoimmune disease? Is PD-1 expression and function different in SLE patients with different disease activity and severity? How does PD-1 expression and function compare to that of other coinhibitory molecules (CTLA-4, BTLA)?
3. Are PD-1/PD-1 ligands involved in tissue injury in SLE? What is the expression of PD-1/PD-1 ligands in affected tissues?
4. How does the lupus inflammatory environment affect the outcome of PD-1/PD-1 ligands pathway?

5 Materials and methods

5.1. Patient and control populations

A case–control study was conducted to test the association between PD-1 polymorphisms and the risk for SLE. A total of 289 SLE patients – diagnosed using the American College of Rheumatology classification criteria [177] – were recruited from the Department of Rheumatology, University Hospital of Heraklion (Crete, Greece). Two hundred fifty six age– and sex–matched healthy blood donors recruited from the Department of Transfusion Medicine, University Hospital of Heraklion, served as controls. Both cases and controls were of Cretan origin. The study was approved by the University Hospital of Heraklion Institutional Review Committee and all subjects gave informed consent.

5.2. DNA extraction and genotyping for the PD-1 gene polymorphisms

Genomic DNA was extracted from EDTA-treated whole blood obtained from patients and controls using the PureGene™ Genomic Whole Blood DNA Purification kit (Gentra Systems) according to the manufacturer's instructions. DNA was stored in Tris-EDTA buffer at a concentration of 100ng/mL. Genotyping for the PD1.3 and PD1.5 SNP was performed by polymerase chain reaction (PCR) and restriction analysis, as described elsewhere [115, 178]. To amplify the PD-1 gene regions encompassing the PD1.3 (G+7146A) and PD1.5 (C+7785T) SNPs the following primers were used: PD1.3-forward 5'-CCC CAG GCA GCA ACC TCA AT-3', PD1.3-reverse 5'-GAC CGC AGG CAG GCA CAT AT-3', PD1.5-forward 5'-GTG CCT GTG TTC TCT GTG GA-3', PD1.5-reverse 5'-CCA AGA GCA GTG TCC ATC CT-3'. PCR was carried out in a total volume of 50μl containing 100ng of genomic DNA, 1× PCR buffer, 200nM of each primer, 1.5mM of MgCl2, 200nM of each dNTP, and 1.0U Taq polymerase (all reagents from Minotech, FORTH, Greece). PCR conditions were denaturation at 95°C for 10 (PD1.3) or 5 (PD1.5) minutes, followed by 45 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C (PD1.3) or 58°C (PD1.5) or for 30 seconds, and extension at 72°C for 15 seconds. An 180-bp fragment was amplified from genomic DNA after the PD1.3 PCR. Ten microliters of the PCR product were used for a 3-hour restriction enzyme digestion at 37ºC with *Pst* I (Minotech), which digested DNA amplified from the A – but not the G – allele into 130-bp and 50-bp fragments. Accordingly, the PD1.5 PCR product (240-bp) was digested with *Pvu* II (Minotech), which digested DNA amplified from the T – but not the C – allele into 180-bp and 60-bp fragments. Both undigested and digested PCR products were visualized in 2% agarose gel stained with ethidium bromide.

5.3. Luciferase assays: plasmid constructions and transient transfection

We generated 180-bp fragments with the two different alleles (G and A) of PD1.3 SNP by PCR using DNA from homozygotes with the corresponding haplotypes. The fragments were inserted in the *Sac* I/*Xho* I site of the pGL3-promoter vector (Promega), upstream of the AdML minimal promoter (MLP). The expression constructs pCMV5-AML1b (pCMV5-RUNX1) and pCMV5-CBFβ were kindly provided by Dr. D-E Zhang (The Scripps Research Institute, California, USA). Jurkat T cells (kindly provided by Dr. J. Papamatheakis, Institute of Molecular Biology and Biotechnology, FORTH, Greece) maintained in RPMI-1640 medium (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco Invitrogen), were seeded at a density of 0.5×10^6 cells/well in 12-well plates (Corning) and were transfected with pGL3-luc (control) or pGL3-luc with the wild-type G allele (PD1.3G-MLP) or the risk A allele (PD1.3A-MLP) (each 0.2μg), with or

without pCMV5-RUNX1 (0.1µg) using the Superfect Reagent (Qiagen). In each transfection, the plasmid pCMV-β-gal was included for normalization of the transfection efficiency. All transfections were carried out in duplicates. After 16 hours of incubation, PMA (10ng/mL) and ionomycin (500ng/mL) (Sigma-Aldrich) was added for another 24 hours. Cells were collected, washed in PBS twice, and analyzed for luciferase activity.

5.4. Preparation of mononuclear cells and isolation of CD4+ T lymphocytes

Peripheral blood mononuclear cells (PBMCs) from 40 SLE patients (38 females [95%], mean ± SD age 41 ± 14 years) and 26 age- and sex-matched healthy blood donors were isolated by Ficoll-Histopaque (10771; Sigma-Aldrich, St. Louis, MO) density-gradient centrifugation of heparinized venous blood and were washed twice in HBSS 1×. Baseline demographic and clinical characteristics of the patients who participated in the immunological studies are shown in **TABLE 5.1**. Twenty four patients (60%) had active disease (defined as SLE Disease Activity Index score ≥8) [179], thirteen patients (33%) had proliferative and/or membranous nephritis, and 16 patients (40%) were receiving corticosteroids. Patients had not taken any lupus medication for 24 hours prior to blood sampling. $CD4^+$ T lymphocytes were isolated from PBMCs by negative selection (Miltenyi Biotec, Bergisch Gladbach, Germany) (purity 92–98%).

Characteristic	N (%) or mean ± SD
Female	38 (95%)
Age (years)	40 ± 13
Disease activity (SLEDAI)	8.8 ± 4.2
Active SLE (SLEDAI ≥8)	30 (60%)
Anti-dsDNA (positive)	12 (30%)
Nephritis (yes)	13 (33%)
Treatment	
Corticosteroids	16 (40%)
Hydroxychloroquine	16 (40%)
Immunosuppressants	10 (25%)

Table 5.1. Baseline characteristics of SLE patients who participated in the immunological studies.

5.5. Antibodies and flow cytometry

All antibodies used in flow cytometry were fluorochrome-conjugated mouse anti-human monoclonal antibodies. PE– and FITC–anti-CD3 (clone UCHT1), PC5–anti-CD8 (OKT8), PE–conjugated anti-PD-L1 (B7-H1) (MIH1), anti-PD-L2 (B7-DC) (MIH18), anti-CTLA-4 (CD152) (14D3), and PE–anti-FoxP3 (PCH101) were obtained from eBioscience (San Diego, CA, USA). PC5–anti-CD19 (J4.119), anti-CD4 (OKT4), and PE–anti-CD69 (TP1.55.3) were from Beckman-Coulter (Miami, USA). PE–anti-PD-1 (CD279) (MIH4), PE–anti-CTLA-4 (BNI3), PE–anti-BTLA (J168-540.90.22), FITC–anti-CD25 (M-A251), anti-CD69 (FN50), anti-HLA-DR (G46-6) were from BD Pharmingen (Franklin Lakes, NJ, USA). FITC–anti-CD40-ligand (24-31) was from Ancell (Bayport, MN, USA). PE– or PC5–IgG1 (679.1Mc7) (Beckman Coulter), PE–IgG2a (eBM2a) and FITC–IgG1 (P3) (eBioscience) were used as IgG isotype controls in all experiments. For staining of freshly isolated PBMCs, 0.5×10^6 cells were incubated in wash buffer (PBS/FBS 2.5%) with appropriate amounts of monoclonal antibodies on ice for 30 minutes. Cells were washed in wash buffer and were immediately analyzed on an EPICS XL–MCL flow cytometer (Miami, USA). For determination of intracellular expression of CTLA-4, cells already stained with anti-CD4 and anti-CD25 were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Pharmingen). Intracellular staining was then performed at 4ºC in saponin-containing perm/wash buffer in the presence PE–conjugated anti-CTLA-4 or IgG2a control (4 µL each). Intracellular FoxP3 staining (8 µL PE–anti-FoxP3 per 0.5×10^6 cells) was performed using the FoxP3 Staining Buffer Set (eBioscience), according to the manufacturer's instructions.

5.6. Quantitative real-time PCR for PD-1

Total RNA was isolated from purified $CD4^+$ T cell pellets using the RNeasy Mini Kit (Qiagen). Genomic DNA was removed using the RNase-Free DNase (Qiagen). First-strand cDNA synthesis was performed by the Thermoscript II RT Kit (Invitrogen) using random hexamers. PD-1 mRNA was determined by real-time PCR using the SYBR Green Master Mix (Applied Biosystems). Thermocycler conditions included an initial holding at 50°C for 2 min and then 95°C for 10 min, which was followed by a two-step PCR program: 95°C for 15 s and 60°C for 60 s for 40 cycles. Data were collected and quantitatively analyzed on an ABI PRISM 7900 sequence detection system (Applied Biosystems). The mean value of the replicates for each sample was calculated and expressed as cycle threshold (C_T, cycle number at which each PCR reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The amount of gene expression was then calculated as the difference (ΔC_T) between the mean C_T value of the sample for the target gene and the mean C_T value of that sample for the endogenous control (GAPDH). Relative expression of genes was expressed as $2^{-\Delta CT}$. PCR primer pairs were as follows: GAPDH, 5′-CAT GTT CCA ATA TGA TTC CAC C-3′ and 5′-GAT GGG ATT TCC ATT GAT GAC-3′; PD-1, 5′-CTC AGG GTG ACA GAG AGA AG-3′ and 5′-GAC ACC AAC CAC CAG GGT TT-3′.

5.7. PD-1 expression on activated $CD4^+$ T cells

Purified $CD4^+$ T cells (1×10^5/well) were incubated in RPMI-1640 medium containing 10% FBS, 2mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate (Sigma-Aldrich), 1% (vol/vol) non-essential amino acids, 100IU/ml penicillin, and 10µg/ml streptomycin (Gibco Invitrogen) on 96-well round-bottomed tissue culture plates (Nunc) coated with 1µg/mL anti-CD3 (UCHT1, R&D Systems). Soluble anti-CD28 (CD28.2, BD Pharmingen) was added at a concentration of 250ng/mL. In some experiments, optimal concentrations of anti-CD3 (5µg/mL) and anti-CD28 (1µg/mL) were used. Cells were harvested at t=12–96 hours, washed in wash buffer and then incubated on ice with PE– or FITC–conjugated anti-CD69, anti-CD25, anti-PD-1, anti-CTLA-4 or IgG1 isotype control. Cells were washed and a total of 10,000 events was collected and analyzed on the flow cytometer. Dead cells were excluded based on forward scatter / side scatter properties.

5.8. Assessment of PD-1 function

To determine the effects of PD-1 crosslinking during T cell activation, purified $CD4^+$ T cells from SLE patients and healthy blood donors (1×10^5 cells/well) were stimulated with plate-bound anti-CD3, soluble anti-CD28 (250ng/mL), in combination with varying concentrations of plate-bound PD-L1.Fc fusion protein (R&D Systems). This fusion protein contains the extracellular part of the human PD-L1 and the Fc part of the human IgG1, and has been shown to specifically activate PD-1. 96-well round-bottomed plates (Nunc) were coated with anti-CD3 (1µg/mL) and PD-L1.Fc (0–5µg/mL) for 2 hours at 37ºC in 100µl PBS solution. Human IgG1 (Sigma) was added as needed to keep the amount of total protein constant at 6µg/mL. Plates were washed twice with PBS before cell culture was initiated. At 24 or 72 hours culture supernatants were collected for cytokine measurement and cells were pulsed with [^{3}H]thymidine (1µCi/well) (Amersham Biosciences, Munich, Germany) for another 16 hours to measure proliferation. BTLA function was assessed using plate-bound HVEM.Fc (R&D, 0–5µg/mL). In some experiments, cells were harvested, washed and analyzed for expression of the activation markers CD69 and CD40-ligand by flow cytometry. To evaluate the effect of SLE serum on PD-1–mediated inhibition of T cell activation, $CD4^+$ T cells from healthy blood donors were stimulated with plate-bound anti-CD3, soluble anti-CD28 and plate-bound PD-L1.Fc (0, 0.1, 1, 2µg/mL) in the presence of 20% heat-inactivated serum from active SLE patients or unrelated healthy donors. Proliferation was measured as previously described.

5.9. Cytokine ELISA

Cytokines in culture supernatants were assayed by ELISA using antibodies to human IFN-γ (Ready-SET-Go! ELISA kit) (eBioscience, San Diego, CA, USA) and IL-10 according to the manufacturer's instructions.

5.10. Autologous mixed lymphocyte reactions

Autologous mixed lymphocyte reactions (AMLR) were set up as previously described [180]. In brief, PBMC $CD3^+$ T and non-T cell fractions from SLE patients (n=17) and healthy blood donors (n=12) were isolated using a magnetic bead positive selection system (Pan T Cell Isolation kit; Miltenyi Biotec). Cells were incubated in RPMI-1640 culture medium containing 10% heat-inactivated autologous human serum, 2mM L-glutamine, 10mM HEPES, 100IU/ml penicillin, and 10μg/ml streptomycin. Non-T stimulator cells were induced to undergo apoptosis by gamma-irradiation with 1000 rad from a cobalt source, and then were co-cultured at a 2:1 ratio with 1×10^5 autologous responder T cells in 96-well round-bottomed plates. On day 4, untreated T cells and AMLR cells were harvested, washed in wash buffer and analyzed for expression of PD-1, PD-L1, BTLA, CTLA-4, CD25, CD69, HLA-DR, and FoxP3 on $CD4^+$ T cells, and PD-L1, HLA-DR, CD40, CD80 on $CD14^+$ cells by flow cytometry. The selection of this time point was based on initial experiments showing the PD-1 peaked during 72–96 hours of AMLR. The proliferative response of T lymphocytes in the AMLR was evaluated after 5 days of culture by addition of [^{3}H]thymidine (1μCi/well) for 24 hours. In some experiments, purified functional grade (low endotoxin) anti-PD-1, anti-PD-L1, anti-CTLA4 (10μg/mL) blocking antibodies or mouse IgG2a (all from eBioscience) were added in culture at initiation to assess the effect of receptor blockade on proliferation.

5.11. Renal tubular epithelial cells

Normal human RTEC were kindly provided by Takashi Kuroiwa (Gunma University Graduate School of Medicine, Japan) and were maintained in renal epithelial basal medium (REBM), supplemented with 0.5% FBS, 10 ng/ml of recombinant human epidermal growth factor, 5 μg/ml of insulin, 0.5 μg/ml of hydrocortisone, 50 μg/ml of gentamicin, 50 ng/ml of amphotericin B, 0.5 μg/ml of epinephrine, 6.5 ng/ml triiodothyronine, and 10 μg/ml of transferrin (Clonetics). RTEC (passages 3–5) were grown until confluent and were treated with recombinant IFN-γ (200 UI/mL), TNF-α (10 ng/mL) or LPS (10 ng/mL). After 48 hours, cells were collected to detect surface PD-L1 expression by flow cytometry. Total RNA was isolated using Trizol Reagent (Life Technologies) to analyze PD-L1 expression by semi-quantitative RT-PCR. The RNA was reverse transcribed using reverse transcriptase and oligod(T)12–18 primers in 20 μl reaction volumes. One-microgram aliquots of the reverse transcription (RT) reaction were then subjected to PCR. The following primers were used: PD-L1 forward, 5V-AATTGTTGGCTTTCACTTT-3V; PD-L1 reverse, 5V-AGCGTCTTT TTCATACTTCA-3V; β-actin forward, 5V-GGCAGGACCAGGAAAAC-3V; β-actin reverse, 5V-CAGCCAATCAAACAGACAAG-3V. An initial PCR denaturation step was performed at 94°C for 4 min. The general cycling parameters for PCR were as follows: denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min for 35 cycles. RT-PCR products were resolved on 2% agarose gels and stained with ethidium bromide.

5.12. Detection of PD-1 and PD-L1 in renal biopsies

Renal biopsies were obtained from lupus nephritis patients (n=15) and from macroscopically unaffected areas of patients with renal cancer (n=9) (control group). Cryostat sections (5μm) were fixed in cold acetone, and detection of PD-1, PD-L1, and CD3 was performed by standard avidin-biotin complex method. After blocking, the sections were incubated with unlabeled goat anti-human PD-1 (C-16, Santa-Cruz Biotechnology), mouse anti-human PD-L1 (29E.2A3, kindly provided by Dr. G. Freeman, Harvard School of Medicine) or mouse anti-

human CD3 monoclonal antibodies (R&D), followed by biotin-conjugated goat or mouse link (Dako). DAB was used as chromogen and sections were counterstained with hematoxylin. A semi-quantitative score was assigned separately for tubular-interstitial and glomerular PD-1/PD-L1 expression according to the following pattern: 0= absent, +1=weak, +2=moderate, +3= strong staining. Since most of the samples displayed weak-to-moderate staining, for simplicity reasons they were categorized as positive or negative.

5.13. Statistical analysis

Allele frequencies were determined and the chi-squared test was used to test the significance of the differences in 2×2 contingency tables. For statistically significant results, an odds ratio (OR) and a Cornfield's 95% confidence interval (95% CI) was calculated. Results were expressed as mean ± standard error of the mean (SEM) and groups of data were compared using the non-parametric Mann-Whitney U test. A p-value (two-tailed) <0.05 was considered as statistically significant.

6 Results

6.1. Increased frequency of the PD1.3A single nucleotide polymorphism in patients with SLE

In certain ethnic populations PD-1 polymorphisms are associated with increased risk for autoimmune disease. To determine whether PD-1 SNPs are associated with susceptibility to SLE in our cohort, we analyzed the genomic DNA of 289 SLE patients (females, 95%; mean age 45 ± 16 years) of Cretan (Greek) origin for the PD1.3 and PD1.5 SNPs and compared their frequencies with those of age– and sex– matched healthy controls (females, 95%; mean age 45 ± 16 years); these two SNPs have been previously associated with SLE in Caucasian populations [115]. We found increased frequency of PD1.3 heterozygosity (G/A) in SLE patients compared to healthy controls (30.1% *versus* 18.4%, p=0.006) **(FIGURE 6.1A-B)**. The presence of A allele was associated with increased odds for SLE (odds ratio [OR] = 2.23; 95% confidence interval [95% CI] 1.55–3.38) but not for lupus nephritis (OR = 1.13; 95% CI 0.43–3.81). In this cohort, we found two patients homozygous for the PD1.3 SNP (i.e. A/A), yielding a frequency of 0.7%, which is similar to the frequency of PD1.3 homozygosity reported for other ethnic groups ranging 0.5–1%. These two patients had hematological, skin and articular manifestations but not major organ involvement. There was no difference in PD1.5 SNP haplotype frequencies between SLE patients and controls (C/C: 35.5% *versus* 35.6%; C/T: 52.5% *versus* 53.9%; T/T: 12.0% *versus* 10.5%, respectively) **(FIGURE 6.1C-D)**. The observed PD1.3 and PD1.5 genotypes in healthy controls were in Hardy-Weinberg equilibrium.

