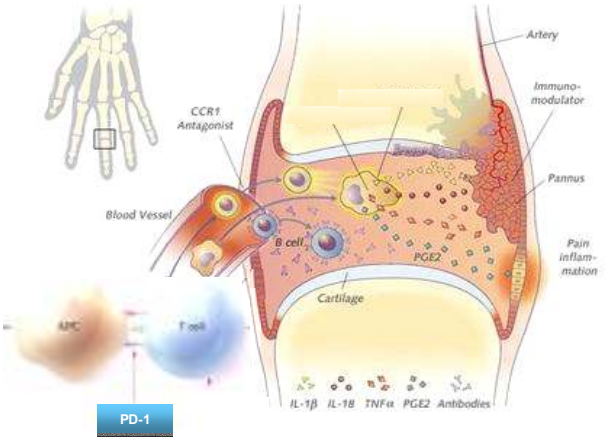
Thesis for doctoral degree (Ph.D)

2010

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



Amalia P. Raptopoulou

Laboratory of Autoimmunity and Inflammation

Department of Rheumatology, Clinical Immunology and Allergy

University of Crete School of Medicine

From the

Laboratory of Autoimmunity and Inflammation

Department of Rheumatology, Clinical Immunology and Allergy

University of Crete School of Medicine

The role of peripheral immunological tolerance in the pathogenesis of rheumatoid arthritis. Study of Programmed death-1 (PD-1) and PD-1 ligands inhibitory pathway

Amalia P. Raptopoulou, MD

Heraklion 2010

Πρόλογος

Η επιλογή της ειδικότητας της ρευματολογίας έγινε σε μια σχετικά «αθώα» εποχή, όπου οι νόμοι της αγοράς δε μας απασχολούσαν ιδιαίτερα και βασικό κριτήριο ήταν η μαγεία που μπορεί να ασκούσαν πάνω μας οι διάφορες παθολογίες. Τα αυτοάνοσα νοσήματα περιβάλλονται από ένα γοητευτικό μυστήριο, κυρίως σε ό,τι αφορά την αιτιολογία και την παθογένειά τους, και αυτό νομίζω ότι ήταν που με ώθησε να ασχοληθώ με τη ρευματολογία. Ερχόμενη στην Κρήτη, η ευκαιρία που μου δόθηκε να ασχοληθώ διεξοδικά με ένα κομμάτι της παθογένειας της ρευματοειδούς αρθρίτιδας και να το μελετήσω τόσο σε ανθρώπους όσο και σε ζωικά πρότυπα, ήταν όντως μοναδική. Αυτό που δεν μπορούσα βέβαια να φανταστώ είναι ότι πίσω από τα μικροσκόπια και τα σωληνάκια, εν μέσω της καθημερινής σκληρής κλινικής δουλειάς, θα δημιουργούνταν γύρω μου ένας ιστός από αγαπημένους ανθρώπους, τους περισσότερους από τους οποίους θεωρώ πλέον οικογένειά μου κι ελπίζω κι αυτοί να αισθάνονται το ίδιο για μένα. Η ακούραστη συμπαράστασή τους σε όλους τους τομείς της επιστημονικής και προσωπικής μου ζωής είναι κάτι που μόνο από πολύ δικά και αγαπημένα πρόσωπα μπορεί κανείς να συναντήσει.

Θα ήθελα λοιπόν, να αφιερώσω το παρακάτω πόνημα, στους δύο «μπαμπάδες» μου της ρευματολογίας, κ Μπούμπα και κ Κρητικό, για τους οποίους ό,τι και να πω είναι λίγο, για την αγάπη, την αφοσίωση και την αυταπάρνηση που έχουν επιδείξει ακούραστα κατά την άσκηση του κλινικού, ερευνητικού κι εκαπιδευτικού τους έργου όλα αυτά τα χρόνια, και που εύχομαι να συνεχίσουν την προσφορά τους για πολλά-πολλά και καλά χρόνια ακόμα. Αποτελούν για μένα πρότυπο ανθρώπων, γιατρών κι επιστημόνων και το θεωρώ τιμή μου που συνεργάστηκα μαζί τους.

Επίσης στους αγαπητούς μου συναδέλφους κι εξαιρετικούς επιστήμονες, κ Μπερτσιά και κ Βεργίνη, η συμβολή των οποίων ήταν κάτι παραπάνω από καθοριστική για την ολοκλήρωση του συγκεκριμένου πονήματος, τόσο στο σχεδιασμό όσο και στην εκτέλεση των πειραμάτων και στον καθηγητή κ Σιδηρόπουλο, που έβαλε τις βάσεις αυτής της εργασίας και συνέβαλε αδιάλειπτα και μεθοδικά στην ολοκλήρωσή της.

Ιδιαίτερες ευχαριστίες στον αδελφικό μου φίλο και αγαπητό συνάδελφο κ Λιναρδάκη, ο οποίος επωμίστηκε πολλά κλινικά βάρη προκειμένου να ολοκληρωθούν από μέρους μου τα πειράματα στο εργαστήριο, στις νοσηλεύτριες κ Παπαδοπούλου, Δημάκα, Χουστουλάκη Μυσιρλάκη και Μελισσουργάκη, για την ανεκτίμητη βοήθειά τους στη συλλογή των δειγμάτων, στις ακούραστες μελισσούλες του εργαστηρίου ρευματολογίας κ Χουλάκη, Νάκου και Καβουσανάκη, για την τεχνική (και όχι μόνο) υποστήριξη, στο εργαστήριο Παθολογίας στον καθηγητή κ Γουλιέλμο, που μαζί με την κ Γιακουπή ασχολήθηκαν διεξοδικά με το γενετικό κομμάτι της μελέτης, στον κ Μακρυγιαννάκη και το ινστιτούτο της Karolinska, για την εξαιρετική τους δουλειά στην ανοσοιστοχημεία, και στην κ Κουταλά για τη βοήθειά της στην ανάλυση των δειγμάτων με κυτταρομετρία ροής.

Επίσης, θερμές ευχαριστίες στον καθηγητή Dr. G. Freeman (Harvard Medical School) για τη χορήγηση της PD-L1.Fc προτεΐνης, στην κ Κρανιδιώτη (Ινστιτούτο Φλέμιγκ), και Vervoordeldonk (University of Amsterdam), στο ζωοκομείο του Πανεπιστημίου Κρήτης και του ΙΤΕ, στην κ Μαμαλάκη και στους συνεργάτες της, καθώς και στην κ Τζαρδή και στο παθολογοανατομικό εργαστήριο του πανεπιστημίου, για τη βοήθειά τους στα πειράματα που έγιναν σε ποντίκια.

Ένα μεγάλο ευχαριστώ στους φίλους και συνειδικευόμενούς μου κ Ντάλη, Κτενιαδάκη, Ρέπα, Καλλιτσάκη και Μαμουλάκη, και στην αγαπητή κα Κουρούμαλλη, για την υποστήριξη και την άψογη συνεργασία τους, καθώς και στην καταπληκτική

μας γραμματέα κ Καμηλάκη, για το κουράγιο της να παλεύει αγόγγυστα τα απάλευτα εντός κι εκτός της ρευματολογικής κλινικής….

Θα ήταν παράλειψη να μην αναφερθώ στο θείο μου Βασίλη, καθηγητή ακτινολογίας στο Harvard, που στήριξε με ενθουσιασμό τις εκάστοτε αποφάσεις μου και που σταθερά με ενθάρρυνε σε κάθε μου νέο βήμα, και στους δασκάλους μου στην παθολογία κ Γαρύφαλλο και κ Ραπτοπούλου, που με εισήγαγαν στις έννοιες των αυτοάνοσων νοσημάτων και που μαζί με την αγαπητή παιδορευματολόγο κ Πρατζίδου και τη φίλη και συνάδελφο κ Σιάκκα, με παρότρυναν να έρθω στην Κρήτη. Την ευγνωμοσύνη μου θα ήθελα επίσης να εκφράσω και προς τις καρδιακές μου φίλες Ιωάννα, Όλγα, Ματίνα, Άνη, Έφη, Μαρία και Μαίρη, που, παρά τη χιλιομετρική απόσταση που μας χωρίζει με τις περισσότερες, είναι πάντα παρούσες στις χαρούμενες αλλά και στις δύσκολες στιγμές μου.

Τέλος, θα ήθελα να ευχαριστήσω το σύζυγό μου Δημήτρη, που υπήρξε η κινητήρια δύναμη και το ασάλευτο στήριγμα πίσω από κάθε μου ενέργεια, και τους γονείς μου, Νίκη, Παύλο και γιαγιά-Άννα για την απύθμενη αγάπη και την ακούραστη συμπαράσταση τους όλα αυτά τα χρόνια. Τους οφείλω το ζειν και το ευζείν μου.

Ἐὰν ταῖς γλώσσαις τῶν ἀνθρώπων λαλῶ καὶ τῶν ἀγγέλων,
ἀγάπην δὲ μὴ ἔχω, γέγονα χαλκὸς ἠχῶν ἢ κύμβαλον ἀλαλάζον.
καὶ ἐὰν ἔχω προφητείαν καὶ εἰδῶ τὰ μυστήρια πάντα καὶ πᾶσαν τὴν γνῶσιν,
καὶ ἐὰν ἔχω πᾶσαν τὴν πίστιν, ὥστε ὄρη μεθιστάνειν,
ἀγάπην δὲ μὴ ἔχω, οὐδέν εἰμι.

(Παύλου, Α' Κορινθίους κεφ. 13)

Η παρούσα εργασία πραγματοποιήθηκε με την οικονομική υποστήριξη της ***Παγκρήτιας Ένωσης Υγείας****, της* ***Ελληνικής Ρευματολογικής Εταιρίας*** *και του προγράμματος της Ευρωπαικής Ένωσης* ***Autocure****. Τους ευχαριστώ πολύ.*


Στην Παυλίνα.....
.....με αγάπη....

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. 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.

Rheumatoid arthritis (RA) is a chronic inflammatory disease of joints and extra-articular tissues, which causes severe disability and premature mortality [1, 2]. Numerous data support a central role of T cells in RA with these cells thought to be triggered locally in an antigen-specific manner resulting in breakdown of tolerance, synovial inflammation, and autoantibody production [3-7]. In the collagen-induced arthritis (CIA animal model of RA, type II collagen (CII)– reactive CD4+ T cells are primary mediators of disease induction driving autoantibody production by B cells and localized chronic inflammatory response [8-12].

Although the role of co-stimulation is well documented in RA and has been further supported by the efficacy of CTLA4-Ig in severe RA, the role of this family of molecules has not been explored in a systematic, organized fashion. We sought to determine the role of PD-1/PD-1 ligands (PD-1L) in RA and test the hypothesis whether defective expression and/or function of this pathway may contribute to T cell hyperactivity within the inflamed joint.

Genomic DNA was extracted from peripheral whole blood obtained from patients and healthy blood donors. Genotyping for the PD1.3A was performed by polymerase chain reaction (PCR). PD-1/PD-1L expression was examined on synovial tissue and synovial fluid (SF) mononuclear cells from RA patients by immunohistochemistry and flow cytometry. PD-1 function was assessed in T cells stimulated with anti-CD3 and PD-L1.Fc to crosslink PD-1. Collagen-induced arthritis (CIA) was induced in *PD-1-/-* C57Bl/6 mice and recombinant PD-L1.Fc was injected to activate PD-1 in vivo.

A. Human studies

To determine whether PD-1 SNPs are associated with susceptibility to RA in our cohort, we analyzed the genomic DNA of 86 RA patients and 227 age- and sex- matched healthy controls for the PD1.3 SNP. No substantial difference in frequencies of PD1.3 heterozygosity (G/A) between RA patients and healthy controls (15,1% versus 19,4%, $p>0,05$) was found.

We have shown that RA synovium and SF were enriched in PD-1+ T cells (24 ± 5% vs. 2 ± 1% in osteoarthritis, $p<0.01$) and PD-1L+ monocytes/macrophages. And that PD-1 crosslinking inhibited T cell

proliferation and IFN-ɣ production in RA patients. Howewver, despite high expression within the joint, PB T cells incubated with RA SF and SF T cells from active RA exhibited reduced PD-1–mediated inhibition at suboptimal –but not optimal– PD-L1.Fc concentrations, indicating a probable diminishing effect of the inflammatory microenvironment of RA on pathway's function.

B. Animal studies

PD-1-/- mice demonstrated increased incidence (73% vs. 36% in wild-type mice, $p<0.05$) and severity (mean maximum arthritis score 4.8 vs. 2.5, $p=0.001$) of CIA, associated with enhanced T cell proliferation and cytokine (IFN-γ, IL-17) production in response to type II collagen. Most interestingly, PD-L1.Fc treatment ameliorated CIA (mean maximum arthritis score 1.8 ± 0.6 vs. 2.5 ± 0.7) and reduced anti-collagen II T cell responses (induction in proliferation [Δcpm] 9594 ± 2147 vs. 4712 ± 2256).

Taken together, our data suggest that тhe negative costimulatory PD-1/PD-1L pathway regulates peripheral T cell responses in both human and murine RA. The PD-1/PD-1L in rheumatoid synovium may represent an additional target for immunomodulatory therapy in RA.

ΠΕΡΙΛΗΨΗ

Τα αυτοάνοσα νοσήματα προκαλούνται ως συνέπεια διαταραχών της ανοσολογικής ανοχής του οργανισμού έναντι αυτοαντιγόνων. Σημαντικό ρόλο στη ρύθμιση των αυτοδραστικών Τ λεμφοκυττάρων στην περιφέρεια έχει η οικογένεια των B7/CD28 μεμβρανικών υποδοχέων. Οι υποδοχείς αυτοί ενεργοποιούνται μετά από πρόσδεση ειδικών συνδετικών μορίων και διαβιβάζουν σήματα ενεργοποίησης ή καταστολής των Τ λεμφοκυττάρων. Η ισορροπία μεταξύ «θετικών» και «αρνητικών» σημάτων ενεργοποίησης καθορίζει τελικά τη δραστικότητα των λεμφοκυττάρων και προστατεύει από την ανάπτυξη αυτοανοσίας. Ο υποδοχέας PD-1 επάγεται στην κυτταρική επιφάνεια ενεργοποιημένων Τ λεμφοκυττάρων και αναστέλλει τον πολλαπλασιασμό και την έκκριση φλεγμονωδών κυτταροκινών. Η ενεργοποίηση του PD-1 πραγματοποιείται μέσω ειδικών συνδετών (PD-1 ligand 1 [PD-L1], PD-L2) που εκφράζονται κυρίως σε αντιγονο-παρουσιαστικά κύτταρα. Πειράματα σε ζωικά πρότυπα έχουν αναδείξει το σημαντικό ρόλο του PD-1 στη ρύθμιση της αυτοδραστικότητας και στην ανάπτυξη αυτοανοσίας.

Η ρευματοειδής αρθρίτιδα (ΡΑ) αποτελεί την πρότυπη φλεγμονώδη αυτοάνοση αρθρίτιδα στον άνθρωπο και χαρακτηρίζεται από χρόνια συστηματική φλεγμονή και καταστροφή διαφόρων ιστών. Τα παθογενετικά χαρακτηριστικά της ΡΑ είναι η παρουσία αυτοδραστικών Τ λεμφοκυττάρων στην περιφέρεια και τους προσβεβλημένους ιστούς, η υπερπαραγωγή αυτοαντισωμάτων από τα Β λεμφοκύτταρα και οι αυξημένες συγκεντρώσεις φλεγμονωδών κυτταροκινών.

Ο κύριος στόχος της παρούσας μελέτης ήταν να διερευνηθεί ο ρόλος του PD-1 και των συνδετών του στη ρύθμιση των Τ λεμφοκυττάρων που συμμετέχουν στην παθογένεια της αρθρικής βλάβης της ρευματοειδούς αρθρίτιδας.

Α. Μελέτες σε ανθρώπους:

Εξετάστηκαν 86 ασθενείς με ΡΑ και 227 υγιείς εθελοντές για την παρουσία του PD1.3 μονονουκλεοτιδικού πολυμορφισμού με τεχνική PCR και χρήση ειδικής νουκλεάσης περιορισμού (PCR-based restriction fragment length polymorphism analysis). Δε διαπιστώθηκε στατιστικά σημαντική διαφορά στη συχνότητα του πολυμορφισμού μεταξύ ασθενών και υγιών μαρτύρων (15,1% vs 19,4%, p>0,05).

Για να διερευνήσουμε τη συμμετοχή του PD-1 στη ρύθμιση των Τ λεμφοκυττάρων στο σημείο της φλεγμονής, μελετήσαμε με ανοσοϊστοχημεία την έκφραση του PD-1/PD-L1 σε βιοψίες αρθρικού υμένα από υγιείς, ασθενείς με οστεοαρθρίτιδα (ΟΑ) και ΡΑ. Διαπιστώθηκε αυξημένη έκφραση του PD-1, κυρίως από Τ CD3+ κύτταρα, σε 8 από τα 9 (89%) δείγματα αρθρικού υμένα ΡΑ, σε σχέση με 2 από τα 8 (25%) και κανένα από τα 9 (0%) δείγματα ΟΑ και υγιούς αρθρικού υμένα αντίστοιχα. Έκφραση του PD-L1, κυρίως από μακροφάγα, ανιχνεύθηκε στις βιοψίες από ΡΑ και ΟΑ ενώ στα δείγματα υγιούς ιστού η έκφρασή του ήταν

μηδαμινή. Επιπλέον, ο βαθμός της έκφρασης των PD-1/PD-L1 ήταν ανάλογος του βαθμού της φλεγμονής. Τα ανωτέρω υποδεικνύουν πιθανή συμμετοχή του PD-1/PD-L1 στη ρύθμιση των φλεγμονωδών Τ κυτταρικών αποκρίσεων σε ιστικό επίπεδο. Στη συνέχεια, μονοπύρηνα κύτταρα αρθρικού υγρού (SFMCs) απομονώθηκαν από ασθενείς με ΡΑ και ΟΑ και μελετήθηκε η έκφραση των PD-1 και PD-L1 στα CD4+ κύτταρα με κυτταρομετρία ροής. Σε αντιστοιχία με τα αποτελέσματα της ανοσοϊστοχημείας, βρέθηκε αυξημένη έκφραση του PD-1 στα Τ κύτταρα ΡΑ (24 ± 5% vs. 4 ± 1%, p=0.003), ιδιαίτερα στους ενεργοποιημένους υποπληθυσμούς (CD69+, CD25+).

Παρά την έκφραση του PD-1 στο περιφερικό αίμα και τους προσβεβλημένους ιστούς (αρθρικός υμένας) των ασθενών με ΡΑ, η λειτουργία του ανοσορρυθμιστικού συστήματος PD-1/PD-L1 ενδεχομένως να τροποποιείται στο φλεγμονώδες περιβάλλον της άρθρωσης. Το ενδεχόμενο αυτό εξετάστηκε κατ'αρχήν, με καλλιέργειες από SFMCs κύτταρα από ΡΑ και ΟΑ, όπου μελετήθηκε η ικανότητα των κυττάρων αυτών να επάγουν την έκφραση του PD-1 μετά από ενεργοποίησή τους με PMA και ionomycin. Βρέθηκε ότι στην ΡΑ η ικανότητα αυτή ήταν μειωμένη (επαγωγή του PD-1 από 18 ± 5% σε 29 ± 4% στην ΡΑ και από 5 ± 2% σε 25 ± 4% στην ΟΑ). Στη συνέχεια, CFSE-σημασμένα CD4+ Τ λεμφοκύτταρα περιφερικού αίματος, καλλιεργήθηκαν απουσία ή παρουσία 15% εκχυλίσματος αρθρικού υγρού από ΡΑ. Μετά από ενεργοποίηση με την πρωτεΐνη PD-L1.Fc, διαπιστώθηκε ότι, παρουσία ορού από ΡΑ, η ικανότητα του PD-1 να αναστέλλει τον Τ κυτταρικό πολλαπλασιασμό ήταν μειωμένη (αδιαίρετα Τ κύτταρα την ημέρα 5: 65.1 ± 4.7% vs. 81.2 ± 7.8%, p=0.028).

Επιπρόσθετα, για να μελετηθεί η ικανότητα του PD-1 να ρυθμίζει την ενεργοποίηση των Τ κυττάρων που βρίσκονται στο αρθρικό υγρό της ΡΑ, πραγματοποιήθηκαν κυτταροκαλλιέργειες CD4+ Τ κυττάρων περιφερικού αίματος και αρθρικού υγρού από ασθενείς με ΡΑ (paired samples), όπου χορηγήθηκαν διάφορες συγκεντρώσεις PD-L1.Fc ώστε να ενεργοποιηθεί ο υποδοχέας PD-1. Η ικανότητα του PD-1 να περιορίσει τον πολ/σμό και την παραγωγή IFN-γ από CD4+ Τ κύτταρα της ρευματοειδούς άρθρωσηςήταν μειωμένη σε σχέση με τα κύτταρα του περιφερικού αίματος, ιδιαίτερα σε χαμηλές συγκεντρώσεις PD-L1.Fc (αναστολή πολ/μού 31 ± 9% vs 53 ± 8% p=0.003).

Β. Μελέτες σε ζώα:

Ποντίκια που δεν εκφράζουν PD-1 (PD-1$^{-/-}$) δημιουργήθηκαν με διαγραφή του διαμεμβρανικού και ενδοκυττάριου τμήματος του γονιδίου του PD-1. Η αρθρίτιδα επαγόμενη από κολλαγόνο (collagen-induced arthritis, CIA) σε ποντικούς αποτελεί ένα κλασσικό και καλά μελετημένο πειραματικό πρότυπο αυτοανοσίας προσομοιάζον στην ανθρώπινη ρευματοειδή αρθρίτιδα.

Η έλλειψη του PD-1 θα μπορούσε να προκαλέσει υπέρμετρη ενεργοποίηση των Τ κυττάρων και επομένως αύξηση της έντασης και του ποσοστού της αρθρίτιδας. Για να εξετάσουμε αυτή την υπόθεση, προκλήθηκε

CIA σε ποντίκια B6-PD-$1^{-/-}$ με χορήγηση κολλαγόνου τύπου II (CII). Παρατηρήθηκε σημαντικά αυξημένο ποσοστό και ένταση αρθρίτιδας, που εκτιμήθηκε μακροσκοπικά και ιστολογικά, σε σχέση με τα wild type B6 ποντίκια (mean ± SEM of maximum arthritis score, B6-PD-$1^{-/-}$: 5.0 ±1.2, *n*=16, vs B6 wt: 2.3 ±1.2, *n*=14, p=0.040).

Η ενεργοποίηση του συστήματος PD-1/PD-L1, θα μπορούσε να προκαλέσει την αναστολή της διέγερσης των Τ λεμφοκυττάρων και επομένως να περιορίσει τον βαθμό της επαγόμενης αρθρίτιδας. Σε B6 ποντίκια, στο πειραματικό πρότυπο της CIA, χορηγήθηκε η συνθετική πρωτεΐνη PD-L1.Fc (που αποτελείται από το εξωκυττάριο τμήμα του PD-L1 και το Fc τμήμα της IgG2a ανοσοσφαιρίνης) που ενεργοποιεί το PD-1 *in vivo,* στο στάδιο πρόκλησης της αρθρίτιδας (πρώτες 10 ημέρες). Στα ποντίκια αυτά παρατηρήθηκε ηπιότερη αρθρίτιδα (μέση ένταση αρθρίτιδας: 1.8 ± 0.6) σε σχέση με τους μάρτυρες, που δεν έλαβαν τη θεραπεία (2.5 ± 0.7) (*n*=5 σε κάθε ομάδα).

Συμπερασματικά, τα ευρήματα της μελέτης αυτής υποστηρίζουν ένα σημαντικό ρόλο του ανασταλτικού συστήματος PD-1/PD-L1 στη ρύθμιση των Τ λεμφοκυττάρων στη ΡΑ. Τεκμηριώνεται διαταραχή της έκφρασης και λειτουργίας του PD-1 ως συνέπεια του φλεγμονώδους περιβάλλοντος της ΡΑ. Η έκφραση του PD-1/PD-L1 στον αρθρικό υμένα της ΡΑ και η έντονη επίδρασή του στην ανάπτυξη αρθρίτιδας σε πειραματικά ζωικά πρότυπα, υποδεικνύουν συμμετοχή του ανοσορρυθμιστικού αυτού συστήματος στην Τ ανοσολογική ανοχή, με δυνατότητα παρέμβασης για θεραπευτικούς σκοπούς.

2. ABBREVIATIONS

APC	Antigen - presenting cell
CIA	Collagen induced arthritis
CII	Collagen type II
MHC	Major histocompatibility complex
OA	Osteoarthritis
PB	Peripheral blood
PD-1	Programmed death-1
PD-L1/2	Programmed death-1 ligand 1/2
RA	Rheumatoid arthritis
SF	Synovial fluid
TCR	T cell receptor

3. INTRODUCTION

3.1 Pathways of immunological self-tolerance

a. Mechanisms of central tolerance

One of the most remarkable properties of the immune system is its ability to recognize, respond to, and eliminate foreign (nonself) antigens while not reacting harmfully to that individual's own (self) antigenic substances. This state of immunologic unresponsiveness to antigens is also called tolerance. **Self-tolerance** is maintained by various mechanisms that prevent the maturation and activation of potentially self-reactive lymphocytes. The primary mechanism of immunological self-tolerance is **central deletion** in which self-reactive T cells are deleted in the thymus during T cell maturation by **negative selection** [13].

Many recent studies suggest that T cell receptor (TCR) editing may play a role in controlling the development of self-reactive T cells in thymus [14] as well. TCRs are selected to recognize a composite ligand comprising peptide fragments of antigen bound to major histocompatibility complex (MHC) molecules. Composites of self-peptides and MHC are displayed on the surface of cortical thymic epithelial cells, and $CD4^+CD8^+$ thymocyte expression of TCRs that weakly bind these ligands, trigger maturation signals that inhibit RAG gene expression, thereby closing off the option of editing [15]. As a result, thymocytes are stimulated to survive. During this process, a minority of self-reactive TCRs triggers an **editing** process; in this case, TCRs are downregulated, RAG expression continues and the offending TCR α-chain is replaced or diluted with a second α-chain that is less self-reactive [14]. The majority of thymocytes whose receptors do not recognize self-MHC molecules are permitted to die. As **positive selected thymocytes** move from the cortex toward the medulla, they continue the maturation process and further test their TCRs for self-reactivity. TCRs that bind strongly to self-peptide/MHC combinations trigger the death (**negative selection**) of thymocytes. These medullary cells express T cell costimulatory molecules, such as CD80 and CD86, the ligands for CD28, and these costimulatory molecules play a crucial role in ensuring self-tolerance [16] **(Figure. 3.1)**.

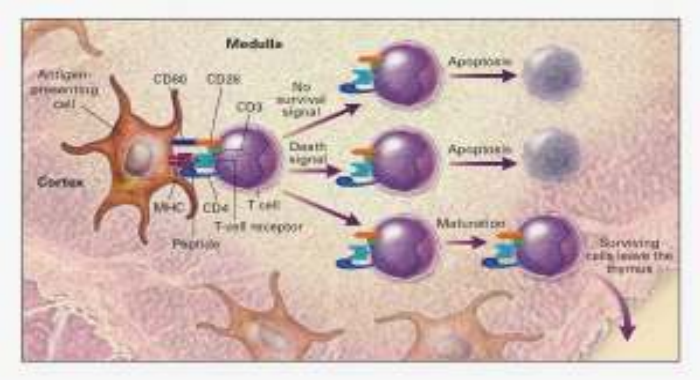


Figure 3.1. Mechanisms of central immune tolerance

b. Mechanisms of peripheral immune tolerance

Although central tolerance is the major mechanism to eliminate self-reactive T cells, such deletion is **incomplete**. Indeed, healthy individuals were shown to harbor self-reactive T cells in the periphery. Therefore, the immune system has developed the mechanisms that deal with tolerance in the peripheral

lymphoid organs, providing the necessary safety net to prevent autoimmunity. The mechanisms responsible for peripheral tolerance can be divided into those that regulate the responding state of T cell intrinsically, such as biochemical and gene-expression changes, and those that provide extrinsic controls, including limiting the supply of essential growth factors, costimuli, and proinflammatory mediators, and active suppression by regulatory T cells (Treg) **(Figure 3.2)**.

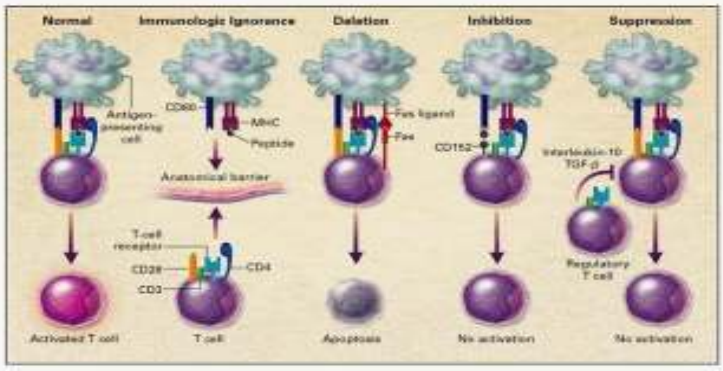


Figure 3.2. Mechanisms of peripheral immune tolerance

b. i. T cell anergy

T cell activation requires recognition of specific antigenic peptides by the TCR, as signal 1, and additional costimulatory signals provided by accessory surface molecules on T cells, as signal 2. When costimulatory signals are absent, T-helper cells become anergic. **Anergy** is the state in which T-helper lymphocytes are alive but are incapable of proliferating and transcribing the interleukin (IL)-2 gene in response to optimal antigenic stimulation provided by professional antigen-presenting cells (APCs) [17]. The molecular pathways involved in the induction of anergy have been recently explored and provide insight for the better understanding of immune regulation mechanisms. Specifically, ligation of the TCR by antigenic peptides presented in the context of MHC proteins induces tyrosine phosphorylation of polypeptides of the TCR-associated CD3 complex. This event is mediated by Src family tyrosine kinases, and leads to the recruitment and activation of ZAP-70. Activated ZAP-70 induces tyrosine phosphorylation of various downstream effector molecules that, in turn, associate with distinct molecules and lead to regulation of activation cascades further downstream. Signaling enzymes and a variety of nonenzymatic adapter proteins, link these receptor-associated events to downstream pathways. Consequences of these proximal events include activation of mitogen-activated protein kinases (MAP kinases) through the Ras pathway, activation of IL-2 transcription through calcineurin phosphatase and nuclear factor of activated T cell (NFAT), and activation of protein kinase C [18]. In anergic cells many of these critical signaling events initiated by T cell activation do not occur, whereas other signaling events predominate [19]. Extensive studies provide cumulating evidence that the balance between stimulatory and inhibitory signals that are initiated via surface receptors and transmitted via specific signaling pathways is crucial to maximize protective immune responses while maintaining immunological tolerance and preventing autoimmunity [13].

b. ii. Regulatory T cells

A significant development in the field of tolerance was the reemergence of the concept that T cell reactivity might be controlled by a distinct subset of T cells with a "regulatory function," named Treg. The majority of these in vivo existing Treg constitutively express CD25 (IL-2 receptor alpha chain) and constitute 5–10% of $CD4^+$ T cells in rodents and 1–2% of $CD4^+$ T cells in humans [20, 21]. The transcription factor Foxp3 belongs to large family of functionally diverse forkhead/ winged-helix transcription factors. Foxp3 binds DNA, localizes to the nucleus and can act as a transcriptional repressor. Identification of consensus forkhead-binding domains adjacent to NFAT transcription factor binding sites in the promoters of several cytokine genes, including those encoding IL-2, IL-4, and tumor necrosis factor, led to the proposal of a model of Foxp3-mediated transcriptional inhibition or repression in which Foxp3 antagonizes NFAT function by competition for DNA-binding sites [22]. Foxp3 is expressed in $CD4^+CD25^+$ Treg in the thymus and the periphery [23]. Foxp3 is highly conserved in humans and appears to have a similar function in regulating immune quiescence. In human T cells, Foxp3 is constitutively expressed in $CD4^+CD25^+$ T cells occurring in vivo, but can also be induced in a subset of $CD4^+CD25^-$ T cells after stimulation in vitro [24]. These in vitro-induced $Foxp3^+$ cells have regulatory functions and suppress immune responses of immune-competent T cells.

b. iii. Antigen-presenting cells

Dendritic cells (DCs), the most potent professional APCs, were shown to contribute to immune tolerance. This function that is apparently incompatible with the conventional view that DCs are primarily involved in innate and adaptive immunity to infections and other antigens in vivo [25, 26]. Evidence accumulating from both animal and human experiments indicates that immature DCs (iDCs) can suppress peripheral T cell responses or induce antigen-specific tolerance. In contrast, specific factors can induce DC maturation and promote a proinflammatory phenotype. [26]. Local cytokine environment, e.g., presence of IL-10 and TFG-β, is another important contributor to the establishment of DC phenotype [27]. A recently emerged concept is that the dialog between Treg and DCs are crucial for regulation of alloimmune responses.

b. iv. Inhibitory receptors

T cells that have exited the thymus remain capable of mounting responses to self-antigen; therefore, biochemical and genetic feedback loops are extremely important for fine tuning of autoreactive T cell responses. Inhibitory molecules expressed on T cells are essential for the maintenance of T cell homeostasis and self-tolerance. CTLA-4 (cytotoxic T lymphocyte antigen 4) and PD-1 (programmed death-1) were recognized as two major coinhibitory receptors of the B7-CD28 family, and will be discussed in detail below **(Figure 3.3)**.

3.2 The B7/CD28 family of co-inhibitory receptors

CTLA-4 is a well-characterized coinhibitory receptor. The strong inhibitory role of CTLA-4 is underscored by the phenotype of CTLA-4−/− mice, that is, the development of a massive accumulation of self-reactive T cells in peripheral lymphoid and nonlymphoid tissues [28, 29]. Major emphasis was recently placed on the evaluation of dynamic changes in CTLA-4 expression, uptake, trafficking, and degradation during T cell activation [30-33]. These careful analyses suggest that CTLA-4 has the potential to influence the activating threshold needed for a T cell to progress to full activation to mediate the attenuation of ongoing proliferative responses and to regulate the development of T-helper subset differentiation. Although the precise mechanism of CTLA-4 function is not clear, competition with CD28 for B7 molecules may have a critical role in mediating this outcome [13, 29, 34]. CTLA-4 polymorphisms appear to differentially influence its inhibitory activity. It is interesting to note that susceptibility to a variety of human autoimmune disorders was mapped to a noncoding 6.1-kb 3′ region of CTLA-4. Allelic variation in this region influenced mRNA levels and soluble alternative splice forms of human CTLA-4 [35]. This information, together with genetic data from diabetes and thyroiditis disease models, suggests that genetic difference in the context of CTLA-4 deficiency may contribute to the development of autoimmune disorders [36].

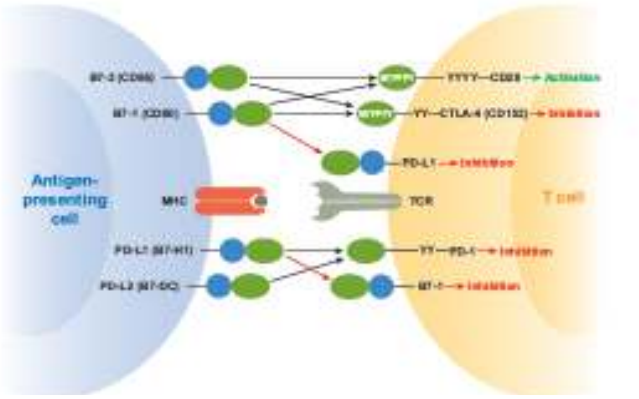


Figure 3.3. Regulation of T cell tolerance by positive and negative costimulation

PD-1 is expressed on activated T cells, B cells, and macrophages. In the presence of a TCR ligation by antigen, PD-1 recognition of its ligands **B7-H1** and **B7-H2** (**PD-L1** and **PD-L2** respectively), results in the phosphorylation of the cytoplasmic ITIM (immunoreceptor tyrosine-based inhibition motif) and the subsequent generation of SHP-2-dependent negative signals **(Figure 3.4)** [37-39]. The genetic loss of PD-1 leads to the development of autoimmune diseases with the genetic background influencing the severity and type of disease [39, 40]. It is important to note that polymorphisms in PD-1 have also recently been associated with susceptibility to systemic lupus erythematosus, type 1 diabetes, Grave's disease rheumatoid arthritis, multiple sclerosis etc [41, 42].

In addition, a third member of coinhibitory receptors, B- and T-lymphocyte-attenuator **(BTLA/CD272**), which belongs to the CD28 immunoglobulin superfamily, was identified [43]. The cytoplasmic region of both human and murine BTLA, similar to that of PD-1 contains an ITIM. Indeed, T cell activation is enhanced in BTLA-deficient mice [43, 44]. BTLA-mediated inhibition of human T cell activation occurred during both

primary $CD4^+$ T cell responses and secondary $CD4^+$ and $CD8^+$ T cell responses, suggesting that BTLA ligation sends a constitutive "off" signal to T cells and thus might play an important role in the maintenance of T cell tolerance [45].

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

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

PD-1 can be expressed on Tcells, B cells, natural killer T cells, activated monocytes, and dendritic cells (DCs). PD-1 is not expressed on resting T cells but is inducibly expressed after activation [46]. Although PD-1 cell surface protein expression can be detected within 24 h of stimulation, functional effects of PD-1 ligation are observed within a few hours following T cell activation [47]. Ligation of TCR or BCR can upregulate PD- 1 on lymphocytes, and the level of mRNA transcription does not strictly correlate with protein production [37]. In normal human reactive lymphoid tissue, PD-1 is expressed on germinal center–associated T cells [48]. PD-1 compartmentalization in intracellular stores has been described in a regulatory T cell population [49, 50]. PD-1 is inducibly expressed on APCs on myeloid CD11c+ DCs and monocytes in humans [51], but its function on these cells is not clear. There are no data to support a function for PD-1 in the absence of antigen receptor signaling.

The two PD-1 ligands differ in their expression patterns. **PD-L1** is constitutively expressed on mouse T and B cells, DCs, macrophages, mesenchymal stem cells, and bone marrow–derived mast cells [52]. PD-L1 expression is also found on a wide range of nonhematopoietic cells and is upregulated on a number of cell types after activation. Both type I and type II interferons (IFNs) upregulate PD-L1 [53, 54]. Several studies have examined which signaling pathways are required for PD-L1 expression by using pharmacological inhibitors. PD-L1 expression in cell lines is decreased when MyD88, TRAF6, and MEK are inhibited [55]. JAK2 has also been implicated in PD-L1 induction [55, 56]. Loss or inhibition of phosphatase and tensin homolog (PTEN), a cellular phosphatase that modifies phosphatidylinositol 3-kinase (PI3K) and Akt signaling, increases post-transcriptional PD-L1 expression in cancers [57].

PD-L2 expression is much more restricted than PD-L1 expression. PD-L2 is inducibly expressed on DCs, macrophages, and bone marrow–derived mast cells. PD-L2 is also expressed on 50% to 70% of resting peritoneal B1 cells [58]. PD-L2+ B1 cells bind phosphatidylcholine and may be important for innate immune responses against bacterial antigens. Less is known about transcriptional regulation of PD-L2. Its induction by IFN- γ is partially dependent on NF-κB [59]. PD-L2 can also be induced on monocytes and macrophages by GM-CSF, IL-4, and IFN-γ [52, 60].

b. Signalling through PD-1/PD-1 ligands and regulation of T cell function

Signaling through costimulatory receptors, primarily functions to modify antigen receptor signaling. PD-1 typically has greater effects on cytokine production than on cellular proliferation, with significant effects on IFN-γ, TNF-α, and IL-2 production. PD-1- mediated inhibitory signals depend on the strength of the TCR signal, with greater inhibition delivered at low levels of TCR stimulation. This reduction can be overcome by costimulation through CD28 [38] or IL-2 [61]. PD-1 may exert its effects on cell differentiation and survival directly by inhibiting early activation events that are positively regulated by CD28 or indirectly through IL-2 [61]. Both CD28 and IL-2 promote cell expansion and survival through effects on antiapoptotic, cell cycle, and cytokine genes. IL-2 withdrawal can lead to cell death, another process in which PD-1 has been implicated.

There is strong evidence that PD-1 ligation inhibits the induction of the cell survival factor Bcl-xL [47]. PD-1 inhibits the expression of transcription factors associated with effector cell function, including GATA-3, Tbet, and Eomes [62]. Further studies are required to determine whether PD-1-mediated inhibition is related to its ability to counteract cell survival signals and effector differentiation mediated through CD28, IL-2, Bcl-xL, or a combination of these factors. PD-1 is phosphorylated on its two intracellular tyrosines upon ligand engagement, and then binds phosphatases that downregulate antigen receptor signaling through direct dephosphorylation of signaling intermediates. Two phosphatases, SH2-domain containing tyrosine phosphatase 1 (SHP-1) and SHP-2, can bind to the ITIM and ITSM motifs of PD-1 [63, 64] **(Figure 3.4)**. PD-1 inhibitory function is lost when the ITSM alone is mutated, demonstrating that this tyrosine plays the primary functional role of PD-1 inhibition [47, 64]. The association between SHP-1 and PD- 1 appears to be weaker than the interaction of PD-1 with SHP-2. Together, these studies suggest that PD-1 functions by recruiting SHP-2, and possibly SHP-1, to the antigen receptor signaling complex [63]. While the binding of SHP-2 to PD-1 is significantly enhanced by PD-1 ligation [47], proximity of PD-1 to the antigen receptor appears to be important for inhibition by PD-1. PD-1 ligation inhibits antigen receptor signaling only in *cis* and not in *trans*, indicating that PD-1 ligation must occur close to the site of antigen receptor engagement [65]. CTLA-4 moves from an intracellular store to the immunological synapse between an APC and lymphocyte after

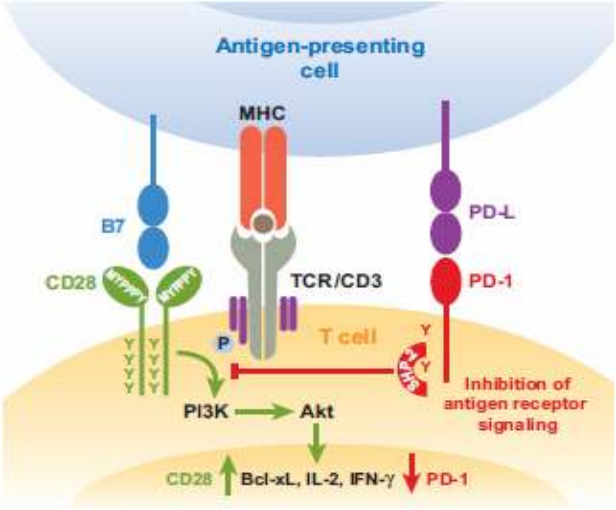


Figure 3.4. Activation of PD-1 results in inhibition of PI-3-kinace-induced activation of Akt. The Erk pathway is also suppressed resulting in decreased Bcl-xL, IL-2 and IFN-γ gene expression

antigen recognition, depending on the strength of signal [30]. In contrast, PD-1 redistributes from uniform cell surface expression to the synapse duringT cell–APC interactions [49].

PD-1 could exert its inhibitory effects by bringing SHP-2 into the synapse during antigen receptor signaling, and cross-linking of PD-1 and CD3 increases the amount of SHP-2, but not SHP- 1, associated with PD-1 [66]. PD-1 ligation inhibits PI3K activity and downstream activation of Akt. **In contrast, CTLA-4** inhibits Akt activation but does not alter PI3K activity, indicating that these coinhibitory receptors function through distinct mechanisms. PD-1 ligation inhibits phosphorylation of CD3ζ, ZAP70, and PKCθ [67]. PD-1 ligation also reduces Erk activation, but this effect can be overcome through cytokine receptor signaling, particularly cytokines that activate STAT5, such as IL-2, IL-7, and IL-15 [65]. SHP-2 positively regulates Er phosphorylation by interacting with Gab2 after IL-2R ligation [68]. Both activationof Erk, which is specifically inhibited by PD-1 ligation, and activation of STAT5, which can overcome PD-1 inhibition, are demonstrated to be involved in the antiapoptotic and proliferative function of IL-2 [69]. This suggests a model whereby the association of PD-1 with SHP-2 serves not only to dephosphorylate signaling intermediates, but also perhaps sequester SHP-2 from its positive signaling role in Erk activation.

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

c. i. PD-1 deficient mice

The first indication that the PD-1 pathway plays a critical role in autoimmunity came from the phenotype of *Pdcd1−/−* mice. Aged *Pdcd1−/−* C57BL/6 mice develop a mild glomerulonephritis with low frequency [39]. *Pdcd1−/−* Balb/c mice develop a dilated cardiomyopathy owing to the production of an autoantibody against cardiac troponin [40, 70] **(Figure 3.6)**. Autoimmunity is accelerated by PD-1 deficiency on autoimmune-prone backgrounds. These findings broadly support a role for PD- 1 in the induction and/or maintenance of tolerance. Subsequent work has examined the mechanisms by which PD-1 and its ligands can control self-reactive T cell responses.

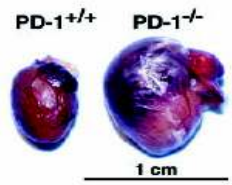


Figure 3.5. *Pdcd1−/−* Balb/c mice develop dilated cardiomyopathy

c. ii. Functional in vivo studies

PD-1 and PD-L1 provide inhibitory signals that regulate both central and peripheral tolerance in multiple ways. PD-1 is expressed on maturing thymocytes in the course of central tolerance induction. PD-L1 is expressed broadly on the thymic cortex and on thymocytes themselves, whereas PD-L2 expression is limited to the thymic medulla [59, 71]. CD4−CD8− (DN) thymocytes start to express PD-1 as they undergo TCRβ rearrangement and begin to display functional pre- TCRs on the cell surface [46]. PD-1:PD-L1 interactions inhibit positive selection during the DN to CD4+CD8+ (DP) maturational stage [72]. PD-1 signaling modifies

positive selection signaling thresholds, and loss of either PD-1 or PD-L1 increases DP thymocyte cell numbers [73]. PD-1 also can contribute to negative selection [74] and has been identified as a candidate gene in a microarray analysis of aberrant central tolerance in nonobese diabetic (NOD) mice [75]. Together, these findings point to a role for PD-1 and PD-L1 in central tolerance induction.

Self-reactive T cells that escape negative selection are controlled in the periphery by mechanisms of peripheral tolerance. Initial interactions between T cells and APCs, such as DCs, can modify potentially self-reactive responses by the display of self-antigen on resting DCs. PD-1 has an important role in controlling the outcome of initial encounters between naive self-reactive T cells and DCs by inhibiting responses of self-reactive T cells. Emerging evidence suggests that immature DCs tolerize T cells, and loss of PD-1 on antigen-specific T cells increases CD8 T cell responses to antigen-bearing resting DCs [76].

c. iii. Experimental disease models

Studies in mouse models of autoimmunity and tolerance have revealed that PD-1:PDL interactions no only are important in the initial phase of activation and expansion of self-reactive T cells, but also influence selfreactive T cell effector function upon antigen reencounter. In the NOD mouse model of autoimmune T cell–mediated diabetes, PDL1 is upregulated in the pancreas on islet cells [59], and loss or blockade of PD-1 or PD-L1 leads to rapid and exacerbated diabetes with accelerated insulitis and proinflammatory cytokine production by T cells [77-79]. 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 [80]. Notably, blockade of PD-1 or PDL1 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. Bone marrow chimera experiments have demonstrated that PD-L1 expression on non–bone marrow–derived cells, including islet cells, inhibits T cell effector function in tissues [80-82]. In the NOD mouse model, *Cd80–/–* NOD mice develop autoimmunity more rapidly and with higher incidence than NOD mice [83], although less quickly than *Pdcd1–/–* and *Cd274–/–* NOD mice. B7-1-blocking antibodies significantly accelerate diabetes onset in NOD mice and induce diabetes in normally resistant male NOD mice, but the two anti-B7-1 mAbs appear to differ in their functional effects. The 16–10A1 mAb (which blocks B7-1:CTLA-4 but not B7-1:PD-L1 interactions) caused more rapid diabetes onset than did the 1G10 mAb (which blocks both B7-1:CTLA- 4 and B7-1:PD-L1 interactions) [84]. Collectively, these findings demonstrate that PD-1: PD-L1 interactions regulate both the initiation and progression of autoimmune diabetes in NOD mice and identify PD-1:PD-L1 interactions as key mediators of T cell tolerance in tissues.

In the experimental autoimmune encephalomyelitis (EAE) model of human multiple sclerosis (MS), PD-1 and its ligands also control self-reactive T cells. PD-1, PD-L1, and PD-L2 are all expressed on cellular infiltrates within the meninges during active EAE disease in C57BL/6 mice [59]. 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 [85] and on microglial cells by IFN-γ [86]. Initial studies described a role for PD-1 and PD-L2 using neutralizing antibody treatment [87]. 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 [88] using blocking antibodies in different mouse strains, such as Balb/c, or gene-deficient animals [89, 90] suggest that PD-1 and PD-L1, but not PD-L2, are predominantly responsible for regulating the severity of disease in most mouse strains. Adoptive transfer studies emphasize the critical function for PD-L1 in limiting myelin-reactive pathogenic effector T cells and show that PD-L1 on both the transferred T cell and in the recipient restrains encephalitogenic T cell responses [90].

Another important mechanism of peripheral tolerance involves regulatory T cells, which can suppress activated T cell proliferation and cytokine production. Both PD-1 and PD-L1 are highly expressed on these populations and may play a role in regulatory T cell function [91]. A number of studies suggest that PD-L1 may be important for inducing regulatory T cell populations, although the mechanism is not yet clear [92]. Experiments in colitis models support the argument for a role of PD-1:PD-L1 on a regulatory cell population and identified a regulatory subpopulation of CD4+CD25−PD-1+ T cells that can inhibit the development of colitis [93]. PD-L1 is important for in vitro inhibition by another suppressive population ofCD4+DX5+ Tcells [94].

In view of its important role in autoimmune disease, the PD-1:PD-L pathway has become **a new therapeutic target**. Therapies that increase the expression of PD-L and trigger PD-1 may ameloriate autoimmune diseases. These approaches are only beginning to be evaluated in animal models, but the results appear promising. DCs genetically modified to overexpress PD-L1 and MOG in the context of MHC II dramatically ameliorate clinical EAE and reduce severity of CNS inflammation [95]. A recombinant adenovirus expressing full-length mouse PD-L1 partially protects against the development of nephritis in lupus-prone mice [96]. IFN-β, an immunemodulatory treatment for MS, can upregulate PD-L1 on APC in vitro and in MS patients in vivo, suggesting that IFN-β may exert its anti-inflammatory effects in part via upregulation of PD-L1 expression [54].

3.4 PD-1 and PD-1 ligands in human autoimmune diseases

a. Expression of PD-1/PD-1 ligands in human autoimmune diseases

Several groups have examined the expression of PD-1 and PD-L1 in patients with autoimmune diseases. In Sjögren's syndrome, PD-1 is expressed by salivary T clls and PD-L1 by salivary epithelial cells, indicating active PD-1/PD-L1 interactions [97, 98]. PD-1 and PD-L1/2 expression has also been detected by

immunohistochemistry, in liver tissue of patients with autoimmune hepatitis and primary biliary cirrhosis [99]. Affected muscle from patients with idiopathic inflammatory myopathies expressed PD-L1 [100] and patients with myasthenia gravis, an autoimmune-mediated neuromuscular disorder, had increased percentage if circulating PD-1+ T cells and PD-L1^{+} monocytes [101]. Enhanced expression of PD-1 on T cells and of PD-L1 on T, B and macrophage/dendritic cells, is seen in inflamed coclofrom inflammatory bowel disease patients [102]. 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 [103]. Other researchers have reported a correlation between circulationg CD4+ CD45R0+CD26^{high} memory T cells and multiple sclerosis disease severity [104]. 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**. Synovial fluid from active RA patients is enriched in PD-1^{+} CD4^{+} T cells, which also express CTLA-4 and produce IL-10 [105]. Similarly, Wan *et al*. reported increased expression of PD-1 and PD-L1 by synovial fluid T cells and macrophages derived from RA patients as opposed to osteoarthritis controls [106]. 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. Furthrmore, the authors were able to characterize a soluble form of PD-1 that corresponded to an alternative 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 [106]. 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. Of note, immobilized anti-PD1 autoantibodies stimulated CD4^{+} T calls proliferation [107]. In SLE Bertsias *et al*, has provided evidence that support a role for the inhibitory PD-1/PD-L1 pathway in regulation of T cell effector function. More specifically, during AMLR, induction of PD-1 on CD4+ T cells was impaired in SLE patients, whereas active SLE serum abrogated the inhibitory function of PD-1, indicating aberrant expression and function of PD-1 in lupus [108].

Taken together, these data –although circumstantial- indicate aberrant expression of PD-1 and PD-1 ligands in patients with autoimmune diseases and suggest that PD-1/PD-L1 interactions participate in regulation of T cell responses in the context of human autoimmunity.

b. Genetic association studies

A role for PD-1:PD-L in humans is suggested by **polymorphisms in PDCD1** that have been associated with human autoimmune diseases, including systemic lupus erythematosis (SLE), type 1 diabetes, rheumatoid arthritis, Grave's disease, and MS [109]. Most of these polymorphisms are found in conserved regions in intronic sequences. One intronic single nucleotide polymorphism (G7146A) in PDCD1 is located in a binding site for Runx1 (AML-1), a transcription factor with an important regulatory role in hematopoiesis [41]. This

polymorphism may alter PD-1 mRNA stability or expression level and is associated with reduced PD-1-mediated inhibition of IFN-γ production in German patients with MS [110]. A recent study suggests that PDCD1 genetic variation may influence the risk and expression of SLE, and effects of PD-1 polymorphisms vary according to ethnic background, similar to the effects of mouse PD- 1 deficiency in different genetic backgrounds [111]. A polymorphism in PDCD1LG2 has been described that correlates with SLE [112], but no polymorphisms in CD274 have been linked to human autoimmune diseases. Bertsias *et al* has reported that the PD1.3 polymorphism is a risk factor for SLE, and is associated with decreased transcriptional activity [108].

3.5 PD-1 and PD-1 ligands in other diseases

a. The role of PD-1 and PD-1 ligands in infections and host defence

PD-1 and its ligands have important roles in regulating immune defenses against **microbes that cause acute and chronic infections**. The PD-1:PD-L pathway appears to be a key determinant of the outcome of infection, regulating the delicate balance between effective antimicrobial immune defenses and immunemediated tissue damage. For example, PD-1−/− mice clear an adenovirus infection more rapidly but develop more severe hepatocellular injury than WT mice [113]. In a mouse model of herpes stromal keratitis, a blocking anti-PD-L1 mAb exacerbated keratitis, increasing HSV-1-specific effector CD4 T cell expansion and IFN-γ production and survival [114]. These studies suggest that the PD-1:PD-L pathway limits the potentially detrimental consequences of vigorous antipathogen effector T cells.

A number of microorganisms that cause chronic infection appear to have exploited the PD-1:PD-L pathway to evade the immune responses and establish persistent infection. Studies in the lymphocytic choriomeningitis virus (LCMV) model of **chronic viral infection** were the first to show a role for the PD-1:PD-L pathway during chronic infection [115]. Viruses that cause chronic infections can render virus-specific T cells nonfunctional and thereby silence the antiviral T cell response [116]. Functional dysregulation, or exhaustion, of CD8 T cells is an important reason for ineffective viral control during chronic infections and is characteristic of chronic LCMV infection in mice, as well as of HIV, HBV, HCV, and HTLV infection in humans and SIV infection in primates. Several groups have shown that PD-1 expression is high o HIV-specific [51, 117, 118], HBV-specific [119, 120], and HCV specific T cells [121]. PD-L1 is als upregulated on peripheral blood CD14+ monocytes and myeloid DCs in patients with chronic HBV infection [122, 123], and on CD14+ cells and Tcells in HIV patients [124]. Blocking PD- 1:PD- interactions in vitro reverses the exhaustion of HIV-specific, HBV-specific [120], HCV-specific, and SIV-specific [125] CD8 and CD4Tcells and restores proliferation and cytokine production [51, 117, 118, 121].

The PD-1:PD-L pathway also may play a key role in the **chronicity of bacterial infections**. *Helicobacter pylori* causes chronic gastritis and gastroduodenal ulcers and is a risk factor for development of gastric cancer [126, 127]. Parasitic worms also have exploited the PD-1:PD-L pathway to induce macrophages with strong suppressive function [128, 129]. PD-L1 and PD-L2 have distinct roles in the immune response to the protozoan parasite *Leishmania mexicana* [130]

The key roles of the PD-1:PD-L pathway in reducing T cell responses during chronic viral infections propel development of strategies to manipulate the interaction of PD-1 and its ligands to restore antiviral T cell responses during chronic viral infections. This pathway may have evolved to limit immune mediated damage to the host during infection by turning off pathogen-specific T cells. **The therapeutic potential of manipulating PD-1 and PD-L1** to enhance immune responses during chronic infection gives impetus to analyzing the relative effects of blocking PD-1:PD-L1 interactions versus B7-1:PD-L1 interactions during chronic infection.

b.The role of PD-1 and PD-1 ligands in transplantation

The role of PD-1, PD-L1, and PD-L2 has been investigated in **models of cardiac and islet transplantation**. PD-1, PD-L1, and PD-L2 expression are induced within cardiac allografts undergoing rejection [131]. Neither PD-L1Ig nor PD-L2Ig alone prolonged cardiac allograft survival. However, PD-L1Ig, but not PD-L2Ig, plus cyclosporine A significantly enhanced allograft survival over that of cyclosporine A or PD-L1Ig alone and led to decreased intragraft expression of IFN-γ as well as CCR5 and CXCR3mRNA. Similarly, PD-L1Ig had synergistic effects when given with rapamycin and led to permanent survival of fully MHCdisparate cardiac allografts. PD-L1Ig also promoted long-term graft survival in CD28−/− recipients [131] and markedly reduced cardiac transplant arteriosclerosis when given in conjunction with anti-CD154 mAb [131]. PD-L1Ig and anti-CD154 similarly synergized and induced long-term survival of islet allografts, whereas either alone failed to prolong islet allograft survival [132]. These findings suggest that PD-1 targeting, when used together with agents in current clinical use or in clinical trails, may strikingly improve the survival of solid organ tranplantation. The exact mechanism by which PD-L1Ig exerts protective effects in these models is not clear; PD-L1Ig could be triggering a negative signal through PD-1 or blocking a positive signal for T cell activation and function. The accelerated rejection of PD-L1–expressing pancreatic islet allografts suggests that PD-L1 expression in pancreatic islets may promote, rather than inhibit, T cell responses [133].

In a murine acute graft-versus-host disease (GVHD) model, GVHD lethality is accelerated when PD-1 engagement is prevented [134]. GVHD was markedly accelerated by a blocking anti-PD-1 mAb or PD-L1.Ig fusion protein. The magnitude of GVHD acceleration was similar when PD-1−/− CD4+ or CD8+ T cells were

infused into sublethally irradiated recipients. Neither perforin nor fas ligand were required for GVHD acceleration, but donor IFN-γ production was necessary. Co-blockade of CTLA-4 and PD-1 synergized and further accelerated GVHD, indicating that the effects of PD-1 and CTLA-4 act independently to down-regulate GVHD lethality. Together, these findings demonstrate that PD-1 ligation downregulates GVHD by modulating IFN-γ production, and they suggest that PD-1 ligation may provide a novel approach for preventing GVHD lethality.

c. The role of PD-1 and PD-1 ligands in tumor immunity

PD-L1 and PD-L2 are expressed on a variety of tumors. PDL1 is expressed on **many murine tumor cell lines**, and IFN-γ enhanced PD-L1 expression on murine tumor cell lines [135]. PD-L1 is highly expressed on **many human carcinomas** [136, 137], including those of the breast, cervix, lung, ovary, and colon, as well as on melanomas [137] and glioblastomas [138]. PD-L1 is not expressed on B cel hematologic malignancies but is strongly expressed in some primaryTcell lymphomas [137]. PD-L1 also is expressed on most thymic epithelial tumors, including benign and invasive thymomas as wel as thymic carcinoma. PDL2 has been identified as the gene that best discriminates primar mediastinal B cell lymphoma (PMBL) from other diffuse large B cell lymphomas, and it may be a **useful molecular diagnostic marker** [139]. PD-L2 also is highly expressed in Hodgkin lymphoma cell lines [139]. The genomic locus containing PD-L1, PD-L2, and several adjacent genes is amplified in PMBL and Hodgkin lymphoma. The upregulation of PD-L1 on tumors has led to the hypothesis that PD-L1 on tumors may be a means by which tumors evade T cell recognition. Indeed, animal models have shown that PD-L1 expression on tumor cells inhibits T cell tumor immunity. Similarly, anti-PD-L1 administration enhanced survival of mice given PD-L1- expressing tumor cells [140]. These findings suggest that PD-L1: PD-1-mediated inhibitory signals give tumors a selective advantage for growth by inhibiting CD8+ T cell responses, and that **PD-L1 blockade may be a valuable approach for cancer immunotherapy**. Currently, a fully human anti-PD-1 mAb has been developed and is in Phase I clinical trials for cancer.

3.6 Rheumatoid Arthritis (RA): the prototype of inflammatory arthritides

a. Overview

Rheumatoid Arthritis (RA) is a chronic inflammatory disorder affecting primarily cartilage and bone of small and middle-sized joints. In addition, larger joints and several organs such as lung, vessels and the hematopoietic system may be involved. Locally, inflammatory cells invade the otherwise relatively acellular synovium leading to hyperplasia and formation of pannus-tissue, which causes destruction of cartilage, erosion of the adjacent bone and ultimately loss of function of the affected joint **(Figure 3.6)**. Systemic

inflammation, in parallel, has long term impact on various organs, considerably increasing the risk for atherosclerosis and lymphoma development. Combined with inevitable side effects of year-long anti-rheumatic medication and the psychological burden of facing early invalidity and social instability, untreated RA has an important socio-economic impact and causes a reduction in life-expectancy of 7 years in average.

From a clinical point of view, there is considerable heterogeneity in both clinical picture as well as course of disease. Although patients with longstanding disease usually present a clinical picture characteristic of RA, overlap with other rheumatic diseases such as mixed connective tissue disease (MCTD) exists, and RA may be accompanied by additional autoimmune diseases (e.g. Sjoegren's syndrome, Hashimoto's thyreoiditis). For reasons largely unknown, the course of disease is highly variable, ranging from mild cases with non-erosive, even sometimes spontaneously remitting disease, to severe, rapidly progressive and destructive RA. Recent analysis of genetic risk factors, autoantibodies and effects of therapies suggests, however, that clinical RA might consist of pathogenetically distinct subgroups, and that different treatment strategies should be applied to patients within these groups.

From an immunological point of view, RA is considered an autoimmune disease, implying breakdown of immunological tolerance towards self at a given point in a patient's life. The trigger initiating this breakdown is so far unknown. Presence of autoantibodies and slowly rising C-reactive protein-levels several years before onset of clinical symptoms indicate that the inflammatory process may be well underway long before patients first consult a physician. Variations between ethnic groups in susceptibility to RA, heterogeneity of disease course and variations in clinical, radiological and laboratory findings within groups strongly suggest that multiple factors, both environmental and genetic, influence onset and progression of RA, presumably with different impact during different stages of disease development. Genetic variations, autoantibodies, cellular immune responses, hormones and gene-environment interactions are among the most studied factors contributing to RA development **(Figure 3.7)**, and shall be discussed in more detail below.

b. RA – Clinical features

Epidemology

RA affects approximately 0.5-1% of European and North-American adults, with considerable regional differences. Annual incidence rates are estimated to be 16.5 cases (per 10^5) in Southern Europe, 29 cases in Northern Europe and 38 cases in North America [141]. The mean age of onset of rheumatoid arthritis is 52 years, although this condition also significantly affects patients between 40 and 70 years of age. In patients <60 years of age, rheumatoid arthritis has a 3 to 5:1 female predominance. However, this ratio becomes more even in patients >60 years of age. Recurrence risk estimates for relatives of affected individuals are 4%

for siblings, around 4.7% for first degree (parent/child) and ~1.9% for second degree relatives. Disease severity in the index case determines recurrence risk. First degree relatives of patients with severe erosive disease can have a risk of up to 15%, whereas the risk for relatives of mildly affected individuals may only be minimally increased [142].

Clinical manifestations

Rheumatoid arthritis is a symmetrical polyarthritis that involves the small joints of the hands and feet, as well as other joints throughout the body. The following is a summary of clinical features that are useful for diagnostic and classification purposes.

- Morning stiffness for at least one hour and present for at least six weeks
- Swelling of three or more joints for at least six weeks
- Swelling of wrist, metacarpophalangeal, or proximal interphalangeal joints for at least six weeks
- Symmetric joint swelling
- Hand x-ray changes typical of RA that must include erosions or unequivocal bony decalcification
- Rheumatoid subcutaneous nodules
- Rheumatoid factors / Antii-CCP antibodies

Many features previously associated with rheumatoid arthritis have become less common, most likely because effective treatment of this condition is initiated earlier, has become increasingly aggressive, and is guided by optimal clinical outcome assessment tools. These features include subcutaneous forearm nodules, development of rheumatoid vasculitis, and the need for joint replacement.

Autoantibodies

RA is accompanied by the occurrence of many autoantibodies. At present, the main clinically useful biologic markers are **rheumatoid factors** and **antibodies to citrullinated peptides. Rheumatoid factor** is an antibody directed to the Fc domain of IgG molecules and is present in approximately 75% of RA patients. In addition to RF, several autoantibodies have been reported to be more specific and to have higher positive predictive value for RA. Some of these highly RA-specific autoantibodies have been found to recognize citrullinated peptides and are called thus **anti-citrullinated protein antibodies (anti-CCP antibodies)**. Such autoantibodies are not only very specific for RA (up to 98%), but also they are detected very early in the disease course or even several years before disease onset. Patients with either rheumatoid factor or anti-CCP antibodies typically have a worse radiologic and functional outcome compared with other patients. Moreover, patients with both of these findings have an even worse outcome compared with patients with only one of these factors and therefore their presence indicates the need for more aggressive therapy.

Diagnosis

There is no single clinical, radiologic, or serologic test that enables a diagnosis of RA to be made with certainty. As with other autoimmune rheumatic diseases, the diagnosis depends upon the aggregation of characteristic symptoms, signs, laboratory data, and radiologic findings.

Treatment – disease monitoring

Early and aggressive disease control is indicated in rheumatoid arthritis to stop development of joint damage and functional limitation, as well as to prevent loss of work and premature atherosclerosis and its associated early mortality. Modern management of rheumatoid arthritis involves an aggressive progressive approach using DMARD therapy early in the disease course and multiple-drug regimens. Methotrexate with folic acid is the most commonly used DMARD, as it has the greatest potential for modifying disease and is therefore central to most treatment regiments for rheumatoid arthritis. In addition, adjunctive therapy with a TNF-α antagonist or other biologic therapy is indicated if the disease continues to be active and progressive.

The goal of therapy should be to achieve no evidence of disease and inflammation, because erosions do not develop when inflammation is not present. Close follow-up is therefore crucial to establish patient response to treatment and is achieved by evaluating disaese activity scores (DAS28) and patient's functional state (HAQ) at every visit to the doctor. Ultrasonography also provides a useful tool for assessing disease activity and progression (detection of active synovial inflammation and bone erosions).