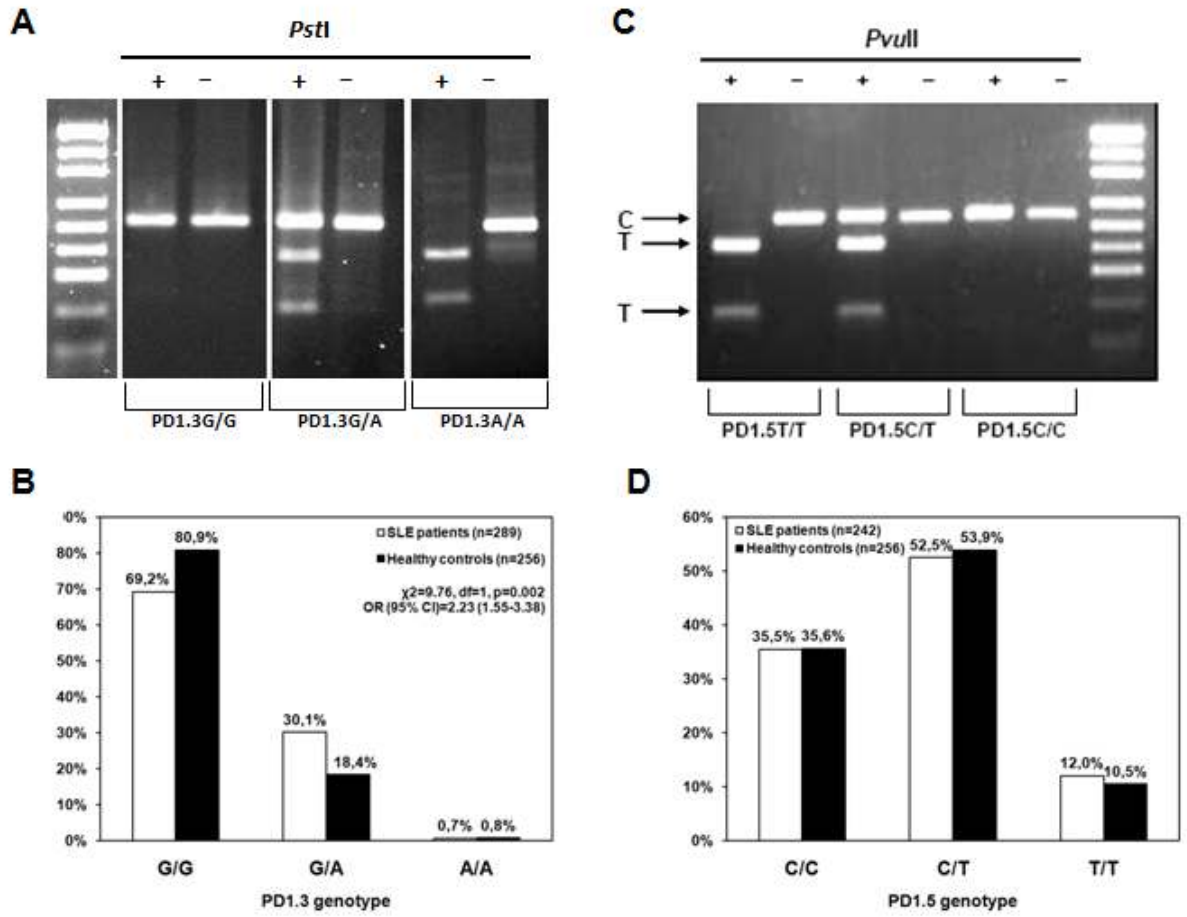


Figure 6.1. Increased frequency of the PD1.3A single nucleotide polymorphism in SLE patients. Genotyping for the PD1.3 **(A)** and PD1.5 **(C)** single-nucleotide polymorphism (SNP) in the PD-1 gene by PCR-RFLP. Increased frequency of the PD1.3A **(B)** – but not the PD1.5T **(D)** – SNP in SLE patients compared to age-/sex-matched healthy controls. PD1.3A was associated with an odds 2.2 (95% confidence interval 1.6–3.4, p=0.002) for SLE.

6.2. The lupus-associated PD1.3A single nucleotide polymorphism is associated with decreased transcriptional activity

The SLE-associated allele (A) of PD1.3 SNP eliminates a putative binding site for the transcriptional factor RUNX1 in an enhancer-like domain in intron 4 of the human PD-1 gene. Electrophoretic mobility shift assays have demonstrated specific binding of nuclear factors from the human Jurkat T cells to the wild-type (G) – but not the risk (A) – binding site with potential impact on PD-1 transcription [115]. To further explore the functional significance of the PD1.3 SNP, we performed transient transfection assays in Jurkat T cells with reporter constructs expressing the firefly luciferase gene under the control of the SLE-associated A allele or the wild-type G allele of PD1.3 SNP **(FIGURE 6.2)**. Overexpression of RUNX1 resulted in increased luciferase activity that was significantly higher with the G than the A allele by 36 ± 4% (results from 5 independent experiments). These results indicate that PD1.3 may represent a functional polymorphism associated with suppressed transcriptional activity.

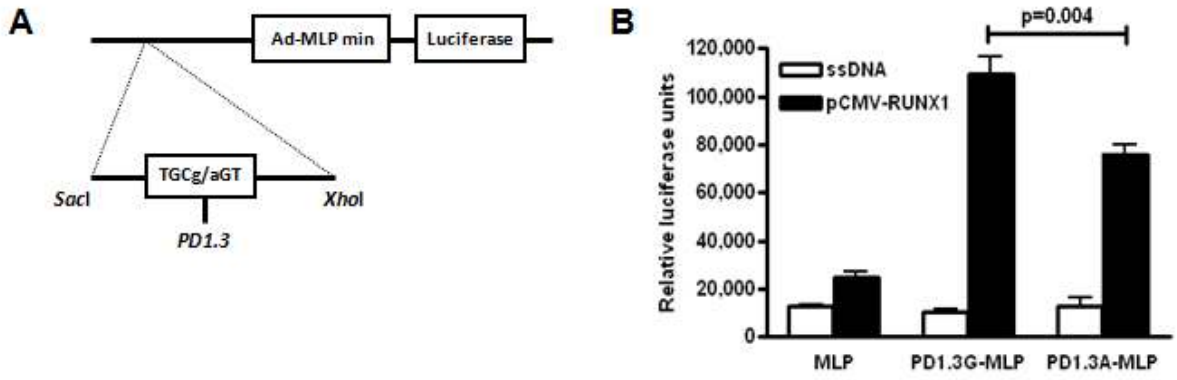
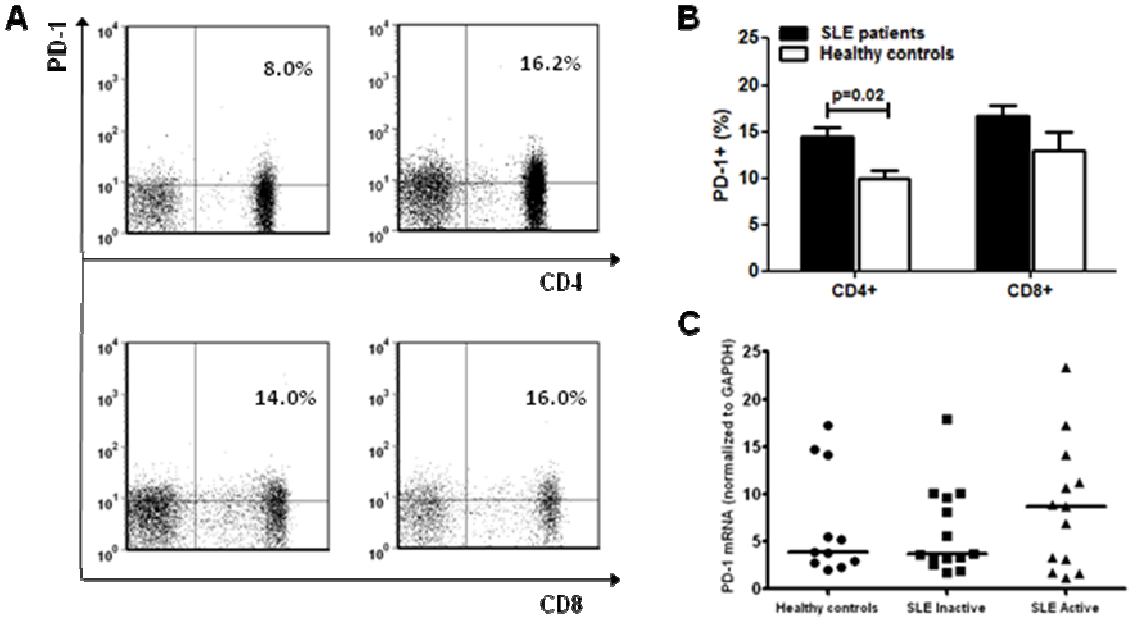


Figure 6.2. **(A)** Investigation of a putative RUNX-1 site in PD1.3 SNP. Schematic representation of the reporter constructs containing the SLE-associated PD1.3A or the wild-type PD1.3G allele of PD1.3 SNP linked to the adenovirus major late minimal promoter (Ad-MLP) and the luciferase gene in the pGL3-basic vector. The sequence of the putative RUNX1 binding site containing the polymorphism is indicated. **(B)** The polymorphic PD1.3A allele is associated with decreased transcriptional activity. Jurkat T-cells were transiently transfected with PD1-luciferase reporter vectors bearing the PD1.3G or the SLE-associated PD1.3A alleles in the absence or in the presence of an expression vector for RUNX1 (pCMV-RUNX1). In each transfection, the plasmid pCMV-β-gal was included for normalization of the transfection efficiency. The normalized mean values (± SEM) of luciferase activity from 4 independent experiments are shown. Overexpression of RUNX1 resulted in increased luciferase activity that was significantly higher in Jurkat T-cells transfected with PD1.3G than with PD1.3A reporter construct (t-test, p=0.004). ssDNA: salmon sperm DNA that was used instead of pCMV-RUNX1 in control experiments.

6.3. Homozygous – but not heterozygous – patients for the PD1.3A polymorphism have reduced basal and induced PD-1 expression on activated $CD4^+$ T cells

6.3.1. PD-1 is overexpressed on peripheral blood T cells in SLE patients

We next examined the expression of PD-1 on freshly isolated peripheral blood lymphocytes in SLE patients. Flow cytometry experiments indicated increased percentage of PD-1$^+$ CD4$^+$ T cells (14.4 ± 1.1% *versus* 10.0 ± 0.8%, p=0.020) and PD-1$^+$ CD8$^+$ T cells (16.7 ± 1.1% *versus* 12.9 ± 2.0%, p=0.061) in SLE patients



compared to healthy blood donors **(FIGURE 6.3A-B)**. Quantitative real-time PCR for PD-1 expression in isolated CD4$^+$ T cells showed increased PD-1 mRNA levels in active SLE (8.6 ± 1.9, n=13) compared to inactive SLE (6.1 ± 1.2, n=14) and healthy donors (6.8 ± 1.7, n=11), although the differences did not reach statistical significance **(FIGURE 6.3C)**.

PD-1 shows basal levels of expression on resting lymphocytes and is induced upon activation [62]. Moreover, there are data implicating PD-1 in the regulatory function of T cells [43, 181, 182]. We therefore studied the expression of PD-1 in various subsets of activated T lymphocytes in SLE patients and healthy controls using triple-color flow cytometry. CD69 was used as early and HLA-DR as late activation marker, CD25 as intermediate activation marker but also to define putative T regulatory subsets, and CD40-ligand (CD40L) as a marker of putative autoreactive T cells as it is important for T–B cell interaction.

With the exception of CD4$^+$ CD25$^+$ T cells, all activated T cell subsets had increased PD-1 expression in both SLE patients and controls. Compared to controls, SLE patients had increased PD-1 on CD4$^+$ CD25$^-$ (16.4 ± 1.4% *versus* 10.3 ± 1.0%, p=0.004), CD4$^+$ CD69$^+$ (19.9 ± 2.0% *versus* 12.7 ± 1.5%, p=0.008), and CD4$^+$ HLA-DR$^+$ cells (26.1 ± 1.5% *versus* 19.4 ± 1.4%, p=0.003). PD-1 expression on CD4$^+$ CD25$^+$ (12.9 ± 0.9% *versus* 10.0 ± 1.0%, p=0.083), CD4$^+$ CD25^{++} (13.6 ± 1.4% *versus* 12.3 ± 2.0%, p=0.456), CD4$^+$ CD40L$^+$ (15.7 ± 2.0% *versus* 12.2 ± 2.3%, p=0.468) and CD8$^+$ HLA-DR$^+$ cells (24.5 ± 1.8% *versus* 20.4 ± 2.8%, p=0.254) was comparable between patients and controls **(Supplementary FIGURE S1)**. Similar results were obtained using PD-1 mean fluorescence intensity values from the flow cytometry analysis.

Further analysis of the flow cytometry data showed a positive correlation between PD-1 expression and disease activity (defined by SLEDAI) and anti-dsDNA autoantibody positive in most CD4$^+$ T cell subsets

Figure 6.3. Increased proportion of peripheral blood PD-1$^+$ T cells in SLE patients. **(A)** Representative flow cytometry histograms of PBMCs stained with anti-CD4 or anti-CD8 and anti-PD1-PE. **(B)** Increased percentage of PD-1$^+$ CD4$^+$ T cells (14.4 ± 1.1% versus 10.0 ± 0.8%) and PD-1$^+$ CD8$^+$ T cells (16.7 ± 1.1% versus 12.9 ± 2.0%) in SLE compared to healthy blood donors. **(C)** Increased PD-1 mRNA levels in purified CD4+ T cells from active SLE patients.

(Supplementary TABLE S1). Renal involvement was not related to PD-1 expression.

Peripheral blood $CD19^+$ B cells from SLE patients had marginally increased PD-1 expression (5.1 ± 0.5% *versus* 4.2 ± 0.5% for total $CD19^+$ B cells; 7.9 ± 1.3% *versus* 5.6 ± 1.4% for $CD19^+$ $CD27^+$ memory B cells) **(FIGURE S2)**. Moreover, PD-1 expression on memory B cells correlated with disease activity (10.9 ± 1.9% in active *versus* 4.9 ± 1.1% in inactive SLE, p=0.022) and anti-dsDNA status (6.0 ± 3.4% in positive *versus* 3.4 ± 1.0% in negative patients, p=0.035).

6.3.2. SLE patients homozygous for the PD1.3A polymorphism have decreased basal and induced PD-1 expression

Based on the results from the transfection assays, we sought to examine whether PD-1A SNP affected PD-1 expression *in vivo*. Although we found no difference between wild-type (i.e. G/G) and heterozygous (i.e. G/A) patients, two patients homozygous for the PD1.3 SNP (i.e. A/A) had minimal PD-1 expression on freshly isolated $CD4^+$ T cells, including the activated $CD4^+$ $CD25^+$, $CD4^+$ $CD69^+$ and $CD4^+$ HLA-DR^+ T cell subsets **(FIGURE 6.4)**. Interestingly, PD-1 levels were also diminished on peripheral blood $CD8^+$ T cells and $CD19^+$ B cells in these two patients (data not shown).

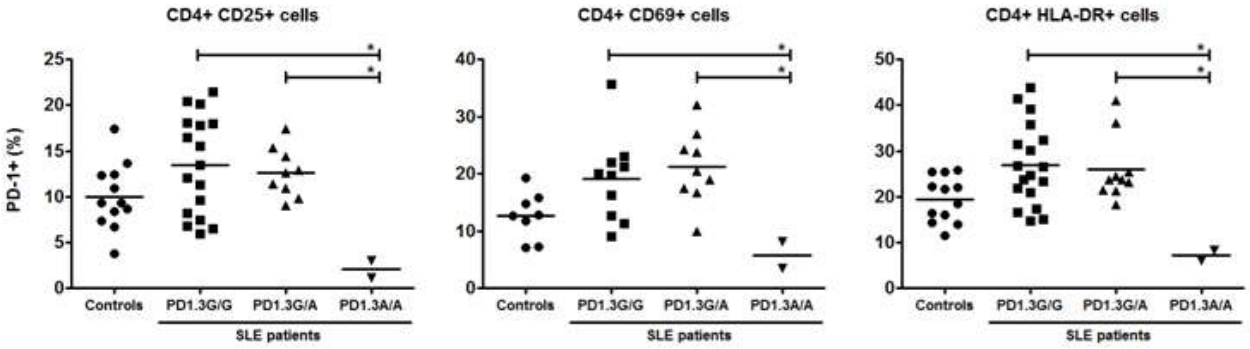
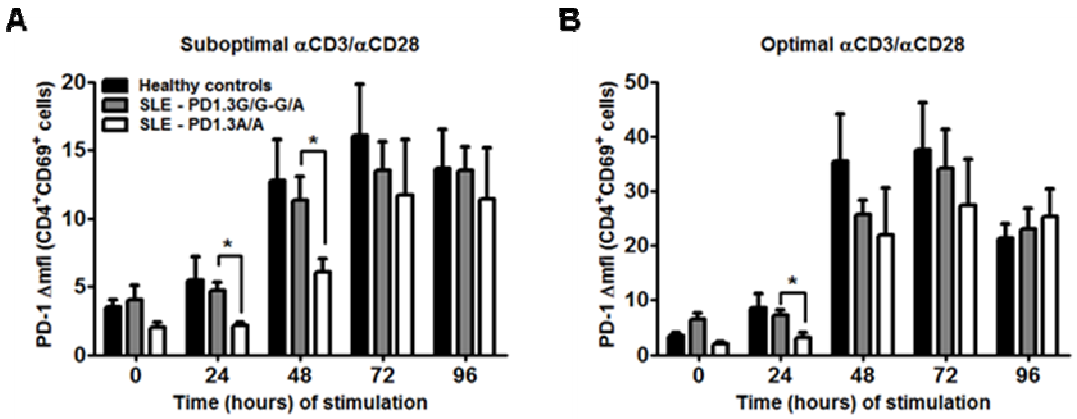


Figure 6.4. SLE patients homozygous for the PD1.3A SNP have decreased basal PD-1 expression on activated $CD4^+$ T cells. SLE patients homozygous for the PD1.3 SNP (A/A) have significantly reduced percentages of PD-1^+ activated $CD4^+$ $CD25^+$, $CD4^+$ $CD69^+$, and $CD4^+$ HLA-DR^+ T cells compared to healthy controls, SLE PD1.3G/G or G/A patients (* p<0.05, Mann-Whitney U test).

Since PD-1 is induced upon lymphocyte activation, we also studied PD-1 expression on anti-CD3/anti-CD28–stimulated $CD4^+$ T cells. PD-1 peaked at 48–72 hours post-stimulation and showed similar kinetics and mean expression levels in SLE patients and healthy controls **(FIGURE S3)**. However, the two patients who were homozygous for PD1.3A had significantly reduced PD-1 levels on activated $CD4^+$ $CD69^+$ T cells at 24–48 hours following stimulation with suboptimal – but not optimal – concentrations of anti-CD3/anti-CD28 antibodies **(FIGURE 6.5)**. Similar results were obtained when PD-1 expression was examined following activation of PBMCs with PMA and ionomycin (data not shown). Collectively, these results suggest that the presence of PD1.3A polymorphism confers reduced basal and induced PD-1 expression at early-to-intermediate stages of $CD4^+$ T cell activation.