Prognosis

If undertreated, and occasionally even with optimal treatment, rheumatoid arthritis is an aggressive disease that targets not only the joints and their surrounding soft tissues but also distant organs and tissues. More than 90% of patients with rheumatoid arthritis develop erosions less than 2 years after disease onset, and 20% of patients with this condition develop erosions even with methotrexate therapy. Moreover, more than 35% of all patients with rheumatoid arthritis stop working within 5 to 10 years of disease onset, and approximately 60% are disabled to some degree after 20 years. Life expectancy for patients with rheumatoid arthritis is decreased by 3 to 10 years compared with the general population, and mortality rates are 25% higher for women. However, methotrexate and anti-TNF therapy has somewhat reversed these effects.

Coronary artery disease is the leading cause of death in patients with rheumatoid arthritis, causing approximately 50% of deaths associated with this condition. Patients with rheumatoid arthritis are also 70% more likely to have a stroke compared with the general population. In addition, these patients have a 70% higher risk for developing infection, which is most likely related to immune dysfunction associated with rheumatoid arthritis and its treatment. Patients with rheumatoid arthritis also are 44 times more likely to develop non-Hodgkin's lymphoma compared with the general population.

c. RA - Pathogenesis

c. i. Autoantibodies in RA

As is the case for other autoimmune diseases, an array of antibodies targeting self-antigens has been characterized in RA. Although the corresponding antigen is usually known, demonstrating pathogenetic relevance of the respective autoantibody at least in animal models has proven far more difficult. The initial notion that mechanisms of autoimmunity might underlie RA pathogenesis came from the discovery of autoantibodies targeting the Fc-part of human IgG (so called **"rheumatoid factors" - RF**) in the blood of affected patients [143] **(Figure 3.6)**. RF, present mostly as **IgM-RF**, but detectable in subgroups of patients also as IgG- and IgA-RF, are thought to form immune complexes activating complement in the joint, which in turn leads to increased vascular permeability and the release of chemotactic factors recruiting immune-competent effector cells to the joint [144]. The mere presence of RF, however, is insufficient to initiate arthritis development, as RF are also found in infectious diseases, autoimmune diseases other than RA and in up to 15% of healthy, mostly elderly individuals. Thus, sensitivity and specificity of RF are, depending on the population studied, 60-70% and 50-90%, respectively. Despite this lack of specificity, presence of RF is one of 7 diagnostic criteria for RA put forward by the American College of Rheumatology in 1987 [145, 146].

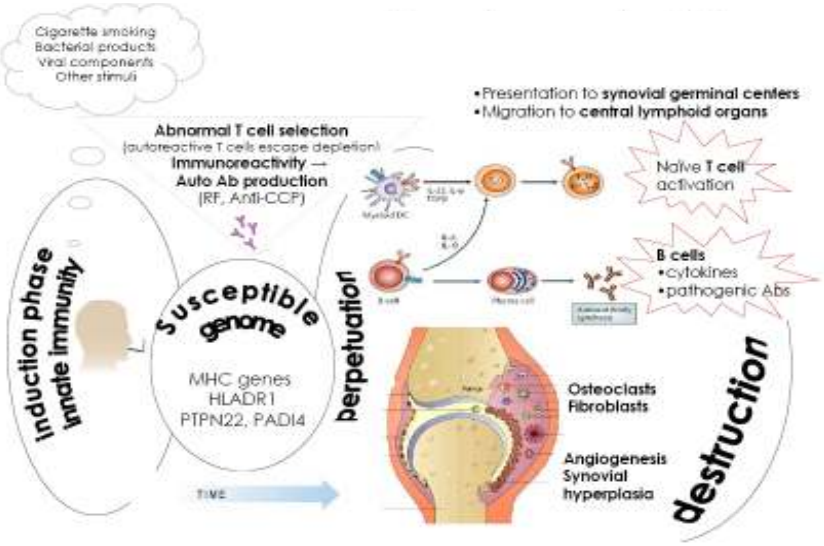


Figure 3.6. Pathogenesis of Rheumatoid Arthritis

The autoantibody most likely directly related to RA-pathogenesis, targets proteins containing the atypical amino-acid citrullin. Citrullination is a process by which arginine residues in a given protein are post-translationally modified ("deiminated") in the presence of high calcium concentrations by an enzyme called PAD (peptidyl arginine deiminase). Citrullination is a physiological process, which is believed to be important for degradation of intracellular proteins during apoptosis. In 1998, two antibodies present in RA-sera, that had been described already years before, were found to both recognize a common target, namely citrullinated fillagrin [147, 148]. This observation placed citrullinated proteins in the center of autoantibody research in RA,

and meanwhile, citrullin-specific reactivities against a number of additional citrullinated proteins (e.g. fibrinogen, vimentin, and α-enolase) have been identified. By the use of novel assays, anti-citrullinated protein antibodies **(ACPA)** are now found in 60-70% of RA-patients, but hardly in other diseases or healthy subjects [149]. Observational findings from the analysis of patient cohorts strongly point to a crucial role for ACPA in RA-pathogenesis, albeit final experimental evidence has yet to be obtained. Nonetheless, ACPA are of important diagnostic value and are useful predictors of disease progression and severity.

c. ii. Genetic Risk Factors

Comparisons between concordance rates in monozygotic twins in several populations indicate that approximately 50% of the variation in prevalence of RA is caused by genetic factors. Many genetic variations that show association with RA have been identified within the last three years due to technical advances in genotyping **(Figure 3.7)**.

The strongest and most relevant genetic risk factor for the development of RA, contributing around 30% to the total genetic effect, is found in HLA class II molecules on chromosomal location 6p21.3, Several **HLA-DRB1** molecules (*0101, *0102, *0401, *0404, *0405, *0408, *1001 and *1402) share a common amino acid sequence at position 70-74 in the third hypervariable region of the DRβ1-chain. This sequence has, therefore, been termed the **"shared epitope".** It is situated in the antigen binding cleft of the respective class II molecule and has thus been implicated in binding of a putative arthritogenic peptide [150]. More detailed analysis of the shared epitope revealed that amino acids at position 70 and 71 flanking positions 72-74 (RAA) seem to modulate the T cell response. Irrespective of the still unknown mechanism by which the shared epitope contributes to the development of RA, multiple studies underline the strong association between shared epitope and susceptibility to as well as severity of RA [151].

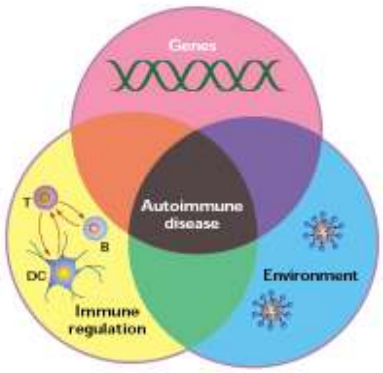


Figure 3.7. Multiple factors, environmental, genetic and immunological, influence onset and progression of RA

Next to genetic variations in HLA-class II molecules, other genetic factors have been identified as important risk factors for the development of RA. A single nucleotide polymorphism (SNP) in tyrosine phosphatase **PTPN22** at position 1858 (C->T) leading to a mis-sense mutation is associated with several autoimmune diseases and has been found to be an HLA-independent risk factor for RF- and ACPA-positive (but not ACPA-negative) RA. As PTPN22 is important in the inhibition of T- and B-cell receptor signalling, the mutation is

thought to lower the threshold for T- and B-cell activation, thus facilitating the development of autoreactive T- and B-lymphocytes [152].

In addition, several whole genome association scans have recently been performed on large sample sets of RA-cohorts originating from different countries. Such genome-wide scans test up to 500.000 SNPs distributed over the entire genome for their association with disease. Next to confirming loci that have previously been associated with RA (i.e. HLA-class II, PTPN22), these scans identified polymorphisms in genetic regions encoding **STAT4,** TNFAIP3/OLIG-3 and **TRAF1-C5** as novel risk factors for ACPA-positive disease [153, 154]. STAT4 (signal transducer and activator of transcription 4) is a transcription factor involved in the differentiation of both type 1 helper T cells (Th1) and Th17-cells (see below). It is in part induced by IL-12 and IL-23, two cytokines that have diverging roles in the differentiation of CD4+ T cells [155].

Several SNPs were also found to be associated with ACPA-positive RA in an intergenic region on chromosome **6q23** between the genes TNFAIP3 (tumor necrosis factor-α induced protein 3) and OLIG-3 (oligodendrocyte lineage transcription factor 3). TNFAIP3 codes for a protein (A20) that is a negative regulator of NFκB and as such involved in the regulation of TNF-α signaling [156, 157]. In a similar fashion, two independent approaches identified a haplotype block encompassing several SNPs in an intergenic region between the genes coding for **C5** (complement component 5) and **TRAF-1** (tumor necrosis factor receptor associated factor-1) on chromosome 9 [153, 158]. While C5 is part of the complement system, TRAF-1 is involved in signalling of TNF-α via TNFR-1.

The relative importance of polymorphisms of other candidate genes (i.e. **CTLA-4, PAD4, PD-1, IL-10, TNFRII, RUNX1**) is somewhat less clear to date, as associations with RA have been found in some populations, but not in others. Such discrepancies, however, largely depend on the size of the respective patient cohort and on the technical approach applied. Alternatively, inconsistent findings might reflect predominance of disease subgroups in different populations. Thus, genetic variations in several genes which need further study might contribute to RA susceptibility and severity independently of the strong effect exerted by HLA-class II molecules. Notably, some of these effects are strongly influenced by environmental factors (gene-environment interactions) [159].

c. iii. Environmental Risk Factors

None of the aforementioned genetic risk factors in itself is sufficient to cause RA. Carrier-ship of one or more of these factors may predispose to either disease development or disease severity, but additional environmental factors are needed for genetic factors to exert their impact. In the context of RA, some environmental factors may have specific effects directly related to RA-pathogenesis (e.g. factors promoting citrullination of proteins), whereas others might have non-specific effects promoting inflammation in general

(e.g. triggers of innate immunity). In addition, these factors might be relevant at distinct time-points during disease development.

Infections are major candidates for the induction of autoimmunity and have, therefore, been intensively studied also in RA. To date, however, no pathogen-derived antigen has been clearly linked to RA-pathogenesis, and convincing evidence for cross-reactivity of self-antigen-specific T- or B-cells with pathogen-derived peptides ("molecular mimicry") in a manner relevant for RA-development is still lacking. Female predominance in various autoimmune diseases including RA suggests that **sex hormones** and reproductive factors influence both RA-development and severity [160-162].

The most prominent example for a gene-environment interaction in RA-pathogenesis, however, is **cigarette smoking (Figure 3.6)**. Several years of smoking confer an increased risk for the development of RA, is associated with more severe disease and the risk increases proportionally with the number of pack-years. Importantly, smoking increases the risk for the development of ACPA-positive, but not for ACPA-negative RA. This risk is further increased in the presence of shared-epitope alleles (up to an estimated 21-fold as compared to shared-epitope negative non-smokers) [163, 164]. As shared-epitope alleles themselves also predispose to the development of ACPA-positive RA only, cigarette smoke could be involved in the induction of protein-citrullination. Supporting this hypothesis, citrullinated proteins have been detected in bronchoalveolar lavage fluid from smokers but not from non-smokers [165]. Thus, smoking might induce apoptosis and subsequently citrullination in alveolar cells, which, by a mechanism so far unknown, induces an anti-citrullin specific immune response. How and why this response eventually targets the joints, however, remains unknown and might require additional environmental factors.

c. iv. Disturbances of the Immune System

During normal embryonic development the immune system is tolerized by various mechanisms against recognizing "self" as "dangerous". T cells, for example, are positively and negatively selected in the thymus to only recognize non-self antigens in the context of MHC-molecules (**central tolerance**). As some autoreactive T cells might escape this selection, and as some self-antigens are only expressed later in life, additional mechanisms of tolerance exist once immune cells enter the periphery (**peripheral tolerance**). Naturally occurring regulatory T cells, for example, efficiently control autoreactive effector T cells. Consequently, lack of such regulatory T cells leads to a syndrome of severe multiorgan autoimmunity including arthritis in both mice and humans [166].

The trigger initiating breakdown of immunological tolerance in RA is still unknown. Rheumatoid synovitis is characterized by massive infiltration of the synovium by various immune effector cells (mainly T- and B-lymphocytes, neutrophils, monocytes, mast cells). As these are absent from a normally very thin synovial layer

in healthy joints, the infiltration is considered to be initiated via an active process of cellular migration. Locally, these cells may proliferate and produce inflammatory cytokines and chemokines. Together with vascular growth factors promoting neovascularization and vascular leakage which facilitates cellular migration, additional effector cells are recruited to the joint. Over time, this process creates a cytokine milieu in the joint that activates synovial fibroblasts and osteoclasts, which in turn degrade cartilage and bone **(Figure 3.7)**. Cellular mediators as well as important cytokines supporting this process are discussed below.

T cells

T cell function in RA can be interpreted from different perspectives. **Effector T cells** are likely to be the first to recognize an autoantigen as foreign, thus being antigen-specifically activated and providing necessary help for B cells to produce autoantibodies. Upon activation, these effector cells proliferate and produce proinflammatory cytokines, thereby driving an inflammatory immune respons e**(Figure 3.8)**. **Regulatory T cells (Tregs)**, on the other hand, appear to be incapable of controlling this autoreactive immune response. Whether Tregs are functionally defective in RA is still a matter of debate, but data indicate that TNF-α is involved in downregulation of Treg-cell function [167].

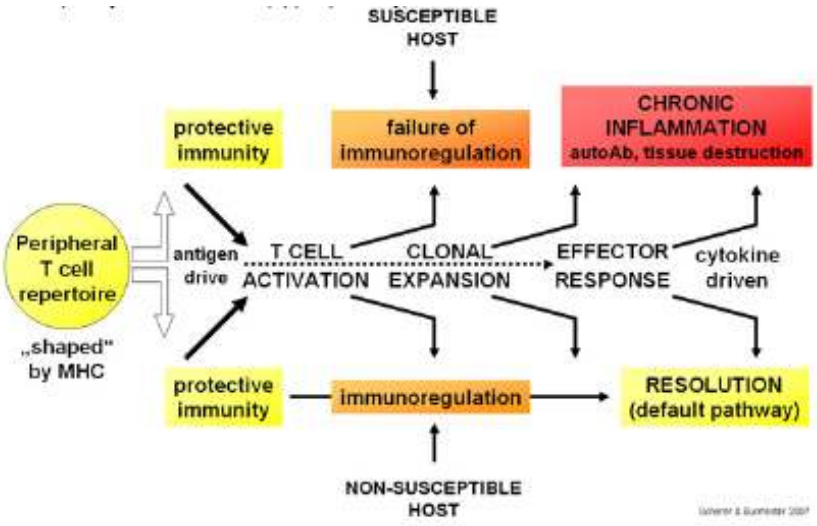


Figure 3. 8. Defective regulation of T-cell immunity leads to chronic inflammation

For many years, rheumatoid arthritis was considered to be a mainly Th1-mediated autoimmune disease. The abundance of Th1-cytokines (especially IFN-γ) and the relative lack of Th2-cytokines (IL-4, IL-5, IL-10) in RA-patients favoured this notion, and the ability of Th1-cells to activate monocytes, the main source of TNF-α in RA, further supported the concept. This view has considerably changed over the last years, mainly due to a new understanding of the original Th1/Th2 paradigm [168]. IL-12, the cytokine driving Th1-development and IFN-γ production, was found to share a common subunit (IL-12*p40*) with recently discovered cytokine IL-23

(consisting of IL-12*p40* and IL23*p19*). IL-23, in turn, is important for IL-17 production by a new subclass of T helper cells termed Th17. Previous models investigating the role of IL-12 in knock-out models of IL-12*p40*, therefore, not only exhibited IL-12 deficiency, but also lack of IL-23.

Importantly, IL-17 producing, but not IFN-γ producing CD4+ T cells proved to be pathogenetically relevant in the murine collagen induced arthritis model (CIA). In line with this observation, mice deficient in IL-23 only (*p19-/-*) were shown to be resistant to CIA, whereas lack of IL-12 (*p35-/-*) induced more severe disease [169]. These findings introduced IL-17 producing T cells as pathogenetic players in RA pathogenesis

The development of **TH17 cells** is not yet fully understood. Murine Th17 cells were found to share a common origin with FoxP3+ regulatory T cells (Treg). Naïve CD4+ T cells of the mouse develop into Treg in response to TGF-β, while additional presence of IL-6 leads to differentiation into Th17 cells. Development of human Th17 cells, however, seems to be distinct from mice, as human naïve CD4+ T cells from umbilical cord blood require IL1-β and IL-23 in order to differentiate into Th17 cells. In addition, lineage commitment of human Th17 cells is more flexible than in the murine system, as subpopulations of human Th17 cells were identified that produce IFN-γ or IL-10 in addition to IL-17. Apart from these cytokines, Th17 cells can produce TNF-α, IL-6, IL-22 and GM-CSF [170].

In RA-patients, both IL-17 and IL23*p19* are found in sera, synovial fluid and synovial biopsies, whereas these cytokines are mostly absent from osteoarthritis patients. IL-17 strongly stimulates macrophages to produce TNF-α as well as IL-1 and has been implicated in the induction of osteoclastogenesis. It also enhances IL-6 production, collagen destruction and collagen synthesis by RA synovial explants. In addition, synovial mRNA levels of IL-17, TNF-α, IL-1β and IL-10 correlate with progressive joint damage. Finally, IL-17 induces production of IL-1β, IL-6, IL-23, IL-8, G-CSF, GM-CSF, VEGF, Cox2 and PGE2 by fibroblast-like synoviocytes [6]. Thus, IL-17 has become an interesting novel therapeutic target in RA, and the efficacy of tocilizumab, an IL-6 antagonist approved for the treatment of RA, might in part be explained by an influence on Th17-development and function.

As described above, naïve T cells develop into effector T cells upon antigen recognition, while the cytokine milieu they encounter during development influences lineage specificity. Recognition of antigen by naïve T cells in the context of MHC-molecules, however, requires additional **costimulatory signals** for efficient T cell activation. Without costimulatory signals, T cells become anergic. Interaction of CD28 and CD80/86 generates one of the most important costimulatory signals, which is controlled by expression of the natural inhibitor CTLA-4. This mechanism is exploited therapeutically by an engineered CTLA4-Ig fusion protein (Abatacept), which efficiently inhibits disease activity in RA. Next to cytokine production, CD4+ effector T cells have been attributed additional functions in RA synovium. By means of direct cell-to-cell contact they provide crucial help

to B cells for antibody production, and by the expression of RANKL they can directly induce osteoclastogenesis [168].

Detailed analysis of human regulatory T cell function in RA is hampered by difficulties in defining a consistent phenotype for these cells. Initially non-regulatory T cells can acquire a regulatory phenotype in the periphery upon stimulation with specific cytokines ("inducible Treg"). "Naturally occurring" regulatory T cells (Treg that have developed in the thymus), on the other hand, also do not form a homogeneous population in the human. This might explain, why conflicting data on functional integrity of Treg cells in RA patients have been published. Whereas an initial report did not find functional impairment of RA-derived Treg cells [167], later work claimed that Treg cells of RA-patients fail to suppress cytokine production by effector T cells. Interestingly, the functional deficit was restored following treatment with TNF-antagonists [171]. This study was later extended to show that treatment with TNF-antagonists gives rise to a newly generated, functionally distinct Treg-cell population that secretes TGF-β and IL-10 [172]. Treg cells express TNFR-II, which makes them susceptible to deleterious effects of TNF-α. Indeed, TNF-α was experimentally shown to inhibit Treg-cell function, and treatment of RA-patients with TNF-antagonists restored Treg cell function also in a second study [173]. The relevance of functionally intact Treg-cells for RA has clearly been shown in murine CIA. Mice depleted of Treg developed more severe disease, and adoptive transfer of Treg in mice with established disease could lower disease severity [174]. So far, however, these useful aspects of Treg cell function have not yet been transferred to human RA.

B cells

The importance of B cells in RA-pathogenesis has only recently received adequate appreciation. The presence of RF and ACPA years before clinical onset of arthritis implicates early involvement of autoantigen-specific B cell activation and plasma cell differentiation. ACPA show high specificity for RA, strong genetic association with shared-epitope alleles, and clearly associate with disease progression and severity. RA synovium frequently exhibits formation of lymphoid follicles with germinal centre-like structure, indicating involvement of B cells in the local immune response. Indeed, T cell activation in RA synovium has recently been shown to be B cell dependent. Finally, depletion of peripheral B cells has proven to be highly effective even in patients with inadequate response to TNF-antagonists.

The role of B cells in RA is multifactorial, including cytokine production (e.g. TNF-α, IL-6, IL-10), antigen presentation, modulation of T cell responses and (auto-) antibody production [175]. By producing TNF-α and IL-6, B cells contribute to the activation of macrophages and directly participate in inflammation. IL-6 is also an important differentiation factor for B cells themselves. IL-10 producing B cells have been implicated in downregulation of the immune response possibly by tolerizing T cells, but their exact phenotype and role in

RA is less well understood. In addition, B cells are responsive to triggers of innate immunity (e.g. immune complexes, bacterial DNA), thus bridging non-specific and specific immune responses [176].

Monocytes/Macrophages

The role of activated macrophages in RA synovium is central to driving and maintaining chronic inflammation. Macrophages are multipotent effector cells that very efficiently integrate innate and adaptive immune responses. They are abundantly present in the rheumatoid synovial membrane and at the cartilage/pannus junction [177]. Important functions include strong phagocytic activity, antigen processing and presentation, secretion of proinflammatory cytokines, expression of Fc-receptors responsive to (auto-) antibodies and immune complexes, complement activation and regulation, toll-like receptor (TLR) expression, tissue degradation and remodelling, and direct interaction with mainly fibroblasts and T cells [177, 178].

Inflammatory cytokines

Despite the therapeutic success of monoclonal antibodies targeting single cytokines, synovial fluid and sera of RA patients contain a dynamic pool of cytokines from different sources with agonistic, synergistic or even opposing effects. In many ways, the cytokine profile of RA synovium reflects the relative contribution of different cell-types to the inflammatory state [179, 180]. Studies evaluating cytokine profiles have tried to assess which cell type predominates during different stages of disease development. Especially the initiation phase of inflammation is thought to be distinct from a chronic, self-perpetuating phase. Recently, patients with early arthritis (symptom duration less than 3 months) that progressed to RA were shown to exhibit a different synovial cytokine profile than patients that remitted or developed other arthritides [181].

Synovial macrophages respond to direct cell-contact with T cells and fibroblasts. Among the most important cytokines secreted by activated macrophages in RA are TNF-α, IL-1, IL-6, IL-12, IL-15, IL-18 and IL-27. In addition, macrophages produce MMP-9 and -12, macrophage migration inhibitory factor (MIF), GM-CSF and thrombospondin [178]. Macrophage function is in itself regulated by various cytokines that in some cases have autocrine effects. IL-4, for example, downregulates macrophage function by reducing the expression of TNF-α, IL-1β and PGE2. IL-10 lowers the expression of HLA-DR and reduces antigen processing and the expression of Fc-receptors. Both IL-4 and IL-10 have strong anti-arthritic properties in murine models of arthritis, and some studies have linked polymorphisms in the IL-10 gene to disease susceptibility [182, 183].

3.7 Animal models and their relevance to rheumatoid arthritis

The value of mapping in animals is dependent on there being good models of human diseases. In this review we focus on RA, a highly heterogeneous autoimmune disease that is known to depend on multiple genes and environmental factors. The disease models should therefore preferably be correspondingly polygenic and dependent on environment. There are a number of available animal models for RA that all mimic various aspects of the disease, possibly reflecting disease pathways that operate in different subgroups of RA patients. Thus, all of these models can be valuable under certain conditions, depending on the question that is to be addressed.

a. Induced arthritis models

If an antigen is known to induce disease, then this permits studies of the antigen-specific response and allows mapping of the genes involved. **Collagen-induced arthritis (CIA)** is induced by the major collagen found in cartilage, namely collagen type II (CII), emulsified in adjuvant [184-186]. Disease develops 2 to 3 weeks after immunization in susceptible strains (H-2q or H-2r) [187]. CIA is the most widely used model for studying arthritis pathology and for testing for novel anti-inflammatory therapeutics [188]. **Proteoglycan (aggrecan)-induced arthritis (PGIA)**, characterized by a progressive disease course, is induced by cartilage proteoglycans. PGIA presents with 100% incidence in BALB/c mice (H-2d), which are normally resistant to CIA [19], and manifest in substrains of C3H (H-2k) [189]. CIA and PGIA are complex highly polygenic diseases that are dependent on both B and T cells [185, 190-192] and are both associated with MHC class II molecules (MHCII) and a large number of both common and unique non-MHC loci [187, 193]. Both CIA and PGIA are believed to have relevance to human disease because antibodies to both CII and proteoglycan in RA patients have been identified [187, 193-196]. Other cartilage structures that can induce arthritis include cartilage oligomeric matrix protein [197, 198] and type XI collagen [199].

Collagen antibody-induced arthritis (CAIA) is induced by injection of specific monoclonal CII antibodies [200]. CAIA resembles CIA but is more acute and has a rapid onset, a few days after injection. Normally, the disease heals after a month and mice remain healthy. The CAIA model is unique because it is independent of MHC and T and B cells [201, 202]. Instead, neutrophils and macrophages are recruited and activated independent of the adaptive immune system, as a result of antibodies binding to the cartilage surface and fixing complement [201]. This allows investigation of effector mechanisms without involvement of the priming phase.

A number of bacteria also have the capacity to induce arthritis in animals. Mice infected with *Borrelia* develop a disease similar to RA (***B. burgdorferi* associated arthritis**) [203] and *Staphylococcus aureus* causes septic arthritis in both rats and mice [204, 205]. Bacterial components, such as cell wall fragments, DNA and heat shock proteins, can also induce arthritis by themselves, one example being the streptococcal cell wall induced arthritis model [206]. In rats, exposure to heat-killed *Mycobacterium tuberculosis* in adjuvant results

in *Mycobacterium* induced-arthritis, often referred to as **adjuvant-induced arthritis** [207]. It was later found that a similar mixture but excluding myco-bacteria (incomplete Freund's adjuvant) also had arthritogenic capacity (**oil-induced arthritis**) [208]. In addition, some mineral oils by themselves had the capacity to induce arthritis, including squalene [209] and pristine [210] **(Pristane-induced arthritis - PIA).**

b. Collagen-induced arthritis (CIA)

Collagen-induced arthritis (CIA) is an animal model of rheumatoid arthritis (RA) that is widely used to address questions of disease pathogenesis and to validate therapeutic targets. Arthritis is normally induced in mice or rats by immunization with autologous or heterologous type II collagen in adjuvant **(Figure 3.9)**. Susceptibility to collagen-induced arthritis is strongly associated with major histocompatibility complex class II genes, and the development of arthritis is accompanied by a robust T- and B-cell response to type II collagen. The chief pathological features of CIA include a proliferative synovitis with infiltration of polymorphonuclear and mononuclear cells, pannus formation, cartilage degradation, erosion of bone, and fibrosis. As in RA, pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin (IL)-1β, are abundantly expressed in the arthritic joints of mice with CIA, and blockade of these molecules results in a reduction of disease severity.

The development of CIA is **strain-dependent**, with H-2q and H-2r haplotypes showing the greatest degree of susceptibility. The DBA/1 strain (H-2q) is the most commonly used strain for pre-clinical testing of potential anti-arthritic drugs and was successfully used to predict the beneficial effects of TNF-α blockade. [211, 212]

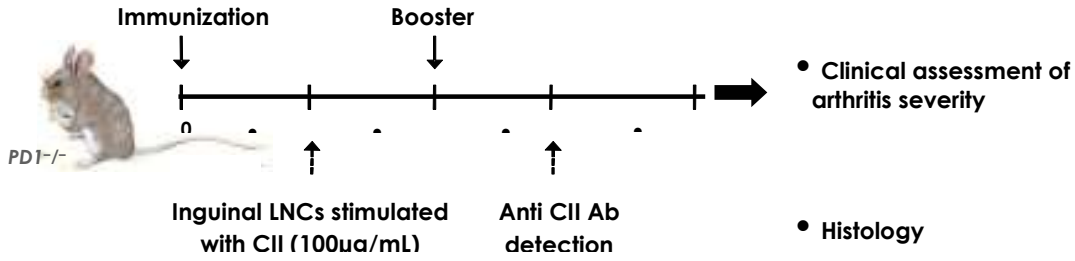


Figure 3.9. Schematic representation of CIA induction

CIA has also been clinically and histologically characterised in the C57BL/6 background. B6 mice develop a chronic form of CIA that closely resembles human RA in terms of its disease course, histological findings, and in its response to commonly used anti-arthritic drugs. B6 mice have also been shown to develop a sustained T-cell response to chicken collagen used for CIA. [213].

Development of CIA is dependent on T cells [191] as well as on B cells [185, 190] , but it is noteworthy that CII-immunization only elicits weak T cell proliferative responses. This could be due to partial tolerance to

CII [214]. The CII-reactive B cell response is characterized suggestive of T cell help. It has been argued that the importance of B cells in this model is at the level of antibody (Ab) production, although a critical role of B cells acting as APCs is also possible [190].

T cell responses in CIA

While T cells are clearly required for the development of CIA, their role in directly mediating the pathogenesis of this experimental disease is less clear. Unlike other autoimmunity models, such as experimental autoimmune encephalomyelitis (EAE), in which T cells are the primary effectors, passive transfer of CII-specific T cells to susceptible strains induces only minor pathological changes in the synovial joints of the recipients [215, 216], while transfer of CII-specific antibody results in severe inflammation [9, 217-220]. However, the fact that mouse CII-specific T cells are present during CIA and T cells can be found in the arthritic joints leaves open the possibility that they are participating directly in the pathogenic response [221, 222]. By using passively transferred transduced T cells, it has been demonstrated that CII-specific T cells do preferentially migrate to arthritic joints [223], and that using CII-specific T cells transduced to constitutively express the IL-12 p40 antagonist, the development of arthritis could be inhibited by targeting the CII-specific T cell response in the joints. In addition, some evidence of restricted TCR expression in arthritic joints has also been described, implying that an oligoclonal population has preferentially homed to these tissues. In all, these data suggest that T cells in the inflamed synovium are participating in the pathogenesis, but it is not clear if this participation is in the form of localized stimulation as a result of murine CII presentation by antigen-presenting cells (APC) in the joint, or if they directly participate in the joint destruction.

CII-specific T cell function

Regardless of their potential role in the pathogenesis, T cells do play a prominent role in shaping the autoimmune response in CIA, both in terms of production and regulation of proinflammatory cytokines, and the modulation of the pathogenic B cell response. Like most class II-restricted models of autoimmunity, the CII-specific T cell response in CIA can be classified as Th1. DBA/1 mice treated with anti-IL-12 as well as mice genetically deficient in IL-12 exhibit a marked reduction in their development of CIA, accompanied by decreased anti-CII immune responses [224, 225]. When CII-specific T cells from CIA susceptible strains are stimulated with antigen in vitro, large amounts of the Th1 cytokines IFN-γ and IL-2 are produced in comparison to the Th2 cytokines IL-4 and IL-10, and elevated numbers of Th1 T cells can be identified in the lymphatic tissue following immunization with CII [226]. In addition, this Th1 characterization is supported by a number of studies in which disease was ameliorated by either reducing the production of Th1 cytokines [223-225, 227, 228] or by increasing the production of Th2 cytokines [229-231]. Consequently, this model has been used by numerous investigators to develop means of down-regulating Th1 cytokine production and/or up-regulating Th2 cytokine production as therapeutic approaches to the regulation of autoimmunity. Despite the Th1 phenotype of the arthritogenic T cell response, the role of IFN-γ in this autoimmunity model is less than

clear. Early attempts to define its role in the autoimmune response by either the treatment of mice with IFN-γ or by the administration of neutralizing antibodies specific for IFN-γ yielded conflicting results [232-235]. In some systems arthritis was accelerated, while in others it was prevented, with the timing of administration also proving to be pivotal to the outcome.

Co-stimulation blockage in CIA

CIA is one of the animal models in which one has observed a potent suppressive effect on disease development by **blocking co-stimulation** [236] The importance of co-stimulation in CIA was recently further highlighted by the complete resistance of CD28-deficient DBA/1 mice to the development of disease [237]. It has been shown that co-stimulation is crucial for the induction of a T cell-dependent B cell response and in the case of CIA, this is possibly a major factor determining disease development. This was performed by using mAbs directed towards CD28, CD80 and CD86 (B7) and CD152. Of these treatments, anti-B7 was the most effective by means of suppressing disease incidence and severity. Although, theoretically, a selective CD28-blockade should be a more efficient way compared to B7-blockade to achieve therapy in CIA, as it would leave the interaction between B7 and CD152 intact, Anti-CD28 only led to a delayed onset of disease and anti-CD152 had no effect [238]

4. RESEARCH QUESTIONS AND AIM OF THE STUDY

Numerous data support a central role of T cells in RA with these cells thought to be triggered locally in an antigen-specific manner resulting in breakdown of tolerance, synovial inflammation, and autoantibody production [3-7]. Regulation of activation of T lymphocytes is mediated by mechanisms involving central and peripheral lymphoid organs. Programmed death-1 (PD-1) is a novel member of the B7 family which plays important role in peripheral tolerance. There is evidence to suggest a distinct role of PD-1 and its ligands (PD-L1/B7-H1, PD-L2/B7-DC) in regulation of T cells.

Although the role of co-stimulation is well documented in RA and has been further supported by the efficacy of CTLA4-Ig in severe RA, the role of this family of molecules has not been explored in a systematic, organized fashion. The aim of this study was to determine the role of PD-1/PD-1 ligands (PD-1L) in RA and test the hypothesis that defective expression and/or function of this pathway contributes to T cell hyperactivity within the inflamed joint.