6.3.3. Comparable expression of PD-L1, BTLA and CTLA-4 on $CD4^+$ T cells in SLE patients and controls. B cells from active SLE patients show decreased BTLA expression

PD-1 mediates its inhibitory function upon engagement with its specific ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC) [53, 64, 183]. In contrast to the restricted expression of PD-L2 on activated macrophages and DCs, PD-L1 is expressed on activated T and B lymphocytes and monocytes/macrophages. We examined expression of PD-L1 on various PBMCs subsets but found no difference between healthy controls and SLE patients **(FIGURE S4A-C)**. We also examined the expression of CTLA-4, a master regulator of T lymphocyte activation that is expressed intracellularly in $CD4^+$ $CD25^+$ cells and mediates T_{REG}–mediated suppression of activated T cells [184]. We found no defective expression of CTLA-4 in $CD4^+$ $CD25^+$ T cells in SLE patients (29.7 ± 3.4% *versus* 26.5 ± 5.5% in healthy controls, p=0.559) **(FIGURE S4D)**.

Figure 6.5. SLE patients homozygous for the PD1.3A SNP display impaired induction of PD-1 on activated $CD4^+$ T cells. PD-1 induction on healthy and SLE $CD4^+$ T cells stimulated with sub-optimal (1µg/mL / 250 ng/mL) and optimal (5µg/mL / 1µg/mL) anti-CD3/anti-CD28 mAbs. Results are presented as PD-1 Δmfi (i.e. PD-1 mfi minus IgG mfi) gated on $CD4^+$ $CD69^+$ cells. The two patients homozygous for the PD1.3 SNP had decreased PD-1 expression at 24–48 hours following activation with sub-optimal anti-CD3/anti-CD28.

BTLA, another coinhibitory receptor, was comparably expressed on lupus and normal $CD4^+$ T cells **(FIGURE 6.6A)**. In contrast, we found decreased BTLA expression on B cells from SLE patients with active *versus* inactive disease. Results were more pronounced on $CD19^+$ $CD27^+$ memory B cells and BTLA was inversely related to SLEDAI (spearman's rho = –0.65, p=0.005) **(FIGURE 6.6B-D)**.

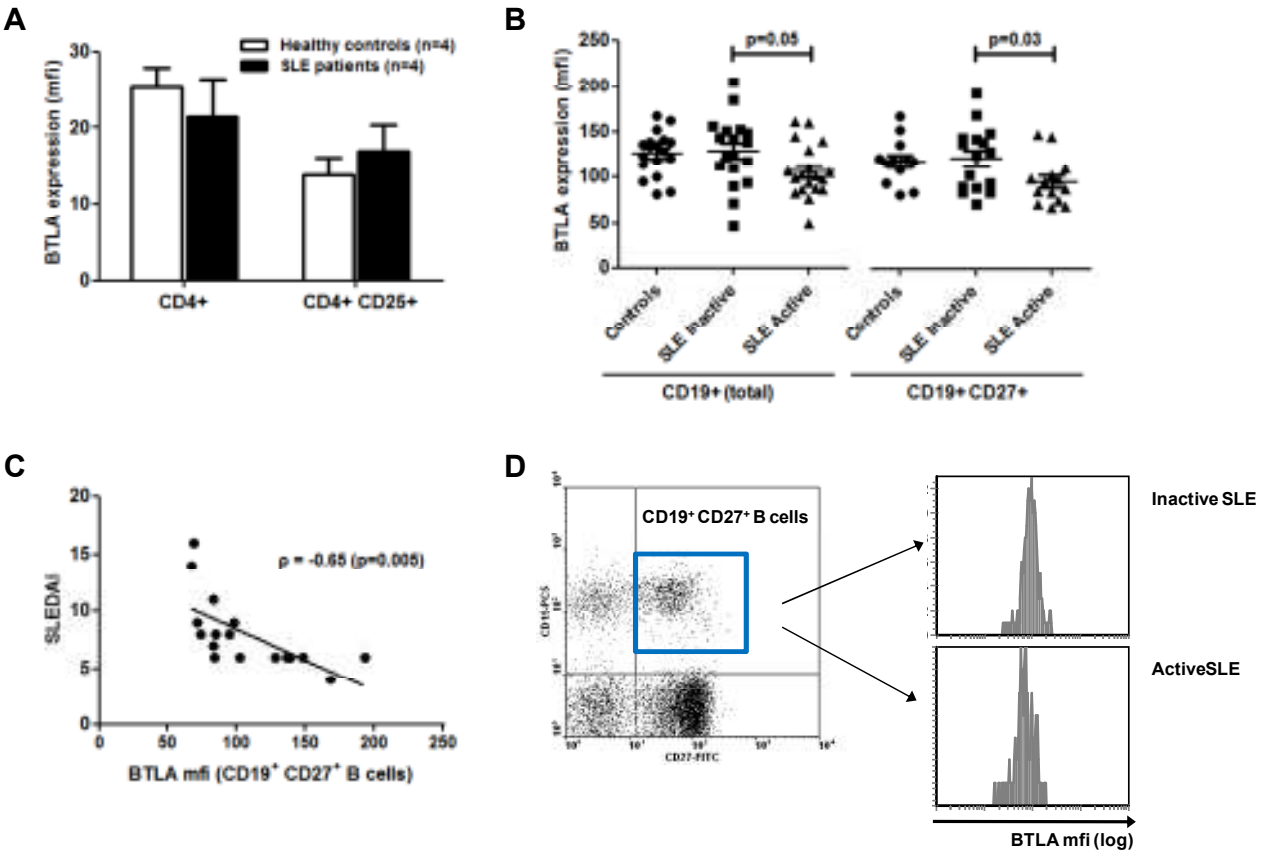


Figure 6.6. Decreased BTLA expression on memory B cells in active SLE patients. **(A)** Comparable BTLA expression on peripheral blood $CD4^+$ and $CD4^+$ $CD25^+$ T cells in SLE and healthy controls (bars represent mean ± SEM BTLA mean fluorescence intensity). **(B)** Active SLE patients have decreased BTLA expression on $CD19^+$ and $CD19^+$ $CD27^+$ memory B cells, compared to inactive SLE and healthy controls. **(C)** Inverse correlation between disease activity (SLEDAI) and BTLA expression (mfi) on memory B cells in SLE patients (spearman's rho = -0.65, p=0.005). **(D)** Representative flow cytometry histogram of BTLA expression on $CD19^+$ $CD27^+$ - gated cells in active and inactive SLE.

6.4. Homozygous SLE patients for the PD1.3A polymorphism show decreased PD-1–mediated inhibition at early-to-intermediate stages of $CD4^+$ T cell activation

PD-1 activation results in suppression of lymphocyte proliferation, IL-2 and IFN-γ production via decreased ERK activation, and suppression of IL-10 production via decreased AKT/PKB activation [60, 61, 185]. To explore whether PD-1 exerts normal suppressive activity in SLE patients, $CD4^+$ T cells were activated with anti-CD3/anti-CD28 antibodies using plate-bound PD-L1.Fc to crosslink PD-1; T cell proliferation and IFN-γ production were measured at 48 and 96 hours of stimulation. Optimal PD-1 crosslinking (PD-L1.Fc 2–5 μg/mL) resulted in profound (70–80%) suppression of T cell proliferation, IFN-γ and IL-10 production in both healthy controls and SLE patients, regardless of the PD1.3 genotype **(FIGURE S5A-C)**. However, when suboptimal PD-L1.Fc concentrations were used (0.1–0.5μg/mL), the two SLE patients who were homozygous for PD1.3A SNP displayed defective PD-1–mediated inhibition of T cell function at 48 hours. Specifically, T cell proliferation was increased by 5.0 ± 8.0% in the two homozygous patients, whereas it was decreased by 13.1 ± 7.8% in healthy controls (p=0.07) and by 15.4 ± 10.0% in PD1.3G/G or G/A SLE patients. Similarly, IFN-γ production was decreased by only 9.0 ± 9.0% in the PD1.3A/A patients, compared to 58.0 ± 4.6% in healthy controls (p=0.02) and 28.0 ± 9.0% in PD1.3G/G or G/A patients **(FIGURE 6.7)**. In conjunction with our

previous studies, these results suggest that the PD1.3A polymorphism is associated with reduced expression and function of PD-1 at early-to-intermediate stages of $CD4^+$ T cell activation.

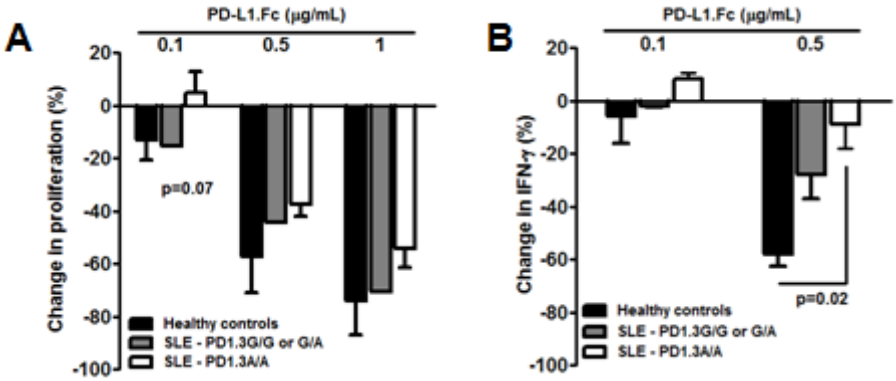


Figure 6.7. $CD4^+$ T cells were stimulated with anti-CD3/anti-CD28 mAb and PD-L1.Fc to crosslink PD-1. At 24 hours cell cultures were pulsed with thymidine to assess cell proliferation and supernates were collected for IFN-γ measurement. Results are shown as percentage of change compared to IgG-treated T cells. Under suboptimal PD-1 crosslinking (PD-L1.Fc 0.1–0.5µg/mL) the two patients homozygous for PD1.3A SNP had defective PD-1–mediated inhibition of T cell proliferation **(A)** and IFN-γ production **(B)**, compared to healthy controls (n=4) and SLE PD1.3G/G or G/A patients (n=4).

In parallel experiments, we assessed the suppressive function of BTLA in isolated $CD4^+$ T cells in SLE patients and healthy controls. BTLA crosslinking by plate-bound HVEM.Fc resulted in dose-dependent suppression of anti-CD3/anti-CD28–mediated proliferation that did not differ between SLE patients and healthy controls (80 ± 5% vs. 63 ± 3% at HVEM.Fc 0.5µg/mL; 93 ± 2% vs. 83 ± 9% at HVEM.Fc 2µg/mL) **(FIGURE S5D-E)**. BTLA–mediated suppression of IFN-γ production was also comparable between SLE patients and healthy controls (56 ± 11% vs. 57 ± 8% at HVEM.Fc 0.5µg/mL; 80 ± 11% vs. 72 ± 8% at HVEM.Fc 2µg/mL). Results did not differ according to the presence of PD1.3A SNP. Therefore, BTLA is effective in downregulating $CD4^+$ T cell activation in SLE patients.

6.5. Deregulated PD-1 expression in autologous mixed lymphocyte reaction (AMRL) in SLE patients

6.5.1. SLE T cells show defective AMLR-induced upregulation of PD-1

In mouse studies, PD-1 is an important regulator of autoreactive T cells and defective expression of PD-1 results in breakdown of self-tolerance and development of autoimmunity. Autologous mixed lymphocyte reaction (AMLR) is a well-described *ex vivo* model of autoreactivity against apoptotic self-antigens [180, 186, 187]. During AMLR, regulatory circuits suppress effector T cells and as a result, only a small degree of cell proliferation ensues. We examined the expression of PD-1 on $CD4^+$ T cells, and the expression of PD-L1 on $CD14^+$ monocytes participating in AMLR in SLE patients and healthy controls. Compared to untreated T cells, PD-1 was significantly induced on AMLR $CD4^+$ $CD25^+$ cells (PD-1 mfi 5.8 ± 0.6 *versus* 3.5 ± 0.3, paired t-test p=0.001) in healthy donors **(FIGURE 6.8A-B)**. In SLE, PD-1 induction was less pronounced (4.5 ± 0.3 *versus* 3.8 ± 0.2, p=0.020). Similarly, PD-1 was significantly induced on AMLR $CD4^+$ $CD69^+$ cells (6.5 ± 0.6 *versus* 3.7 ± 0.3, p=0.042) in healthy controls but not in SLE patients (5.2 ± 0.4 *versus* 4.9 ± 0.5, p=0.502). Accordingly, healthy controls had significantly higher PD-1 levels on AMLR $CD4^+$ $CD25^+$ and $CD4^+$ $CD69^+$

activated cells (p=0.024 for both) – but not in $CD4^+$ $FoxP3^+$ regulatory-enriched T cells – compared to SLE patients. In contrast, the expression of CTLA-4 – another negative regulator of T cell function – was comparable between patients and controls (data not shown).

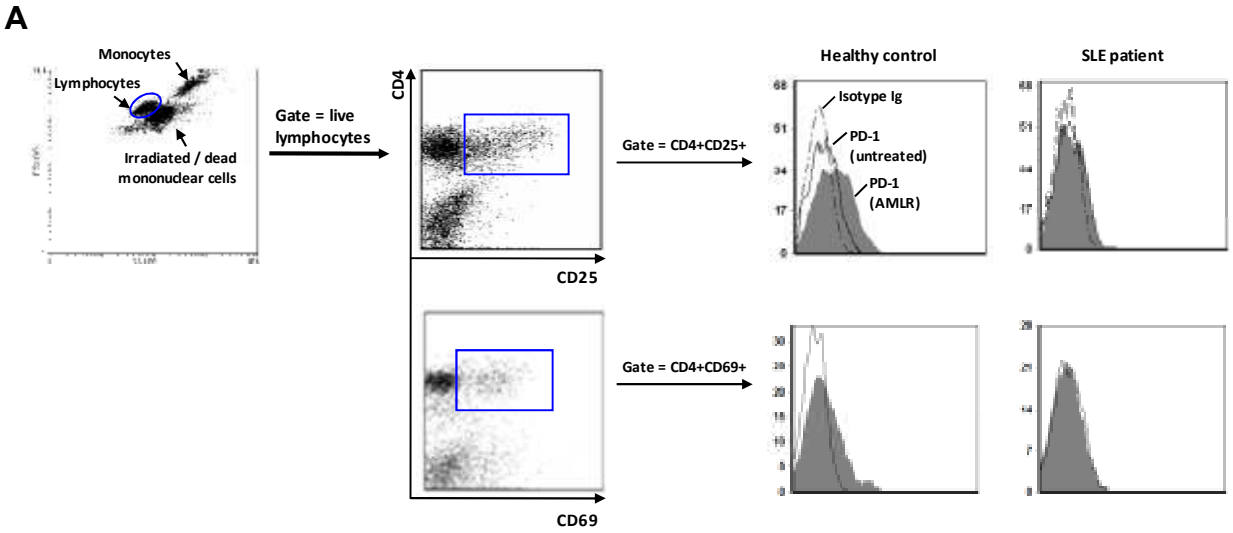


Figure 6.8. Defective induction of PD-1 expression on SLE activated $CD4^+$ T cells participating in autologous mixed lymphocyte reaction (AMLR), an ex vivo model of human autoreactivity against self-antigens. **(A)** Representative flow cytometry analysis of PD-1 expression in AMLR in one healthy control and a patient with SLE (see Materials and Methods) (dashed line = isotype control, black line = PD-1 expression on untreated $CD4^+$ cells, grey histogram = PD-1 expression on AMLR $CD4^+$ cells). **(B)** PD-1 was significantly upregulated on AMLR $CD4^+$ $CD25^+$ and $CD4^+$ $CD69^+$ cells in healthy controls (n=12) but not in SLE patients (n=17). Paired analyses were performed using the paired t-test; the Mann-Whitney test was used for comparisons between independent groups (* p<0.05, ** p<0.01).

T cell activation did not differ between SLE and controls as determined by proliferation results (stimulation index 12 ± 3 in both controls and patients) and expression of HLA-DR on AMLR CD4+ cells (26.4 ± 3.1% in controls *versus* 26.7 ± 3.9% in patients) (data not shown). As a control for the assay we examined the expression of PD-L1 and other costimulatory molecules (HLA-DR, CD40, CD80) on $CD14^+$ monocytes in the AMLR. Although we found decreased expression of CD40 (98 ± 1% *versus* 73 ± 7%, p=0.013) and CD80 (58 ± 8% *versus* 33 ± 7%, p=0.048) on SLE monocytes compared to healthy controls, there was no difference in HLA-DR or PD-L1 expression **(FIGURE S6)**.

6.5.2. SLE patients with the PD1.3A polymorphism have decreased PD-1 expression on AMLR T cells

We next analyzed AMLR data according to the PD1.3 genotype and found that SLE patients homozygous for PD1.3 had significantly reduced PD-1 levels on $CD4^+$ $CD25^+$ and $CD4^+$ $CD69^+$ cells **(FIGURE 6.9)**. Interestingly, PD1.3A was also associated with decreased PD-1 expression in AMRL-induced $CD4^+$ $FoxP3^+$ regulatory-enriched T cells.

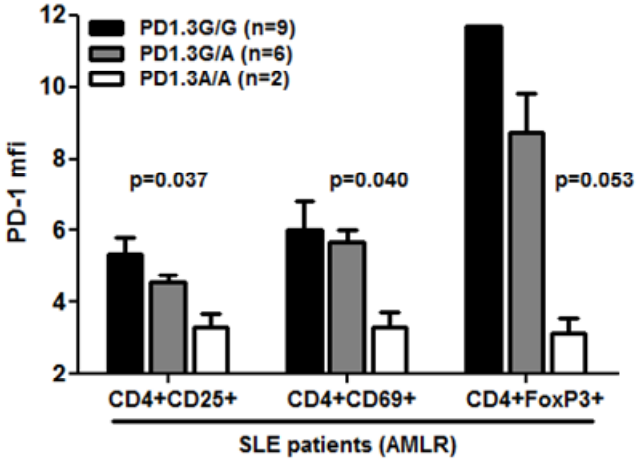


Figure 6.9. Defective PD-1 expression on AMLR CD4+ T cells in SLE patients with the PD1.3A SNP. Patients homozygous for PD1.3A had significantly reduced PD-1 expression on AMLR $CD4^+$ $CD25^+$, $CD4^+$ $CD69^+$, and $CD4^+$ $FoxP3^+$ T cells compared to PD1.3G/G and PD1.3G/A patients. For FoxP3 analysis data were available for n=2 G/G, n=3 G/A and n=2 A/A SLE patients.

Overall, these data suggest that in the AMLR model of human autoreactivity against self-antigens, PD-1 is highly induced on activated $CD4^+$ T cells in healthy donors but its expression is lower in SLE patients; patients with the PD1.3A polymorphism display even lower PD-1 expression.

6.5.3. Regulation of AMLR-induced proliferation by the PD-1/PD-L1 pathway

The role of PD-1/PD-L1 in AMLR was further examined by adding anti-PD-1 and anti-PD-L1 blocking mAbs in culture and measuring the effect on proliferation. In healthy controls anti-PD-1 mAb (10μg/mL) increased proliferation by 59 ± 12% and anti-PD-L1 mAb (10μg/mL) by 89 ± 31% (n=6 independent experiments) **(FIGURE S7)**. In contrast, the respective percentages in SLE patients (n=6) were -2 ± 12% and 8 ± 14%. Anti-CTLA-4 mAb resulted in increased AMLR proliferation comparable to healthy controls (34 ± 22%) and SLE patients (23 ± 27%). These data further support an important role of PD-1/PD-L1 in regulation of T cell responses in humans.

6.6. PD-1 and PD-L1 are expressed in the kidneys of patients with lupus nephritis

In murine models of autoreactivity, PD-1 is involved in regulation of effector T cells at the site of inflammation mostly through interaction with PD-L1 expressed by activated parenchymal cells. We examined PD-1 ligands expression in a human renal tubular epithelial cell (RTEC) line and found that RTEC readily upregulated PD-L1 – but not PD-L2 – mRNA and protein levels after *in vitro* stimulation with IFN-γ **(FIGURE S8)**, suggesting that PD-L1/PD-1 interactions might regulate T cell responses in the kidneys. To this end, we examined PD-1/PD-L1 expression in renal biopsies from patients with lupus nephritis by immunohistochemistry. In 8 out of 13 (62%) lupus nephritis samples, PD-1 staining was detected in the glomeruli compared to 0/9 control samples (unaffected tissue from renal cancer surgeries) **(FIGURE 6.10** and **TABLE S2)**. Similarly, PD-1 was detected in renal tubules of patients with lupus nephritis (6/13, 56%) but not controls (0/9, 0%). All eight PD-1 positive lupus nephritis samples were also stained positive for CD3, expressed in the glomeruli and tubulo-interstitial region, suggesting a correlation between PD-1 expression and CD3+ T cell infiltrates in the lupus nephritis lesions. We found no differences in PD-1 expression according to renal histology or other clinical parameters, and the PD1.3 genotype was available on very few patients to allow for associations with the immunohistochemistry results. PD-L1 expression was restricted to the renal tubules of both SLE patients (10/15, 67%) and controls (5/9, 56%). Thus, molecules of the PD-1/PD-L1 pathway are expressed in the

renal tissue in lupus nephritis patients indicating a potential role in the regulation of immune inflammatory responses at the tissue level.

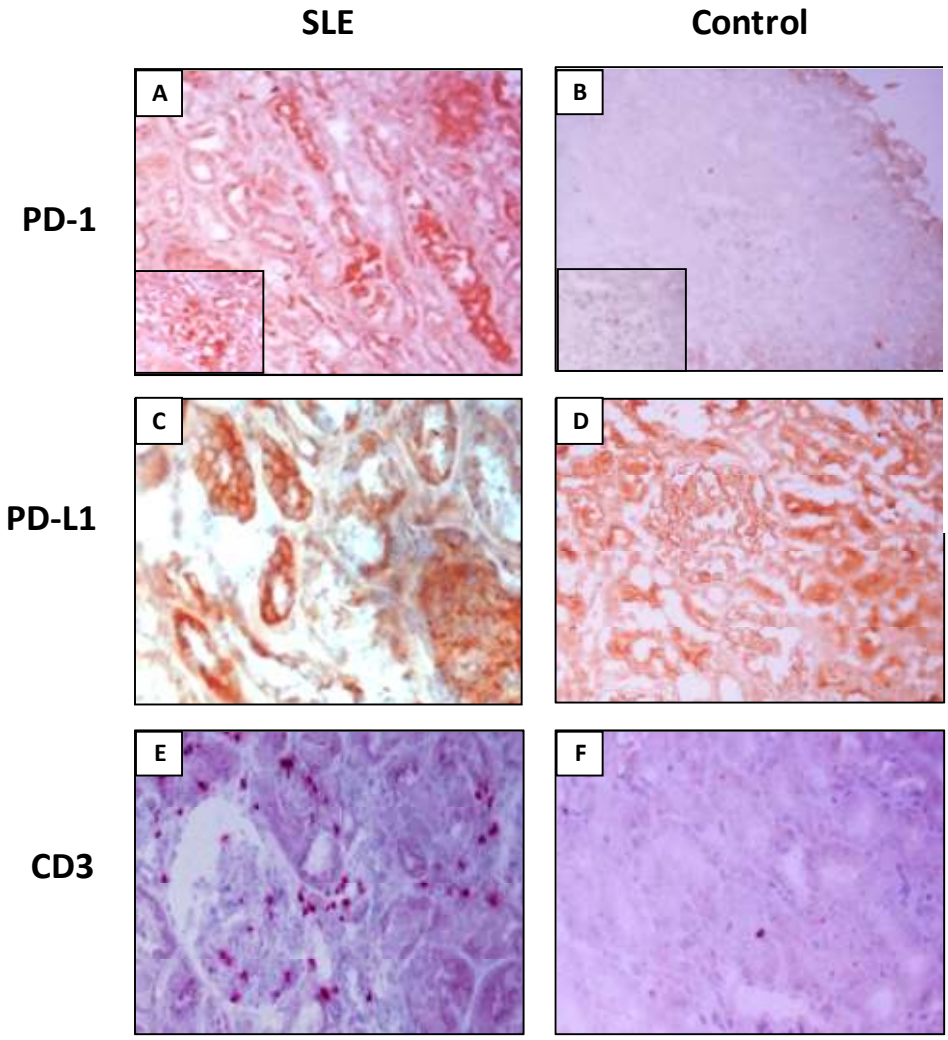


Figure 6.10. Enhanced expression of PD-1 in renal biopsies from SLE patients with nephritis. PD-1 is expressed in the renal glomeruli from patients with lupus nephritis **(A)** but not from unaffected kidney tissue **(B)**. Comparable PD-L1 expression in renal tubules from lupus nephritis **(C)** and normal kidney tissue **(D)**. PD-1 expression in lupus nephritis correlated with $CD3^+$ glomerular and tubulo-interstitial infiltrates **(E)** (the inset represents a single glomerulus).

6.7. Treatment with SLE serum abrogates PD-1–mediated inhibition of $CD4^+$ T cell proliferation

Previous studies have shown that the PD-1/PD-L1 pathway may be influenced by several factors such as the level of costimulation, pro-inflammatory cytokines, and TLR-agonists. SLE serum contains high concentrations of soluble costimulators (e.g. CD40L, CD28), immune complexes, and cytokines which could affect PD-1 function. To explore whether the suppressive function of PD-1 is abrogated within the inflammatory milieu of lupus, we incubated $CD4^+$ T lymphocytes from healthy blood donors with 20% serum from either healthy blood donors or active SLE patients and measured the effect of PD-1 crosslinking on cell proliferation. At suboptimal conditions of PD-1 activation (PD-L1.Fc 0.1µg/mL), incubation with SLE serum resulted in decreased suppression of T cell proliferation compared to normal serum (6.7 ± 3.6% *versus* 16.3 ± 2.7%, n=6 experiments, p=0.027) **(FIGURE 6.11)**. A similar effect was observed at higher PD-L1.Fc concentration (1µg/mL) (27.3 ± 4.2% with SLE serum *versus* 43.0 ± 5.8% with healthy serum, p=0.09) but not at optimal PD-L1.Fc (2µg/mL). Together, these results indicate that soluble factors in the SLE serum may represent an additional distinct mechanism contributing to aberrant regulation of PD-1 function and T cell hyperactivity in SLE.

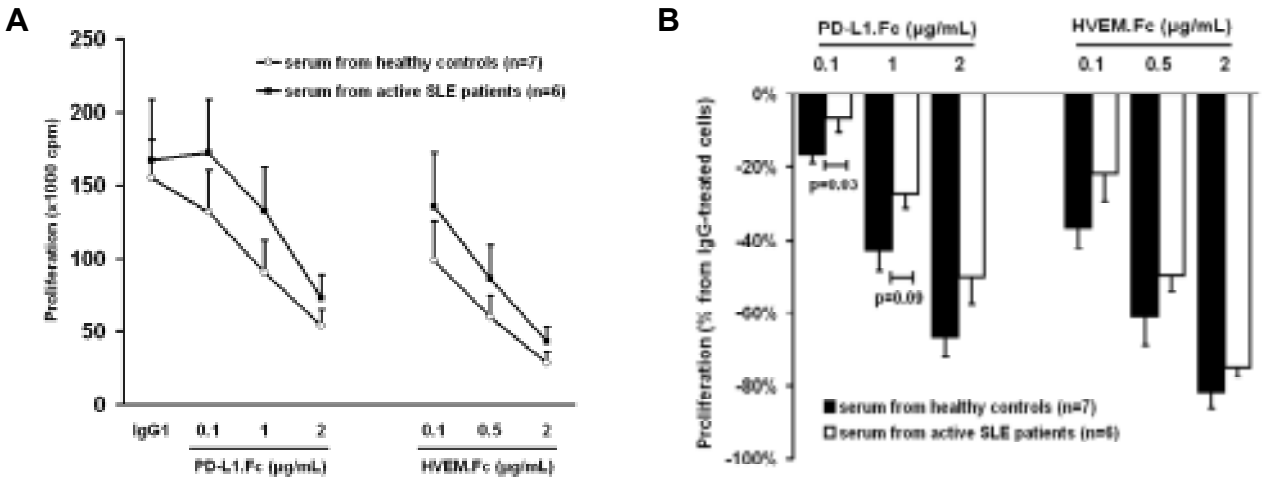


Figure 6.11. SLE serum abrogates the inhibitory function of PD-1. To assess the effect of serum on PD-1-mediated inhibition, $CD4^+$ T cells from healthy blood donors were stimulated with anti-CD3/anti-CD28 mAb and PD-L1.Fc or IgG1 in culture medium supplemented with 20% serum from non-homologous healthy donors or active SLE patients. Results are presented as raw thymidine counts **(A)** or percentage of inhibition compared to IgG1-treated cells **(B)**, and represent the mean ± SEM from seven independent experiments. At the lowest PD-L1.Fc concentrations (0.1 and 1µg/mL) addition of active SLE serum resulted in decreased PD-1-mediated inhibition of cell proliferation (p=0.03 and p=0.09, respectively). In control experiments, HVEM.Fc was used to evaluate BTLA function in SLE T cells.

7 Discussion

The present work provides evidence to support a role for the inhibitory PD-1/PD-L1 pathway in regulation of T cell function in human SLE. Our experiments indicate aberrant expression and function of PD-1 in lupus, attributed to both intrinsic and extrinsic factors.

7.1. The role of PD1.3A polymorphism as a regulatory polymorphism in SLE

We confirmed the role of PD1.3 – but not PD1.5 – SNP as a genetic risk factor for SLE in Cretan (Greek) patients. A recent meta-analysis of 13 independent studies concluded that PD1.3A was significantly associated with SLE in Latin Americans but not in Europeans and Africans; in contrast, PD1.3A was a risk factor for lupus nephritis in Europeans (estimated OR 2.2). The PD1.5C allele also conferred increased risk for SLE in Europeans (estimated OR 1.3)[113]. As with other genetic associations, results are highly variable across different ethnic groups owing to genetic heterogeneity. To this end, Ferreiros-Vidal *et al.* [188] have reported a significant gradient of PD1.3A allele frequency across Europe, ranging from 5-7% in Northern to 11-15% in Southern regions. In our cohort, PD1.3A frequency in SLE patients was 31%, which is one the highest rates reported and approximates those of multi-case Nordic families. Crete has been considered a genetically homogeneous region where several genetic traits have aggregated over hundreds of years [189]. Of note, PD1.3A was not a risk factor for nephritis or other major organ involvement in SLE.

Previous work by Prokunina *et al.* has shown the PD1.3A SNP to disrupt a RUNX-1 binding site in an enhancer-like domain in intron 4 of the PD-1 gene with possible alteration in gene expression [115]. Of interest, RUNX-1 is implicated in regulation of genes which are associated with rheumatoid and psoriatic, and polymorphisms of the RUNX-1 itself confer increased susceptibility to psoriasis arthritis [117, 118, 190]. We found that the A (risk) allele of PD1.3 is associated with decreased (~30–40%) luciferase activity in T cells overexpressing RUNX-1. RUNX-1 is a relatively weak transcription factor that enhances target gene expression in lymphoid tissues. Our results are in accordance with those of other groups who have studied the effect of SNPs in other genes that disrupt RUNX1-binding sites residing in introns, and have reported decreased binding of RUNX-1 and decreased transcriptional activity when the polymorphic allele is present [117, 118]. Indeed, two patients homozygous (A/A) for the PD1.3 SNP had very low basal and induced PD-1 levels, conferring decreased PD-1–mediated inhibition of T cell proliferation and IFN-γ production. Of note, there was no difference in PD-1 expression between wild-type (G/G) and heterozygous (G/A) patients which could reflect the weak contribution of endogenous RUNX-1 on PD-1 transcription under basal, non-induced state, as indicated by our transactivation data **(FIGURE 6.2)**. Thus, PD1.3 SNP could affect PD-1 expression in cases of increased RUNX-1 levels or under certain conditions of lymphocyte activation *in vivo*, as indicated by the reduced PD-1 expression in AMLR in SLE patients with the PD1.3A risk allele.

7.2. PD-1 is a potent T cell regulator in humans

PD-1 was significantly induced on human T cells following TCR/CD28 and PMA/ionomycin stimulation. Importantly, PD-1 was upregulated on $CD4^+$ T cells participating in AMRL, an *ex vivo* model of autoreactivity against apoptotic self-antigens, suggesting an important role for PD-1/PD-L1 in regulation of T cell tolerance in humans. There is evidence to support a 'dual' role for PD-1 with regard to T cell function. Thus, PD-1 expression has been shown to correlate with the activation status of T cells and the surface expression of CD25 and CD69 [64, 191]. During antigen recall responses, however, previously stimulated T cells induce higher PD-1 at low antigen levels, implying that PD-1 preferentially attenuate weak antigen responses that are especially relevant to self-tolerance [53]. In humans, this is further supported by findings in chronic viral

infections where antigen-specific T cells have high PD-1 levels associated with cellular exhaustion and anergy [125, 126]. Crosslinking of PD-1 effectively inhibited effector T cell functions, including proliferation and cytokine production. Apart from PD-1, CTLA-4 and BTLA are also negative lymphocyte regulators which act to increase the TCR threshold for potent T cell responses. Thus, the outcome of T cell activation is determined by the combination and relative strength of costimulatory *versus* coinhibitory signals [63] and according to the current paradigm, anergy may be due to lack of costimulation or strong coinhibitory signaling.

7.3. Defective PD-1 expression and function in SLE as a result of both intrinsic and extrinsic factors

Our experiments showed impaired expression and function of PD-1 in SLE patients in a series of experimental settings. First, patients homozygous for the PD1.3A SNP had significantly reduced basal and induced PD-1 expression on activated $CD4^+$ T cells, associated with decreased PD-1–mediated inhibition of T cell proliferation and IFN-γ secretion. These findings were more prominent at early-to-intermediate stages of T cell activation suggesting that PD-1 might determine early T cell fate. Next, we assessed the role of PD-1 during AMLR, which is a well established *ex vivo* model of autoreactivity against apoptotic self-antigens and has been used to study the immunological processes involved in generation of autoimmunity. Nucleosome-primed T cells show enhanced AMLR–induced proliferation and thus, AMLR represents a suitable experimental setting to study immune responses pertinent to lupus [186]. PD-1 was highly induced on AMLR $CD4^+$ $CD25^+$ and $CD4^+$ $CD69^+$ T cells in healthy donors but not in SLE patients. In line with this finding, blocking of the PD-1/PD-L1 pathway increased AMLR proliferation in healthy controls but not in SLE patients. The decreased expression of PD-1 in SLE might contribute to aberrant T cell responses such as cytokine production and memory cell differentiation. Of note, although the expression of PD-L1 and HLA-DR on AMLR monocytes/macrophages was comparable between patients and controls, patients had decreased expression of the costimulatory molecules CD40 and CD80, which are important in monocyte–T cell interaction and initiation of the AMLR [187]. This finding is in accordance with previous reports demonstrating impaired stimulatory capacity of lupus non-T cells participating in AMLR and thus, the decreased stimulatory capacity of SLE monocytes might contribute to defective induction of PD-1 on $CD4^+$ T cells.

Consistent with its role in maintaining tolerance, PD-1 is more effective at suboptimal conditions of TCR activation and CD28 costimulation [62]. Optimal PD-1 crosslinking suppressed anti-CD3/anti-CD28–induced T cell proliferation and cytokine production in both SLE patients and controls. However, the outcome of PD-1 activation is affected by factors such as cytokines (e.g. IL-6, IL-10), the level of costimulation, and TLR signaling [45, 62]. Serum from SLE patients contains high levels of pro-inflammatory cytokines, complexes of self DNA with autoantibodies, and soluble costimulators which could all influence PD-1 function. To test this hypothesis, we examined the function of PD-1 in normal T cells incubated with either healthy or active SLE serum. SLE serum abrogated PD-1 at suboptimal – but not optimal – dose of plate-bound PD-L1.Fc. These results underscore the importance of the level of PD-L1 expression in determining the outcome of the PD-1/PD-L1. Therefore, within the lupus inflammatory milieu (for instance in the kidneys of lupus nephritis patients) and in cases of low PD-1/PD-L1 expression, the inhibitory function of PD-1 could be abrogated. Of note, higher PD-L1 concentrations effectively inhibited T cell proliferation, overcoming the inhibitory effect of SLE serum. Collectively, these data suggest aberrant regulation of PD-1 expression and function in human SLE as a result of both direct (presence PD1.3A polymorphism) and indirect (inflammatory milieu, AMLR interactions) effects.

7.4. PD-1/PD-L1 pathway modulates T cell responses at the tissue level

In murine models of autoreactivity, PD-1 is involved in regulation of effector T cells at the site of inflammation mostly through interaction with PD-L1 expressed by activated parenchymal cells. We indirectly addressed this issue by examining PD-1/PD-1 ligands expression in kidney biopsies from patients with lupus nephritis. PD-1

staining was detected in the glomeruli and tubules of SLE patients but not controls, and correlated with CD3 T cell expression. In contrast, PD-L1 expression was restricted to the renal tubules of both SLE patients and controls. These results indicate that PD-1/PD-L1 interactions may be important for the regulation of T cell responses in affected kidney tissue in SLE. Furthermore, it is conceivable that variations in factors affecting the expression and outcome of PD-1/PD-L1 (e.g. PD1.3A SNP, inflammatory cytokines) could determine the severity of tissue damage and the disease phenotype.

7.5. Manipulation of PD-1/PD-L1 pathway as a potential therapeutic target

Our results showed that optimal PD-1 activation using high PD-L1.Fc concentrations effectively downregulated effector T cell responses and could overcome the deleterious effects of SLE serum. Modulation of the PD-1/PD-L1 pathway has been proposed as a possible therapeutic target in autoimmune disorders where aberrant immune responses are encountered. A similar approach has been used with CTLA4-Ig which inhibits T cells by disruption of the CD28/B7 interaction [192]. To this end, PD-L1.Fc has been used either alone or with immunosuppressive therapy in mouse transplantation studies and has been shown to prolong graft survival [142, 193]. Combined administration of PD-L1 expressing adenovirus and anti-ICOS-ligand mAb results in amelioration of lupus-like manifestations in the murine BXSB model [194]. In view of our immunohistochemistry studies which showed significant upregulation of PD-1/PD-L1 in lupus-affected kidneys, manipulation of PD-1/PD-L1 to deactivate peripheral and tissue-infiltrating T cells appears as a promising therapeutic modality in SLE. Of note, the benefits of such therapy should be balanced against possible risks for infections and carcinogenesis due to disturbed anti-viral and anti-tumor T cell responses.