5. MATERIALS AND METHODS

5.1. Genotyping

DNA extraction and genotyping for PD1.3

Genomic DNA was extracted from EDTA-treated peripheral whole blood obtained from patients and healthy blood donors 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 PD1.3A was performed by polymerase chain reaction (PCR) and restriction analysis, as described elsewhere. To amplify the PD-1 gene regions encompassing the PD1.3 (G+7146A) SNP 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'. 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 $MgCl_2$, 200nM of each dNTP, and 1.0U Taq polymerase (all reagents from Minotech, FORTH, Greece). PCR conditions were denaturation at 95°C for 10 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. A 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. Both undigested and digested PCR products were visualized in 2% agarose gel stained with ethidium bromide.

Statistical analysis

The genotype and allele frequencies were determined by direct counting 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). A p-value (two-tailed) <0.05 was considered as statistically significant.

7.2. Preparation of mononuclear cells and isolation of $CD4^+$ and $CD3^+$ T lymphocytes

Synovial fluid (SF) and peripheral blood (PB) were obtained from RA (n=67, mean ± SD age 62 ± 11 years) and osteoarthritis (OA) (n=32, 71 ± 6 years) patients. RA synovial fluid serum was collected as a supernatant from RA synovial fluid after hyaluronidase treatment and centrification. All RA patients had active arthritis (DAS28 6.2 ± 0.8), 46 (69%) were rheumatoid factor positive, 46 (69%) were on DMARDs and 21 (31%) on anti-TNF agents. SF was treated with hyalouronidase (Sigma-Aldrich, St. Louis, MO) and washed in PBS. SF and PB mononuclear cells (SFMCs and PBMCs) were isolated by Ficoll-Histopaque (Sigma-Aldrich, St. Louis, MO) density-gradient centrifugation and washed in PBS. $CD4^+$ T lymphocytes (purity 92–98%) were isolated by positive selection with magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany).

5.3. Antibodies and flow cytometry

The following mouse anti-human antibodies were used as PE−, FITC−, or PC5−conjugates: anti-CD3 (clone UCHT1), anti-PD-L1 (B7-H1) (MIH1), anti-PD-L2 (B7-DC) (MIH18) and anti-PD-1 (J116) (all from eBioscience, San Diego, CA, USA). Anti-CD4 (OKT4), and anti-CD69 (TP1.55.3) were from Beckman-Coulter (Miami, USA). Anti-CD25 (M-A251) and anti-HLA-DR (G46-6), were from BD Pharmingen (Franklin Lakes, NJ, USA). PE− or PC5−conjugated IgG1 (679.1Mc7) (Beckman Coulter) and FITC−conjugated IgG1 (P3) (eBioscience) were used as IgG isotype controls in all experiments. PBMCs or SFMCs (0.5×10^6 cells) were incubated in wash buffer with appropriate amounts of monoclonal antibodies on ice for 30 minutes. Cells were washed and were immediately analyzed on an EPICS XL−MCL flow cytometer (Miami, USA). The CellTrace CFSE cell proliferation kit (Invitrogen, Eugene, Oregon, USA) was used for CFSE labeling of T cells.

5.4. Stimulation of PB and SF $CD4^+$ T cells and assessment of PD-1 function

PB and SF $CD4^+$ T cells (1×10^5/well) were incubated in RPMI-1640 complete medium (containing 10% FBS, 2mM L-glutamine, 10mM HEPES, 100 IU/ml penicillin and 10 µg/ml streptomycin) (Gibco Invitrogen) in 48-well tissue culture plates (Nunc) and were stimulated with PMA (10 ng/mL) and ionomycin (500 ng/ mL). After 48 hours cells were harvested, washed, 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 events was analyzed. To assess PD-1 function, $CD4^+$ T cells from RA patients were stimulated with plate-bound anti-CD3 mAb (UCHT1) in combination with plate-bound PD-L1.Fc to crosslink PD-1 (both from R&D Systems). Briefly, 96-well flat-bottomed plates (Nunc) were coated with anti-CD3 (1µg/mL), PD-L1.Fc (0–5µg/mL) for 4 hours at 37°C in 100µl PBS solution. Human IgG1 (Sigma) was added as needed to keep the amount of total protein constant. Plates were washed twice with PBS before cell culture was initiated. At 72 hours cells were pulsed with [^{3}H]thymidine (1µCi/well) (Amersham Biosciences, Munich, Germany) for another 16 hours to measure proliferation. In studies using CFSE–labeled T cells, proliferation was determined based on CFSE dilution analyzed with WinMDI software. In some experiments, culture medium was supplemented with non-homologous hyaluronidase–treated RA SF (15% vol/vol) to evaluate effects on PD-1 function.

5.5. Immunochemistry for PD-1 and PD-1 ligands expression

Synovial biopsy specimens were obtained by open surgery in RA and OA patients and by arthroscopy in healthy controls. All procedures were approved by the Northern Stockholm Ethical Review Board and informed consent was obtained from all participants. Tissues were snap frozen in dry ice–cooled isopentane. Serial cryostat sections (7 µm) were fixed for 20 minutes with 2% (vol/vol) formaldehyde and stored at -70 ºC. Immunohistochemistry was performed using mouse IgG1 anti-human PD-1 antibody (MIH4), anti-PD-L1 (MIH1), anti-PD-L2 (MIH18) (eBioscience, San Diego, CA), mouse IgG1 anti-human CD3 (SK7; BD Biosciences, San Jose, CA), mouse IgG1 anti-human CD163 (Ber-MAC3; DakoCytomation, Glostrup, Denmark) and mouse IgG1 anti-human CD19 (HD37; DakoCytomation) as previously described (28). Isotype and concentration

matched controls were used. Stained biopsy sections were evaluated semiquantitatively by 2 independent observers (AIC, DM) who were unaware of the sample's identity, for expression of PD-1/PD-1L and the degree of inflammation [239]. Analysis of serial sections stained with cell-type specific markers (CD3 for T cells, CD19 for B cells, CD163 for macrophages) was performed.

5.6. Collagen-induced arthritis

PD-1$^{-/-}$ mice bred on C57Bl/6 (B6) background were a kind gift of Dr. Zhang (Department of Orthopedic Surgery, University of Chigaco, IL, USA) [39]. Wild-type and *PD1*$^{-/-}$ B6 mice were maintained under pathogen-free conditions at the Institute of Molecular Biology and Biotechnology (FORTH) facilities. CIA was induced according to standard protocol. Briefly, an emulsion was formed by dissolving 2 mg/ml chick CII (Sigma, St. Louis, MO) overnight at 4°C in 10 mM acetic acid and combining it with an equal volume of CFA containing 5 mg/ml heat-killed Mycobacterium tuberculosis (H37 Ra, Difco, Detroit, MI). 8-week mice were injected intradermally at two sites into the base of the tail with a total of 100 μl of emulsion; this was repeated as a boost 21 days later. In some experiments wild-type B6 mice were injected intraperitoneally PD-L1.Fc (0.1mg/mouse) on days 0, 2, 3, 5, and 10 post–immunization. PD-L1.Fc protein (1873, kindly provided by Dr. G. Freeman, Harvard School of Medicine) consists of the extracellular domains of murine PD-L1 linked to the hinge-CH2-CH3 domains of a mutated murine IgG2a to reduce FcR and complement binding, and has been shown to stimulate PD-1 *in vivo* (30-31). Animals were assessed for redness and swelling of all four limbs, and a clinical score rangin from 0 (no inflammation) to 4 (extensive swelling and erythema of the entire paw) was allocated for each mouse two to three times per week for up to 42 days as previously described [240, 241]. At termination, the rear paws of the mice were removed, fixed, decalcified, and paraffin embedded [11, 213]. Frontal sections (5 mm) were stained with hematoxylin and eosin and evaluated according to the presence or absence of inflammatory cell infiltrates (defined as focal accumulations of leukocytes).

5.7. Anti-CII T cell responses

Inguinal lymph nodes were excised from CIA mice on day 10 after immunization (early T cell responses). Lymph node cells (LNCs) were cultured in complete RPMI 1640 medium in the presence or absence of two different concentrations of CII (10μg/ml, 50μg/ml). After 48 hours, 100 μL of culture medium was removed for measurement of cytokines and 24 hours later, the remaining cells were pulsed with 1 μCi ^{3}H thymidine per well for a further 16 hours. Cells were then harvested and plates were assessed for ^{3}H thymidine incorporation. Each assay was performed on a minimum of three occasions. Secreted IFN-γ, IL-10, and IL-17 were measured in the culture supernatant by sandwich ELISA using capture and detection antibody pairs (BD Biosciences).

5.8. Production of anti-CII antibodies

Anti-CII IgG production was measured in mouse serum , 39 days after the first immunization, by ELISA. Briefly, serum samples from immunized mice were added in a serial dilutions (1:500, 1:1000, 1:2000) on plates precoated with 10μg/ml chicken CII. CII specific Abs were detected with peroxidase-conjugated goat anti-mouse IgG Abs (Chondrex, USA).

5.9. Statistical analysis

The non-parametric Mann-Whitney and Kruskal-Wallis tests were used for comparisons between two or more groups, respectively. The chi-squared test was used to compare proportions. The correlation between PD-1/PD-L expression and degree of synovial tissue inflammatory was tested by the Spearman rank order test. The paired t-test was used for comparisons in PD-1 expression and/or function in paired PB and SF samples. P values <0.05 were considered statistically significant.

6. RESULTS

6.1. Comparable PD1.3A single nucleotide polymorphism frequency in RA patients and healthy controlls

In certain ethnic populations PD-1 polymporphims are associated with increased risc for autoimmune disease. To determine whether PD-1 SNPs are associated with susceptibility to RA in our cohort, we analyzed the genomic DNA of 86 RA patients (females, 58%, mean age 67 ± 12 years) of Creatan (Greek) origin for the PD1.3 SNP and compared their frequency with those of 227 age- and sex- matched healthy controls (females, 60%, mean age 59 ± 17 years); this SNP has been previously associated with RA in asian populations [242]. We found no substantial difference in frequencies of PD1.3 heterozygosity (G/A) between RA patients and healthy controls (15,1% versus 19,4%, p>0,05) **(Figure 6.1)**, although there was a trend towards decreased frquency in the male subpopulation, that didn't reach statistical significance (p=0.29).

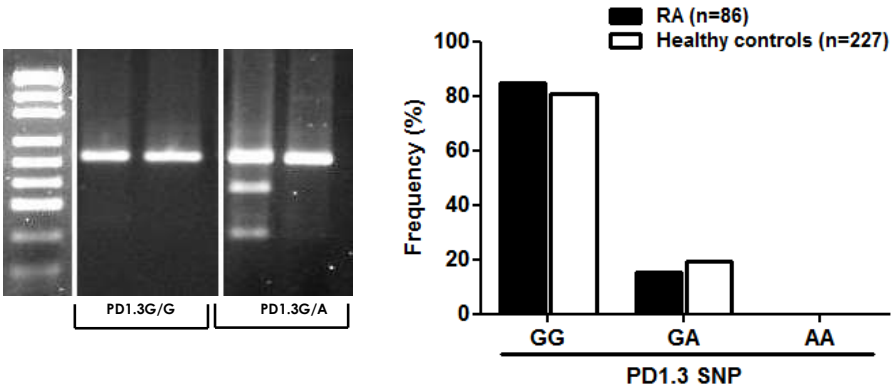


FIGURE 6. 1. No substantial difference in frequencies of PD1.3 heterozygosity (G/A) between RA patients and healthy controls

6.2. Incresed expression of PD-1/PD-1L in human RA synovial fluid and tissue

We first performed immunohistochemistry in RA synovial tissue sections using OA and healthy synovial tissues as controls. Eight out of nine (8/9, 89%) RA samples showed PD-1 expression, as compared to 2/8 (25%) OA patients and 0/8 (0%) healthy individuals **(Figure 6.2A)**. All RA and OA synovial samples were PD-L1 positive compared to only 1/8 (12.5%) of healthy individuals. Similarly, 9/9 (100%) RA and 6/8 (75%) OA synovial tissue were PD-L2 positive compared to only 2/8 (25%) of healthy individuals. In semi-quantitative analysis, RA biopsies had higher expression of PD-1 (median 1.5, range 0-3) than both OA (median 0, range 0-1) and healthy individuals' biopsies (no PD-1 expression). PD-L1 was highly expressed in both RA (median 3, range 1-3) and OA (median 2, range 1-3) biopsies, with minimal expression in healthy individuals (median 0, range 0-1). As for PD-L2, the only difference detected was between RA (median 2, range 1-3) and healthy synovial tissue (median 0, range 0-2) **(Figure 6.2B)**. Synovial expression of PD-1/PD-L1/PD-L2 was significantly

increased in synovia with high degree of inflammation, defined as total synovial inflammation score equal or above the median value of 4.5 **(Figure 6.2C)**

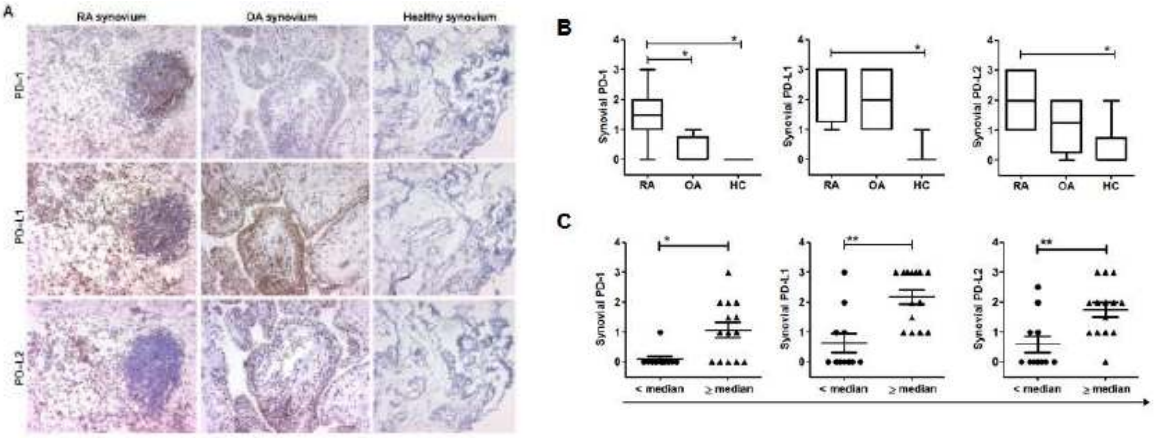


FIGURE 6.2. Expression of PD-1/PD-1L in synovium in RA, OA patients, and healthy controls.

A. RA patients display increased PD-1 expression compared to OA patients and healthy controls (HC) (*upper lane*). PD-L1 (*middle lane*) and PD-L2 (*lower lane*) expression is enhanced in both RA and OA patients compared to HC. In RA, PD-1 is mainly expressed in lymphoid aggregates of the sublining layer and also in few scattered inflammatory cells residing in the sublining and lining layer. Positive immunostaining is indicated with the brown colour represeting DAB staining (×100magnification).

B. Graphs represent box plots of mean immunostaining intensity (0 = none, 4 = maximum) of PD-1/PD-1L in RA (n=10), OA (n=9), and HC (n=9) *p<0.05 for pairwise comparisons.

C. Synovial expression of PD-1/PD-L correlates with the degree of inflammation. Scatter dot plot analysis of PD-1/PD-1L expression in synovia with low (< median) *versus* high (≥ median) inflammatory score. Lines represent mean ± SEM immunostaining intensity *p<0.05, **p<0.01.

We also examined the localization of PD-1/PD-1L in the rheumatoid synovium. PD-1 was expressed in lymphoid aggregates of the sublining layer and in few scattered inflammatory cells residing in the sublining and lining layer. Examination of the immunohistochemistry figures revealed similar immunostaining patterns for PD-1 and the T-cell marker CD3, suggesting that PD-1 is most likely expressed by synovial T cells **(Figure 6.3A)**. PD-L1 and PD-L2 were expressed by synovial cells of the lining and sublining layers, while PD-L1 by sublining endothelial cells. Most cells expressing both PD-L1 and PD-L2 were macrophages but few lymphocytes also expressed small amounts of PD-L2 **(Figure 6.3B)**. Taken together, PD-1 and PD-1L are expressed in the rheumatoid synovium suggesting a role of this pathway in RA.

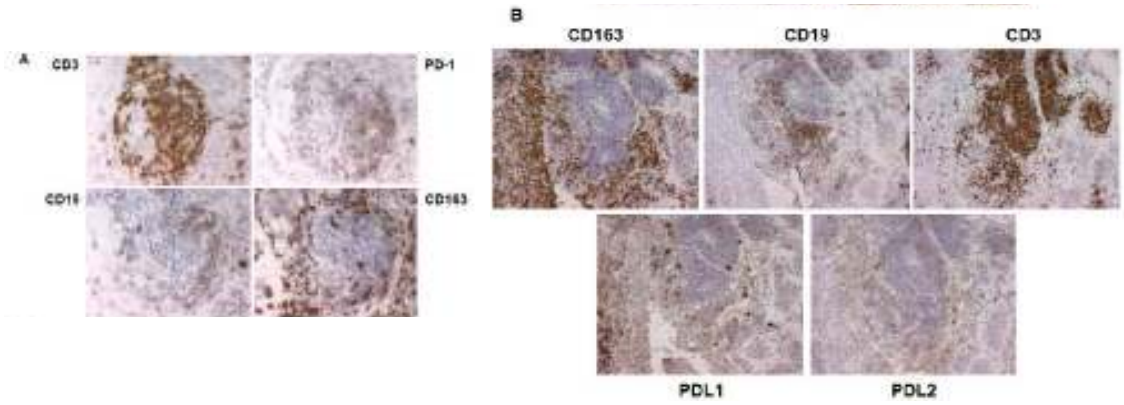


FIGURE 6. 3. Localization of PD-1/PD-1L in the rheumatoid synovium

A. PD-1 is mainly expressed by infiltrating CD3+ T cells in rheumatoid synovium (×250 magnification).

B The majority of cells expressing both PD-L1 and PDL-2 are CD163+macrophages but few lymphocytes may also express small amounts of PD-L2 (×100 magnification).

6.3. SF T lymphocytes from RA patients is enriched in PD-1+ expressing T lymphocytes

We next examined the expression of PD-1 in SF T cells from RA patients. In both RA and OA, SF was enriched in PD-1+ CD4+ T cells compared to PB. RA patients had higher percentage of SF PD-1+ CD4+ T cells compared to OA patients (24 ± 5% vs. 4 ± 1%, p=0.003) **(Figures 6.4A-B).**

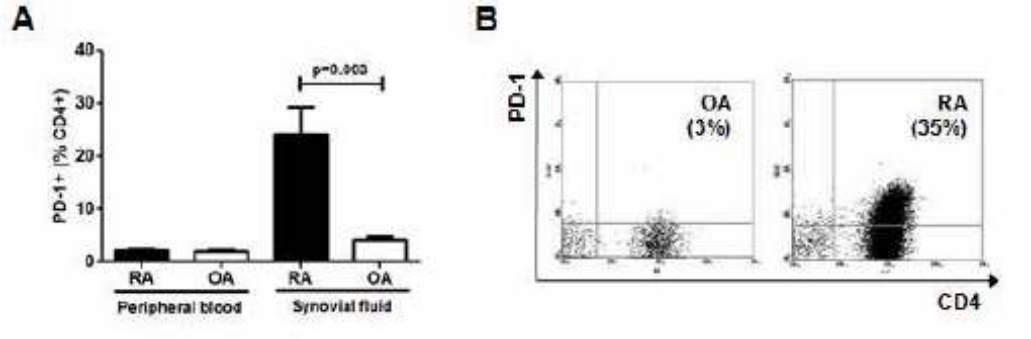


FIGURE 6.4. RA synovial fluid is enriched in PD-1+ CD4+ T cells with reduced capacity to further upregulate PD-1 upon stimulation

A. PD-1 expression on PB and SF mononuclear cells by flow cytometry. In PB, RA and OA patients had comparable levels of PD-1 on CD4+ T cells. In SF, RA patients had significantly increased PD-1 expression in CD4+ T cells.

B. Representative flow cytometry for PD-1 expression in SF CD4+ T cells from one OA and one RA patient.

PD-1 was also overexpressed on SF CD4+ CD69+ and CD4+ CD25+ activated cells in RA patients (data not shown). In contrast, there was no difference in PD-L1 expression in SF lymphocytes and monocytes between RA and OA patients **(Figure 6.4C)**. We also evaluated the capacity of T cells to further upregulate PD-1 upon activation with PMA and ionomycin. PD-1 was significantly upregulated on SF T cells from OA patients (25 ±

4% vs. 5 ± 2% at baseline, paired t-test p=0.006), whereas a less profound upregulation was observed on RA SF T cells (29 ± 4% vs. 18 ± 5% at baseline, p=0.067) **(Figure 6.4D)**.

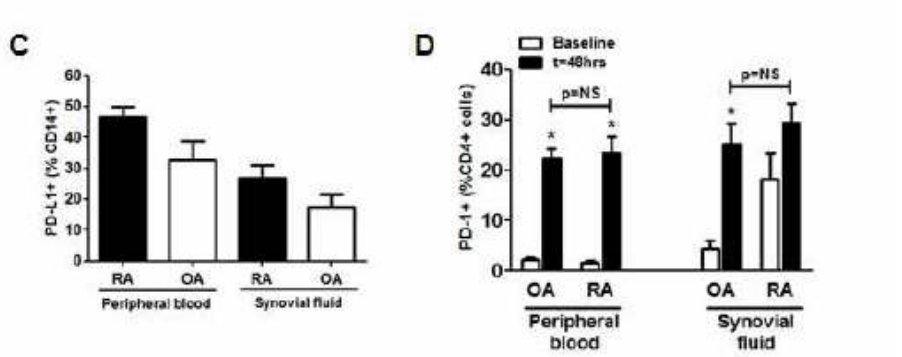


FIGURE 6.4. RA synovial fluid is enriched in PD-1+ CD4+ T cells with reduced capacity to further upregulate PD-1 upon stimulation

C. Comparable expression of PD-L1 on PB and SF CD14+ monocytes between RA and OA patients.

D. SF CD4+ T cells from RA patients have increased baseline PD-1 expression but reduced capacity to further increase PD-1 expression following stimulation with PMA and ionomycin. Comparable induction and expression of PD-1 on PB CD4+ T cells in OA and RA patients following stimulation with PMA and ionomycin. *p<0.05 for the difference between baseline and induced PD-1 expression (paired t-test).

Overall, these data corroborate the immunohistochemistry results showing enhanced expression of PD-1 – but not of PD-L1 – within the rheumatoid joint.

6.4. PD-1 regulates T cell proliferation and cytokine production in RA patients; decreased PD-1 mediated suppression in peripheral blood T cells incubated with RA synovial fluid and in RA synovial fluid T cells

PD-1 activation results in suppression of lymphocyte proliferation and cytokine production via decreased ERK and AKT/PKB activation. To assess whether PD-1 regulates T cell responses in RA patients, PB CD4+ T cells were activated with plate-bound anti-CD3 mAb and PD-L1.Fc to crosslink PD-1; IFN-γ production and T cell proliferation were assessed at 48 and 96 hours of stimulation, respectively.

Using CFSE–labeled T cells, we found that PD-1 crosslinking resulted in significant suppression of proliferation in RA patients **(Figure 6.5A)**, which was comparable to that in OA patients and healthy controls (data not shown). Moreover, PD-1 activation by PD-L1.Fc caused a dose-dependent decrease in anti-CD3–induced IFN-γ production by RA CD4+ T cells **(Figure 6.5B)**.

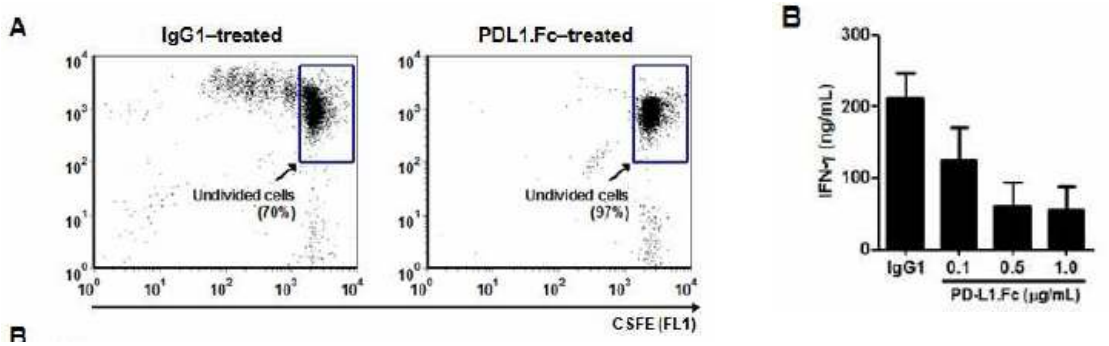


FIGURE 6.5. PD-1 regulates T cell proliferation and cytokine production in RA patients; decreased PD-1mediated suppression in peripheral blood T cells incubated with RA synovial fluid and in RA synovial fluid T cells.

A. PB CSFE–labeled CD4+ T cells from RA patients were stimulated with anti-CD3 (1µg/mL) and PD-L1.Fc (0-2µg/mL) to crosslink PD-1. Proliferation was assessed by CFSE dilution on day 5. Representantive flow cytometry of RA T cells treated with IgG1 (control) or PD-L1.Fc (0.1µg/mL). The proportion of undivided T cells is shown. At least 5 cellular divisions are noticed in IgG1– treated T cells, as compared to none in PD-L1.Fc–treated cells.

B. PB CD4+ T cells from RA were stimulated with anti-CD3/PD-L1.Fc. After 48 hours, IFN-γ was measured in culture supernates by ELISA. PD-1 crosslinking significantly reduced IFN-γ production by stimulated T cells.

RA is characterized by chronic on-going T cell activation within the joints, resulting in joint destruction and disability. Previous studies have shown that the PD-1/PD-L pathway may be influenced by several factors such as the level of costimulation, pro-inflammatory cytokines, and TLR-agonists (33-34). To explore whether the suppressive function of PD-1 is abrogated within the rheumatoid joint inflammatory milieu, we incubated CFSE–labeled PB CD4+ T lymphocytes from RA patients with 15% RA SF and measured the effect of PD-1 crosslinking on cell proliferation. Incubation with RA SF significantly decreased suppression of T cell

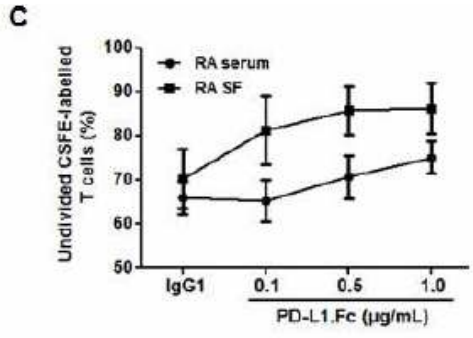


FIGURE 6.5.C. PD-1 regulates T cell proliferation and cytokine production in RA patients

C. PB CSFE–labeled CD4+ T cells from RA were stimulated in culture medium supplemented with 15% RA SF or serum. Results are the percentage of undivided T cells on day 5. RA SF reduced PD-1– mediated inhibition of T cell proliferation, especially at suboptimal PD-L1.Fc (0.1µg/mL). Dots and error bars represent the mean ± SEM of 4 independent experiments using 3 different RA SF samples.

proliferation compared to RA serum, especially at PD-L1.Fc 0.1µg/mL (undivided T cells on day 5: 65.1 ± 4.7% vs. 81.2 ± 7.8%, *n*=5 experiments, paired t-test p=0.028) **(Figure 6.5C)**.

We next examined whether SF CD4+ T cells have normal PD-1 function, and compared its inhibitory function in paired PB and SF samples from RA patients, by measuring T cell proliferation and IFN-γ production. Activation of PD-1 by plate-bound PD-L1.Fc resulted in suppression of anti-CD3–induced T cell proliferation,

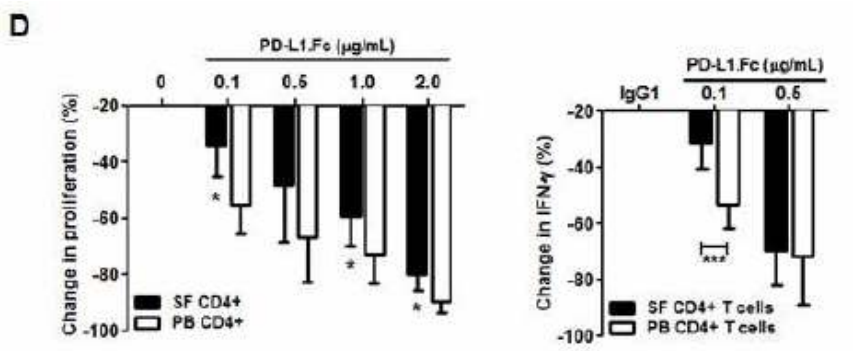


FIGURE 6.5.D. PD-1 regulates T cell proliferation and cytokine production in RA patients

D. SF and PB CD4+ T cells from the same RA patient were stimulated with anti-CD3/PD-L1.Fc. After 48 hours IFN-γ was measured in culture supernate; after 72 hours thymidine was added for another 16 hours to measure proliferation. The percentage of inhibition of anti-CD3–induced proliferation and IFN-γ production was calculated. Compared to PB, SF CD4+ T cells from RA had decreased PD-1□mediated T cell inhibition, especially at PD-L1.Fc concentration 0.1µg/mL *p<0.05,***p<0.001 (paired *t*-test).

which was less pronounced in SF than PB CD4+ T cells, especially at the lowest PD-L1.Fc concentrations (0.1µg/mL) (inhibition by 55 ± 10% in PB vs. 34 ± 11% in SF, paired t-test p=0.022) **(Figure 6.5D**). At optimal PD-L1.Fc concentrations (5µg/mL), inhibition of anti-CD3–induced proliferation was fully restored in both SF and PB T cells (83 ± 9% *vs.* 95 ± 3%, p=0.076) (data not shown). PD-1–mediated suppression of IFN-γ production was also decreased in SF compared to PB CD4+ T cells from RA patients at suboptimal PD-L1.Fc concentration (0.1µg/mL) (inhibition by 53 ± 8% in PB vs. 31 ± 9% in SF, paired t-test p=0.003) **(Figure 6.5D**). Higher PD-L1.Fc concentrations (0.5µg/mL) resulted in comparable inhibition of IFN-γ production in PB and SF CD4+ T cells. Together, these data suggest that within the inflammatory milieu of the rheumatoid joint, RA T cells exhibit impaired PD-1−mediated inhibition at suboptimal –but not optimal– PD-L1 concentrations.

6.5. PD-1 knockout mice are susceptible to collagen-induced arthritis and develop severe disease

Our results in RA patients indicated that PD-1/PD-1L is upregulated and may play a role in regulating T cell activation within the inflamed joint. To directly assess the significance of PD-1/PD- 1L I arthritis, CIA was induced in mice deficient for *PD-1*. Our hypothesis was that *PD-1* deficiency would result in disturbed T cell tolerance and increased prevalence and/or severity of CIA. To this end, we used wild-type and *PD-1-/-* mice

bred on the autoimmune-resistant C57Bl/6 rather than the susceptible DBA/1J strain. Mice were immunized at the base of the tail on day 0 and 21 with CII in CFA **(Figure 3.9)**.

In agreement with previous studies [213], 36% of wild-type B6 mice developed CIA of mild-to-moderate severity (mean ± SEM of maximum arthritis score 2.3 ±1.2, *n*=14). In contrast, 73% of *PD-1-/-* mice developed arthritis (p=0.028 vs. wild- type mice) with severe joint inflammation (maximum arthritis score 5.0 ±1.2, *n*=16, p=0.040 vs. wild-type mice) early in the course of CIA (19th day, **Figure 6.6A**), as evidenced by marked swelling and erythema of the hindpaws and forepaws. Inflammation included the wrist and ankle and extended distally through the limb and digits **(Figure 6.6B)**.

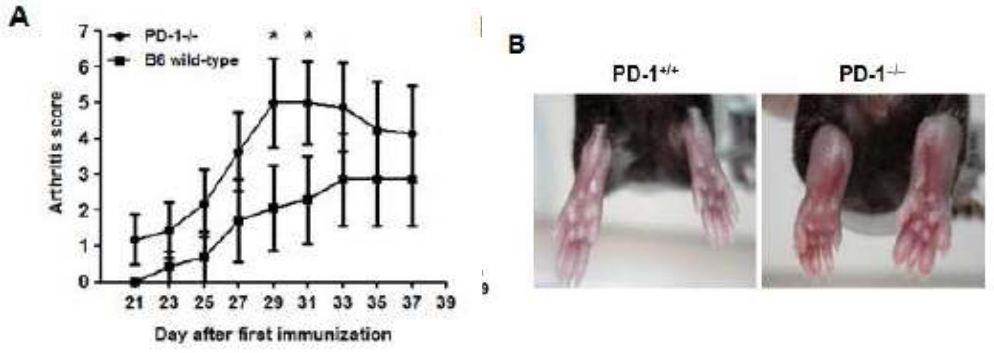


FIGURE 6.6. Increased susceptibility and severity to collagen-induced arthritis (CIA) in C57Bl/6 PD-1-/- mice.

PD-1–/– and wild-type (PD-1+/+) C57Bl/6 mice were immunized with type II chicken collagen in CFA, and after day 21, disease severity was scored by visual inspection of mouse paws, as described in Materials-Methods.

A. PD-1-/- mice demonstrate increased CIA severity compared to wild-type littermates *p<0.05.

B. Representative photos of forepaws and hindpaws of wild-type and PD1–/– mice 40 days after first immunization with CII.