7.6. Conclusions and future projects

In summary, our data suggest aberrant regulation of PD-1 expression and function in human SLE as a result of direct and indirect effects. The expression of PD-1/PD-L1 in the affected tissues and during AMLR indicates a role for this pathway in maintenance of peripheral T cell tolerance. These data provide a basis to better elucidate the association of PD-1 polymorphisms with human lupus. Importantly, PD-1 cross-linking on lupus T cells has a significant effect on effector T cell function implying that modulation of the PD-1/PD-L1 pathway may represent an additional therapeutic target in SLE. Ongoing experiments in PD-1 deficient New Zealand Black lupus-prone mice will examine its effects on disease severity and will further delineate its role in T cell regulation.

8 Supplementary material

Figure S1

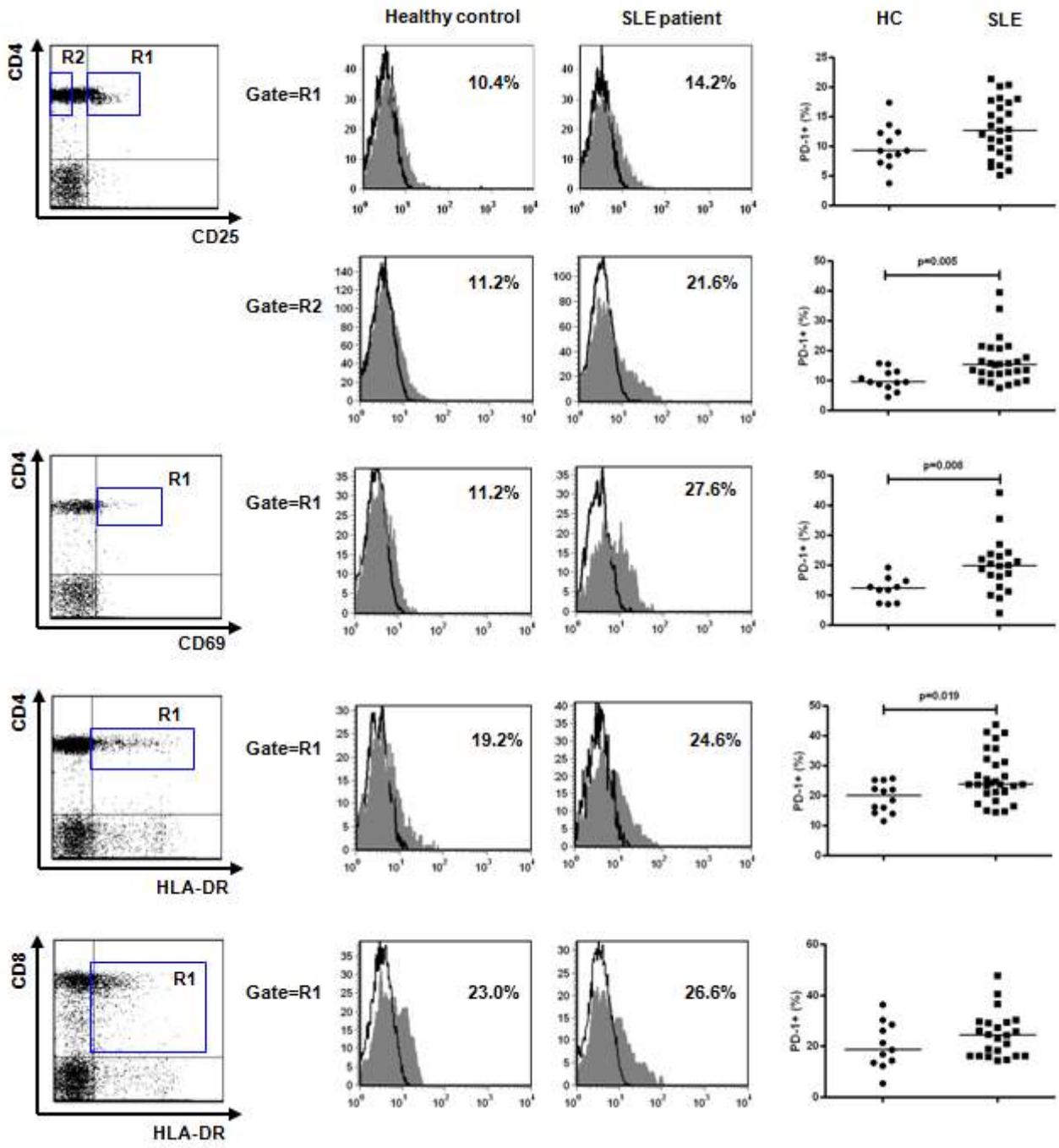


Figure S1. Increased proportion of PD-1$^+$ activated T cells in SLE patients. PBMCs were stained with CD4 or CD8, CD25, CD69 or HLA-DR, and either IgG1-PE or PD-1-PE. Lymphocyte subsets (CD4$^+$ CD25$^+$, CD4$^+$ CD25$^-$, CD4$^+$ CD69$^+$, CD4$^+$ HLA-DR$^+$, CD8$^+$ HLA-DR$^+$) were gated and PD-1 expression was analyzed as histogram (grey shaded) over its Ig isotype (black line). Individual values from healthy controls and SLE patients are also shown.

Figure S2

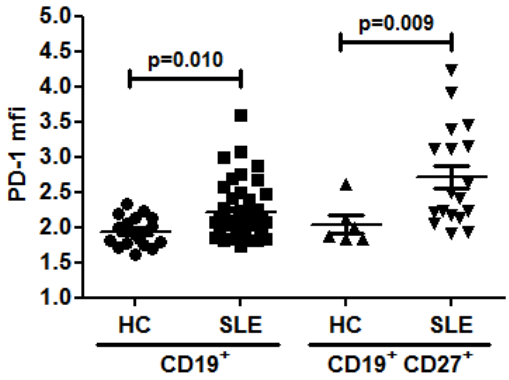


Figure S2. Increased proportion of peripheral blood PD-1+ B cells in SLE patients. Dots represent individual values of PD-1 expression (mean fluorescence intensity, mfi) on total CD19+ and CD19+ CD27+ memory B cells from healthy controls (HC) and SLE patients (SLE).

Figure S3

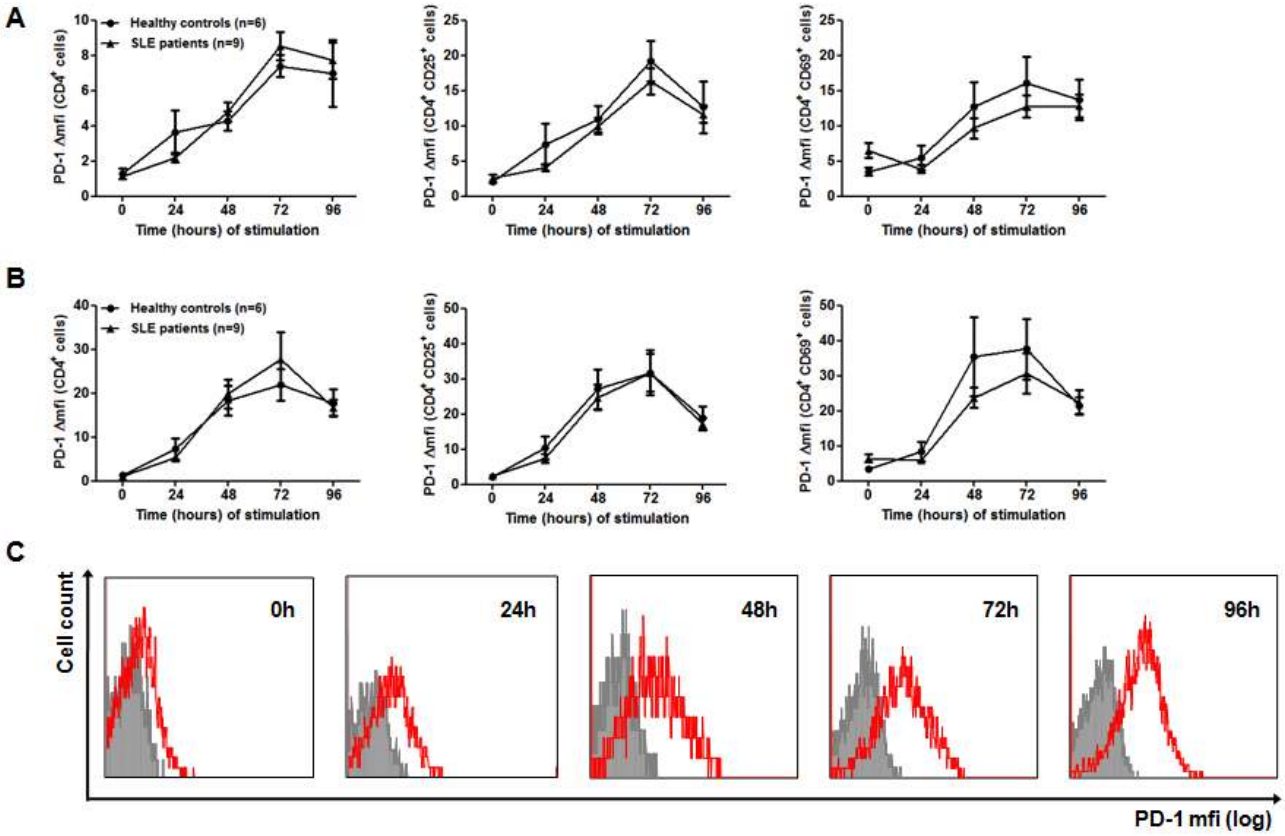


Figure S3. Similar kinetics of PD-1 induction on isolated peripheral blood CD4+ T cells in SLE patients and healthy controls. **(A)** CD4+ T cells were stimulated with plate-bound anti-CD3 (1µg/mL) and soluble anti-CD28 (250ng/mL), were harvested at t=24–96h, and stained with PD-1-PE and CD25 or CD69-FITC. Results are presented as PD-1 Δmfi (i.e. PD-1 mfi minus IgG mfi) on total (*left panel*), CD25+ (*middle panel*), and CD69+ (*right panel*) activated CD4+ T cells. **(B)** The same experiment using optimal concentration of anti-CD3 (5µg/mL) and anti-CD28 (1µg/mL) antibodies. **(C)** Representative flow cytometry histogram from a healthy control. Grey shadows areas represent the IgG isotype and red line represent PD-1 staining.

Figure S4

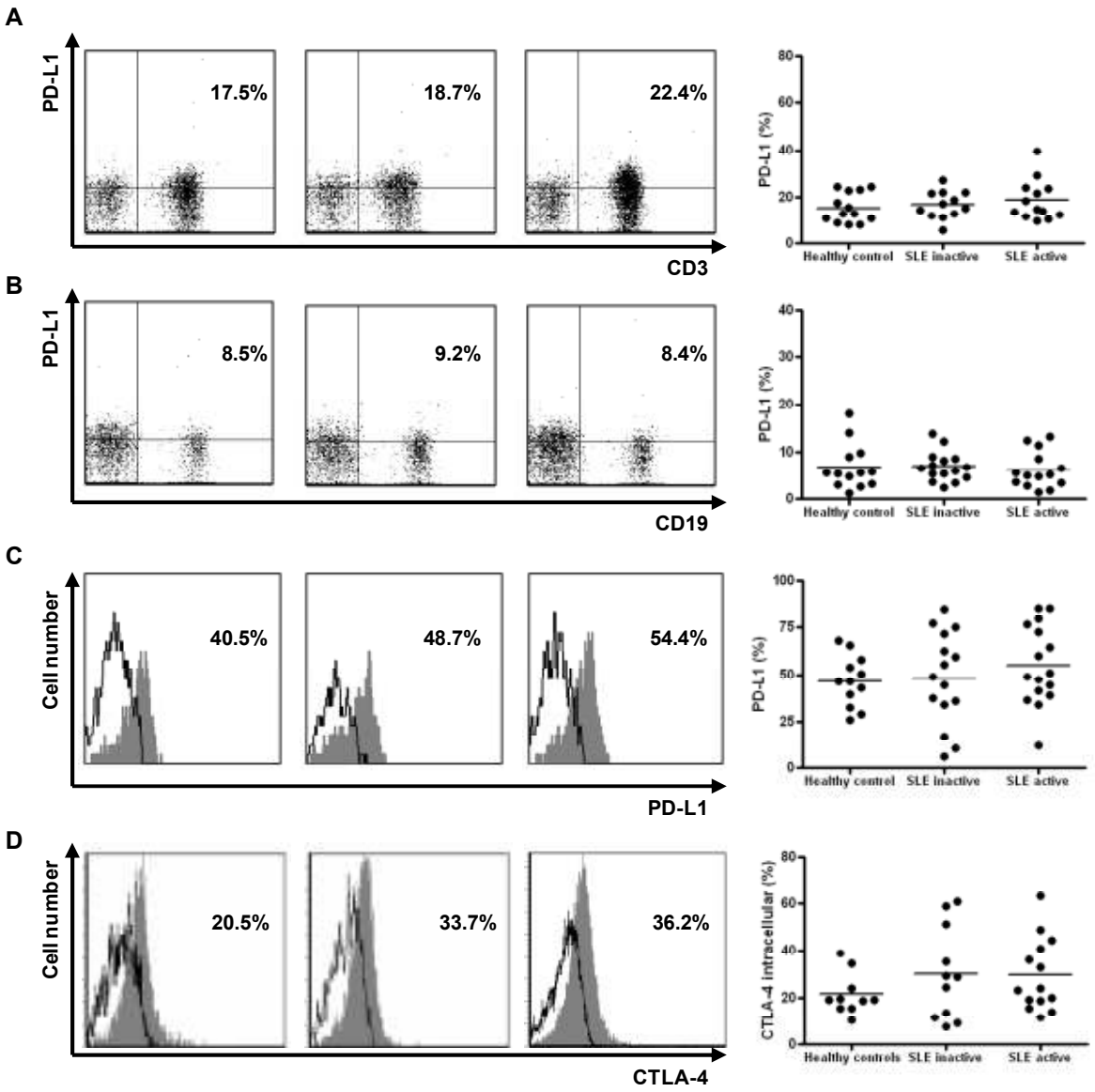


Figure S4. Comparable expression of the immunoregulatory receptors PD-L1 and CTLA-4 in PBMCs in SLE patients and healthy controls. Peripheral blood CD3+ T cells **(A)**, $CD19^{+}$ B cells **(B)** and monocyte/macrophage-gated cells **(C)** was examined for PD-L1 expression by flow cytometry. Intracellular CTLA-4 expression in $CD4^{+}$ $CD25^{+}$ T cells was also analyzed **(D)**. Individual values and representative flow cytometry histograms from healthy controls, inactive SLE, and active SLE patients are shown.

Figure S5

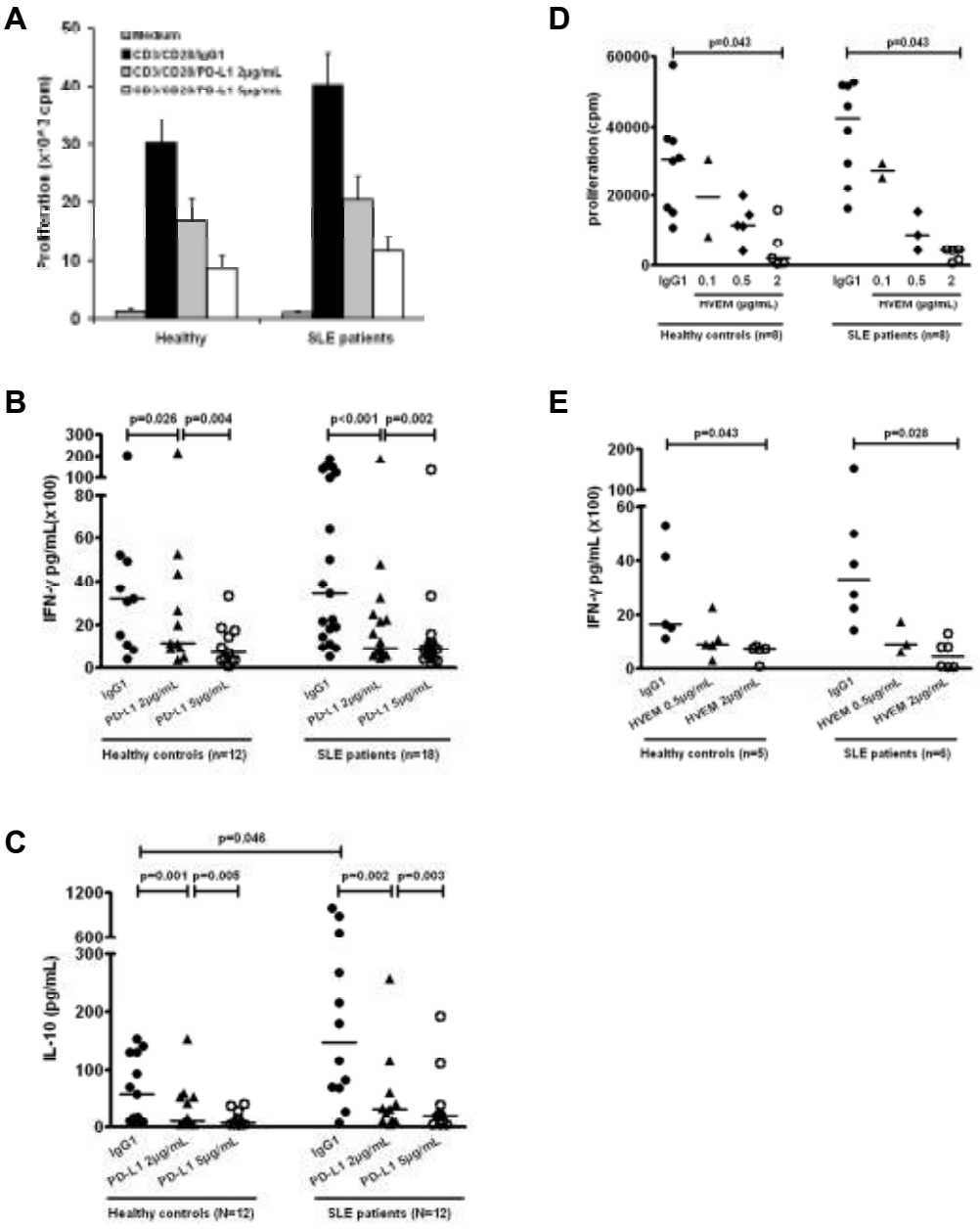


Figure S5. Normal suppressive function of the immunoregulatory receptors PD-1 and BTLA in lupus T cells. $CD4^+$ T cells were stimulated with anti-CD3/anti-CD28 mAb and various concentration plate-bound PD-L1.Fc or HVEM.Fc to crosslink PD-1 and BTLA respectively. At 72 hours cell cultures were pulsed with thymidine for another 16 hours to assess cell proliferation **(A, D)** and supernates were collected for IFN-γ **(B, E)** and IL-10 **(C)** measurement. PD-1 and BTLA crosslinking resulted in significant reduction in T cell proliferation and cytokine secretion in both patients and controls. T cells from both patients and controls had minimal proliferation and cytokine production when cultured in medium alone (not shown).

Figure S6

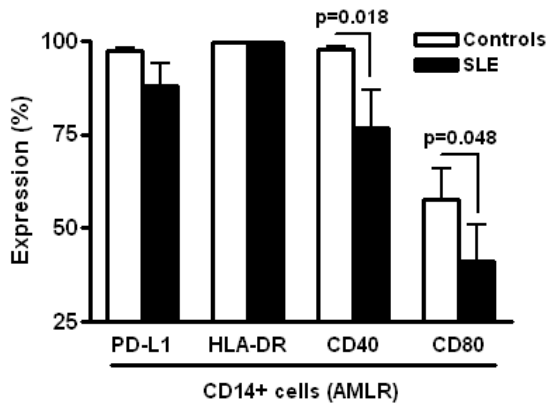


Figure S6. PD-L1 and HLA-DR expression on CD14⁺ monocytes participating in the AMLR was comparable between controls and SLE patients but SLE monocytes had decreased expression of CD40 and CD80. Results are mean ± SEM from 5-6 independent experiments in each group.

Figure S7

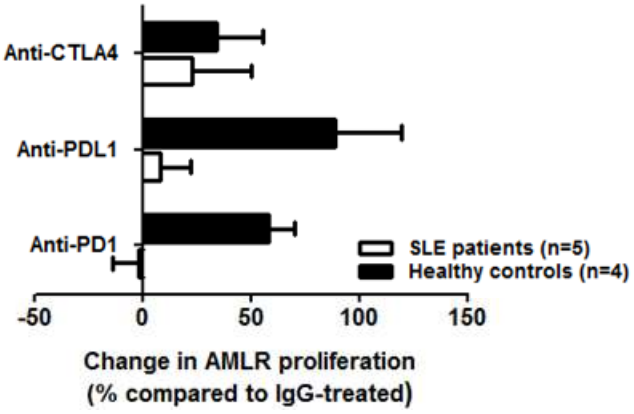


Figure S7. Regulation of AMLR by the PD-1/PD-L1 and CTLA-4 pathways. Blocking anti-CTLA4, anti-PDL1, anti-PD1 mAb or mouse IgG2a (all 10µg/mL) were added in AMLR and proliferation was assessed as described in *Materials and Methods*. PD-1/PD-L1 blockade significantly enhanced AMLR-induced proliferation in healthy controls but not in SLE patients. Comparable effect of CTLA-4 blockade on AMLR-induced proliferation in patients and controls.

Figure S8

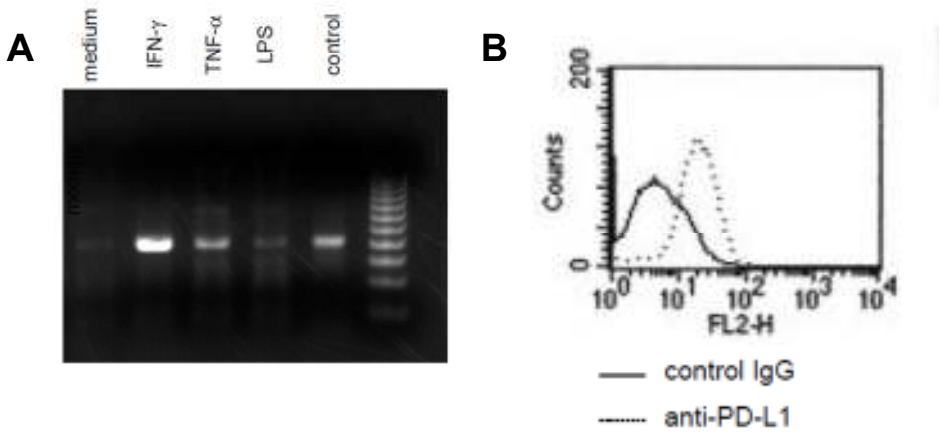


Figure S8. Human renal tubular epithelial cells (RTECs) express PD-L1 following activation with IFN-γ. RTECs (passage 3-5) were treated with recombinant IFN-γ (200 UI/mL) for 48 hours and PD-L1 mRNA and protein expression was examined by semi-quantitative RT-PCR **(A)** and flow cytometry **(B)**.

Table S1. Correlation of PD-1 expression with disease activity and serum anti-dsDNA antibodies in SLE patients.

Figures represent the proportion (mean ± SEM) of PD-1$^+$ CD4$^+$ and CD8$^+$ peripheral blood T cell subsets in SLE patients with inactive or active (SLEDAI ≥8) disease and according to the presence of serum anti-dsDNA antibodies. Statistically significant associations (p-value <0.05, Mann-Whitney U test) are highlighted.

	Disease activity			Anti-dsDNA		
PD-1 (%)	**Inactive**	**Active**	p-value	**Negative**	**Positive**	p-value
CD4$^+$	13.6 ± 1.3	15.2 ± 1.8	0.627	12.3 ± 0.8	18.4 ± 2.7	**0.040**
CD4$^+$ CD25$^+$	13.2 ± 1.3	12.6 ± 1.3	0.734	11.7 ± 1.0	14.2 ± 1.8	0.293
CD4$^+$ CD25^{++}	13.8 ± 2.3	13.4 ± 1.6	0.886	11.1 ± 1.1	15.8 ± 2.2	0.075
CD4$^+$ CD25$^-$	15.6 ± 1.3	17.1 ± 2.5	0.846	13.6 ± 0.8	20.9 ± 3.5	0.080
CD4$^+$ CD69$^+$	19.0 ± 1.4	20.9 ± 3.9	0.940	17.4 ± 1.8	22.3 ± 4.2	0.055
CD4$^+$ HLA-DR$^+$	23.3 ± 1.8	28.8 ± 2.3	**0.040**	22.2 ± 1.2	30.8 ± 2.9	**0.012**
CD4$^+$ CD40L$^+$	12.6 ± 2.1	19.6 ± 3.4	0.155	12.7 ± 2.3	23.5 ± 1.6	**0.021**
CD8$^+$	17.1 ± 1.9	16.3 ± 1.2	0.982	17.1 ± 1.5	16.7 ± 1.7	0.777
CD8$^+$ HLA-DR$^+$	22.7 ± 1.8	25.6 ± 2.8	0.801	21.5 ± 1.5	29.5 ± 3.6	0.102

Table S2. Expression of PD-1 and PD-L1 in renal biopsies from patients with lupus nephritis and controls

	Lupus nephritis	**Controls** [1]
PD-1 staining		
Glomerular	8/13 (61.5%)[2]	0/9 (0%)
Tubular	6/13 (56.2%)	0/9 (0%)
PD-L1 staining		
Glomerular	1/15 (6.7%)	0/9 (0%)
Tubular	10/15 (66.7%)	5/9 (55.5%)

[1] Control tissue was unaffected renal tissue from renal cancer surgeries

[2] Percentage of samples positively stained for PD-1 or PD-L1 (see Materials-Methods)

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Genetic, Immunologic, and Immunohistochemical Analysis of the Programmed Death 1/Programmed Death Ligand 1 Pathway in Human Systemic Lupus Erythematosus

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Objective. **A putative regulatory intronic polymorphism (PD1.3) in the programmed death 1 (PD-1) gene, a negative regulator of T cells involved in peripheral tolerance, is associated with increased risk for systemic lupus erythematosus (SLE). We undertook this study to determine the expression and function of PD-1 in SLE patients.**

Methods. **We genotyped 289 SLE patients and 256 matched healthy controls for PD1.3 by polymerase chain reaction–restriction fragment length polymorphism analysis. Expression of PD-1 and its ligand, PDL-1, was determined in peripheral blood lymphocytes and in renal biopsy samples by flow cytometry and immunohistochemistry. A crosslinker of PD-1 was used to assess its effects on anti-CD3/anti-CD28–induced T cell proliferation and cytokine production.**

Results. **SLE patients had an increased frequency of the PD1.3 polymorphism (30.1%, versus 18.4% in controls; $P = 0.006$), with the risk A allele conferring decreased transcriptional activity in transfected Jurkat cells. Patients homozygous for PD1.3—but not patients heterozygous for PD1.3—had reduced basal and induced PD-1 expression on activated CD4+ T cells. In autologous mixed lymphocyte reactions (AMLRs), SLE patients had defective PD-1 induction on activated CD4+ cells; abnormalities were more pronounced among homozygotes. PD-1 was detected within the glomeruli and renal tubules of lupus nephritis patients, while PDL-1 was expressed by the renal tubules of both patients and controls. PD-1 crosslinking suppressed proliferation and cytokine production in both normal and lupus T cells; addition of serum from patients with active SLE significantly ameliorated this effect on proliferation.**

Conclusion. **SLE patients display aberrant expression and function of PD-1 attributed to both direct and indirect effects. The expression of PD-1/PDL-1 in renal tissue and during AMLRs suggests an important role in regulating peripheral T cell tolerance.**

Systemic lupus erythematosus (SLE) is characterized by a wide spectrum of clinical manifestations and aberrant production of autoantibodies against nuclear or cell surface antigens and serum proteins. Although the initiating events are not completely understood, it is generally accepted that deregulated/autoreactive T lymphocytes play an important role in disease pathogenesis (1,2).

Under physiologic conditions, self-reactive T cells are strictly controlled by mechanisms involving both central and peripheral lymphoid organs. In this context, B7 family molecules provide signals that are critical for both stimulating and inhibiting T cell activation (3,4). The balance between costimulatory and inhibitory sig-

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naling is important for maintenance of peripheral tolerance, and breakdown of self tolerance results in pathogenesis of autoimmunity (4–6).

Programmed death 1 (PD-1) is a homolog of CD28 and CTLA-4 and belongs to the immunoglobulin superfamily. PD-1 has 2 tyrosines in its cytoplasmic tail which form an immunoreceptor tyrosine–based inhibition motif and an immunoreceptor tyrosine–based switch motif (4,7). Both PD-1 and CTLA-4 inhibit T cells, although through distinct mechanisms; PD-1 inhibits Akt phosphorylation by preventing CD28-mediated activation of phosphatidylinositol 3-kinase, whereas CTLA-4 inhibits Akt phosphorylation by recruiting the protein phosphatase 2A (8,9).

Cumulative data suggest a distinct role of PD-1 and its ligands (programmed death ligand 1 [PDL-1], also called B7-H1; PDL-2, also called B7-DC) in T cell regulation. PD-1 is important for the "fine tuning" of lymphocyte activation at the tissue level based upon the expression of PDL-1 in nonlymphoid organs such as heart, lung, placenta, kidney, and liver (4,7,10,11). A broader role of PD-1 in immune regulation has also been suggested considering its induction not only on activated T cells, but also on B cells and monocytes (4). Of interest, PD-1 ligation is more effective than CTLA-4 in suppressing anti-CD3/anti-CD28–induced changes in the T cell transcriptional profile (8).

The critical role of PD-1 in maintenance of peripheral tolerance is highlighted by gene disruption studies demonstrating strain-specific autoimmune phenotypes. PD-1–deficient C57BL/6 mice develop lupus-like proliferative arthritis and glomerulonephritis, and PD-1–deficient BALB/c mice develop autoimmune dilated cardiomyopathy (7). Deficiency of PD-1 in the NOD background results in accelerated diabetes (12). These phenotypes are milder than the massive lymphadenopathy and infiltration of T cells into multiple organs observed in CTLA-4 deficiency and are more reminiscent of the human autoimmunity (13).

In humans, a role for PD-1 in tolerance and autoimmunity was first proposed by investigators in genetic studies reporting associations between PD-1 gene polymorphisms and development of autoimmunity (14–19). Specifically, a regulatory single-nucleotide polymorphism (SNP) designated PD1.3, with a G-to-A change at position +7146, located in an enhancer-like domain in intron 4 of the PD-1 gene, shows strong association with SLE in Northern Europeans and Mexican Americans (relative risk 2.6–3.5) (15). The risk allele A of the PD1.3 SNP disrupts the binding of the RUNX-1 transcription factor to the enhancer, with potential impact on gene expression (15). However, to date its effect on gene transcription has not been studied.

In view of the important role of PD-1 in T cell function, we sought to examine the role of PD-1 in human SLE. To this end, we genotyped our patients for 2 common SNPs of the PD-1 gene and examined the effect of the PD1.3 SNP on gene transcription. In addition, we examined the expression of PD-1 and its ligands in the peripheral blood and in the kidneys of patients with lupus nephritis and assessed PD-1 function in lupus T cells. To our knowledge, this represents the first comprehensive evaluation of the PD-1/PDL-1 pathway in this disease.

PATIENTS AND METHODS

Patient and control populations. A case–control study including 289 SLE patients and 256 age- and sex-matched healthy blood donors (both of Cretan origin) was conducted to test the association between PD-1 polymorphisms and the risk for SLE. SLE patients were diagnosed (20) and recruited from the University Hospital of Heraklion (Crete, Greece). The study was approved by the Institutional Review Committee, and all subjects gave written informed consent.

DNA extraction and genotyping for PD1.3 and PD1.5. Genomic DNA was extracted from EDTA-treated blood (PureGene Genomic Whole Blood DNA Purification kit; Gentra Systems, Minneapolis, MN). Genotyping for the PD1.3 and PD1.5 SNPs was performed by polymerase chain reaction (PCR)–restriction fragment length polymorphism (*Pst* I and *Pvu* II, respectively) analysis, according to published protocols (14,15).

Plasmid constructions and transient transfection in luciferase assays. We generated 180-bp fragments with the 2 different alleles (G and A) of the PD1.3 SNP by PCR using DNA from homozygotes with the corresponding haplotypes. The fragments were inserted in the *Sac* I/*Xho* I site of the pGL3-basic vector (Promega, Madison, WI), upstream of the AdML minimal promoter (MLP). The expression construct pCMV-RUNX1 was kindly provided by Dr. D.-E. Zhang (The Scripps Research Institute, La Jolla, CA). Jurkat T cells were seeded in 12-well plates and were transfected in duplicates with pGL3-Luc (control) or pGL3-Luc with the wild-type G allele (PD1.3G-MLP) or the risk A allele (PD1.3A-MLP) (each 0.2 μg), with or without pCMV-RUNX1 (0.1 μg) using the Superfect Reagent (Qiagen, Chatsworth, CA). In each transfection, the plasmid pCMV-β-gal was included for normalization of the transfection efficiency. After 16 hours of incubation, phorbol myristate acetate (10 ng/ml) and ionomycin (500 ng/ml) (Sigma-Aldrich, St. Louis, MO) were added for another 24 hours. Cells were washed twice in phosphate buffered saline (PBS) and analyzed for luciferase activity.

Preparation of mononuclear cells and isolation of CD4+ T lymphocytes. Peripheral blood mononuclear cells (PBMCs) from 40 SLE patients (38 female, mean ± SD age 41 ± 14 years) and 26 age- and sex-matched healthy blood

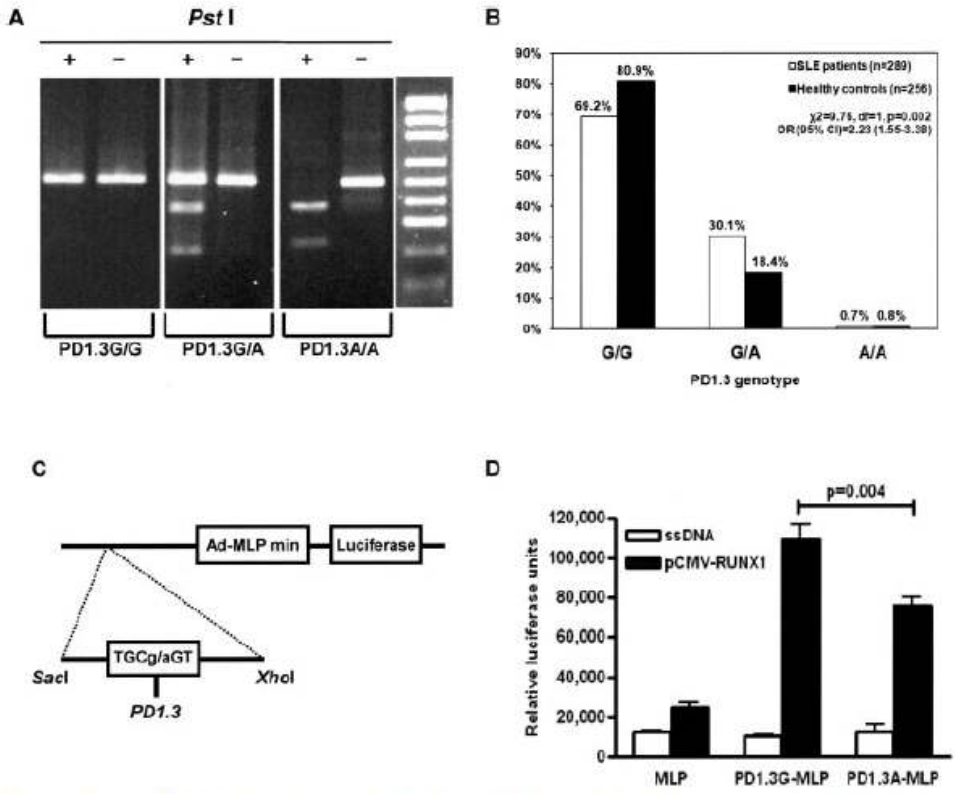

Figure 1. Association of decreased transcriptional activity with increased frequency of the PD1.3A single-nucleotide polymorphism (SNP) in patients with systemic lupus erythematosus (SLE). **A,** Genotyping for the PD1.3 SNP in intron 4 of the programmed death 1 gene by polymerase chain reaction–restriction fragment length polymorphism analysis. **B,** Frequencies of PD1.3 genotypes in SLE patients and age- and sex-matched healthy controls, showing increased frequency of the PD1.3A SNP in SLE patients. The presence of the A allele was associated with increased risk for SLE (odds ratio [OR] 2.23, 95% confidence interval [95% CI] 1.55–3.38, $P = 0.002$). **C,** Investigation of a putative RUNX-1 site in the PD1.3 SNP. Shown is a schematic representation of the reporter constructs containing the SLE-associated PD1.3A allele or the wild-type PD1.3G allele of the PD1.3 SNP linked to the adenovirus major late minimal promoter (MLP) and the luciferase gene in the pGL3-basic vector. The sequence of the putative RUNX-1 binding site containing the polymorphism is indicated. **D,** Association of decreased transcriptional activity with the polymorphic PD1.3A allele. Jurkat T cells were transiently transfected with PD1-luciferase reporter vectors bearing the PD1.3G or the SLE-associated PD1.3A alleles in the absence or in the presence of an expression vector for RUNX-1 (pCMV-RUNX1). In each transfection, the plasmid pCMV-β-gal was included for normalization of the transfection efficiency. The normalized mean and SEM values of luciferase activity from 5 independent experiments are shown. Overexpression of RUNX-1 resulted in increased luciferase activity that was significantly higher in Jurkat T cells transfected with PD1.3G reporter construct than in those transfected with PD1.3A reporter construct ($P = 0.004$ by t-test). ssDNA = salmon sperm DNA that was used instead of pCMV-RUNX1 in control experiments.

donors were isolated by Ficoll-Histopaque (Sigma-Aldrich) density-gradient centrifugation of heparinized venous blood. Twenty-four patients (60%) had active disease (SLE Disease Activity Index score ≥8) (21), 13 patients (33%) had proliferative or membranous nephritis, and 16 patients (40%) were receiving corticosteroids. Patients had not taken any lupus medication for 24 hours prior to blood sampling. CD4+ T lymphocytes (98% purity) were isolated from PBMCs by negative selection (Miltenyi Biotec, Bergisch Gladbach, Germany).