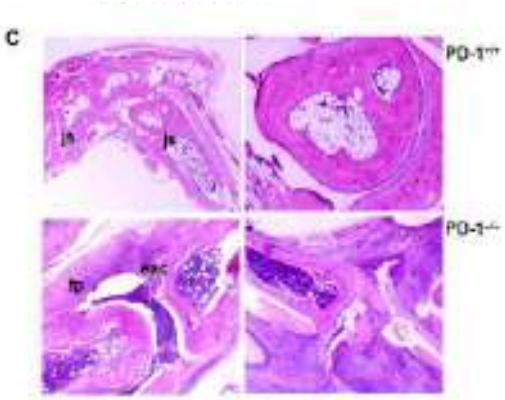


FIGURE 6.6C. H&E staining of pedal joints in CIA. Wild-type (upper lanes) mice had no signs of joint tissue degeneration and inflammation with even and clear joint space (js) and smooth articular cartilage (ac) (magnification ×40, ×100). Most PD-1-/- mice (lower lances) had severe lesions characterized by fibrovascular synovial and periarticular proliferation (fp) and erosion of articular cartilage (eac).

We next examined the histology of pedal joints in CIA mice. Wild-type B6 joints had minimal or no signs of tissue degeneration and inflammation, whereas most *PD-1-/-* B6 mice had severe lesions of extensive fibrovascular and proliferative synovitis composed of abundant fibroblasts, hypertrophic synoviocytes, and infiltration of inflammatory cells **(Figure 6.6C)**, which extended into the joint space. In severely affected joints, there was moderate to severe cartilage destruction, and marked remodeling of bone. Often, the fibrovascular proliferation and inflammation extended into the periarticular connective tissues and adjacent musculature.

6.6. Amelioration of collagen-induced arthritis by administration of PD-L1.Fc

To directly assess the role of therapeutic modulation of PD-1 in inflammatory arthritis, CIA was induced in wild-type B6 mice, which were next injected intraperitonealy with either soluble murine PD-L1.Fc fusion protein or PBS **(Figure 6.7)**. PD-L1.Fc has been shown to crosslink PD-1 *in vivo* [240, 241] and our hypothesis was that PD-1 activation would de-activate T cells and inhibit CIA development.

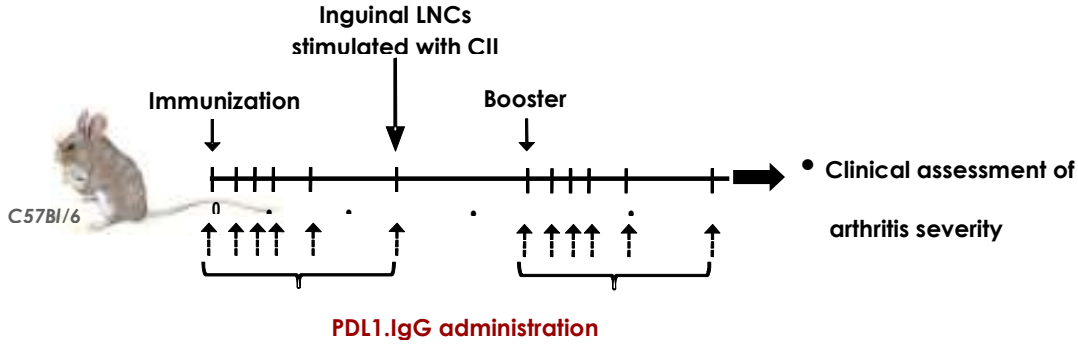


Figure 6.7. Schematic representation of intraperitoneal injection of PD-L1.Fc fusion protein during CIA induction

Indeed, PD-L1.Fc–treated mice developed less severe arthritis (mean arthritis score on day 35 postimmunization: 1.8 ± 0.6 vs. 2.5 ± 0.7 in control mice, *n*=5 in each group) **(Figure 6.8A)**, the effect being more pronounced within the male subpopulation (not shown). Antigen-specific T cell responses in CIA mice were analyzed and T cells from control mice exhibited increased proliferation in response to CII compared to T cells from PD-L1.Fc–treated mice (induction in proliferation [Δcpm] 9594 ± 2147 vs. 4712 ± 2256, *n*=3 in each group) **(Figure 6.8B)**. These results further support a role for PD-1 in the regulation of anti-CII T cell responses and the development of CIA.

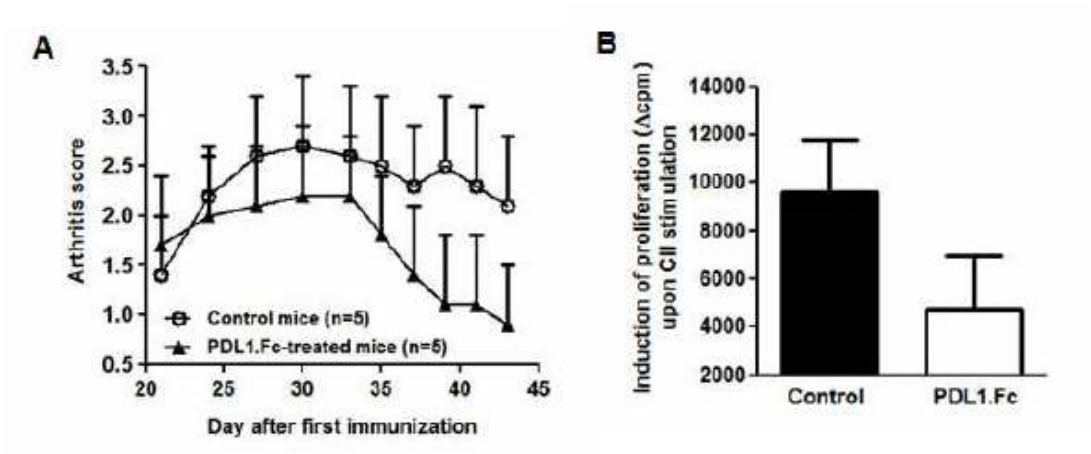


FIGURE 6.8. PD-L1.Fc treatment ameliorates CIA in in C57Bl/6 mice

CIA was induced in wild-type C57Bl/6 mice as previously described. Mouse PD-L1.IgG2a fusion protein (PD-L1.Fc) or PBS (control mice) was injected intraperitonealy at 0.1 mg/mouse on days 0, 2, 3, 5, and 10 post-immunization. Disease severity was assessd as previously described.

A. PDL1.Fc–treated mice demonstrate decreased susceptibility and severity to CIA compared to control littermates.

B. CII-specific T cell responses in CIA mice were assessed in inguinal LNCs stimulated with CII (100µg/mL) as previously described. Induction of proliferation was lower in PD-L1.Fc– treated than in control mice (mean ± SEM cpm 9594 ± 2147 versus 4714 ± 2256, *n*=3 independent experiments).

6.7. Enhanced anti-CII T cell responses in the immunized PD-1$^{-/-}$ mice

To analyze antigen-specific T cell responses in CIA mice, we immunized mice with CII and their inguinal LNCs were harvested 10 days later and were stimulated with different doses of CII. T cells from *PD-1-/-* mice exhibited increased proliferation in response to CII compared to T cells fro wildtype mice ([3H]thymidine incorporation assay: 10949 ± 3673 cpm vs. 4730 ± 1786 cpm, p=0.144) **(Figure 6.9)**. Stimulation with CII (50 µg/ml) resulted in production of IFN-γ and IL-17 which was also significantly higher by *PD-1-/-* compared to wild-type T cells (IFN-γ: 833 ± 281 ng/mL vs. 268 ± 76 ng/mL, IL-17: 134 ± 18 pg/mL vs. 50 ± 11 pg/mL, $p<0.05$). Since PD-1 is also expressed by activated B cells, *PD-1* deficiency could affect production of anti-CII antibodies in CIA *PD-1-/-* mice. To better characterize the immune mechanisms underlying *PD-1-/-* mice susceptibility to CIA, we measured anti-CII IgG production in mice serum (day 39). Levels of IgG Abs to anti-CII in both strains (*PD-1-/-* and wild-type) were comparable **(Figure 6.9)**, indicating that the increased susceptibility and severity of CIA in *PD-1-/-* mice is predominantly due to aberrant T cell activation rather to an effect on B cell-mediated autoantibody production.

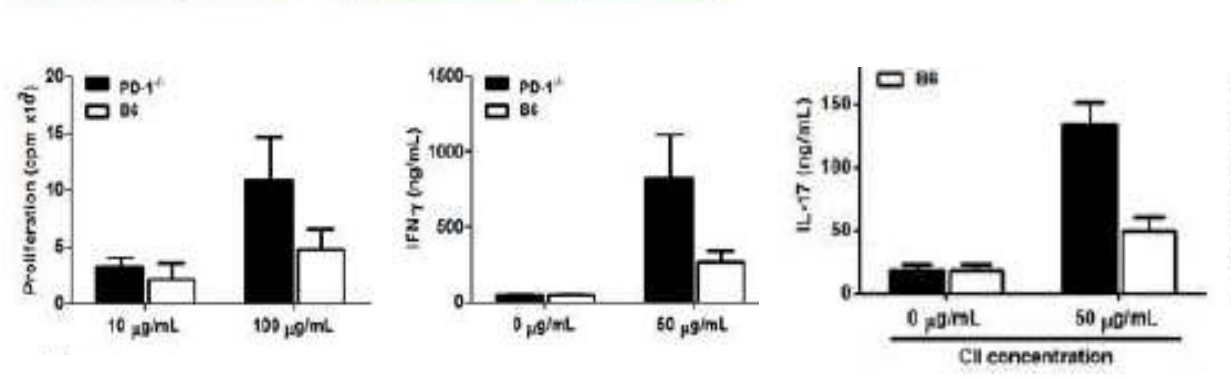


FIGURE 6.9. Increased susceptibility and severity to collagen-induced arthritis (CIA) in C57Bl/6 PD-1-/- mice.

To analyze CII-specific T cell responses in CIA, mice were immunized with CII and their inguinal LNCs were harvested 10 days later and were stimulated with different doses of CII. Compared to T cells from wild-type mice, PD-1-/- T cells exhibited increased proliferation, IFN-γ, and IL-17 production ($p<0.05$). Anti-CII IgG levels were comparable between PD-1-/- and wild-type mice. Bars represent mean ± SEM (n=4 independent experiments).

6.8. Comparable anti-CII humoral responses in PD-1$^{-/-}$ and wild-type mice

Since PD-1 is also expressed by activated B cells, *PD-1* deficiency could affect production of anti-CII antibodies in CIA *PD-1*$^{-/-}$ mice. To better characterize the immune mechanisms underlying *PD-1*$^{-/-}$ mice susceptibility to CIA, we measured anti-CII IgG production in mice serum (day 39). Levels of IgG Abs to anti-CII in both strains (*PD-1*$^{-/-}$ and wild-type) were comparable **(Figure 6.10)**, indicating that the increased susceptibility and severity of CIA in *PD-1*$^{-/-}$ mice is mostly due to aberrant T cell activation rather than B cell-mediated autoantibody production.

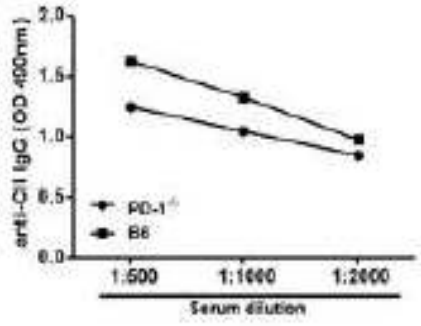


FIGURE 6.10 Increased susceptibility and severity to collagen-induced arthritis (CIA) in C57Bl/6 PD-1-/- mice.

To analyze CII-specific T cell responses in CIA, mice were immunized with CII and their inguinal LNCs were harvested 10 days later and were stimulated with different doses of CII. Compared to T cells from wild-type mice, PD-1-/- T cells exhibited increased proliferation, IFN-γ, and IL-17 production ($p<0.05$). Anti-CII IgG levels were comparable between PD-1-/- and wild-type mice. Bars represent mean ± SEM (n=4 independent experiments).

7. DISCUSSION

The present work provides evidence to support a key role for the inhibitory PD-1/PD-1L pathway in regulating T cell function in RA.

Although in certain ethnic populations PD-1 polymporphims are associated with increased risc for RA [242], our studies did not confirm a role of PD1.3 as a genetic factor for RA in Cretan (Greek) populations. It is of note however, that the sample size was small (86 patinets and 227 healthy controls) and that there was a trend towards decreased frequency in the male subpopulation, which could represent an area for future investigation.

PD-1/PD-1L is upregulated in the synovium of RA patients and PD-1 inhibits RA SF T cell proliferation under optimal –but not suboptimal– PD-L1.Fc concentrations. To our knowledge, this is the first study to examine the role of PD-1/PD-1L in the CIA model of RA. We found that in these mice, PD-1 is a potent regulator of T cell responses, and *PD-1-/-* mice demonstrate increased susceptibility and severity of arthritis. Importantly, PD-L1.Fc treatment ameliorates anti-CII T cell responses and development of CIA.

In agreement with the results of other studies [105, 106], we found enhanced expression of PD1 in RA synovial T lymphocytes, indicating that PD-1/PD-1L interactions may be involved in the regulation of T cell effector function at the site of inflammation. PD-1 upregulation most likely reflects the ongoing activation due to continuous antigen stimulation of synovial mononuclear cells, as indicated by the correlation of PD-1 expression with the histological degree of synovial inflammation. Accordingly, the synovial membrane from RA patients contains CD4+ T cells with an activated/memory phenotype [243]. In our stimulation experiments RA SF T cells had decreased capacity to further upregulate PD-1, probably due to exhaustion caused by the chronic inflammation in the joint. Alternatively, upregulation of PD-1 might be compensatory to the well described overexpression of several costimulatory molecules such as CD80/CD86 and ICOS in RA synovium [244-249].

Consistent with its role in maintaining self-tolerance, PD-1 regulates T cell function only at suboptimal conditions of TCR activation and CD28 costimulation [65, 241]. We found that PD-1 activation through plate-bound PD-L1.Fc could efficiently inhibit anti-CD3–induced PB T cell proliferation and IFN-γ production in RA patients. However, the outcome of PD-1 activation is also affected by factors such as cytokines, the level of costimulation, and TLR signaling [65]. It is conceivable that T lymphocytes in the rheumatoid joint are exposed to an inflammatory milieu that renders them hyperreactive and resistant to PD-1 activation. To explore this hypothesis, we stimulated PB CD4+ T cells from RA patients with anti-CD3 and PD-L1.Fc, and RA SF was added in culture to evaluate effects on PD-1 function. RA SF inhibited PD-1–mediated suppression of T cell proliferation at suboptimal –but not optimal– doses of PD-L1.Fc. We also assessed the function of PD-1 in

paired PB and SF CD4+ T lymphocytes from RA patients. Although PDL1. Fc could efficiently inhibit anti-CD3–induced proliferation and IFN-γ production in PB T lymphocytes, SF T lymphocytes required higher concentrations of PD-L1.Fc to achieve the same level of inhibition. This suggests that, in spite of higher PD-1 expression, RA SF T lymphocytes are relatively resistant to PD-1–mediated suppression. This finding in conjunction with our immunohistochemical study showing expression of PD-1 by T lymphocyte aggregates and PD-1L by macrophages infiltrating the rheumatoid synovium, indicate that synovial PD-1L concentrations might not be adequate to effectively downregulate T cells. This is further supported by the fact that expression of PD-1L was comparable between RA and OA patients. Nonetheless, in the presence of excess PD-1 stimulation – as in the case of exogenous PD-L1 fusion protein administration – RA SF T lymphocytes may be efficiently inhibited.

CIA is an established model of RA which is dependent on CII–reactive CD4+ T cells infiltrating the rheumatoid synovium and producing inflammatory cytokines. Various B7 costimulatory molecules such as CD28/ICOS/B7h, have been implicated in the pathogenesis of CIA; absence or blocking of either of these molecules results in amelioration of arthritis and CIIγmediated immune responses [11, 12]. This study demonstrates an important role for the negative costimulator PD-1 in CIA; C57Bl/6 *PD-1-/-* mice were more susceptible to CIA, had higher arthritis severity scores and more extended histopathological lesions in the affected joints compared to wild-type littermates. T cell proliferative responses to CII and production of IFN-γ and IL-17 were significantly increased in *PD-1-/-* mice, whereas IL-10 and anti-CII IgG production was not affected. These results suggest that PD-1 regulates the Th1/Th17 rather than the Th2 or humoral responses against CII and resemble findings described in EAE, where *PD-1-/-* T cells produced increased amounts of IFN-γ and IL-17 in recall responses to myelin antigen [89].

Modulation of T cell costimulatory pathways has been used to treat CIA and costimulation blockade with CTLA4-Ig is an effective therapy in patients with severe RA [250]. Based on our findings that PD-1/PD-1L regulates T cell responses in human and murine RA, we treated CIA mice after arthritis induction with PD-L1.Fc fusion protein to activate PD-1. PD-L1.Fc treatment resulted in reduced CIA severity, which was associated with suppressed anti-CII T cell proliferative responses. Thus, targeting PD-1/PD-1L represents a potential therapeutic option in RA [251]. PD-1 activation mediated by PD-L1.Fc has been shown to inhibit T cell-dependent pathologic immune responses and prolong allograft survival in experimental transplantation models [240, 252, 253].

In summary, this study delineates the role of the negative costimulatory pathway PD-1/PD-1L in the homeostatic control of inflammation in the rheumatoid joint. PD-1/PD-1L is upregulated in the synovium of active RA patients and regulates T cell responses in both human and murine RA, emphasized by the enhanced

susceptibility and severity of CIA in *PD-1*–deficient mice. Importantly, synovial T cells from RA patients are inhibited by optimal PD-1 crosslinking and PD-1 activation with PD-L1.Fc ameliorates CIA, providing an additional therapeutic aim to deactivate pathogenic T cells in RA. Ongoing experiments will assess the therapeutic efficacy of PD-L1.Fc administration in mice after arthritis has occurred.

8. ACKNOWLEDGMENTS

We wish to thank Christianna Choulaki, PhD, Magda Nakou, PhD, Eleni Koutala, Bsc, Melanie Rittirich, BSc, Melina Kavousanaki, Margriet Vervoordeldonk, PhD (Academic Medical Center/ University of Amsterdam, The Netherlands) and Xanthi Kranidioti, PhD (Fleming Institute, Greece) for technical assistance, George Goulielmos and Hara Yiakoupi for PD.1 genotyping Charalampos Linardakis, MD, Zacharenia Papadopoulou, Xara Misirlaki and Eva Choustoulaki, RN, for help in collecting blood samples. We also acknowledge Dr. G. Freeman (Harvard Medical School) for providing PD-L1.Fc fusion protein. This work was supported by funds from the Hellenic Society of Rheumatology, the Pancretan Health Association, the Hellenic Ministry of Education, Hellenic Republic and European Union (EPEAEK fund), and the FP6 European AUTOCURE Program.

9. REFERENCES

1. Feldmann, M., Brennan, F, Maini, R, *Rheumatoid arthritis.* Cell, 1996. 85(3): p. 307-10.
2. Firestein, G., *Evolving concepts of rheumatoid arthritis.* Nature, 2003. 423(6937): p. 356-61.
3. Bennett, J., *The role of T lymphocytes in rheumatoid arthritis and other autoimmune diseases.* Arthritis Rheum, 2008. 58(2 Suppl): p. 53-7.
4. Joosten, L.A., Abdollahi-Roodsaz, S, Heuvelmans-Jacobs, M, Helsen, M.M, van den Bersselaar, L.A, Oppers-Walgreen, B, et al, *T cell dependence of chronic destructive murine arthritis induced by repeated local activation of Toll-like receptor-driven pathways: crucial role of both interleukin-1beta and interleukin-17.* Arthritis Rheum, 2008. 58(1): p. 98-108.
5. Klareskog, L., Catrina, A.I, Paget, S *Rheumatoid arthritis.* Lancet, 2009. 373(9664): p. 659-72.
6. Lundy, S.K., Sarkar, S, Tesmer, L.A, Fox, D.A., *Cells of the synovium in rheumatoid arthritis. T lymphocytes.* Arthritis Res Ther, 2007. 9(1): p. 202.
7. McInnes, I.B., Schet G, *Cytokines in the pathogenesis of rheumatoid arthritis.* Nat Rev Immunol, 2007. 7(6): p. 429-42.
8. Chiocchia G, B.M., Ronziere MC, Herbage D, Fournier C, *T cell regulation of collagen-induced arthritis in mice. I. Isolation of Type II collagen-reactive T cell hybridomas with specific cytotoxic function.* J Immunol, 1990. 145(2): p. 519-25.
9. Holmdahl R, J.L., Larsson A, Jonsson R *Arthritis in DBA/1 mice induced with passively transferred type II collagen immune serum. Immunohistopathology and serum levels of anti-type II collagen auto-antibodies.* Scand J Immunol 1990. 31(2): p. 147-157.
10. Holmdahl R, K.L., Rubin K, Bjork J, Smedegard G, Jonsson R, et al, *Role of T lymphocytes in murine collagen induced arthritis.* Agents Actions, 1986. 19(5-6): p. 295-305.
11. Williams, R.O., *Collagen-induced arthritis in mice.* Methods Mol Med, 2007. 136: p. 191-9.
12. Rosloniec, E.F., Cremer, M, Kang, A, Myers, L.K, *Collagen-induced arthritis.* Curr Protoc Immunol, 2001. Chapter 15: p. Unit 15 5.
13. Walker, L.S., Abbas, A.K, *The enemy within: keeping self-reactive T cells at bay in the periphery.* Nat Rev Immunol, 2002. 2: p. 11-19.
14. Nemazee, D., Hogquist, K.A, *Antigen receptor selection by editing or downregulation of V(D)J recombination.* Curr Opin Immunol, 2003. 15: p. 182-189.
15. Gotter, J., Kyewski, B, *Regulating self-tolerance by deregulating gene expression.* Curr Opin Immunol, 2004. 16: p. 741-745.
16. Goodnow, C.C., Sprent J, Fazekas de St Groth B, Vinuesa C.G, *Cellular and genetic mechanisms of self tolerance and autoimmunity.* Nature, 2005. 435: p. 590-597.
17. Schwartz, R.H., *T cell clonal anergy.* Curr Opin Immunol, 1997. 9: p. 351-357.
18. Saito, T., Yamasaki, S, *Negative feedback of T cell activation through inhibitory adapters and costimulatory receptors.* Immunol Rev, 2003. 192: p. 143-160.
19. Appleman, L.J., Boussiotis, VA, *T cell anergy and costimulation.* Immunol Rev, 2003. 191: p. 1-20
20. Sakaguchi, S., *Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self.* Nat Immunol, 2005(6): p. 345-352.
21. von Boehmer, H., *Mechanisms of suppression by suppressor T cells.* Nat Immunol, 2005. 6: p. 338-344.
22. Schubert, L.A., Jeffery E, Zhang Y, Ramsdell F, Ziegler SF, *Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation.* J Biol Chem, 2001. 276: p. 37672-37679.
23. Sakaguchi, S., *The origin of FOXP3-expressing CD4+ regulatory T cells: thymus or periphery.* J Clin Invest, 2003. 112: p. 1310-1312.
24. Walker, M.R., Kasprowicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH, Ziegler SF, *Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25− T cells.* J Clin Invest, 2003. 112: p. 1437-1443.
25. Steinman, R.M., Hawiger D, Nussenzweig MC, *Tolerogenic dendritic cells.* Annu Rev Immunol, 2003. 21: p. 685-711.
26. Wallet, M.A., Sen P, Tisch R, *Immunoregulation of dendritic cells.* Clin Med Res, 2005. 3: p. 166-175.

27. Allavena, P., Piemonti L, Longoni D, Bernasconi S, Stoppacciaro A, Ruco L, Mantovani A, *IL-10 prevents the differentiation of monocytes to dendritic cells but promotes their maturation to macrophages.* Eur J Immunol, 1998. 28: p. 359-369.
28. Waterhouse, P., Penninger JM, Timms E, Wakeham A, Shahinian A, Lee, KP, Thompson CB, Griesser H, Mak TW, *Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4.* Science, 1995. 270: p. 985-988.
29. Tivol, E.A., Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH, *Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4.* Immunity, 1995. 3: p. 541-547.
30. Egen, J.G., Allison, J.P *Cytotoxic T lymphocyte antigen-4 accumulation in the immunological synapse is regulated by TCR signal strength.* Immunity 2002. 16: p. 23-35.
31. Darlington, P.J., Baroja ML, Chau TA, Siu E, Ling V, Carreno BM, Madrenas J, *Surface cytotoxic T lymphocyte-associated antigen 4 partitions within lipid rafts and relocates to the immunological synapse under conditions of inhibition of T cell activation.* J Exp Med, 2002. 195: p. 1337-1347.
32. Chikuma, S., Imboden JB, Bluestone JA, *Negative regulation of T cell receptor-lipid raft interaction by cytotoxic T lymphocyte-associated antigen 4.* J Exp Med, 2003. 197: p. 129-135.
33. Egen, J.G., Kuhns MS, Allison JP, *CTLA-4: new insights into its biological function and use in tumor immunotherapy.* Nat Immunol, 2002. 3: p. 611-618.
34. Sharpe, A.H., Freeman GJ, *The B7-CD28 superfamily.* Nat Rev Immunol, 2002. 2: p. 116-126.
35. Ueda, H., Howson JM, Esposito L, Heward J, Snook H, Chamberlain G, Rainbow DB, Hunter KM et al., *Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease.* Nature 2003. 423: p. 506-511.
36. Levin, L., Tomer Y, *The etiology of autoimmune diabetes and thyroiditis: evidence for common genetic susceptibility.* Autoimmun Rev, 2003. 2: p. 377-386.
37. Agata, Y., Kawasaki, A, Nishimura, H, Ishida, Y, Tsubata, T, et al, *Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes.* Int. Immunol, 1996. 8: p. 765-72.
38. Freeman, G.J., Long, A.J, Iwai, Y, Bourque, K, Chernova, T, et al. . , *Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. .* J. Exp. Med. , 2000. 192: p. 1027-34.
39. Nishimura, H., Nose, M, Hiai, H, Minato, N, Honjo, T, *Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor.* Immunity, 1999. 11(2): p. 141-51.
40. Nishimura, H., Okazaki, T, Tanaka, Y, Nakatani, K, Hara, M, et al. . , *Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. .* Science 2001. 291: p. 319-22.
41. Prokunina, L., Castillejo-Lopez, C, Oberg, F, Gunnarsson, I, Berg, L, et al, *Aregulatory polymorphism in PDCD1 is associated with susceptibility to systemic lupus erythematosus in humans. .* Nat. Genet 2002. 32: p. 666-69.
42. Nielsen, C., Laustrup H, Voss A, Junker P, Husby S, Lillevang ST, *A putative regulatory polymorphism in PD-1 is associated with nephropathy in a population-based cohort of systemic lupus erythematosus patients.* Lupus, 2004. 13: p. 510-516.
43. Watanabe, N., Gavrieli M, Sedy JR, Yang J, Fallarino F, Loftin SK, Hurchla MA, Zimmerman N, Sim J, Zang X, Murphy TL, Russell JH, Allison JP, Murphy KM, *BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1.* Nat Immunol, 2003. 4: p. 670-679.
44. Krieg, C., Han P, Stone R, Goularte OD, Kaye J, *Functional analysis of B and T lymphocyte attenuator engagement on CD4+ and CD8+ T cells.* J Immunol, 2005. 175: p. 6420-6427.
45. Otsuki, N., Kamimura Y, Hashiguchi M, Azuma M, *Expression and function of the B and T lymphocyte attenuator (BTLA/CD272) on human T cells.* Biochem Biophys Res Commun, 2006. 344: p. 1121-1127.
46. Nishimura, H., Agata, Y, Kawasaki, A, Sato, M, Imamura, S, et al., *Developmentally regulated expression of the PD-1 protein on the surface of double-negative (CD4−CD8−) thymocytes.* Int. Immunol, 1996. 8: p. 773-80.
47. Chemnitz, J.M., Parry, R.V, Nichols, K.E, June, C.H, Riley, J.L. . , *SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation.* J. Immunol, 2004. 173: p. 945-54.

48. Dorfman, D.M., Brown, J.A, Shahsafaei, A, Freeman, G.J *Programmed death-1 (PD-1) is a marker of germinal center-associated T cells and angioimmunoblastic T-cell lymphoma.* Am. J. Surg. Pathol, 2006. 30: p. 802-10.
49. Pentcheva-Hoang, T., Chen, L, Pardoll, D.M, Allison, J.P, *Programmed death-1 concentration at the immunological synapse is determined by ligand affinity and availability.* Proc. Natl. Acad. Sci. USA 2007. 104: p. 17765-70.
50. Raimondi, G., Shufesky, W.J, Tokita, D, Morelli, A.E, Thomson, A.W. , *Regulated compartmentalization of programmed cell death-1 discriminates CD4+CD25+ resting regulatory T cells from activated T cells. .* J. Immunol, 2006. 176: p. 2808-16.
51. Petrovas, C., Casazza, J.P, Brenchley, J.M, Price, D.A, Gostick, E, et al. , *PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. .* J. Exp. Med. , 2006. 203: p. 2281-92.
52. Yamazaki, T., Akiba, H, Iwai, H, Matsuda, H, Aoki, M, et al. , *Expression of programmed death 1 ligands by murine T cells and APC. .* J. Immunol. , 2002. 169: p. 5538-45.
53. Eppihimer, M.J., Gunn, J, Freeman, G.J, Greenfield, E.A, Chernova, T, et al. . , *Expression and regulation of the PD-L1 immunoinhibitory molecule on microvascular endothelial cells. .* Microcirculation 2002. 9: p. 133-45.
54. Schreiner, B., Mitsdoerffer, M, Kieseier, B.C, Chen, L, Hartung, H.P, et al, *Interferon-β enhances monocyte and dendritic cell expression of B7-H1 (PD-L1), a strong inhibitor of autologous T-cell activation: relevance for the immune modulatory effect in multiple sclerosis. .* J. Neuroimmunol, 2004. 155: p. 172-82.
55. Liu, J., Hamrouni, A, Wolowiec, D, Coiteux, V, Kuliczkowski, K, et al. . , *Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-γ and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway. .* Blood, 2007. 110: p. 296-304.
56. Lee, S.J., Jang, B.C, Lee, S.W, Yang, Y.I, Suh, S.I, et al. , *Interferon regulatory factor-1 is prerequisite to the constitutive expression and IFN-γ-induced upregulation of B7-H1 (CD274).* FEBS Lett. , 2006. 580: p. 755-62.
57. Parsa, A.T., Waldron, J.S, Panner, A, Crane, C.A, Parney, I.F, et al. . *Loss of tumor suppressorPTENfunction increases B7-H1 expression and immunoresistance in glioma.* Nat. Med, 2007. 13: p. 84-88.
58. Zhong, X., Tumang J.R, Gao, W, Bai, C, Rothstein, T.L, *PD-L2 expression extends beyond dendritic cells/macrophages to B1 cells enriched for VH11/VH12 and phosphatidylcholine binding. .* Eur. J. Immunol. , 2007. 37: p. 2405-10.
59. Liang, S.C., Latchman, Y.E, Buhlmann, J.E, Tomczak, M.F, Horwitz, B.H, et al. , *Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses.* Eur. J. Immunol., 2003. 33: p. 2706-16.
60. Loke, P., Allison, J.P, *PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells.* Proc. Natl. Acad. Sci. USA, 2003. 100: p. 5336-41.
61. Carter, L., Fouser, L.A, Jussif, J, Fitz, L, Deng, B, et al., *PD-1:PD-L inhibitory pathway affects bothCD4+ andCD8+ Tcells and is overcome by IL-2. .* Eur. J. Immunol. , 2002. 32: p. 634-43.
62. Nurieva, R., Thomas, S, Nguyen, T, Martin-Orozco, N, Wang, Y, et al. , *T-cell tolerance or function is determined by combinatorial costimulatory signals. .* EMBO J, 2006. 25: p. 2623-33.
63. Sheppard, K.A., Fitz, L.J, Lee, J.M, Benander, C, George J.A, Wooters J, et al, *PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta.* FEBS Lett,, 2004. 574(1-3): p. 37-41.
64. Okazaki, T., Maeda, A, Nishimura, H, Kurosaki, T, Honjo, T. , *PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domaincontaining tyrosine phosphatase 2 to phosphotyrosine. .* Proc. Natl. Acad. Sci. USA, 2001. 98: p. 13866-71.
65. Bennett, F., Luxenberg, D, Ling, V, Wang, I.M, Marquette, K, Lowe, D, et al, *Program death-1 engagement upon TCR activation has distinct effects on costimulation and cytokine-driven proliferation: attenuation of ICOS, IL-4, and IL-21, but not CD28, IL-7, and IL-15 responses.* J Immunol, 2003. 170(2): p. 711-18.