Antibodies and flow cytometry. All antibodies were mouse anti-human monoclonal antibodies (mAb). Phycoerythrin (PE)–conjugated anti–PDL-1 (anti–B7-H1) (MIH1) was from eBioscience (San Diego, CA). PE-Cy5–conjugated anti-CD4 (OKT4) and PE-conjugated anti-CD69 (TP1.55.3) were from Beckman Coulter (Miami, FL). PE-conjugated anti–PD-1 (MIH4), fluorescein isothiocyanate (FITC)–conjugated anti-CD25 (M-A251), anti-CD69 (FN50), and anti–HLA–DR (G46-6) were from BD PharMingen (Franklin Lakes, NJ). PE- or PE-Cy5–conjugated IgG1 (679.1Mc7) (Beckman Coulter), PE-conjugated IgG2a (eBM2a; eBioscience), and FITC-conjugated IgG1 (P3) (eBioscience) were used as IgG isotype controls. PBMCs (0.5×10^6) were incubated in wash buffer (PBS/2.5% fetal bovine serum [FBS])

with appropriate amounts of mAb on ice for 30 minutes. Cells were washed and were immediately analyzed on an EPICS XL-MCL flow cytometer (Beckman Coulter).

PD-1 expression on activated CD4+ T cells. CD4+ T cells (1×10^5/well) were incubated in RPMI 1640 medium containing 10% FBS, 2 m*M* L-glutamine, 10 m*M* HEPES, 100 IU/ml penicillin, and 10 μg/ml streptomycin (Gibco Invitrogen, Karlsruhe, Germany) on 96-well round-bottomed tissue culture plates (Nunc, Naperville, IL) coated with 1 μg/ml anti-CD3 (UCHT1; R&D Systems, Minneapolis, MN), and 250 ng/ml soluble anti-CD28 (CD28.2; BD PharMingen) was added. In some experiments, optimal concentrations of anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml) were used. Cells were harvested at 12–96 hours and analyzed for PD-1 and CD69 expression by flow cytometry. Dead cells were excluded based on forward scatter/side scatter properties, and a total of 10,000 live cells were analyzed.

Assessment of PD-1 function. To determine the effects of PD-1 crosslinking during T cell activation, CD4+ T cells (1×10^5/well) were stimulated in 96-well plates with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (250 ng/ml) in combination with various concentrations of plate-bound PDL-1.Fc (0–5 μg/ml) (R&D Systems), a chimeric protein containing the extracellular part of human PDL-1 linked to the Fc fragment of human IgG1. Human IgG1 was added to keep the amount of total protein constant at 6 μg/ml. At 24 or 72 hours culture supernatants were collected for cytokine measurement, and cells were pulsed with ^{3}H-thymidine (1 μCi/well) (Amersham Biosciences, Munich, Germany) for another 16 hours to measure proliferation. To evaluate the effect of SLE serum on PD-1–mediated inhibition, CD4+ T cells from healthy blood donors were stimulated with anti-CD3/anti-CD28 and plate-bound PDL-1.Fc in the presence of 20% heat-inactivated serum from patients with active SLE or unrelated healthy donors. Proliferation was measured as previously described.

Cytokine enzyme-linked immunosorbent assay (ELISA). Cytokines in supernatants were assayed by ELISA using antibodies to human interferon-γ (IFNγ) and interleukin-10 (IL-10) (Ready-SET-Go! ELISA kit; eBioscience) according to the manufacturer's instructions.

Autologous mixed lymphocyte reactions (AMLRs). AMLRs were set up as previously described (22). PBMC CD3+ T and non–T cell fractions from SLE patients (n = 17) and healthy donors (n = 12) were isolated using a magnetic bead positive selection system (Pan T Cell Isolation kit; Miltenyi Biotec). Cells were incubated in RPMI 1640 medium containing 10% heat-inactivated autologous human serum. Non–T stimulator cells were induced to undergo apoptosis by gamma irradiation with 1,000 rads from a cobalt source and were then cocultured at a 2:1 ratio with 1×10^5 autologous responder T cells in 96-well round-bottomed plates. On day 4, untreated T cells and AMLR cells were harvested, washed, and analyzed for PD-1 and other activation/costimulatory markers by flow cytometry. The selection of this time point was based on initial experiments showing peak PD-1 expression during 72–96 hours of AMLRs. The proliferative response of T lymphocytes in AMLRs was evaluated after 5 days of culture by addition of ^{3}H-thymidine for 24 hours.

Detection of PD-1 and PDL-1 in renal biopsy samples. Renal biopsy samples were obtained from lupus nephritis patients (n = 15) and from macroscopically unaffected tissues of patients with renal cancer (n = 9) (control group). Cryostat sections (5 μm) were fixed in cold acetone, and detection of PD-1, PDL-1, and CD3 was performed by standard avidin–biotin complex method. After blocking, the sections were incubated with unlabeled goat anti-human PD-1 (C-16; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-human PDL-1 (29E.2A3; kindly provided by Dr. G. Freeman, Harvard School of Medicine), or mouse anti-human CD3 mAb, followed by biotin-conjugated goat or mouse secondary antibody (Dako, Carpinteria, CA). We used 3,3′-diaminobenzidine as chromogen, and sections were counterstained with hematoxylin. A semiquantitative score was assigned separately for tubulointerstitial and glomerular PD-1/PDL-1 expression according to the following staining pattern: 0 = absent; +1 = weak; +2 = moderate; +3 = strong. Since most of the samples displayed weak-to-moderate staining, for simplicity they were categorized as either positive or negative for PD-1/PDL-1 expression.

Statistical analysis. The genotype and allele frequencies were determined, and the chi-square test was used to test the significance of the differences in 2×2 contingency tables. The nonparametric Mann-Whitney U test was used for comparisons among groups with small or unequal sample sizes. Results were expressed as the mean ± SEM, and 2-tailed *P* values less than 0.05 were considered significant.

RESULTS

Increased frequency of the PD1.3 SNP in patients with SLE. In certain ethnic populations PD-1 polymorphisms are associated with increased risk for autoimmune disease (14,15,23,24). To determine whether PD-1 SNPs are associated with susceptibility to SLE in our cohort, we analyzed the genomic DNA of 289 SLE patients (95% women, mean ± SD age 45 ± 16 years) of Cretan (Greek) origin for the PD1.3 (Figure 1A) and PD1.5 SNPs and compared their frequencies with those in age- and sex-matched healthy controls (95% women, mean ± SD age 45 ± 16 years); these 2 SNPs have previously been associated with SLE in Caucasian populations (15).

We found an increased frequency of PD1.3 heterozygosity (G/A) in SLE patients compared with healthy controls (30.1% versus 18.4%; $P = 0.006$) (Figure 1B). The presence of the A allele was associated with increased risk for SLE (odds ratio [OR] 2.23, 95% confidence interval [95% CI] 1.55–3.38) but not for lupus nephritis (OR 1.13, 95% CI 0.43–3.81). We found 2 patients who were homozygous for the PD1.3 SNP (A/A), yielding a frequency of 0.7%, which is similar to the frequency of PD1.3 homozygosity reported for other ethnic groups, ranging from 0.5% to 1% (15,17,25). These 2 patients had hematologic, skin, and articular manifestations but no nephritis or other major organ

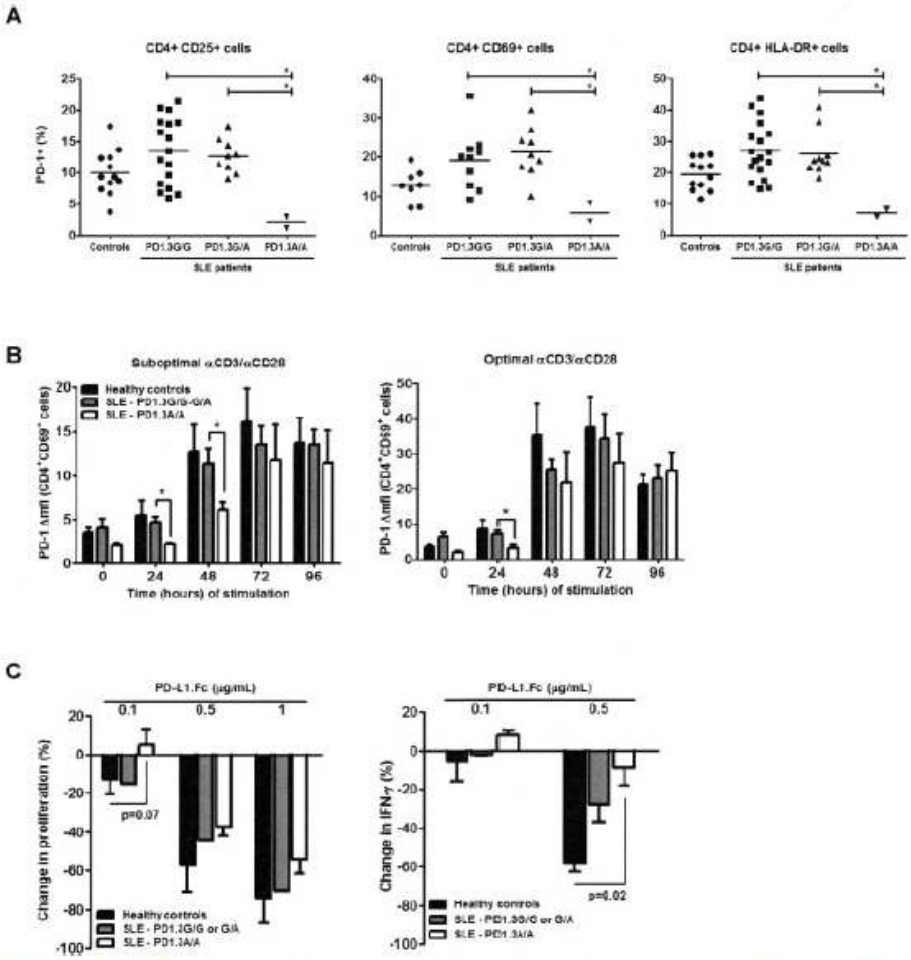

Figure 2. Decreased basal and induced programmed death 1 (PD-1) expression on activated CD4+ T cells in SLE patients homozygous for the PD1.3 SNP, and decreased PD-1–mediated suppression of T cell function. **A,** SLE patients homozygous for the PD1.3 SNP (A/A) have significantly reduced percentages of PD-1+ activated CD4+CD25+, CD4+CD69+, and CD4+HLA–DR+ T cells compared with healthy controls, PD1.3 G/G SLE patients, or PD1.3 G/A SLE patients (* = $P < 0.05$ by Mann-Whitney U test). Horizontal bars indicate the mean. **B,** PD-1 is induced on healthy and SLE CD4+ T cells stimulated with suboptimal (1 μg/ml and 250 ng/ml, respectively) and optimal (5 μg/ml and 1 μg/ml, respectively) concentrations of anti-CD3 and anti-CD28 monoclonal antibodies (mAb). Results are presented as PD-1 ΔMFI (PD-1 mean fluorescence intensity [MFI] − IgG MFI) gated on CD4+CD69+ cells. Values are the mean and SEM. The 2 patients who were homozygous for the PD1.3 SNP had significantly decreased PD-1 expression at 24 and 48 hours following activation with suboptimal concentrations of anti-CD3/anti-CD28 mAb (* = $P < 0.05$ by Mann-Whitney U test). **C,** CD4+ T cells were stimulated with anti-CD3/anti-CD28 mAb and plate-bound PDL-1.Fc (a chimeric protein containing the extracellular part of human programmed death ligand 1 [PDL-1] linked to the Fc fragment of human IgG1) to crosslink PD-1. At 24 hours cell cultures were pulsed with ^{3}H-thymidine to assess cell proliferation, and supernatants were collected for interferon-γ (IFNγ) measurement. Results are shown as the percentage of change compared with IgG-treated CD4+ T cells. Values are the mean and SEM. Under conditions of suboptimal PD-1 crosslinking (0.1–0.5 μg/ml PDL-1.Fc), the 2 patients who were PD1.3 A/A had defective PD-1–mediated inhibition of T cell proliferation and IFNγ production compared with healthy controls (n = 4), PD1.3 G/G SLE patients (n = 4), or PD1.3 G/A SLE patients (n = 4). See Figure 1 for other definitions.

involvement. There was no difference in PD1.5 SNP haplotype frequencies between patients and controls (data not shown). The observed frequencies of PD1.3 and PD1.5 genotypes in controls were in Hardy-Weinberg equilibrium.

The SLE-associated allele (A) of PD1.3 elimi-

nates a putative binding site for the transcriptional factor RUNX-1 in an enhancer-like domain in intron 4 of the human PD-1 gene. Electrophoretic mobility shift assays have demonstrated specific binding of nuclear factors from the human Jurkat T cells to the wild-type (G) binding site—but not to the risk (A) binding site—with potential impact on PD-1 transcription (15). To explore the functional significance of the PD1.3 SNP, we performed transient transfection assays in Jurkat T cells with reporter constructs expressing the firefly luciferase gene under the control of the SLE-associated A allele or the wild-type G allele of the PD1.3 SNP (Figure 1C). Overexpression of RUNX-1 resulted in increased luciferase activity that was significantly higher with the G allele than with the A allele (by 36 ± 4%) (n = 5 independent experiments) (Figure 1D). Thus, PD1.3 may represent a functional polymorphism associated with suppressed transcriptional activity.

Reduced basal and induced PD-1 expression on activated CD4+ T cells in patients homozygous but not in those heterozygous for the PD1.3 polymorphism, and decreased PD-1–mediated inhibition at early-to-intermediate stages of activation. We next examined the expression of PD-1 on PBMCs in SLE patients. Although we found no difference between wild-type (G/G) and heterozygous (G/A) patients, the 2 patients who were homozygous for the PD1.3 SNP (A/A) had minimal PD-1 expression on freshly isolated CD4+ T cells, including the activated CD25+, CD69+, and HLA–DR+ T cell subsets (Figure 2A). PD-1 was also decreased on CD8+ T cells and CD19+ B cells in these 2 patients (data not shown). PD1.3 A/A patients also had significantly reduced PD-1 levels on CD4+CD69+ T cells 24 and 48 hours following stimulation with suboptimal—but not with optimal—concentrations of anti-CD3/anti-CD28 antibodies (Figure 2B). In contrast, the expression of PDL-1 and CTLA-4 was not affected (data not shown).

PD-1 activation results in suppression of lymphocyte proliferation and cytokine production via decreased ERK and Akt/protein kinase B activation (8,9,26). To explore whether PD-1 exerts normal suppressive activity in SLE patients, CD4+ T cells were activated with anti-CD3/anti-CD28 antibodies using plate-bound PDL-1.Fc to crosslink PD-1; T cell proliferation and IFNγ production were measured at 48 and 96 hours of stimulation.

Optimal PD-1 crosslinking (at 2–5 μg/ml PDL-1.Fc) resulted in profound (70–80%) suppression of proliferation and IFNγ production both in controls and in SLE patients, regardless of the PD1.3 genotype (data not shown). However, when suboptimal PDL-1.Fc concentrations were used (0.1–0.5 μg/ml), the 2 PD1.3 A/A patients displayed defective PD-1–mediated inhibition of T cell function. Specifically, T cell proliferation at 48 hours was increased by 5.0 ± 8.0% in the PD1.3 A/A patients, whereas it was decreased by 13.1 ± 7.8% in healthy controls ($P = 0.07$ versus PD1.3 A/A patients) and by 15.4 ± 10.0% in PD1.3 G/G or G/A SLE patients (Figure 2C). Similarly, IFNγ production was decreased by only 9.0 ± 9.0% in PD1.3 A/A patients, compared with decreases of 58.0 ± 4.6% in controls ($P = 0.02$) and 28.0 ± 9.0% in PD1.3 G/G or G/A patients (Figure 2C). Collectively, these results suggest that the PD1.3 polymorphism confers reduced basal and induced PD-1 expression at early-to-intermediate stages of CD4+ T cell activation, and that this is associated with reduced PD-1–mediated inhibition of T cell proliferation and IFNγ production.

Deregulated expression of PD-1 in AMLR in SLE patients. In mouse studies PD-1 is an important regulator of autoreactive T cells, while defective expression of PD-1 results in breakdown of self tolerance and development of autoimmunity (7,10,11,27,28). AMLR is a well-described ex vivo model of autoreactivity against apoptotic self antigens (22,29,30). During AMLR, regulatory circuits suppress effector T cells, and only a small degree of cell proliferation ensues.

We examined the expression of PD-1 on CD4+ T cells as well as the expression of PDL-1 on CD14+ monocytes participating in AMLR in SLE patients and healthy controls. Compared with untreated T cells, PD-1 was significantly induced on AMLR CD4+CD25+ cells (PD-1 mean fluorescence intensity [MFI] 5.8 ± 0.6 versus 3.5 ± 0.3; $P = 0.001$ by paired t-test) in healthy controls (Figures 3A and B). In SLE patients PD-1 induction on CD4+CD25+ cells was less pronounced (PD-1 MFI 4.5 ± 0.3 on AMLR cells versus 3.8 ± 0.2 on untreated cells; $P = 0.020$). Similarly, PD-1 was significantly induced on AMLR CD4+CD69+ cells in controls (PD-1 MFI 6.5 ± 0.6 versus 3.7 ± 0.3; $P = 0.042$) but not in SLE patients (PD-1 MFI 5.2 ± 0.4 versus 4.9 ± 0.5; $P = 0.502$). Accordingly, controls had significantly higher PD-1 expression on CD4+CD25+ and CD4+CD69+ activated cells (but not in CD4+, forkhead box P3–positive regulatory-enriched cells [data not shown]) compared with SLE patients ($P = 0.024$ for both). The expression of CTLA-4, another negative regulator of T cell function, was comparable between patients and controls (data not shown).

Of interest, SLE patients homozygous for the PD1.3 polymorphism had significantly reduced PD-1

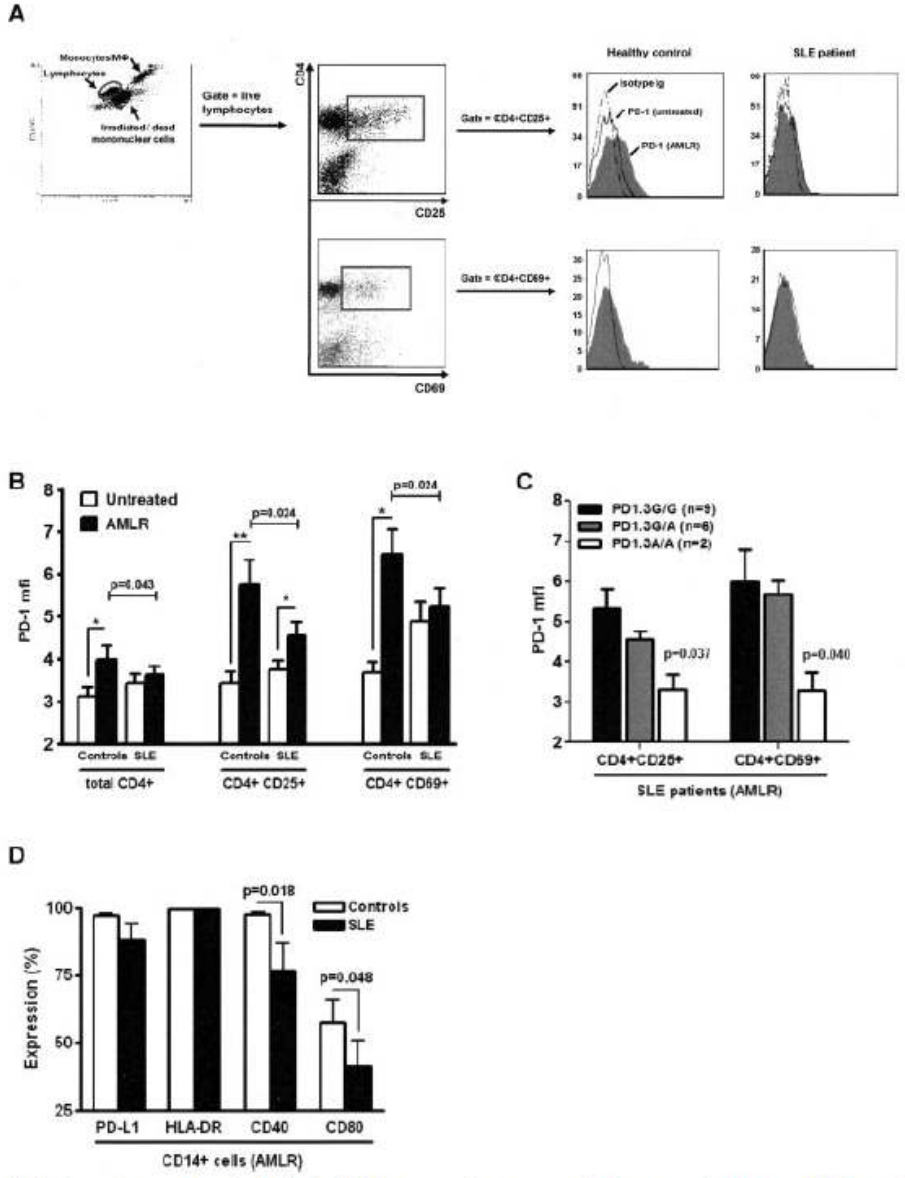

Figure 3. Defective induction of programmed death 1 (PD-1) expression on systemic lupus erythematosus (SLE) activated CD4+ T cells participating in autologous mixed lymphocyte reactions (AMLRs), an ex vivo model of human autoreactivity against self antigens. **A,** Shown is a representative flow cytometry analysis of PD-1 expression in an AMLR in 1 healthy control and 1 SLE patient (see Patients and Methods). Dashed line represents isotype control; solid line represents PD-1 expression on untreated CD4+ cells; shaded histogram represents PD-1 expression on AMLR CD4+ cells. **B,** PD-1 was significantly up-regulated on AMLR CD4+CD25+ and CD4+CD69+ cells in healthy controls (n = 12); a smaller but significant up-regulation of PD-1 was observed on AMLR CD4+CD25+ cells, but not on AMLR CD4+CD69+ cells, in SLE patients (n = 17). Healthy controls had significantly higher PD-1 expression on AMLR CD4+CD25+ and CD4+CD69+ cells than did SLE patients. Paired analyses were performed using the paired *t*-test; the Mann-Whitney U test was used for comparisons between independent groups ($* = P < 0.05$; $** = P < 0.01$). **C,** Patients who were PD1.3 A/A had significantly reduced PD-1 expression on AMLR CD4+CD25+ and CD4+CD69+ T cells compared with PD1.3 G/G and PD1.3 G/A patients. **D,** Programmed death ligand 1 (PDL-1) and HLA–DR expression on CD14+ monocytes participating in the AMLR was comparable between controls and SLE patients, but SLE monocytes had decreased expression of CD40 and CD80. Values in **B–D** are the mean and SEM from 5–6 independent experiments in each group. Mϕ = macrophages; MFI = mean fluorescence intensity.

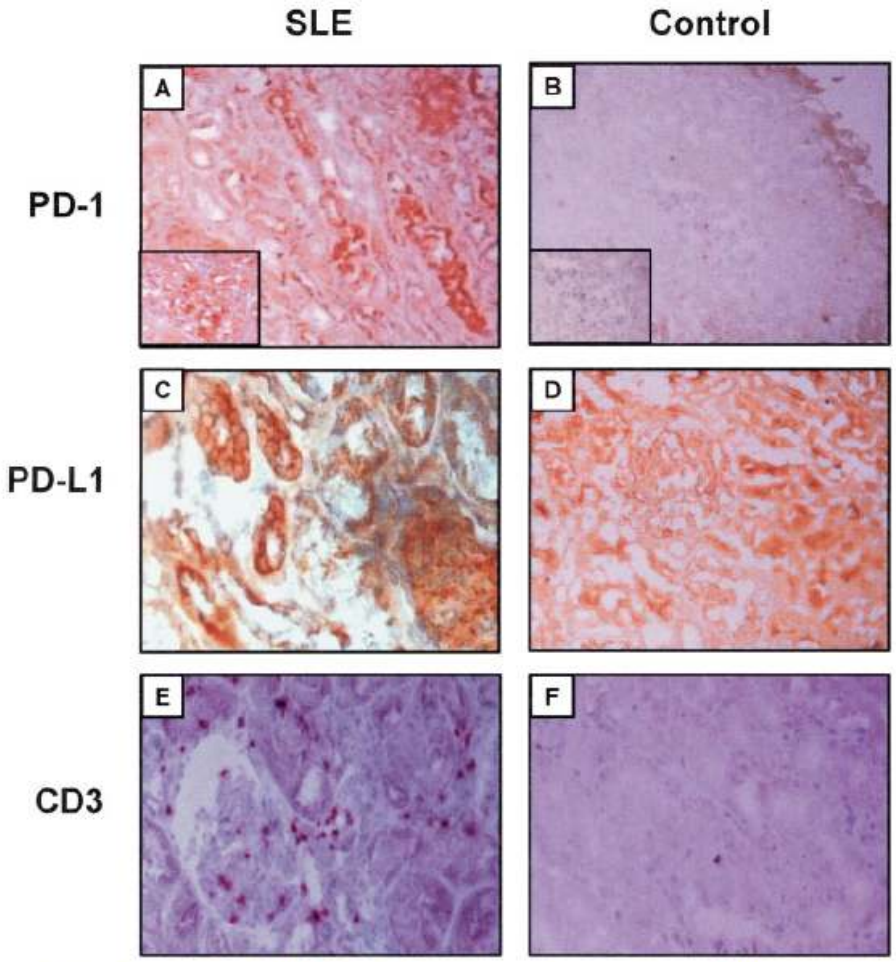

Figure 4. Enhanced expression of programmed death 1 (PD-1) in renal biopsy samples from systemic lupus erythematosus (SLE) patients with nephritis. PD-1 is expressed in the renal glomeruli from patients with lupus nephritis (**A**) but not in the renal glomeruli of unaffected kidney tissue (**B**). **Insets** represent a single glomerulus. Comparable programmed death ligand 1 (PDL-1) expression is shown in renal tubules from patients with lupus nephritis (**C**) and in renal tubules of normal kidney tissue (**D**). PD-1 expression in lupus nephritis correlated with CD3+ glomerular and tubulointerstitial infiltrates (**E**), whereas control kidney tissue showed no CD3+ infiltrates (**F**). (Original magnification × 100 in **A–F**; × 250 in **insets**.)

levels on AMLR CD4+CD25+ and CD4+CD69+ cells (Figure 3C). T cell activation was similar in patients and controls as determined by proliferation results (stimulation index 12 ± 3 both in controls and in patients) and by expression of HLA–DR on CD4+ cells (26.4 ± 3.1% in controls versus 26.7 ± 3.9% in patients). We also examined the expression of PDL-1 and other costimulatory molecules (HLA–DR, CD40, CD80) on CD14+ monocytes in the AMLR. Although we found decreased expression of CD40 (73 ± 7% versus 98 ± 1%; $P = 0.018$) and CD80 (33 ± 7% versus 58 ± 8%; $P = 0.048$) on monocytes in SLE patients compared with those in healthy controls, there was no difference in HLA–DR or PDL-1 expression (Figure 3D).

The role of PD-1/PDL-1 in AMLR was further examined by adding anti–PD-1 and anti–PDL-1 blocking mAb in culture and measuring the effect on proliferation. In healthy controls anti–PD-1 mAb (10 μg/ml) increased proliferation by 59 ± 12%, and anti–PDL-1 mAb (10 μg/ml) increased proliferation by 89 ± 31% (n = 6 independent experiments) (data not shown). In contrast, the respective percentages in SLE patients (n = 6) were –2 ± 12% and 8 ± 14%. Anti–CTLA-4 mAb resulted in increased AMLR proliferation that was

comparable between healthy controls (34 ± 22%) and SLE patients (23 ± 27%) (data not shown).

Taken together, these data suggest that in the AMLR model of human autoreactivity against self antigens, PD-1 is highly induced on activated CD4+ T cells in healthy donors, but its expression is lower in SLE patients. Patients homozygous for the PD1.3 polymorphism display even lower PD-1 expression.

Expression of the PD-1/PDL-1 pathway in renal biopsy samples from SLE patients. In murine models of autoreactivity, PD-1 is involved in regulation of effector T cells at the site of inflammation mostly through interaction with PDL-1 expressed by activated parenchymal cells (4,7,10,11). We therefore examined the expression of the PD-1/PDL-1 pathway in renal biopsy samples from patients with lupus nephritis by immunohistochemistry. PD-1 staining was detected in the glomeruli in 8 of 13 samples from patients with lupus nephritis (62%) compared with 0 of 9 control samples (Figure 4 and Table 1). Similarly, PD-1 was detected in renal tubules in 6 of 13 samples from patients with lupus nephritis (46%) but in 0 of 9 control samples (0%). All 8 PD-1–positive lupus nephritis samples were also stained positive for CD3, expressed in the glomeruli and tubulointerstitial region, suggesting a correlation between PD-1 and CD3+ T cell infiltrates in lupus nephritis. We found no differences in PD-1 expression according to renal histology or other clinical parameters, and the PD1.3 genotype was available for very few patients to allow for associations. PDL-1 expression was detected in the renal tubules of both SLE patients (10 of 15 [67%]) and controls (5 of 9 [56%]). Thus, molecules of the PD-1/PDL-1 pathway are expressed in the renal tissue in lupus nephritis patients, indicating a potential role in the regulation of immune inflammatory responses at the tissue level.

Table 1. Expression of PD-1 and PDL-1 in renal biopsy samples from patients with lupus nephritis and controls*

	Patients with lupus nephritis	Controls†
PD-1 staining		
Glomerular	8/13 (61.5)	0/9 (0)
Tubular	6/13 (46.2)	0/9 (0)
PDL-1 staining		
Glomerular	1/15 (6.7)	0/9 (0)
Tubular	10/15 (66.7)	5/9 (55.6)

* Values are the number/total number (%) of samples staining positive for programmed death 1 (PD-1) or programmed death ligand 1 (PDL-1) (see Patients and Methods).
† Control tissue was unaffected renal tissue from renal cancer surgeries.

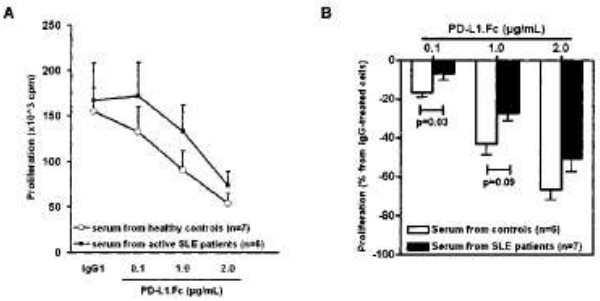

Figure 5. Systemic lupus erythematosus (SLE) serum abrogates the inhibitory function of programmed death 1 (PD-1). To assess the effect of serum on PD-1–mediated inhibition, CD4+ T cells from healthy blood donors were stimulated with anti-CD3/anti-CD28 monoclonal antibodies and PDL-1.Fc (a chimeric protein containing the extracellular part of human programmed death ligand 1 [PDL-1] linked to the Fc fragment of human IgG1) or IgG1 in culture medium supplemented with 20% serum from unrelated healthy donors or patients with active SLE. Results are presented as raw ^{3}H-thymidine counts (**A**) or as percentage of inhibition compared with IgG1-treated cells (**B**) and represent the mean and SEM from 6 independent experiments. At the lowest PDL-1.Fc concentrations (0.1 μg/ml and 1 μg/ml), addition of serum from patients with active SLE resulted in decreased PD-1–mediated inhibition of cell proliferation.

Decrease in PD-1–mediated inhibition of CD4+ T cell proliferation by treatment with SLE serum. Previous studies have shown that the PD-1/PDL-1 pathway may be influenced by several factors such as the level of costimulation, proinflammatory cytokines, and Toll-like receptor (TLR) agonists (31,32). SLE serum contains high concentrations of soluble costimulators (e.g., CD40L, CD28), immune complexes, and cytokines that could affect PD-1 function. To explore whether the suppressive function of PD-1 is abrogated within the inflammatory milieu of lupus, we incubated CD4+ T lymphocytes from healthy blood donors with 20% serum from either healthy blood donors or patients with active SLE and measured the effect of PD-1 crosslinking on cell proliferation. At suboptimal PD-1 activation (0.1 μg/ml PDL-1.Fc), incubation with SLE serum resulted in decreased suppression of T cell proliferation compared with normal serum (6.7 ± 3.6% versus 16.3 ± 2.7%; $P = 0.027$) (n = 6 experiments) (Figures 5A and B). A similar effect was observed at a higher PDL-1.Fc concentration (1 μg/ml) (27.3 ± 4.2% with SLE serum versus 43.0 ± 5.8% with normal serum; $P = 0.09$) but not at the optimal concentration of PDL-1.Fc (2 μg/ml). Together, these results indicate that soluble factors in SLE serum may represent an additional distinct mechanism contributing to aberrant regulation of PD-1 function and T cell hyperactivity in SLE.

DISCUSSION

In this report, we provide evidence in support of a role for the inhibitory PD-1/PDL-1 pathway in regulation of T cell function in human SLE. Our experiments indicate aberrant expression and function of PD-1 in lupus. Specifically, during AMLR, induction of PD-1 on CD4+ T cells was impaired in SLE patients, whereas serum from patients with active SLE abrogated the inhibitory function of PD-1. To our knowledge, this is the first study to demonstrate that the PD1.3 polymorphism risk factor for SLE is a regulatory polymorphism associated with decreased transcriptional activity.

Prokunina et al (15) have shown that the PD1.3 SNP disrupts a RUNX-1 binding site in an enhancer-like domain in intron 4 of the PD-1 gene with possible alteration in gene expression. Interestingly, RUNX-1 is implicated in regulation of genes that are associated with rheumatoid arthritis and psoriatic arthritis (33–35), and polymorphisms of RUNX-1 itself confer increased susceptibility to psoriasis (33).

We found that the A (risk) allele of PD1.3 is associated with decreased (~30–40%) luciferase activity in T cells overexpressing RUNX-1. RUNX-1 is a relatively weak transcription factor that enhances target gene expression in lymphoid tissues. Our results are in accordance with those of other groups that have studied the effect of SNPs in other genes that disrupt RUNX-1–binding sites residing in introns and that have reported decreased binding of RUNX-1 and decreased transcriptional activity when the polymorphic allele is present (34,35). Indeed, 2 patients homozygous for PD1.3 A/A had minimal basal and induced PD-1 levels, conferring decreased PD-1–mediated inhibition of T cell proliferation and IFNγ production. We found no difference in PD-1 expression between wild-type (G/G) and heterozygous (G/A) patients. The lack of effect on PD-1 expression within heterozygotes may reflect the weak contribution of endogenous RUNX-1 on PD-1 transcription in the basal, noninduced state, as indicated by our transactivation data (Figure 1D). The PD1.3 SNP could affect PD-1 expression in cases of increased RUNX-1 levels or under certain conditions of lymphocyte activation, as indicated by the reduced PD-1 expression in AMLR in SLE patients with the PD1.3A risk allele (Figure 3C).

AMLR is an in vitro model of autoreactivity against apoptotic self antigens and has been used to study the immunologic processes involved in generation of autoimmunity (22,29,30,36). During AMLR, immunoregulatory circuits suppress T cell autoreactivity, and only a small degree of proliferation ensues (22,29). Nucleosome-primed T cells show enhanced AMLR-induced proliferation, and thus AMLR represents a suitable experimental setting in which to study immune responses pertinent to lupus (30). PD-1 was highly induced on activated AMLR CD4+CD25+ and CD4+CD69+ T cells in healthy donors but not in SLE patients. In line with this, blocking of PD-1/PDL-1 increased AMLR proliferation in controls but not in SLE patients. The decreased expression of PD-1 in SLE might contribute to aberrant T cell responses not captured in this assay such as cytokine production and memory cell differentiation. PDL-1 and HLA–DR on AMLR monocytes was comparable between patients and controls, although patients had decreased expression of the costimulatory molecules CD40 and CD80, which are important in monocyte–T cell interaction and initiation of the AMLR (36). This is in accordance with previous reports of impaired stimulatory capacity of lupus non–T cells in AMLR (29). Thus, decreased stimulation by lupus non–T cells during AMLR could contribute to the defective induction of PD-1 on T cells in SLE patients.

Consistent with its role in maintaining self tolerance, PD-1 regulates T cell function only in suboptimal conditions of T cell receptor activation and CD28 costimulation (32,37). Optimal PD-1 crosslinking inhibited anti-CD3/anti-CD28–induced T cell proliferation and cytokine production both in SLE patients and in healthy donors. However, the outcome of PD-1 activation is also affected by factors such as cytokines (e.g., IL-6, IL-10), the level of costimulation, and TLR signaling (31,32). Accordingly, SLE serum contains high levels of inflammatory cytokines, complexes of self DNA with autoantibodies, and soluble costimulators (23,38–40), all of which could influence the function of PD-1. To test this hypothesis, we examined the function of PD-1 in normal T cells incubated with serum from either healthy controls or patients with active SLE. SLE serum inhibited the function of PD-1 at a suboptimal—but not at an optimal—dose of PDL-1.Fc. These results underscore the importance of the level of PDL-1 expression in determining the outcome of the PD-1/PDL-1 pathway. Therefore, within the lupus inflammatory milieu (for instance, kidneys with lupus nephritis) and in cases of low PD-1/PDL-1 expression, the inhibitory function of PD-1 could be abrogated. Of interest, at higher PDL-1 concentrations efficient inhibition of T cell proliferation was observed, overcoming the inhibitory effect of SLE serum.

In conclusion, our data suggest aberrant regulation of PD-1 expression and function in human SLE as a result of direct (PD1.3 polymorphism) and indirect (inflammatory milieu) effects. The expression of PD-1/PDL-1 in the affected tissues and during AMLRs indicate a role of this pathway in maintenance of peripheral T cell tolerance. These data provide a basis to better elucidate the association of PD-1 polymorphisms with human lupus.

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AUTHOR CONTRIBUTIONS

Dr. Boumpas had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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