66. Saunders, P.A., Hendrycks, V.R, Lidinsky, W.A, Woods, M.L. , *PD-L2:PD-1 involvement in T cell proliferation, cytokine production, and integrin-mediated adhesion.* Eur.J. Immunol, 2005. 35: p. 3561-69.
67. Parry, R.V., Chemnitz, J.M, Frauwirth, K.A, Lanfranco, A.R, Braunstein, I, Kobayashi, S.V, et al, *CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms.* Mol Cell Biol, 2005. 25(21): p. 9543-53.
68. Arnaud, M., Crouin, C, Deon, C, Loyaux, D, Bertoglio, J, *Phosphorylation of Grb2- associated binder 2 on serine 623 by ERK MAPK regulates its association with the phosphatase SHP-2 and decreases STAT5 activation.* J. Immunol, 2004. 173: p. 3962-71.
69. Van Parijs, L., Refaeli, Y, Lord, J.D, Nelson, B.H, Abbas, A.K, Baltimore, D., *Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activationinduced cell death. .* Immunity 1999. 11: p. 281-88.
70. Okazaki, T., Tanaka, Y, Nishio, R, Mitsuiye, T, Mizoguchi, A, et al. , *Autoantibodies against cardiac troponin I are responsible for dilated cardiomyopathy in PD-1-deficient mice. .* Nat. Med. , 2003. 9: p. 1477-83.
71. Brown, J.A., Dorfman, D.M, Ma, F.R, Sullivan, E.L, Munoz, O, et al, *Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production.* J. Immunol., 2003. 170: p. 1257-66.
72. Nishimura, H., Honjo, T, Minato, N., *Facilitation of beta selection and modification of positive selection in the thymus of PD-1-deficient mice.* J. Exp. Med., 2000. 191: p. 891-98.
73. Keir, M.E., Latchman, Y.E, Freeman, G.J, Sharpe, A.H. , *Programmed death-1 (PD-1): PD-ligand 1 interactions inhibit TCR-mediated positive selection of thymocytes.* J. Immunol. , 2005. 175: p. 7372-79.
74. Blank, C., Brown, I, Marks, R, Nishimura, H, Honjo, T, Gajewski, T.F, *Absence of programmed death receptor 1 alters thymic development and enhances generation of CD4/CD8 double-negative TCR-transgenic T cells.* J. Immunol. , 2003. 171: p. 4574-81.
75. Zucchelli, S., Holler, P, Yamagata, T, Roy, M, Benoist, C, Mathis, D. , *Defective central tolerance induction in NOD mice: genomics and genetics. .* Immunity 2005. 22: p. 385-96.
76. Probst, H.C., McCoy, K, Okazaki, T, Honjo, T, van den Broek, M. , *Resting dendritic cells induce peripheral CD8+ Tcell tolerance through PD-1 and CTLA-4. .* Nat. Immunol., 2005. 6: p. 280-86.
77. Ansari, M.J., Salama, A.D, Chitnis, T, Smith, R.N, Yagita, H, et al, *The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice.* J. Exp. Med., 2003. 198: p. 63-69.
78. Wang, J., Yoshida, T, Nakaki, F, Hiai, H, Okazaki, T, Honjo, T, *Establishment of NOD-Pdcd1-/- mice as an efficient animal model of type I diabetes.* Proc Natl Acad Sci U S A, 2005. 102(33): p. 11823-8.
79. Keir, M.E., Liang, S.C, Guleria, I, Latchman, Y.E, Qipo, A, Albacker, L.A, et al, *Tissue expression of PD-L1 mediates peripheral T cell tolerance.* J Exp Med, 2006. 203(4): p. 883-95.
80. Fife, B.T., Guleria, I, Gubbels Bupp, M, Eagar ,T.N, Tang, Q, et al, *Insulin-induced remission in new-onset NOD mice is maintained by the PD-1-PD-L1 pathway.* J. Exp. Med, 2006. 203: p. 2737-47.
81. Grabie, N., Gotsman, I, Dacosta, R, Pang, H, Stravrakis, G, et al, *Endothelial PD-L1 regulates CD8+ T cell mediated injury in the heart.* Circulation 2007. 116: p. 2062-71.
82. Keir, M.E., Freeman, G.J, Sharpe, A.H. . , *PD-1 regulates self-antigen specific responses in lymph nodes and tissues.* J. Immunol, 2007. 179: p. 5064-70.
83. Yadav, D., Fine, C, Azuma, M, Sarvetnick, N., *B7-1 mediated costimulation regulates pancreatic autoimmunity.* Mol. Immunol, 2007. 44: p. 2616-24.
84. Lenschow, D.J., Ho, S.C, Sattar, H, Rhee, L, Gray, G, et al, *Differential effects of anti-B7-1 and anti-B7-2 monoclonal antibody treatment on the development of diabetes in the nonobese diabetic mouse.* J. Exp. Med, 1995. 181: p. 1145-55.
85. Cheng, X., Zhao, Z, Ventura, E, Gran, B, Shindler, K.S, Rostami, A, *The PD-1/PD-L pathway is up-regulated during IL-12-induced suppression of EAE mediated by IFN-γ.* J. Neuroimmunol, 2007. 185: p. 75-86.

86. Magnus, T., Schreiner, B, Korn, T, Jack, C, Guo, H, et al. . , *Microglial expression of the B7 family member B7 homolog 1 confers strong immune inhibition: implications for immune responses and autoimmunity in the CNS.* J. Neurosci, 2005. 25: p. 2537-46.
87. Salama, A.D., Chitnis, T, Imitola, J, Ansari, M.J, Akiba, H, et al, *Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis.* J. Exp. Med, 2003. 198: p. 71-78.
88. Zhu, B., Guleria, I, Khosroshahi, A, Chitnis, T, Imitola, J, et al, *Differential role of programmed death-ligand 1 and programmed death-ligand 2 in regulating the susceptibility and chronic progression of experimental autoimmune encephalomyelitis.* J. Immunol, 2006. 176: p. 3480-89.
89. Carter, L.L., Leach, M.W, Azoitei, M.L, Cui, J, Pelker, J.W, Jussif, J, et al, *PD-1/PD-L1, but not PD-1/PD-L2, interactions regulate the severity of experimental autoimmune encephalomyelitis.* J Neuroimmunol, 2007. 182 (1-2): p. 124-34.
90. Latchman, Y.E., Liang, S.C, Wu, Y, Chernova, T, Sobel, R.A, et al, *PD-L1-deficient mice show that PD-L1 on T cells, antigen-presenting cells, and host tissues negatively regulates T cells.* Proc. Natl. Acad. Sci. USA 2004. 101: p. 10691-96.
91. Baecher-Allan C, B.J., Freeman GJ, Hafler DA. . , *CD4+CD25+ regulatory cells from human peripheral blood express very high levels of CD25 ex vivo. .* Novartis Found. Symp. , 2003. 252: p. 67-88.
92. Krupnick, A.S., Gelman, A.E, Barchet, W, Richardson, S, Kreisel, F.H, et al *Murine vascular endothelium activates and induces the generation of allogeneicCD4+25+Foxp3+ regulatory T cells. .* J. Immunol, 2005. 175: p. 6265-70.
93. Totsuka, T., Kanai, T, Makita, S, Fujii, R, Nemoto, Y, et al *Regulation of murine chronic colitis by CD4+CD25− programmed death-1+ Tcells.* Eur. J. Immunol, 2005. 35: p. 1773-85.
94. Hornung, M., Farkas, S.A, Sattler, C, Schlitt, H.J, Geissler, E.K, *DX5+ NKT cell induce the death of colitis-associated cells: involvement of programmed death ligand-1.* Eur. J. Immunol, 2006. 36: p. 1210-21.
95. Hirata, S., Senju, S, Matsuyoshi, H, Fukuma, D, Uemura, Y, Nishimura, Y., *Prevention of experimental autoimmune encephalomyelitis by transfer of embryonic stem cellderived dendritic cells expressing myelin oligodendrocyte glycoprotein peptide along with TRAIL or programmed death-1 ligand.* J. Immunol, 2005. 174(1888-97).
96. Ding, H., Wu, X, Wu, J, Yagita, H, He, Y, et al, *Delivering PD-1 inhibitory signal concomitant with blocking ICOS costimulation suppresses lupus-like syndrome in autoimmune BXSB mice.* Clin. Immunol, 2006. 118: p. 258-67.
97. Kobayashi, M., Kawano, S, Hatachi, S, Kurimoto, C, Okazaki, T, Iwai, Y, Honjo, T, Tanaka, Y, Minato, N, Komori, T, Maeda, S, Kumagai, S., *Enhanced expression of programmed death-1 (PD-1)/PD-L1 in salivary glands of patients with Sjögren's syndrome.* J Rheumatol, 2005. 32(11): p. 2156-63.
98. Bolstad, A.I., Eiken, H.G, Rosenlund, B, Alarcón-Riquelme, M.E, Jonsson, R, *Increased salivary gland tissue expression of Fas, Fas ligand, cytotoxic T lymphocyte-associated antigen 4, and programmed cell death 1 in primary Sjögren's syndrome.* Arthritis Rheum, 2003. 48(1): p. 174-85.
99. Mataki, N., Kikuchi, K., Kawai, T, Higashiyama M, Okada Y, Kurihara C, Hokari R, Kawaguchi A, Nagao S, Kondo T, Itoh K, Miyakawa H, Miura S., *Expression of PD-1, PD-L1, and PD-L2 in the liver in autoimmune liver diseases.* Am J Gastroenterol, 2007. 102(2): p. 302-12.
100. Wiendl, H., Mitsdoerffer, M., Schneider, D., Chen L, Lochmüller H, Melms A, Weller M., *Human muscle cells express a B7-related molecule, B7-H1, with strong negative immune regulatory potential: a novel mechanism of counterbalancing the immune attack in idiopathic inflammatory myopathies.* FASEB J, 2003. 17(13): p. 1892-4.
101. Sakthivel, P., Ramanujam, R., Wang XB, Pirskanen R, Lefvert AK., *Programmed Death-1: from gene to protein in autoimmune human myasthenia gravis.* J Neuroimmunol, 2008. 193(1-2): p. 149-55.
102. Kanai, T., Totsuka, T., Uraushihara K, Makita S, Nakamura T, Koganei K, Fukushima T, Akiba H, Yagita H, Okumura K, Machida U, Iwai H, Azuma M, Chen L, Watanabe M., *Blockade of B7-H1 suppresses the development of chronic intestinal inflammation.* J Immunol, 2003. 171(8): p. 4156-63.
103. Tsutsumi, Y., Jie, X., Ihara K, Nomura A, Kanemitsu S, Takada H, Hara T., *Phenotypic and genetic analyses of T-cell-mediated immunoregulation in patients with Type 1 diabetes.* Diabet Med, 2006. 23(10): p. 1145-50.

104. Krakauer, M., Sorensen, P.S, Sellebjerg F., *CD4(+) memory T cells with high CD26 surface expression are enriched for Th1 markers and correlate with clinical severity of multiple sclerosis.* J Neuroimmunol., 2006. 181(1-2): p. 157-64.
105. Hatachi S, I.Y., Kawano S, Morinobu S, Kobayashi M, Koshiba M, et al, *CD4+ PD-1+ T cells accumulate as unique anergic cells in rheumatoid arthritis synovial fluid.* J Rheumatol, 2003. 30(7): p. 1410-9.
106. Wan, B., Nie, H., Liu A, Feng G, He D, Xu R, et al, *Aberrant regulation of synovial T cell activation by soluble costimulatory molecules in rheumatoid arthritis.* J Immunol, 2006. 177(12): p. 8844-50.
107. Dong, H., Strome, S.E, Matteson, E.L, Moder, K.G, Flies, D.B, et al, *Costimulating aberrant T cell responses by B7-H1 autoantibodies in rheumatoid arthritis.* J. Clin. Invest, 2003. 111(363-70).
108. Bertsias, G.K., Nakou, M, Choulaki, C, Raptopoulou, A, Papadimitraki, E, Goulielmos, G, et al, *Genetic, immunologic, and immunohistochemical analysis of the programmed death 1/programmed death ligand 1 pathway in human systemic lupus erythematosus.* Arthritis Rheum, 2009. 60(1): p. 207-218.
109. Okazaki, T., Honjo, T, *PD-1 and PD-1 ligands: from discovery to clinical application.* Int. Immunol, 2007. 19: p. 813-24.
110. Kroner, A., Mehling, M, Hemmer, B, Rieckmann, P,Toyka, K.V et al *A PD-1 polymorphism is associated with disease progression in multiple sclerosis.* Ann. Neurol, 2005. 58: p. 50-57.
111. Thorburn, C.M., Prokunina-Olsson, L, Sterba, K.A, Lum, R.F, Seldin, M.F, et al, *Association of PDCD1 genetic variation with risk and clinical manifestations of systemic lupus erythematosus in a multiethnic cohort.* Genes Immun, 2007. 8: p. 279-87.
112. Wang, S.C., Lin, C.H, Ou, T.T,Wu, C.C, Tsai, W.C, et al, *Ligands for programmed cell death 1 gene in patients with systemic lupus erythematosus.* J. Rheumatol, 2007. 34: p. 721-25.
113. Iwai, Y., Terawaki, S, Ikegawa, M, Okazaki, T, Honjo, T., *PD-1 inhibits antiviral immunity at the effector phase in the liver.* J. Exp. Med, 2003. 198: p. 39-50.
114. Jun, H., Seo, S.K, Jeong, H.Y, Seo, H.M, Zhu, G, et al, *B7-H1 (CD274) inhibits the development of herpetic stromal keratitis (HSK).* FEBS Lett, 2005. 579: p. 6259-64.
115. Barber, D.L., Wherry, E.J, Masopust, D, Zhu, B, Allison, J.P, et al, *Restoring function in exhausted CD8 T cells during chronic viral infection.* Nature 2006. 439: p. 682-87.
116. Wherry, E.J., Ahmed. R, *Memory CD8 T-cell differentiation during viral infection.* J. Virol, 2004. 78: p. 5535-45.
117. Day, C.L., Kaufmann, D.E, Kiepiela, P, Brown, J.A, Moodley, E.S, et al, *PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression.* Nature 2006. 443: p. 350-54.
118. Trautmann, L., Janbazian, L, Chomont, N, Said, E.A, Gimmig, S, et al, *Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction.* Nat. Med, 2006. 12: p. 1198-202.
119. Boettler, T., Panther, E, Bengsch, B, Nazarova, N, Spangenberg, H.C, et al, *Expression of the interleukin-7 receptor α chain (CD127) on virus-specific CD8+ T cells identifies functionally and phenotypically defined memory T cells during acute resolving hepatitis B virus infection.* J. Virol, 2006. 80: p. 3532-40.
120. Boni, C., Fisicaro, P, Valdatta, C, Amadei, B, Di Vincenzo, P, et al, *Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection.* J. Virol., 2007. 81: p. 4215-25.
121. Urbani, S., Amadei, B, Tola, D, Massari, M, Schivazappa, S, et al, *PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion.* J. Virol, 2006. 80: p. 11398-403.
122. Chen, L., Zhang, Z, Chen, W, Zhang, Z, Li, Y, et al, *B7-H1 up-regulation on myeloid dendritic cells significantly suppresses T cell immune function in patients with chronic hepatitis B.* J. Immunol, 2007. 178: p. 6634-41.
123. Geng, L., Jiang, G, Fang, Y, Dong, S, Xie, H, et al, *B7-H1 expression is upregulated in peripheral blood CD14+ monocytes of patients with chronic hepatitis B virus infection, which correlates with higher serum IL-10 levels.* J. Viral Hepat, 2006. 13: p. 725-33.
124. Trabattoni, D., Saresella, M, Biasin, M, Boasso, A, Piacentini, L, et al, *B7-H1 is upregulated in HIV infection and is a novel surrogate marker of disease progression.* Blood, 2003. 101: p. 2514-20.

125. Velu, V., Kannanganat, S, Ibegbu, C, Chennareddi, L, Villinger, F, et al, *Elevated expression levels of inhibitory receptor programmed death 1 on simian immunodeficiency virus-specific CD8 T cells during chronic infection but not after vaccination.* J. Virol, 2007. 81: p. 5819-28.
126. Das, S., Suarez, G, Beswick, E.J, Sierra, J.C, Graham, D.Y, Reyes, V.E, *Expression of B7-H1 on gastric epithelial cells: its potential role in regulatingTcells during Helicobacter pylori infection.* J. Immunol. , 2006. 176: p. 3000-9.
127. Beswick, E.J., Pinchuk, I.V, Das, S, Powell, D.W, Reyes, V.E, *Expression of the programmed death ligand 1, B7-H1, on gastric epithelial cells after Helicobacter pylori exposure promotes development of CD4+ CD25+ FoxP3+ regulatory T cells.* Infect. Immun, 2007. 75: p. 4334-41.
128. Terrazas, L.I., Montero, D, Terrazas, C.A, Reyes, J.L, Rodriguez-Sosa, M., *Role of the programmed Death-1 pathway in the suppressive activity of alternatively activated macrophages in experimental cysticercosis.* Int. J. Parasitol, 2005. 35: p. 1349-58.
129. Smith, P., Walsh, C.M, Mangan, N.E, Fallon, R.E, Sayers, J.R, et al, *Schistosoma mansoni worms induce anergy of T cells via selective up-regulation of programmed death ligand 1 on macrophages.* J. Immunol, 2004. 173: p. 1240-48.
130. Liang, S.C., Greenwald, R.J, Latchman, Y.E, Rosas, L, Satoskar, A, et al, *PD-L1 and PD-L2 have distinct roles in regulating host immunity to cutaneous leishmaniasis.* Eur.J. Immunol, 2006. 36: p. 58-64.
131. Thompson, R.H., Gillett, M.D, Cheville, J.C, Lohse, C.M, Dong, H, et al, *Costimulatory B7-H1 in renal cell carcinoma patients: indicator of tumor aggressiveness and potential therapeutic target.* Proc. Natl. Acad. Sci. USA, 2004. 101: p. 17174-79.
132. Wu, C., Zhu, Y, Jiang, J, Zhao, J, Zhang, X.G, Xu, N, *Immunohistochemical localization of programmed death-1 ligand-1 (PD-L1) in gastric carcinoma and its clinical significance.* Acta Histochem, 2006. 108: p. 19-24.
133. Blazar, B.R., Carreno, B.M, Panoskaltsis-Mortari, A, Carter, L, Iwai, Y, et al, *Blockade of programmed death-1 engagement accelerates graft-versus-host disease lethality by an IFN-γ-dependent mechanism.* J. Immunol, 2003. 171: p. 1272-77.
134. Rosenwald, A., Wright, G, Leroy, K, Yu, X, Gaulard, R.D, et al, *Molecular diagnosis of primary mediastinal B cell lymphoma identifies a clinically favorable subgroup of diffuse large B cell lymphoma related to Hodgkin lymphoma.* J. Exp. Med, 2003. 198: p. 851-62.
135. Chemnitz, J.M., Eggle, D, Driesen, J, Classen, S, Riley, J.L, et al, *RNA-fingerprints provide direct evidence for the inhibitory role of TGFβ and PD-1 on CD4+ T cells in Hodgkin's lymphoma. .* Blood 2007. 110: p. 3226-33.
136. Shimauchi, T., Kabashima, K, Nakashima, D, Sugita, K, Yamada, Y, et al, *Augmented expression of programmed death-1 in both neoplastic and non-neoplastic CD4+ T-cells in adult T-cell leukemia/lymphoma. .* Int. J. Cancer 2007. 121: p. 2585-90.
137. Hirano, F., Kaneko, K, Tamura, H, Dong, H, Wang, S, et al, *Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity.* Cancer Res, 2005. 65: p. 1089-96.
138. Iwai, Y., Ishida, M, Tanaka, Y, Okazaki, T, Honjo, T, Minato, N, *Involvement of PDL1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade.* Proc. Natl. Acad. Sci. USA, 2002. 99: p. 12293-97.
139. Iwai, Y., Terawaki, S, Honjo, T, *PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. .* Int. Immunol., 2005. 17: p. 133-44.
140. Wong, R.M., Scotland, R.R, Lau, R.L,Wang, C, Korman, A.J, et al, *Programmed death-1 blockade enhances expansion and functional capacity of human melanoma antigenspecific CTLs.* Int. Immunol. , 2007. 19: p. 1223-34.
141. Alamanos, Y., Voulgari, P.V, Drosos, A.A, *Incidence and prevalence of rheumatoid arthritis, based on the 1987 American College of Rheumatology criteria: a systematic review.* Semin.Arthritis Rheum. , 2006. 36: p. 182-188.
142. Brown, M.A., Newton, J. L, Wordsworth, B. P., *Genetics for Rheumatologists - The molecular genetic basis of rheumatological disorders.* Remedica, 2002.
143. Waaler, E., *On the occurrence of a factor in human serum activating the specific agglutination of sheep blood corpuscles.* Acta Pathol Microbiol Scand, 1940. 17: p. 172-188.

144. Zvaifler, N.J., *The immunopathology of joint inflammation in rheumatoid arthritis.* Adv.Immunol, 1973. 16: p. 265-336.
145. Firestein, G.S., *Evolving concepts of rheumatoid arthritis.* Nature 2003. 423: p. 356-361.
146. Arnett, F.C., Edworthy, S. M, Bloch, D. A, McShane, D. J, Fries, J. F, Cooper, N. S, Healey, L. A, Kaplan, S. R, Liang, M. H, Luthra, H. S, et al, *The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis.* Arthritis Rheum, 1988. 31: p. 315-324.
147. Schellekens, G.A., de Jong, B. A, van den Hoogen, F. H, van de Putte, L. B, van Venrooij, W. J, *Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies.* J.Clin.Invest 1998(101): p. 273-281.
148. Girbal-Neuhauser, E., Durieux, J. J, Arnaud, M, Dalbon, P, Sebbag, M, Vincent, C, Simon, M, Senshu, T, Masson-Bessiere, C, Jolivet-Reynaud, C et al *The epitopes targeted by the rheumatoid arthritis-associated antifilaggrin autoantibodies are posttranslationally generated on various sites of (pro filaggrin by deimination of arginine residues.* J.Immunol, 1999. 162: p. 585-594.
149. van Gaalen, F., Ioan-Facsinay, A, Huizinga, T. W, Toes, R. E, *The devil in the details: the emerging role of anticitrulline autoimmunity in rheumatoid arthritis.* J.Immunol., 2005. 175: p. 5575-5580.
150. Gregersen, P.K., Silver, J, Winchester, R. J, *The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis.* Arthritis Rheum, 1987. 30: p. 1205-1213.
151. Gourraud, P.A., Dieude, P, Boyer, J. F, Nogueira, L, Cambon-Thomsen, A, Mazieres, B, Cornelis, F, Serre, G, Cantagrel, A, Constantin, A, *A new classification of HLA-DRB1 alleles differentiates predisposing and protective alleles for autoantibody production in rheumatoid arthritis.* Arthritis Res.Ther, 2007. 9: p. R27.
152. Wesoly, J., van der Helm-van Mil, A. H, Toes, R. E, Chokkalingam, A. P, Carlton, V. E, Begovich, A. B, Huizinga, T. W, *Association of the PTPN22 C1858T single-nucleotide polymorphism with rheumatoid arthritis phenotypes in an inception cohort.* Arthritis Rheum, 2005. 52: p. 2948-2950.
153. Kurreeman, F.A.S., Padyukov, L., Marques, R.B., Schrodi, S.J, Seddighzadeh, M, Stoeken-Rijsbergen, G, van der Helm-van Mil A H, Allaart, C.F, Verduyn, W., Houwing-Duistermaat, J. et al. , *A Candidate Gene Approach Identifies the TRAF1/C5 Region as a Risk Factor for Rheumatoid Arthritis.* Plos Medicine, 2007. 4(9): p. e278.
154. Consortium, W.T.C.C., *Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls.* Nature 2007. 447: p. 661-678.
155. Remmers, E.F., Plenge,R.M., Lee,A.T., Graham,R.R., Hom,G., Behrens,T.W., de Bakker,P.I., Le,J.M., Lee,H.S., Batliwalla,F. et al, *STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus.* N.Engl.J.Med., 2007. 357: p. 977-986.
156. Plenge, R.M., Cotsapas,C., Davies,L., Price,A.L., de Bakker,P.I., Maller,J., Pe'er,I., Burtt,N.P., Blumenstiel,B., DeFelice,M. et al, *Two independent alleles at 6q23 associated with risk of rheumatoid arthritis.* Nat Genet, 2007. 39: p. 1477-1482.
157. Thomson, W., Barton,A., Ke,X., Eyre,S., Hinks,A., Bowes,J., Donn,R., Symmons,D., Hider,S., Bruce,I.N. et al, *Rheumatoid arthritis association at 6q23.* Nat Genet, 2007. 39: p. 1431-1433.
158. Plenge, R.M., Seielstad,M., Padyukov,L., Lee,A.T., Remmers,E.F., Ding,B., Liew,A., Khalili,H., Chandrasekaran,A., Davies,L.R. et al., *TRAF1-C5 as a Risk Locus for Rheumatoid Arthritis -- A Genomewide Study. .* N.Engl.J.Med., 2007.
159. van der Helm-van Mil, A.H., Wesoly, J. Z., Huizinga, T. W., *Understanding the genetic contribution to rheumatoid arthritis.* Curr.Opin.Rheumatol, 2005. 17: p. 299-304.
160. Pedersen, M., Jacobsen,S., Klarlund,M., Pedersen,B.V., Wiik,A., Wohlfahrt,J., Frisch,M, *Environmental risk factors differ between rheumatoid arthritis with and without auto-antibodies against cyclic citrullinated peptides.* Arthritis Res.Ther, 2006: p. 8:R133.
161. Jorgensen, C., Picot,M.C., Bologna,C., Sany,J., *Oral contraception, parity, breast feeding, and severity of rheumatoid arthritis.* Ann.Rheum.Dis, 1996. 55: p. 94-98.
162. Cutolo, M., Lahita, R.G., *Estrogens and arthritis.* Rheum.Dis.Clin.North Am, 2005(31): p. 19-27, vii.
163. Klareskog, L., Padyukov,L., Ronnelid,J., Alfredsson,L., *Genes, environment and immunity in the development of rheumatoid arthritis.* Curr.Opin.Immunol, 2006. 18: p. 650-655.

164. Klareskog, L., Padyukov,L., Alfredsson,L, *Smoking as a trigger for inflammatory rheumatic diseases.* Curr.Opin.Rheumatol., 2007. 19: p. 49-54.
165. Klareskog, L., Stolt,P., Lundberg,K., Kallberg,H., Bengtsson,C., Grunewald,J., Ronnelid,J., Harris,H.E., Ulfgren,A.K., Rantapaa-Dahlqvist,S. et al., *A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination.* Arthritis Rheum, 2006. 54: p. 38-46.
166. Sakaguchi, S., Ono,M., Setoguchi,R., Yagi,H., Hori,S., Fehervari,Z., Shimizu,J., Takahashi,T., Nomura,T. , *Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease.* Immunol.Rev, 2006. 212: p. 8-27.
167. van Amelsfort, J.M., Jacobs,K.M., Bijlsma,J.W., Lafeber,F.P, Taams,L.S, *CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid.* Arthritis Rheum, 2004. 50: p. 2775-2785.
168. Toh, M.L., Miossec, P., *The role of T cells in rheumatoid arthritis: new subsets and new targets.* Curr.Opin.Rheumatol, 2007. 19: p. 284-288.
169. Murphy, C.A., Langrish,C.L., Chen,Y., Blumenschein,W., McClanahan,T., Kastelein,R.A., Sedgwick,J.D., Cua,D.J., *Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. . :.* J.Exp.Med, 2003. 198: p. 1951-1957.
170. Furuzawa-Carballeda, J., Vargas-Rojas,M.I., Cabral,A.R, *Autoimmune inflammation from the Th17 perspective.* Autoimmun.Rev, 2007. 6: p. 169-175.
171. Ehrenstein, M.R., Evans,J.G., Singh,A., Moore,S., Warnes,G., Isenberg,D.A., Mauri,C., *Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy.* J.Exp.Med, 2004. 200: p. 277-285.
172. Nadkarni, S., Mauri,C., Ehrenstein,M.R, *Anti-TNF-alpha therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-beta.* J.Exp.Med, 2007. 204: p. 33-39.
173. Valencia, X., Stephens,G., Goldbach-Mansky,R., Wilson,M., Shevach,E.M., Lipsky,P.E., *TNF downmodulates the function of human CD4+CD25hi T-regulatory cells.* Blood 2006. 108: p. 253-261.
174. Morgan, M.E., Flierman,R., van Duivenvoorde,L.M., Witteveen,H.J., van Ewijk,W., van Laar,J.M., de Vries,R.R., Toes,R.E. , *Effective treatment of collagen-induced arthritis by adoptive transfer of CD25+ regulatory T cells.* Arthritis Rheum., 2005. 52: p. 2212-2221.
175. Dorner, T., *Crossroads of B cell activation in autoimmunity: rationale of targeting B cells.* J.Rheumatol.Suppl 2006. 77: p. 3-11.
176. Mauri, C., Ehrenstein,M.R., *Cells of the synovium in rheumatoid arthritis. B cells.* Arthritis Res.Ther, 2007. 9: p. 205.
177. Kinne, R.W., Stuhlmuller,B., Burmester,G.R., *Cells of the synovium in rheumatoid arthritis. Macrophages.* Arthritis Res.Ther, 2007. 9: p. 224.
178. Szekanecz, Z.K., A.E, *Macrophages and their products in rheumatoid arthritis.* Curr.Opin.Rheumatol, 2007. 19: p. 289-295.
179. Brennan, F., Beech, J, *Update on cytokines in rheumatoid arthritis.* Curr.Opin.Rheumatol, 2007. 19: p. 296-301.
180. Karouzakis, E., Neidhart, M., Gay, R.E., Gay,S., *Molecular and cellular basis of rheumatoid joint destruction.* Immunol.Lett, 2006. 106: p. 8-13.
181. Raza, K., Falciani,F., Curnow,S.J., Ross,E.J., Lee,C.Y., Akbar,A.N., Lord,J.M., Gordon,C., Buckley,C.D., Salmon,M, *Early rheumatoid arthritis is characterized by a distinct and transient synovial fluid cytokine profile of T cell and stromal cell origin.* Arthritis Res.Ther, 2005. 7: p. R784-R795.
182. Padyukov, L., Hytonen,A.M., Smolnikova,M., Hahn-Zoric,M., Nilsson,N., Hanson,L.A., Tarkowski,A., Klareskog,L, *Polymorphism in promoter region of IL10 gene is associated with rheumatoid arthritis in women.* J.Rheumatol.Suppl, 2004. 31: p. 422-425.
183. Eskdale, J., McNicholl,J., Wordsworth,P., Jonas,B., Huizinga,T., Field,M., Gallagher,G., *Interleukin-10 microsatellite polymorphisms and IL-10 locus alleles in rheumatoid arthritis susceptibility.* Lancet 1998. 352: p. 1282-1283.
184. Trentham DE, T.A., Kang AH, *Autoimmunity to type I collagen an experimental model of arthritis.* J Exp Med 1977(146): p. 857-868.

185. Svensson L, J.J., Holmdahl R, Jansson L, *B cell-deficient mice do not develop type II collagen induced arthritis (CIA).* Clin Exp. Immunol, 1998. 111: p. 521-526.
186. Courtenay JS, D.M., Dayan AD, Martin A, Mosedale B *Immunisation against heterologous type II collagen induces arthritis in mice.* Nature 1980. 283: p. 666-668.
187. Wooley PH, L.H., Stuart JM, David CS, *Type II collagen induced arthritis in mice. I. Major histocompatibility complex (I region) linkage and antibody correlates.* J Exp Med, 1981(154): p. 688-700.
188. Jirholt J, L.A., Holmdahl R: , *The genetics of rheumatoid arthritis and the need for animal models to find and understand the underlying genes.* Arthritis Res, 2001(3): p. 87-97.
189. Glant TT, B.T., Vermes C, Chandrasekaran R, Valdéz JC, Otto JM, Gerard D, Velins S, Lovász G, Zhang J, Mikecz K, Finnegan A, *Variations in susceptibility to proteoglycan induced arthritis and spondylitis among C3H substrains of mice: evidence of genetically acquired resistance to autoimmune disease.* Arthritis Rheum, 2001(44): p. 682-692.
190. O'Neill SK, S.M., Glant TT, Cao Y, Doodes PD, Finnegan A, *Antigen-specific B cells are required as APCs and autoantibody- producing cells for induction of severe autoimmune arthritis.* J Immunol 2005(174): p. 3781-3788.
191. Corthay A., J.A., Vestberg M., Holmdahl R, *Collagen-induced arthritis development requires alpha beta T cells but not gamma delta T cells: studies with T cell-deficient (TCR mutant) mice.* Int. Immunol, 1999. 11: p. 1065-1073.
192. Adarichev VA, V.J., Bardos T, Finnegan A, Mikecz K, Glant TT, *Combined autoimmune models of arthritis reveal shared and independent qualitative (binary) and quantitative trait loci.* J Immunol 2003(170): p. 2283-2292.
193. Banerjee S, W.C., Poole AR, *The induction of arthritis in mice by the cartilage proteoglycan aggrecan: roles of CD4+ and CD8+ T cells.* Cell Immunol, 1992. 144: p. 347-357.
194. Glant T, C.J., Szucs T: . , :. *Immunopathologic role of proteoglycan antigens in rheumatoid joint disease.* Scand J Immunol 1980(11): p. 247-252.
195. Cook AD, R.M., Stockman A, Muirden KD, Mackay IR, *Specificity of antibodies to type II collagen in early rheumatoid arthritis.* J Rheumatol, 1994(21): p. 1186-1191.
196. Cook AD, G.R., Ramshaw J, Mackay IR, Rowley MJ, *Antibodies against the CB10 fragment of type II collagen in rheumatoid arthritis.* Arthritis Res Ther, 2004(6): p. R477-R483.
197. Carlsen S, N.K., Backlund J, Holmberg J, Hultqvist M, Vestberg M, Holmdahl R: , : . *Cartilage oligomeric matrix protein induction of chronic arthritis in mice.* Arthritis Rheum, 2008. 58: p. 2000-2011.
198. Carlsen S, H.A., Olsson H, Heinegard D, Holmdahl R, *Cartilage oligomeric matrix protein (COMP)-induced arthritis in rats.* Clin Exp Immunol, 1998. 114: p. 477-484.
199. Cremer MA, G.M., Terato K, Kang AH, *Type XI and II collagen- induced arthritis in rats: characterization of inbred strains of rats for arthritis-susceptibility and immune-respon-siveness to type XI and II collagen.* Autoimmunity 1995. 20: p. 153-161.
200. Holmdahl R, R.K., Klareskog L, Larsson E, Wigzell H *Characterization of the antibody response in mice with type II collagen- induced arthritis, using monoclonal anti-type II collagen antibodies.* Arthritis Rheum, 1986. 29: p. 400-410.
201. Nandakumar KS, S.L., Holmdahl R, *Collagen type II-specific monoclonal antibody-induced arthritis in mice: description of the disease and the influence of age, sex, and genes.* Am J Pathol 2003. 163: p. 1827-1837.
202. Nandakumar KS, B.J., Vestberg M, Holmdahl R, *Collagen type II (CII)-specific antibodies induce arthritis in the absence of T or B cells but the arthritis progression is enhanced by CII-reactive T cells.* Arthritis Res Ther 2004. 6: p. R544-R550.
203. Schaible UE, K.M., Wallich R, Tran T, Simon MM, *Experimental Borrelia burgdorferi infection in inbred mouse strains: antibody response and association of H-2 genes with resistance and susceptibility to development of arthritis.* Eur J Immunol 1991. 21: p. 2397-2405.
204. Bremell T, L.S., Holmdahl R, Ryden C, Hansson GK, Tarkowski A, *Immunopathological features of rat Staphylococcus aureus arthritis.* Infect Immun 1994. 62: p. 2334-2344.

205. Bremell T, L.S., Yacoub A, Ryden C, Tarkowski A, *Experimental Staphylococcus aureus arthritis in mice.* Infect Immun 1991. 59: p. 2615-2623.
206. Kimpel D, D.T., Kannan K, Wolf RE, *Streptococcal cell wall arthritis: kinetics of immune cell activation in inflammatory arthritis.* Clin Immunol, 2002. 105: p. 351-362.
207. Pearson, C., *Development of arthritis, periarthritis and periostitis in rats given adjuvants.* Proc Soc Exp Biol Med, 1956. 91: p. 95-101.
208. Kleinau S, E.H., Holmdahl R, Klareskog L, *Adjuvant oils induce arthritis in the DA rat. I. Characterization of the disease and evidence for an immunological involvement.* J Autoimmun, 1991. 4: p. 871-880.
209. Carlson BC, J.A., Larsson A, Bucht A, Lorentzen JC, *The endogenous adjuvant squalene can induce a chronic T-cellmediated arthritis in rats.* Am J Pathol, 2000. 156: p. 2057-2065.
210. Vingsbo C, S.P., Brun JG, Jonsson R, Saxne T, Holmdahl R, *Pristane-induced arthritis in rats: a new model for rheumatoid arthritis with a chronic disease course influenced by both major histocompatibility complex and non-major histocompatibility complex genes.* Am J Pathol 1996. 149: p. 1675-1683.
211. Williams RO, F.M., Maini RN *Anti-tumor necrosis factorameliorates joint disease in murine collagen-induced arthritis.* Proc Natl Acad Sci USA 1992(89): p. 9784-9788.
212. Feldmann M, M.R., *TNF defined as a therapeutic target for rheumatoid arthritis and other autoimmune diseases.* Nat Med 2003. 9: p. 1245-1250.
213. Inglis, J.J., Criado, G, Medghalchi, M, Andrews, M, Sandison, A, Feldmann, M, Williams, R.O, *Collagen-induced arthritis in C57BL/6 mice is associated with a robust and sustained T-cell response to type II collagen.* Arthritis Res Ther, 2007. 9(5): p. 113.
214. Malmstrom V., M.E., Burkhardt H., Mattsson R., Vuorio E., Holmdahl R. , *Systemic versus cartilage-specific expression of a type II collagenspecific T-cell epitope determines the level of tolerance and susceptibility to arthritis.* Proc. Natl. Acad. Sci. U.S.A., 1996. 93: p. 4480-4485.
215. Trentham DE, D.R., David JR, *Passive transfer by cells of type II collagen-induced arthritis in rats.* J Clin Invest, 1978. 62: p. 359-361.
216. Holmdahl R, K.L., Rubin K, *T-cell in collagen II induced arthritis in mice: characterization of arthritogenic collagen II specific T-lymphocyte lines an clones that can transfer arthritis.* Scand J Immunol 1985. 22: p. 295-298.
217. Wooley PH, L., S. H, *Passive transfer of arthritis in mice by human anti-type II collagen antibody.* Mayo Clinic Proc, 1984. 59: p. 737.
218. Stuart JM, T.K., Townes AS, *Serum transfer of collagen induced arthritis. II. Identification and localization of autoantibody to type II collagen in donor and recipient rats. :,.* Arthritis Rheum 1983. 26: p. 1237-1239.
219. Stuart JM, C.M., Townes AS, *Type II collagen induced arthritis in rats. Passive transfer with serum and evidence that IgG anticollagen antibodies can cause arthritis.* J Exp Med, 1982. 155.
220. Englert ME, F.K., Suarez CR, *Passive transfer of collagen arthritis: heterogeneity of anti-collagen IgG.* Cell Immunol 1986. 101: p. 373-375.
221. Mussener A, L.M., Lindroos E, *Cytokin production in synovial tissue of mice with collagen-induced arthritis (CIA). :.* Clin Exp Immunol. 107: p. 485-488.
222. Holmdahl R, J.R., Larsson P, *Early appearance of activated CD4+ T lymphocytes and class II antigen-expressing cells in joints of DBA/1 mice immunized with type II collagen. .* Lab Invest 1988. 58: p. 53-56.
223. Nakajima A, S.C., Sandora MR, *Antigen-specific T cell-mediated gene therapy in collagen-induced arthritis. .* J Clin Invest, 2001. 107: p. 1293-1296.
224. McIntyre KW, S.D., Gillooly KM, *Reduced incidence and severity of collagen induced arthritis in interleukin-12-deficient mice* Eur J Immunol 1996. 26: p. 2933-2937.
225. Malfait AM, B.D., Presky DH, *Blockade of IL-12 during the induction of collagen induced arthritis (CIA) markedly attenuates the severity of the arthritis. .* Clin Exp Immunol, 1998. 111: p. 377-380.
226. Okamoto Y, G.Y., Tokui H, *Characterization of the cytokine network at a single cell level in mice with collagen-induced arthritis using a dual color ELISPOT assay.* J Interferon Cytokine Res 2000. 20: p. 55-58.

227. Ross SE, W.R., Mason LJ, *Suppression of TNF-alpha expression, inhibition of Th1 activity, and amelioration of collagen-induced arthritis by rolipram.* J Immunol 1997. 159: p. 6253-6256.
228. Butler DM, M.A., Maini RN, *Anti-IL-12 and anti-TNF antibodies synergistically suppress the progression of murine collagen-induced arthritis.* Eur J Immunol 1999. 29: p. 2205-2207.
229. Kim KN, W.S., Ma Y, *Viral IL-10 and soluble TNF receptor act synergistically to inhibit collagen-induced arthritis following adenovirus-mediated gene transfer.* J Immunol 2000. 164: p. 1576-1579.
230. Joosten LA, L.E., Helsen MM, *Protection against cartilage and bone destruction by systemic interleukin-4 treatment in established murine type II collagen-induced arthritis.* Arthritis Res, 1999. 1: p. 81-84.
231. Joosten LA, L.E., Durez P, *Role of interleukin-4 and interleukin-10 in murine collagen-induced arthritis. Protective effect of interleukin-4 and interleukin-10 treatment on cartilage destruction.* Arthritis Rheum, 1997. 40: p. 249-252.
232. Nakajima H, T.H., Hiyama Y, *The effect of treatment with interferon-gamma on type II collagen-induced arthritis.* Clin Exp Immunol 1990. 81: p. 441.
233. Mauritz NJ, H.R.J.R., *Treatment with gamma-interferon triggers the onset of collagen arthritis in mice.* Arthritis Rheum, 1988. 31: p. 1297-1300.
234. Cooper SM, S.S., Ranges GE, *Suppression of murine collagen-induced arthritis with monoclonal anti-Ia antibodies and augmentation with IFN- gamma. .* J Immunol, 1988. 141: p. 1958-1961.
235. Chiocchia, G., Boissier MC, Ronziere MC, Herbage D, Fournier C, *T cell regulation of collagen-induced arthritis in mice. I. Isolation of Type II collagen-reactive T cell hybridomas with specific cytotoxic function.* J Immunol, 1990. 145(2): p. 519-25.
236. Webb L.M., W.M.J., Feldmann M, *Prevention and amelioration of collagen-induced arthritis by blockade of the CD28 co-stimulatory pathway: requirement for both B7-1 and B7-2. .* Eur. J.Immunol, 1996. 26: p. 2320-2328.
237. Tada Y., N.K., Ho A., Morito F., Ushiyama O., Suzuki N., Ohta H., Mak T.W., *CD28-deficient mice are highly resistant to collagen-induced arthritis.* J. Immunol. , 1999. 162: p. 203-208.
238. Tellander AC, P.U., Runström A, Andersson M, Michaëlsson E, *Interference with CD28, CD80, CD86 or CD152 in collagen-induced arthritis. Limited role of IFN-gamma in anti-B7-mediated suppression of disease. .* J Autoimmun, 2001. 17: p. 39-50.
239. Makrygiannakis, D., af Klint, E, Catrina, S.B, Botusan, I.R, Klareskog, E, Klareskog, L, et al, *Intraarticular corticosteroids decrease synovial RANKL expression in inflammatory arthritis.* Arthritis Rheum, 2006. 54(5): p. 1463-72.
240. Gao, W., Demirci, G, Strom, T.B, Li, XC, *Stimulating PD-1-negative signals concurrent with blocking CD154 co-stimulation induces long-term islet allograft survival. .* Transplantation, 2003. 76(6): p. 994-9.
241. Latchman, Y., Wood, C.R, Chernova, T, Chaudhary, D, Borde, M, Chernova, I, et al, *PD-L2 is a second ligand for PD-1 and inhibits T cell activation.* Nat Immunol, 2001. 2(3): p. 261-8.
242. Kong, E.K., Prokunina-Olsson, L, Wong, W.H, Lau, C.S, Chan, T.M, Alarcon-Riquelme, M, et al, *A new haplotype of PDCD1 is associated with rheumatoid arthritis in Hong Kong Chinese.* Arthritis Rheum, 2005. 52(4): p. 1058-62.
243. Panayi, G.S., Lanchbury JS, Kingsley GH, *The importance of the T cell in initiating and maintaining the chronic synovitis of rheumatoid arthritis.* Arthritis Rheum, 1992. 35(7): p. 729-35.
244. Liu, M.F., Kohsaka, H, Sakurai H, Azuma M, Okumura K, Saito I, et al, *The presence of costimulatory molecules CD86 and CD28 in rheumatoid arthritis synovium.* Arthritis Rheum, 1996. 39(1): p. 110-4.
245. Namekawa, T., Wagner UG, Goronzy JJ, Weyand CM, *Functional subsets of CD4 T cells in rheumatoid synovitis.* Arthritis Rheum, 1998. 41(12): p. 2108-16.
246. Shimoyama, Y., Nagafuchi H, Suzuki N, Ochi T, Sakane T, *Synovium infiltrating T cells induce excessive synovial cell function through CD28/B7 pathway in patients with rheumatoid arthritis.* J Rheumatol, 1999. 26(10): p. 2094-101.
247. Berner, B., Wolf, G, Hummel KM, Muller GA, Reuss-Borst MA, *Increased expression of CD40 ligand (CD154) on CD4+ T cells as a marker of disease activity in rheumatoid arthritis.* Ann Rheum Dis, 2000. 59(3): p. 190-5.

248. Okamoto, T., Saito S, Yamanaka H, Tomatsu T, Kamatani N, Ogiuchi H, *Expression and function of the co-stimulator H4/ICOS on activated T cells of patients with rheumatoid arthritis. .* J Rheumatol, 2003. 30(6): p. 1157-63.
249. Ruth, J.H., Rottman JB, Kingsbury GA, Coyle AJ, Haines GK, 3rd, Pope RM, et al, *ICOS and B7 costimulatory molecule expression identifies activated cellular subsets in rheumatoid arthritis.* Cytometry A, 2007. 71(5): p. 317-26.
250. Goeb, V., Buch, M.H, Vital, E.M, Emery, P, *Costimulation blockade in rheumatic diseases: where we are? .* Curr Opin Rheumatol, 2009. 21: p. 244-50.
251. Vincenti, F., Luggen, M, *T cell costimulation: a rational target in the therapeutic armamentarium for autoimmune diseases and transplantation.* Annu Rev Med, 2007. 58: p. 347-58.
252. Dudler, J., Li, J, Pagnotta, M, Pascual, M, von Segesser, L.K, Vassalli, G, *Gene transfer of programmed death ligand-1.Ig prolongs cardiac allograft survival.* Transplantation, 2006. 82(12): p. 1733-7.
253. Ozkaynak, E., Wang, L, Goodearl, A, McDonald, K, Qin, S, O'Keefe, T, et al, *Programmed death-1 targeting can promote allograft survival.* J Immunol, 2002. 169(11): p. 6546-53.

10. PUBLICATION

ARTHRITIS & RHEUMATISM
Vol. 62, No. 7, July 2010, pp 1870–1880
DOI 10.1002/art.27500

The Programmed Death 1/Programmed Death Ligand 1 Inhibitory Pathway Is Up-Regulated in Rheumatoid Synovium and Regulates Peripheral T Cell Responses in Human and Murine Arthritis

Amalia P. Raptopoulou,[1] George Bertsias,[1] Dimitrios Makrygiannakis,[2] Panagiotis Verginis,[1] Iraklis Kritikos,[1] Maria Tzardi,[1] Lars Klareskog,[2] Anca I. Catrina,[2] Prodromos Sidiropoulos,[1] and Dimitrios T. Boumpas[1]

Objective. **T cells play a major role in the pathogenesis of rheumatoid arthritis (RA). The programmed death 1 (PD-1)/programmed death ligand 1 (PDL-1) pathway is involved in peripheral tolerance through inhibition of T cells at the level of synovial tissue. The aim of this study was to examine the role of PD-1/PDL-1 in the regulation of human and murine RA.**

Methods. **In synovial tissue and synovial fluid (SF) mononuclear cells from patients with RA, expression of PD-1/PDL-1 was examined by immunohistochemistry and flow cytometry, while PD-1 function was assessed in RA peripheral blood (PB) T cells after stimulation of the cells with anti-CD3 and PDL-1.Fc to crosslink PD-1. Collagen-induced arthritis (CIA) was induced in *PD-1*$^{-/-}$ C57BL/6 mice, and recombinant PDL-1.Fc was injected intraperitoneally to activate PD-1 in vivo.**

Results. **RA synovium and RA SF were enriched with PD-1+ T cells (mean ± SEM 24 ± 5% versus 4 ± 1% in osteoarthritis samples; $P = 0.003$) and enriched with PDL-1+ monocyte/macrophages. PD-1 crosslinking inhibited both T cell proliferation and production of interferon-γ (IFNγ) in RA patients; PB T cells incubated with RA SF, as well as SF T cells from patients with active RA, exhibited reduced PD-1–mediated inhibition of T cell proliferation at suboptimal, but not optimal, concentrations of PDL-1.Fc. *PD-1*$^{-/-}$ mice demonstrated increased incidence of CIA (73% versus 36% in wild-type mice; $P < 0.05$) and greater severity of CIA (mean maximum arthritis score 5.0 versus 2.3 in wild-type mice; $P = 0.040$), and this was associated with enhanced T cell proliferation and increased production of cytokines (IFNγ and interleukin-17) in response to type II collagen. PDL-1.Fc treatment ameliorated the severity of CIA and reduced T cell responses.**

Conclusion. **The negative costimulatory PD-1/PDL-1 pathway regulates peripheral T cell responses in both human and murine RA. PD-1/PDL-1 in rheumatoid synovium may represent an additional target for immunomodulatory therapy in RA.**

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the joints that causes severe disability and premature mortality (1,2). Numerous data support a central role of T cells in RA. These cells are thought to be triggered locally in an antigen-specific manner, resulting in breakdown of tolerance, synovial inflammation, and autoantibody production (3–7). In the collagen-induced arthritis (CIA) animal model of RA, type II collagen (CII)–reactive CD4+ T cells are primary mediators of disease induction, by driving autoantibody production in B cells and enhancing the localized chronic inflammatory response (8–12).

Regulation of activation of T lymphocytes is

Supported by the Hellenic Society of Rheumatology, the Pancretan Health Association, the Hellenic Ministry of Education, Hellenic Republic, and the European Union (EPEAEK Fund and Sixth Framework Programme AutoCure program).

[1]Amalia P. Raptopoulou, MD, George Bertsias, MD, PhD, Panagiotis Verginis, PhD, Iraklis Kritikos, MD, PhD, Maria Tzardi, MD, PhD, Prodromos Sidiropoulos, MD, Dimitrios T. Boumpas, MD, FACP: University of Crete School of Medicine, Heraklion, Greece; [2]Dimitrios Makrygiannakis, MD, PhD, Lars Klareskog, MD, Anca I. Catrina, MD, PhD: Karolinska University Hospital and Karolinska Institutet, Stockholm, Sweden.

Drs. Raptopoulou and Bertsias contributed equally to this work. Drs. Sidiropoulos and Boumpas contributed equally to this work.

Address correspondence and reprint requests to Dimitrios T. Boumpas, MD, FACP, Laboratory of Autoimmunity and Inflammation, Department of Rheumatology, Clinical Immunology and Allergy, University of Crete School of Medicine, PO Box 2208, Heraklion 71003, Greece. E-mail: boumpasd@med.uoc.gr.

Submitted for publication May 6, 2009; accepted in revised form March 30, 2010.

mediated by mechanisms involving central and peripheral lymphoid organs. The B7 family of molecules is critical for stimulating or inhibiting T cells; engagement of CD28 and inducible costimulator (ICOS) by CD80/CD86 and B7h (ICOS ligand), respectively, stimulates T cell responses, whereas engagement of CTLA-4 by CD80/CD86 inhibits T cell responses (13). Programmed death 1 (PD-1) is a novel member of the B7 family that plays an important role in peripheral tolerance. Both PD-1 and CTLA-4 inhibit T cells, albeit through different mechanisms (14); PD-1 inhibits Akt phosphorylation by preventing CD28-mediated activation of phosphatidylinositol 3-kinase, whereas CTLA-4 acts by recruiting the PP2A phosphatase (15,16).

There is evidence to support a distinct role of PD-1 and its ligands (PDL-1/B7-H1 and PDL-2/B7-DC) in the regulation of T cells. PD-1 is thought to be important for the "fine tuning" of lymphocyte activation at the level of synovial tissue, considering the wide pattern of expression of one of its ligands, PDL-1, in activated endothelial and epithelial cells (17–19). A broader role of PD-1 in immune regulation has also been suggested, based on its induction not only on activated T cells, but also on B cells and monocytes. Of interest, PD-1 ligation is more effective than CTLA-4 in suppressing CD3/CD28-induced changes in the T cell transcriptional profile (15). The critical role of PD-1 in immune regulation is highlighted by gene disruption studies demonstrating strain-specific autoimmune phenotypes (20,21). In humans, a role for PD-1 in the regulation of self tolerance and autoimmunity was suggested by associations between polymorphisms in the PD-1 gene and autoimmune diseases such as systemic lupus erythematosus, RA, type 1 diabetes mellitus, and multiple sclerosis (22–27).

Although the role of costimulation is well documented in RA and has been further supported by the efficacy of CTLA-4Ig in severe RA, the role of this family of molecules has not been explored in a systematic, organized manner. We sought to determine the role of PD-1/PDL-1 in RA and to test the hypothesis that defective expression and/or function of this pathway may contribute to T cell hyperactivity within the inflamed joint. To this end, we examined PD-1/PDL-1/PDL-2 expression and function in both human RA and murine CIA. The role of PD-1 in RA was further studied by inducing CIA in *PD-1*–deficient (*PD-1*$^{-/-}$) mice and by using PDL-1.Fc to crosslink PD-1 in vivo. Our data suggest that the PD-1/PDL-1 pathway regulates T cell responses within the rheumatoid joint, and may therefore represent a potential therapeutic target for RA.

MATERIALS AND METHODS

Preparation of mononuclear cells and isolation of T lymphocytes. Synovial fluid (SF) and peripheral blood (PB) were obtained from patients with RA (n = 67; mean ± SD age 62 ± 11 years) and patients with osteoarthritis (OA) (n = 32; mean ± SD age 71 ± 6 years). All RA patients had active arthritis (mean ± SD Disease Activity Score in 28 joints 6.2 ± 0.8) (28), of whom 46 (69%) were rheumatoid factor positive, 46 (69%) were receiving disease-modifying antirheumatic drugs, and 21 (31%) were receiving anti–tumor necrosis factor agents. SF samples were treated with hyaluronidase (Sigma-Aldrich) and washed in phosphate buffered saline (PBS). SF and PB mononuclear cells (SFMCs and PBMCs, respectively) were isolated by Ficoll-Histopaque (Sigma-Aldrich) density-gradient centrifugation and washed in PBS. CD4+ T lymphocytes (purity 92–98%) were isolated by positive selection with magnetic beads (Miltenyi Biotec).

Antibodies and flow cytometry. The following mouse anti-human antibodies were used as phycoerythrin (PE), fluorescein isothiocyanate (FITC), or peridinin chlorophyll protein (PerCP)–Cy5.5 conjugates: anti-CD3 (clone UCHT1), anti–PDL-1/B7-H1 (MIH1), anti–PDL-2/B7-DC (MIH18), and anti–PD-1 (J116) (all from eBioscience). Anti-CD4 (OKT4) and anti-CD69 (TP1.55.3) were from Beckman Coulter. Anti-CD25 (M-A251) and anti–HLA–DR (G46-6) were from BD PharMingen. PE- or PerCP-Cy5.5–conjugated IgG1 (679.1Mc7) (Beckman Coulter) and FITC-conjugated IgG1 (P3) (eBioscience) were used as IgG isotype controls in all experiments. PBMCs or SFMCs (0.5×10^6 cells) were incubated in wash buffer with appropriate amounts of monoclonal antibodies (mAb) on ice for 30 minutes. Cells were washed and were immediately analyzed on an Epics XL-MCL flow cytometer. The CellTrace 5,6-carboxyfluorescein succinimidyl ester (CFSE) cell proliferation kit (Invitrogen) was used for CFSE labeling of T cells.

Stimulation of PB and SF CD4+ T cells and assessment of PD-1 function. PB and SF CD4+ T cells (1×10^5/well) were incubated in RPMI 1640 complete medium (containing 10% fetal bovine serum, 2 m*M* L-glutamine, 10 m*M* HEPES, 100 IU/ml penicillin, and 10 μg/ml streptomycin) (Gibco Invitrogen) in 48-well tissue culture plates (Nunc) and were stimulated with phorbol myristate acetate (PMA) (10 ng/ml) and ionomycin (500 ng/ml). After 48 hours, cells were harvested, washed, and analyzed for expression of PD-1 and CD69 by flow cytometry. Dead cells were excluded based on forward scatter/side scatter properties and a total of 10,000 events were analyzed. To assess PD-1 function, CD4+ T cells from RA patients were stimulated with plate-bound anti-CD3 mAb (UCHT1) and PDL-1.Fc (both from R&D Systems) to crosslink PD-1. Briefly, 96-well flat-bottomed plates (Nunc) were coated with anti-CD3 (1 μg/ml) or PDL-1.Fc (0–5 μg/ml) for 4 hours at 37°C in 100 μl PBS solution. Human IgG1 (Sigma) was added as needed to keep the amount of total protein constant. Plates were washed twice before cell culture was initiated.

After 48 hours, culture supernatants were collected and the levels of interferon-γ (IFNγ) were measured by enzyme-linked immunosorbent assay (ELISA) (eBioscience). At 72 hours, cells were pulsed with ^{3}H-thymidine (1 μCi/well) (Amersham Biosciences) for another 16 hours to measure T cell proliferation. In studies using CFSE-labeled T cells, pro-

liferation was determined based on the CFSE dilution, with results analyzed using WinMDI software. In some experiments, culture medium was supplemented with nonhomologous, hyaluronidase-treated RA SF (15% volume/volume) to evaluate the effects on PD-1 function.

Determination of PD-1 and PDL-1 expression by immunohistochemistry. Synovial tissue biopsy specimens were obtained by open surgery from patients with RA and patients with OA and by arthroscopy from healthy control subjects. All procedures were approved by the Northern Stockholm Ethics Review Board and informed consent was obtained from all participants. Tissue sections were snap frozen in dry ice–cooled isopentane. Serial cryostat sections (7 μm) were fixed for 20 minutes with 2% (volume/volume) formaldehyde and stored at −70°C. Immunohistochemistry was performed using mouse IgG1 anti-human PD-1 antibody (MIH4), anti–PDL-1 (MIH1), anti–PDL-2 (MIH18) (eBioscience), mouse IgG1 anti-human CD3 (SK7; BD Biosciences), mouse IgG1 anti-human CD163 (Ber-MAC3; DakoCytomation), and mouse IgG1 anti-human CD19 (HD37; DakoCytomation), as previously described (29). Isotype and concentration-matched controls were used. Stained biopsy sections were evaluated semiquantitatively by 2 independent observers (DM and AIC), who were unaware of each sample's identity, for the expression of PD-1/PDL-1 (assessed as a score for immunostaining intensity, where 0 = none and 4 = maximum) and for the degree of synovial inflammation (29). In addition, analyses of serial sections stained with cell-type specific markers (CD3 for T cells, CD19 for B cells, CD163 for macrophages) were performed.

Induction of CIA and treatment of wild-type mice with PDL-1.Fc. *PD-1*$^{-/-}$ mice bred on the C57BL/6 (B6) background (30) were a kind gift from Dr. Zhang (Department of Orthopedic Surgery, University of Chicago). Wild-type and *PD-1*$^{-/-}$ B6 mice were maintained under pathogen-free conditions at the Institute of Molecular Biology and Biotechnology facilities in Greece. CIA was induced according to the standard protocol. Briefly, an emulsion was formed by dissolving 2 mg/ml chick CII (Sigma) overnight at 4°C in 10 m*M* acetic acid and combining it with an equal volume of Freund's complete adjuvant (CFA) containing 5 mg/ml heat-killed *Mycobacterium tuberculosis* (H37Ra; Difco). Eight-week-old mice were injected intradermally at 2 sites into the base of the tail with a total of 100 μl of emulsion; this was repeated as a booster injection 21 days later. In some experiments, wild-type B6 mice were injected intraperitoneally with PDL-1.Fc (0.1 mg/mouse) on days 0, 2, 3, 5, and 10 postimmunization. PDL-1.Fc protein (1873; kindly provided by Dr. G. Freeman, Harvard School of Medicine, Boston, Massachusetts) consists of the extracellular domains of murine PDL-1 linked to the hinge CH2–CH3 domains of a mutated murine IgG2a, to reduce Fc receptor and complement binding, and has been shown to stimulate PD-1 in vivo (31,32).

Animals were assessed for redness and swelling of all 4 limbs, and a clinical score ranging from 0 (no inflammation) to 4 (extensive swelling and erythema of the entire paw) was allocated for each mouse 2–3 times per week for up to 42 days, as previously described (12,33). After the mice were killed, the rear paws were removed, fixed, decalcified, and paraffin embedded (12,33). Frontal sections of the paw tissue (5 mm) were stained with hematoxylin and eosin and evaluated according to the presence or absence of inflammatory cell infiltrates (defined as focal accumulations of leukocytes).

Anti-CII T cell responses. Inguinal lymph nodes were excised from mice with CIA on day 10 after immunization. Lymph node cells (LNCs) were cultured in the presence or absence of varying concentrations of CII (10–100 μg/ml). After 48 hours, 100 μl of culture medium was removed for measurement of cytokines, and 24 hours later, the remaining cells were pulsed with 1 μCi ^{3}H-thymidine per well for a further 16 hours. Each assay was performed on a minimum of 3 occasions. The levels of IFNγ and interleukin-17 (IL-17) were measured by ELISA (BD Biosciences).

Production of anti-CII antibodies. Anti-CII IgG production was measured by ELISA in mouse serum, 39 days after the first immunization. Briefly, serum samples were added in serial dilutions (1:500, 1:1,000, 1:2,000) on plates precoated with 10 μg/ml chicken CII. CII-specific IgG was detected with peroxidase-conjugated goat anti-mouse IgG antibodies (Chondrex).

Statistical analysis. The nonparametric Mann-Whitney and Kruskal-Wallis tests were used for comparisons between ≥2 groups. The chi-square test was used to compare proportions. The paired *t*-test was used for comparisons of PD-1 expression and/or function in paired PB and SF samples. *P* values less than 0.05 were considered statistically significant.

RESULTS

Increased expression of PD-1/PDL-1 in human RA synovial tissue and RA SF. We first performed immunohistochemical analyses of RA synovial tissue sections, in comparison with OA and healthy synovial tissue sections as controls. Eight (89%) of 9 RA synovial tissue samples showed PD-1 expression, as compared with 2 (25%) of 8 samples from OA patients and none of the samples from healthy individuals (Figure 1A). All RA and OA synovial tissue samples were PDL-1 positive, compared with only 1 (12.5%) of 8 healthy tissue samples. Similarly, 100% of the RA synovial tissue samples and 6 (75%) of 8 OA synovial tissue samples were PDL-2 positive, compared with only 2 (25%) of 8 synovial tissue samples from healthy individuals.

In semiquantitative analyses, RA synovial tissue displayed higher expression of PD-1 (median immunostaining intensity 1.5, range 0–3; n = 10) than did either OA synovial tissue (median 0, range 0–1; n = 9) or healthy synovial tissue (no PD-1 expression; n = 9). PDL-1 was highly expressed in both RA biopsy tissue (median immunostaining intensity 3, range 1–3) and OA biopsy tissue (median 2, range 1–3), whereas minimal expression of PDL-1 was observed in biopsy specimens from healthy individuals (median 0, range 0–1). With regard to the expression of PDL-2, the only significant difference in expression was between RA synovial tissue (median immunostaining intensity 2, range 1–3) and healthy synovial tissue (median 0, range 0–2) (Figure

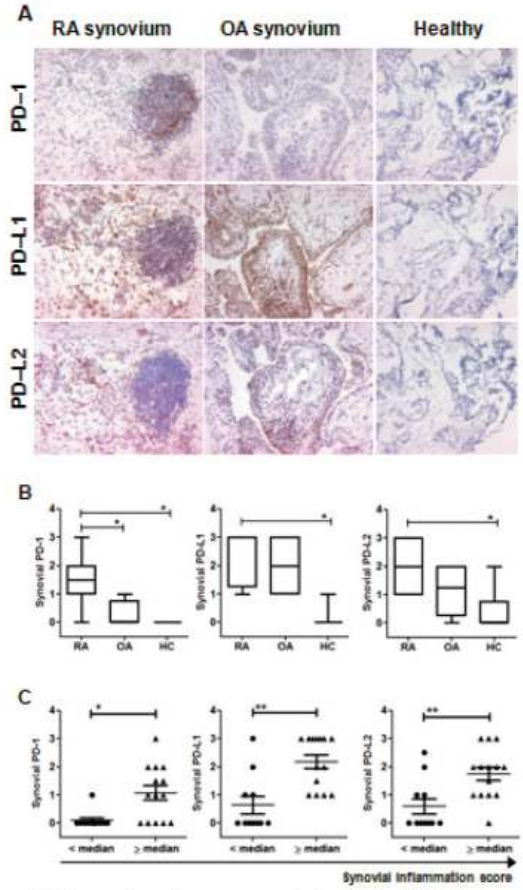


Figure 1. Expression of programmed death 1 (PD-1)/programmed death ligand 1 (PDL-1) in the synovium of patients with rheumatoid arthritis (RA), patients with osteoarthritis (OA), and healthy controls (HC). **A,** Increase in expression of PD-1 (**top**) in RA patients compared with OA patients and healthy controls, and increase in expression of PDL-1 (**middle**) and PDL-2 (**bottom**) in both RA and OA patients compared with healthy controls. In RA, PD-1 is mainly expressed in lymphoid aggregates of the sublining layer and also in a few scattered inflammatory cells residing in the sublining and lining layer. Positive immunostaining is indicated with the brown color, representing staining with diaminobenzidine (original magnification × 100). **B,** Semiquantitative analysis of PD-1/PDL synovial tissue expression. Results are expressed as the median immunostaining intensity score (where 0 = none and 4 = maximum) in RA (n = 10), OA (n = 9), and healthy control (n = 9) synovial tissue; data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles. **C,** Correlation of the synovial expression of PD-1/PDL with the degree of inflammation. Results are presented as scatter dot plots of PD-1/PDL expression in the synovia according to samples with low total synovial inflammation scores (below the median of 4.5) versus those with high total synovial inflammation scores (greater than or equal to the median of 4.5). Bars show the mean ± SEM (see Materials and Methods for details on the microscopic analysis of PD-1/PDL-1 expression and calculation of total synovial inflammation score). $* = P < 0.05$; $** = P < 0.01$, for pairwise comparisons.

1B). Synovial expression of PD-1/PDL-1/PDL-2 was significantly increased in all synovial tissue samples that were assessed as displaying a high degree of inflammation, defined as those with a total synovial inflammation score greater than or equal to the median value of 4.5 (Figure 1C).

We also examined the localization of PD-1/PDL-1 in the rheumatoid synovium. PD-1 was expressed in lymphoid aggregates of the sublining layer and in a few scattered inflammatory cells residing in the sublining and lining layer. Examination of the immunohistochemical findings revealed similar immunostaining patterns for PD-1 and the T cell marker CD3, suggesting that PD-1 is most likely expressed by synovial T cells

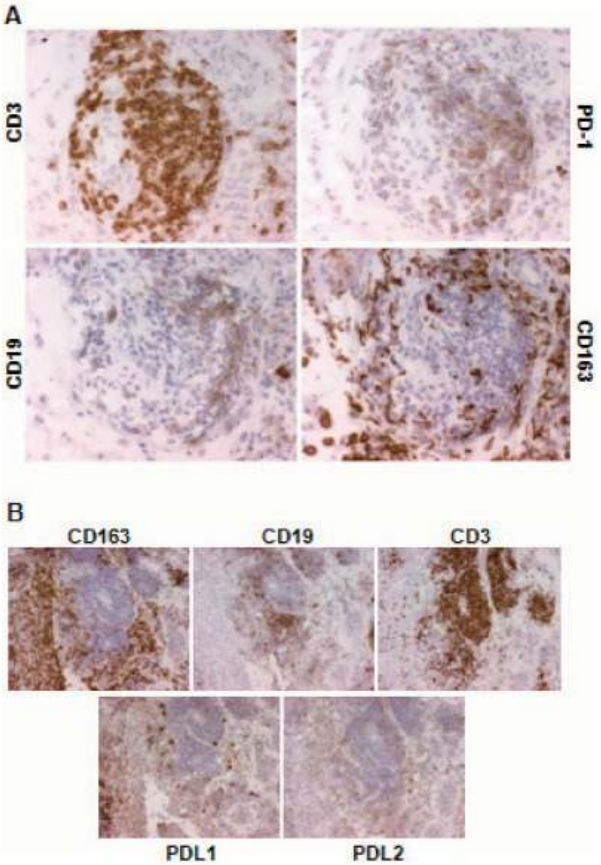


Figure 2. Immunostaining patterns of expression of programmed death 1 (PD-1) (**A**) and programmed death ligand 1 (PDL-1) and PDL-2 (**B**) in relation to cell type–specific markers in synovium from a representative patient with rheumatoid arthritis. **A,** PD-1 was mainly expressed by infiltrating CD3+ T cells in the rheumatoid synovium (original magnification × 250). **B,** The majority of cells expressing both PDL-1 and PDL-2 were CD163+ macrophages, but a few lymphocytes also expressed small amounts of PDL-2 (original magnification × 100).

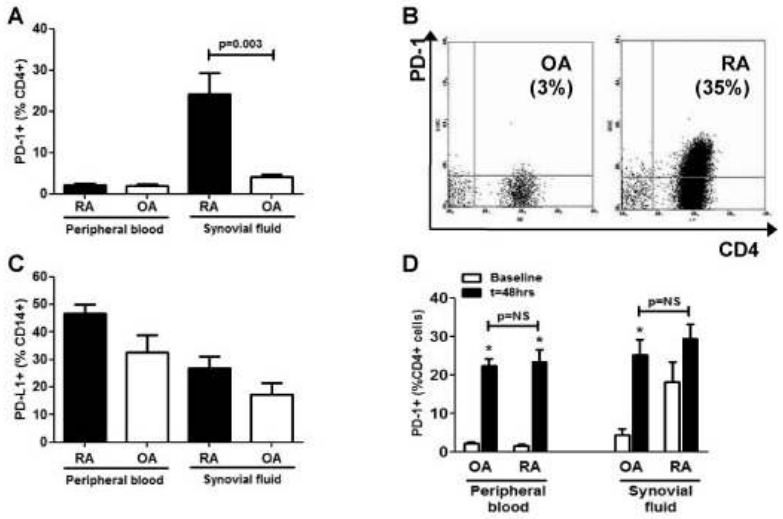


Figure 3. Enrichment of programmed death 1–positive (PD-1+) CD4+ T cells in rheumatoid arthritis (RA) synovial fluid (SF), as compared with osteoarthritis (OA) SF, with a reduced capacity to further up-regulate PD-1 upon stimulation. **A,** PD-1 expression in peripheral blood (PB) and SF mononuclear cells, as determined by flow cytometry. PB CD4+ T cells from RA patients and those from OA patients had comparable levels of PD-1, while in the SF, only CD4+ cells from RA patients had increased PD-1 expression. **B,** Representative results of flow cytometry analyses of PD-1 expression in SF CD4+ T cells from 1 patient with OA and 1 patient with RA. **C,** Comparable expression of programmed death ligand (PDL-1) in PB and SF CD14+ monocytes between patients with RA and patients with OA. **D,** Increased PD-1 expression in SF CD4+ T cells from patients with RA at baseline, but with reduced capacity to further increase PD-1 expression following 48 hours of stimulation with phorbol myristate acetate/ionomycin. Bars show the mean and SEM results in samples from 15 RA patients and 10 OA patients. * = $P < 0.05$ versus baseline. NS = not significant.

(Figure 2A). Both PDL-1 and PDL-2 were expressed by synovial cells of the lining and sublining layers, while PDL-1 was also expressed by sublining endothelial cells. Most cells expressing both PDL-1 and PDL-2 were macrophages, but a few lymphocytes also expressed small amounts of PDL-2 (Figure 2B). Taken together, these results indicating that PD-1 and PDL-1 display increased expression in the rheumatoid synovium suggest that this pathway may play a role in the pathogenesis of RA.

Enrichment of PD-1–expressing T lymphocytes in RA SF. We next examined the expression of PD-1 in SF T cells from RA patients. Both in SF samples from RA patients and in those from OA patients, the SF was enriched with PD-1+CD4+ T cells, as compared with only minimal expression in the PB. RA patients had a higher percentage of SF PD-1+CD4+ T cells as compared with OA patients (mean ± SEM 24 ± 5% versus 4 ± 1%; $P = 0.003$) (Figures 3A and B). PD-1 was also overexpressed in CD4+CD69+ and CD4+CD25+ activated T cells in the SF from patients with RA (results not shown). In contrast, no significant difference in PDL-1 expression was observed in SF lymphocytes and monocytes between RA and OA patients (Figure 3C).

We also evaluated the capacity of T cells to further up-regulate PD-1 expression upon activation with PMA and ionomycin. PD-1 was significantly up-regulated on SF T cells from OA patients (mean ± SEM 25 ± 4% at 48 hours of stimulation versus 5 ± 2% at baseline [n = 10]; $P = 0.006$ by paired t-test), whereas a less profound up-regulation was observed in RA SF T cells (29 ± 4% at 48 hours of stimulation versus 18 ± 5% at baseline [n = 15]; $P = 0.067$) (Figure 3D). Overall, these data corroborate the results from immunohistochemistry, showing enhanced expression of PD-1, but not of PDL-1, within the rheumatoid joint.

Suppression of T cell proliferation and cytokine production by PD-1 in RA, and abrogation of PD-1 regulation in PB T cells incubated with RA SF and in RA SF T cells. PD-1 activation results in suppression of lymphocyte proliferation and cytokine production via decreased ERK and Akt/protein kinase B activation. To

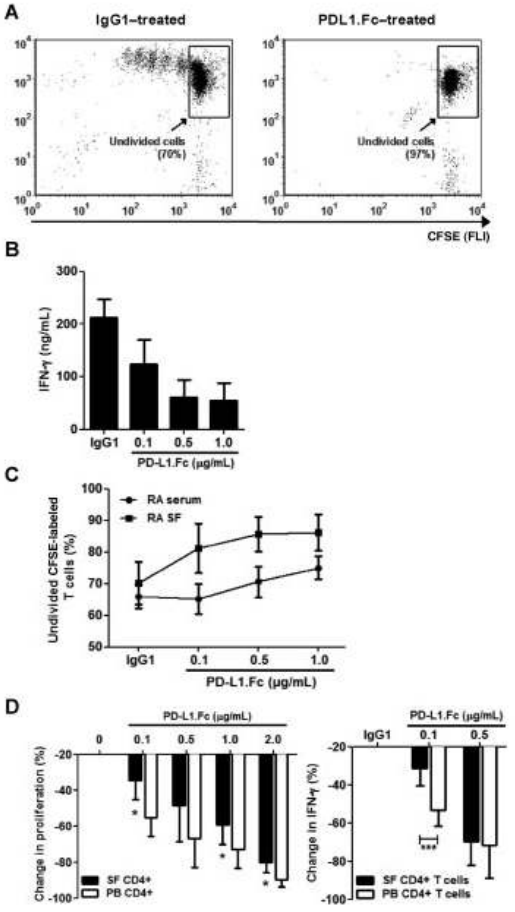


Figure 4. Regulation of T cell proliferation and cytokine production by programmed death 1 (PD-1) in patients with rheumatoid arthritis (RA), and abrogation of PD-1–mediated suppression of proliferation in peripheral blood (PB) T cells incubated with RA synovial fluid (SF) and in RA SF T cells. **A,** PB T cells labeled with 5,6-carboxyfluorescein succinimidyl ester (CFSE) from patients with RA were stimulated with anti-CD3, and T cell proliferation was assessed by flow cytometry, according to CFSE dilution, on day 5 in RA T cells treated with IgG1 (control) or PDL-1.Fc (0.1 μg/ml). The proportion of undivided T cells was determined. **B,** PB T cells from patients with RA were stimulated with anti-CD3/PDL-1.Fc, and after 48 hours, levels of interferon-γ (IFNγ) were measured in the culture supernatants. PD-1 crosslinking significantly reduced IFNγ production. Bars show the mean and SEM in 4 samples. **C,** CFSE-labeled PB T cells from patients with RA were stimulated in culture medium supplemented with 15% RA SF or RA serum. RA SF reversed the PD-1–mediated inhibition of T cell proliferation. Bars show the mean ± SEM of 4 independent experiments using 3 different RA SF samples. **D,** Paired SF and PB T cells from patients with RA were stimulated with anti-CD3/PDL-1.Fc, and T cell proliferation, assessed by ^{3}H-thymidine incorporation, and IFNγ production in the culture supernatants were compared between the paired samples. SF T cells, compared with PB T cells, show a reversal of PD-1–mediated T cell inhibition. Bars show the mean and SD. * = $P < 0.05$; *** = $P < 0.001$, versus PB CD4+ T cells.

assess whether PD-1 regulates T cell responses in RA patients, PB CD4+ T cells were activated with plate-bound anti-CD3 mAb and PDL-1.Fc to crosslink PD-1, and the production of IFNγ and extent of T cell proliferation were assessed following 48 hours and 96 hours of stimulation, respectively. Using CFSE-labeled T cells, we found that PD-1 crosslinking resulted in significant suppression of T cell proliferation in RA patients (Figure 4A), which was comparable with that in OA patients and healthy controls (results not shown). Moreover, PD-1 activation by PDL-1.Fc caused a dose-dependent decrease in anti-CD3–induced IFNγ production by RA CD4+ T cells (Figure 4B).

RA is characterized by chronic ongoing T cell activation within the joints, resulting in joint destruction and disability. 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 (34,35). To explore whether the suppressive function of PD-1 is abrogated within the rheumatoid joint inflammatory milieu, we incubated CFSE-labeled PB CD4+ T lymphocytes from RA patients with 15% RA SF and measured the effect of PD-1 crosslinking on cell proliferation. Incubation with RA SF significantly reversed the PD-1–mediated suppression of T cell proliferation as compared with that in cultures with RA serum, especially after treatment of the cells with PDL-1.Fc at 0.1 μg/ml (mean ± SEM proportion of undivided T cells on day 5, 65.1 ± 4.7% in RA SF–treated cultures versus 81.2 ± 7.8% in RA serum–treated cultures [n = 4 experiments]; $P = 0.028$ by paired *t*-test) (Figure 4C).

We next examined whether SF CD4+ T cells have normal PD-1 function, and compared the inhibitory function of PD-1 in paired PB and SF samples from RA patients by measuring the extent of T cell proliferation and level of IFNγ production. Activation of PD-1 by plate-bound PDL-1.Fc resulted in suppression of anti-CD3–induced T cell proliferation, which was less pronounced in SF CD4+ T cells than in PB CD4+ T cells, especially at the lowest concentration of PDL-1.Fc (0.1 μg/ml) (mean ± SD inhibition of proliferation 55 ± 10% in PB versus 34 ± 11% in SF; $P = 0.022$ by paired *t*-test) (Figure 4D). At optimal PDL-1.Fc concentrations (5 μg/ml), inhibition of anti-CD3–induced proliferation was fully restored in both SF T cells and PB T cells (inhibition of proliferation 83 ± 9% versus 95 ± 3%; $P = 0.076$) (results not shown).

PD-1–mediated suppression of IFNγ production was abrogated to a greater extent in SF CD4+ T cells compared with PB CD4+ T cells from RA patients, at the suboptimal PDL-1.Fc concentration (0.1 μg/ml)

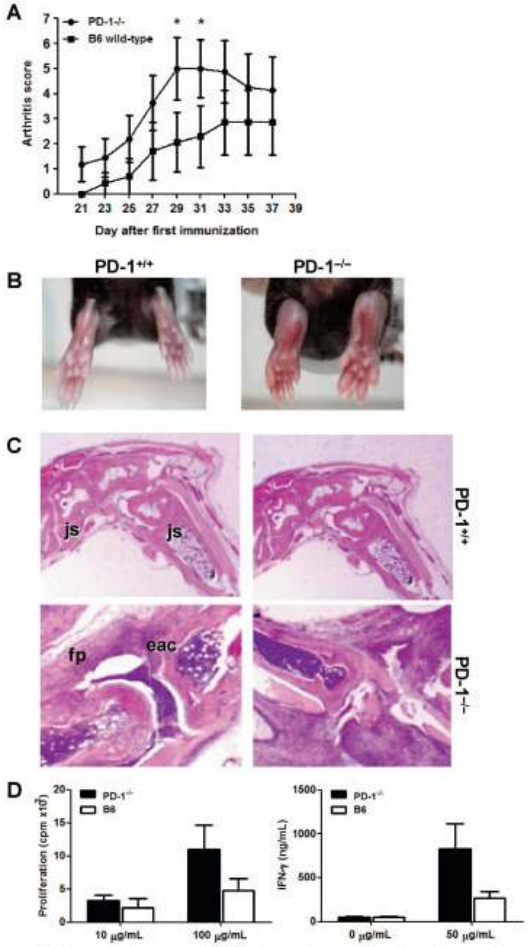


Figure 5. Increased susceptibility to and severity of collagen-induced arthritis (CIA) in C57BL/6 mice deficient in the programmed death 1 gene (*PD-1*$^{-/-}$). *PD-1*$^{-/-}$ and wild-type (*PD-1*$^{+/+}$) C57BL/6 mice were immunized with type II chicken collagen (CII) in Freund's complete adjuvant, and after day 21, disease severity was scored by visual inspection of the mouse paws. **A,** Increased severity of CIA in *PD-1*$^{-/-}$ mice compared with wild-type littermates. * = $P < 0.05$ versus wild-type. Bars show the mean ± SEM of 5 mice per group. **B,** Representative findings of inflammation in the fore paws and hind paws of *PD-1*$^{-/-}$ mice compared with wild-type mice 40 days after immunization. **C,** Hematoxylin and eosin staining of the mouse pedal joints. Wild-type mice (**top**) had no signs of joint tissue inflammation, with even and clear joint space (**js**) and smooth articular cartilage, while *PD-1*$^{-/-}$ mice (**bottom**) had severe fibrovascular synovial and periarticular proliferation (**fp**) and erosion of articular cartilage (**eac**) (original magnification × 40). **D,** T cell proliferation and interferon-γ (IFNγ) production in *PD-1*$^{-/-}$ mice compared with wild-type (B6) mice. Mice were immunized with CII, and 10 days later, their inguinal lymph node cells were harvested and stimulated with different doses of CII. Compared with T cells from wild-type mice, *PD-1*$^{-/-}$ T cells showed increased proliferation and increased IFNγ production ($P <$ 0.05). Bars show the mean and SEM of 4 mice per group. Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org.

(inhibition of IFNγ production 53 ± 8% in PB versus 31 ± 9% in SF; $P = 0.003$ by paired *t*-test) (Figure 4D). Higher PDL-1.Fc concentrations (0.5 μg/ml) resulted in comparable inhibition of IFNγ production in PB and SF CD4+ T cells. Taken together, these data suggest that within the inflammatory milieu of the rheumatoid joint, RA T cells exhibit impaired PD-1–mediated inhibition in the presence of suboptimal, but not optimal, concentrations of PDL-1.

Susceptibility of PD-1–knockout mice to CIA and to the development of severe disease. Our results in RA patients indicated that PD-1/PDL-1 expression is upregulated and this may play a role in regulating T cell activation within the inflamed joint. To directly assess the significance of PD-1/PDL-1 in arthritis, CIA was induced in mice deficient in *PD-1*. Our hypothesis was that *PD-1* deficiency would result in disturbed T cell tolerance and increased prevalence and/or severity of CIA. To this end, we used wild-type and *PD-1*$^{-/-}$ mice bred on the autoimmune-resistant C57BL/6 strain rather than on the susceptible DBA/1J strain. Mice were immunized with CII in CFA at the base of the tail on days 0 and 21.

Consistent with the findings in previous studies (33), 36% of wild-type B6 mice developed CIA of mild-to-moderate severity (mean ± SEM maximum arthritis score 2.3 ± 1.2; n = 14). In contrast, 73% of *PD-1*$^{-/-}$ mice developed arthritis ($P = 0.028$ versus wild-type mice) with severe joint inflammation (maximum arthritis score 5.0 ± 1.2 [n = 16]; $P = 0.040$ versus wild-type mice) early in the course of CIA (on day 19) (Figure 5A), as evidenced by marked swelling and erythema of the hind paws and fore paws. Sites of inflammation included the wrist and ankle and extended distally through the limb and digits (Figure 5B).

We next examined the histologic features of the pedal joints in mice with CIA. Wild-type B6 joints had minimal or no signs of tissue degeneration and inflammation, whereas most *PD-1*$^{-/-}$ B6 mice had severe lesions of extensive fibrovascular and proliferative synovitis, composed of abundant fibroblasts, hypertrophic synoviocytes, and infiltration of inflammatory cells (Figure 5C), which extended into the joint space. In severely affected joints, there was moderate-to-severe cartilage destruction and marked remodeling of bone. Often, the fibrovascular proliferation and inflammation extended into the periarticular connective tissue and adjacent musculature.

Enhanced anti-CII T cell responses in the immunized *PD-1*$^{-/-}$ mice. To analyze antigen-specific T cell responses in the mice with CIA, we immunized the mice with CII, and 10 days later, their inguinal LNCs were harvested and were stimulated with different doses of

CII. T cells from *PD-1*$^{-/-}$ mice exhibited increased proliferation in response to CII as compared with T cells from wild-type mice (^{3}H-thymidine incorporation in assays with 100 μg/ml CII, mean ± SEM 10,949 ± 3,673 counts per minute versus 4,730 ± 1,786 cpm; $P = 0.144$) (Figure 5D). Moreover, stimulation with CII (50 μg/ml) resulted in production of IFNγ (Figure 5D) and IL-17 (results not shown), and the levels of these cytokines were also significantly higher in *PD-1*$^{-/-}$ T cells compared with wild-type T cells (mean ± SEM 833 ± 281 ng/ml versus 268 ± 76 ng/ml for IFNγ and 134 ± 18 pg/ml versus 50 ± 11 pg/ml for IL-17; $P < 0.05$ for each).

Since PD-1 is also expressed by activated B cells, *PD-1* deficiency could affect the production of anti-CII antibodies in *PD-1*$^{-/-}$ mice with CIA. To better characterize the immune mechanisms underlying the susceptibility of *PD-1*$^{-/-}$ mice to CIA, we measured anti-CII IgG production in the mouse serum (on day 39 postimmunization). Levels of IgG antibodies to anti-CII in both strains (*PD-1*$^{-/-}$ and wild-type mice) were comparable (results not shown), indicating that the increased susceptibility to and severity of CIA in *PD-1*$^{-/-}$ mice is predominantly due to aberrant T cell activation rather than to an effect on B cell–mediated autoantibody production.

Amelioration of CIA by administration of PDL-1.Fc. To directly assess the role of therapeutic modulation of PD-1 in inflammatory arthritis, CIA was induced in wild-type B6 mice, followed by intraperitoneal injection with either soluble murine PDL-1.Fc fusion protein or PBS as control. PDL-1.Fc has been shown to crosslink PD-1 in vivo (31,32) and our hypothesis was that PD-1 activation would deactivate T cells and thus inhibit the development of CIA. Indeed, PDL-1.Fc–treated mice developed less severe arthritis (mean ± SEM arthritis score on day 35 postimmunization, 1.8 ± 0.6 versus 2.5 ± 0.7 in control mice; n = 5 in each group) (Figure 6A), with the effect being more pronounced within the male subpopulation (results not shown). Antigen-specific T cell responses in the mice with CIA were analyzed, and T cells from control mice exhibited increased proliferation in response to CII as compared with T cells from PDL-1.Fc–treated mice (mean ± SEM induction of proliferation [expressed as the change in cpm] 9,594 ± 2,147 versus 4,712 ± 2,256; n = 3 in each group) (Figure 6B). These results further support a role for PD-1 in the regulation of anti-CII T cell responses and the development of CIA.

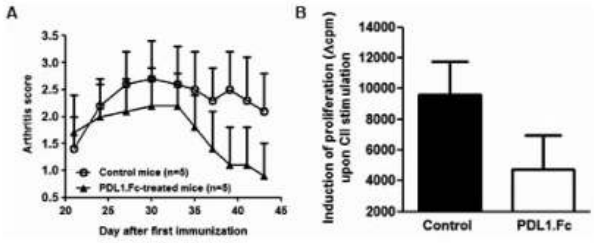


Figure 6. Amelioration of collagen-induced arthritis (CIA) by PDL-1.Fc treatment in C57BL/6 mice. CIA was induced with type II chicken collagen (CII) in wild-type C57BL/6 mice, followed by intraperitoneal injection of mouse PDL-1.IgG2a fusion protein, or phosphate buffered saline as control, at 0.1 mg/mouse on days 0, 2, 3, 5, and 10 postimmunization. **A,** Disease severity was scored as previously described. PDL-1.Fc–treated mice demonstrated decreased susceptibility to and severity of CIA compared with control littermates. Bars show the mean and SEM in 5 mice per group. **B,** CII-specific T cell responses in mice with CIA were assessed in inguinal lymph node cells stimulated with CII (100 μg/ml). Induction of proliferation (expressed as the change in counts per minute) was lower in PDL-1.Fc–treated mice than in control mice (mean ± SEM Δcpm 9,594 ± 2,147 versus 4,712 ± 2,256; n = 3 independent experiments).

DISCUSSION

In this study, we provide evidence to support a key role for the inhibitory PD-1/PDL-1 pathway in regulating T cell function in RA. PD-1/PDL-1 is up-regulated in the synovium of RA patients, and PD-1 inhibits RA SF T cell proliferation under optimal, but not suboptimal, concentrations of PDL-1.Fc. To our knowledge, this is the first study to examine the role of PD-1/PDL-1 in the CIA model of RA. We found that in these mice, PD-1 is a potent regulator of T cell responses, and *PD-1*$^{-/-}$ mice demonstrate increased susceptibility to and severity of arthritis. Importantly, PDL-1.Fc treatment ameliorates anti-CII T cell responses and inhibits the development of CIA.

In accordance with the results of other studies (36,37), we observed enhanced expression of PD-1 in RA synovial T lymphocytes, indicating that PD-1/PDL-1 interactions may be involved in the regulation of T cell effector function at the site of inflammation. PD-1 up-regulation most likely reflects the ongoing activation due to continuous antigen stimulation of SFMCs, as indicated by the correlation of PD-1 expression with the histologic degree of synovial inflammation (Figure 1C). Accordingly, the synovial membrane of RA patients contains CD4+ T cells with an activated/memory phenotype (38). In our stimulation experiments, RA SF T cells had a decreased capacity to further up-regulate PD-1, probably due to exhaustion caused by the chronic inflammation in the joint. Alternatively, up-regulation of PD-1 might be compensatory for the well-described overexpression of several costimulatory molecules, such as CD80/CD86 and ICOS, in RA synovium (39–44).

Consistent with its role in maintaining self tolerance, PD-1 regulates T cell function only at suboptimal

conditions of T cell receptor activation and CD28 costimulation (32,34). We found that PD-1 activation through plate-bound PDL-1.Fc could efficiently inhibit anti-CD3–induced PB T cell proliferation and IFNγ production in RA patients. However, the outcome of PD-1 activation is also affected by factors such as cytokines, the level of costimulation, and TLR signaling (34). It is conceivable that T lymphocytes in the rheumatoid joint are exposed to an inflammatory milieu that renders them hyperreactive and resistant to PD-1 activation.

To explore this hypothesis, we stimulated PB CD4+ T cells from RA patients with anti-CD3 and PDL-1.Fc, and RA SF was added to the cultures to evaluate its effects on PD-1 function. RA SF inhibited PD-1–mediated suppression of T cell proliferation at suboptimal, but not optimal, doses of PDL-1.Fc (Figure 4C). We also assessed the function of PD-1 in paired PB and SF CD4+ T lymphocytes from RA patients. Although PDL-1.Fc could efficiently inhibit anti-CD3–induced proliferation and IFNγ production in PB T lymphocytes, SF T lymphocytes required higher concentrations of PDL-1.Fc to achieve the same level of inhibition. This suggests that, in spite of higher PD-1 expression, RA SF T lymphocytes are relatively resistant to PD-1–mediated suppression. This finding, in conjunction with the results from our immunohistochemical study showing expression of PD-1 by T lymphocyte aggregates and PDL-1 by macrophages infiltrating the rheumatoid synovium, indicates that synovial PDL-1 concentrations might not be adequate to effectively down-regulate T cells. This is further supported by the fact that expression of PDL-1 was comparable between RA and OA patients. Nonetheless, in the presence of excess PD-1 stimulation, as in the case of exogenous administration of PDL-1 fusion protein, RA SF T lymphocytes may be efficiently inhibited.

CIA is an established model of RA, and its development is dependent on CII-reactive CD4+ T cells infiltrating the rheumatoid synovium and producing inflammatory cytokines. Various B7 costimulatory molecules, such as CD28/ICOS/B7h, have been implicated in the pathogenesis of CIA; absence or blocking of either of these molecules results in the amelioration of arthritis and CII-mediated immune responses (11,12). This study demonstrates an important role for the negative costimulator PD-1 in CIA, in that *PD-1*$^{-/-}$ C57BL/6 mice were more susceptible to CIA, had higher arthritis severity scores, and had more extended histopathologic lesions in the affected joints as compared with their wild-type littermates. T cell proliferative responses to CII and production of IFNγ and IL-17 were significantly increased in *PD-1*$^{-/-}$ mice, whereas production of IL-10 and that of anti-CII IgG were not affected. These results suggest that PD-1 regulates the Th1/Th17 pathway rather than the Th2 or humoral responses against CII. Our findings are similar to those described in experimental autoimmune encephalomyelitis, in which *PD-1*$^{-/-}$ T cells produced increased amounts of IFNγ and IL-17 in recall responses to myelin antigen (45).

Modulation of T cell costimulatory pathways has been used to treat CIA, and costimulation blockade with CTLA-4Ig is an effective therapy in patients with severe RA (46). Based on our findings that PD-1/PDL-1 regulates T cell responses in human and murine RA, we treated mice with CIA, after arthritis induction, with PDL-1.Fc fusion protein to activate PD-1. PDL-1.Fc treatment resulted in reduced severity of CIA, which was associated with suppressed anti-CII T cell proliferative responses. Thus, targeting PD-1/PDL-1 represents a potential therapeutic option in RA (47). PD-1 activation mediated by PDL-1.Fc has been shown to inhibit T cell–dependent pathologic immune responses and prolong allograft survival in experimental transplantation models (31,48,49).

In summary, this study delineates the role of the negative costimulatory pathway PD-1/PDL-1 in the homeostatic control of inflammation in the rheumatoid joint. PD-1/PDL-1 is up-regulated in the synovium of patients with active RA and regulates T cell responses in both human and murine RA, emphasized by the enhanced susceptibility to and severity of CIA in *PD-1*–deficient mice. Importantly, synovial T cells from RA patients are inhibited by optimal PD-1 crosslinking, and PD-1 activation with PDL-1.Fc ameliorates CIA, providing an additional therapeutic strategy to deactivate pathogenic T cells in RA.

ACKNOWLEDGMENTS

We wish to thank Christianna Choulaki, PhD, Magda Nakou, PhD, Eleni Koutala, BSc, Melanie Rittirich, BSc, Melina Kavousanaki, Margriet Vervoordeldonk, PhD (Academic Medical Center/University of Amsterdam, The Netherlands), and Xanthi Kranidioti, PhD (Fleming Institute, Greece) for technical assistance, as well as Charalampos Linardakis, MD and Eva Choustoulaki, RN for help in collecting blood samples. We also acknowledge Dr. G. Freeman (Harvard Medical School) for providing the PDL-1.Fc fusion protein.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. 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.

Study conception and design. Raptopoulou, Bertsias, Verginis, Sidiropoulos, Boumpas.

Acquisition of data. Raptopoulou, Makrygiannakis, Verginis, Kritikos, Tzardi, Klareskog, Catrina, Boumpas.

Analysis and interpretation of data. Raptopoulou, Bertsias, Makrygiannakis, Verginis, Sidiropoulos, Boumpas.

REFERENCES

1. Feldmann M, Brennan F, Maini R. Rheumatoid arthritis. Cell 1996;85:307–10.
2. Firestein GS. Evolving concepts of rheumatoid arthritis. Nature 2003;423:356–61.
3. Lundy SK, Sarkar S, Tesmer LA, Fox DA. Cells of the synovium in rheumatoid arthritis: T lymphocytes. Arthritis Res Ther 2007; 9:202.
4. Bennett JC. The role of T lymphocytes in rheumatoid arthritis and other autoimmune diseases. Arthritis Rheum 2008;58 Suppl 2:S53–7.
5. Joosten LA, Abdollahi-Roodsaz S, Heuvelmans-Jacobs M, Helsen MM, van den Bersselaar LA, Oppers-Walgreen B, et al. T cell dependence of chronic destructive murine arthritis induced by repeated local activation of Toll-like receptor–driven pathways: crucial role of both interleukin-1β and interleukin-17. Arthritis Rheum 2008;58:98–108.
6. Klareskog L, Catrina AI, Paget S. Rheumatoid arthritis. Lancet 2009;373:659–72.
7. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol 2007;7:429–42.
8. Holmdahl R, Jansson L, Larsson A, Jonsson R. Arthritis in DBA/1 mice induced with passively transferred type II collagen immune serum: immunohistopathology and serum levels of anti-type II collagen auto-antibodies. Scand J Immunol 1990;31:147–57.
9. Holmdahl R, Klareskog L, Rubin K, Bjork J, Smedegard G, Jonsson R, et al. Role of T lymphocytes in murine collagen induced arthritis. Agents Actions 1986;19:295–305.
10. Chiocchia G, Boissier MC, Ronziere MC, Herbage D, Fournier C. T cell regulation of collagen-induced arthritis in mice. I. Isolation of type II collagen-reactive T cell hybridomas with specific cytotoxic function. J Immunol 1990;145:519–25.
11. Rosloniec EF, Cremer M, Kang A, Myers LK. Collagen-induced arthritis. Curr Protoc Immunol 2001;Chapter 15:Unit 15.5.
12. Williams RO. Collagen-induced arthritis in mice. Methods Mol Med 2007;136:191–9.
13. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. Annu Rev Immunol 2005;23:515–48.
14. Fife BT, Pauken KE, Eagar TN, Obu T, Wu J, Tang Q, et al. Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. Nat Immunol 2009;10: 1185–92.
15. Parry RV, Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi SV, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. Mol Cell Biol 2005;25: 9543–53.
16. Sheppard KA, Fitz LJ, Lee JM, Benander C, George JA, Wooters J, et al. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3ζ signalosome and downstream signaling to PKCθ. FEBS Lett 2004;574:37–41.
17. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 2008;26:677–704.
18. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA, et al. Tissue expression of PD-L1 mediates peripheral T cell tolerance. J Exp Med 2006;203:883–95.
19. Martin-Orozco N, Wang YH, Yagita H, Dong C. Cutting edge: programmed death (PD) ligand-1/PD-1 interaction is required for CD8+ T cell tolerance to tissue antigens. J Immunol 2006;177: 8291–5.
20. Wang J, Yoshida T, Nakaki F, Hiai H, Okazaki T, Honjo T. Establishment of NOD-Pdcd1$^{-/-}$ mice as an efficient animal model of type 1 diabetes. Proc Natl Acad Sci U S A 2005;102: 11823–8.
21. Okazaki T, Honjo T. The PD-1-PD-L pathway in immunological tolerance. Trends Immunol 2006;27:195–201.
22. Alarcon-Riquelme ME. The genetics of shared autoimmunity. Autoimmunity 2005;38:205–8.
23. Bertsias GK, Nakou M, Choulaki C, Raptopoulou A, Papadimitraki E, Goulielmos G, et al. Genetic, immunologic, and immunohistochemical analysis of the programmed death 1/programmed death ligand 1 pathway in human systemic lupus erythematosus. Arthritis Rheum 2009;60:207–18.
24. James ES, Harney S, Wordsworth BP, Cookson WO, Davis SJ, Moffatt MF. PDCD1: a tissue-specific susceptibility locus for inherited inflammatory disorders. Genes Immun 2005;6:430–7.
25. Kong EK, Prokunina-Olsson L, Wong WH, Lau CS, Chan TM, Alarcon-Riquelme M, et al. A new haplotype of PDCD1 is associated with rheumatoid arthritis in Hong Kong Chinese. Arthritis Rheum 2005;52:1058–62.
26. Lee SH, Lee YA, Woo DH, Song R, Park EK, Ryu MH, et al. Association of the programmed cell death 1 (PDCD1) gene polymorphism with ankylosing spondylitis in the Korean population. Arthritis Res Ther 2006;8:R163.
27. Pearce SH, Merriman TR. Genetic progress towards the molecular basis of autoimmunity. Trends Mol Med 2006;12:90–8.
28. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight–joint counts: development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995;38:44–8.
29. Makrygiannakis D, af Klint E, Catrina SB, Botusan IR, Klareskog E, Klareskog L, et al. Intraarticular corticosteroids decrease synovial RANKL expression in inflammatory arthritis. Arthritis Rheum 2006;54:1463–72.
30. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 1999;11:141–51.
31. Gao W, Demirci G, Strom TB, Li XC. Stimulating PD-1-negative signals concurrent with blocking CD154 co-stimulation induces long-term islet allograft survival. Transplantation 2003;76:994–9.
32. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol 2001;2:261–8.
33. Inglis JJ, Criado G, Medghalchi M, Andrews M, Sandison A, Feldmann M, et al. Collagen-induced arthritis in C57BL/6 mice is associated with a robust and sustained T-cell response to type II collagen. Arthritis Res Ther 2007;9:R113.
34. Bennett F, Luxenberg D, Ling V, Wang IM, Marquette K, Lowe D, et al. Program death-1 engagement upon TCR activation has distinct effects on costimulation and cytokine-driven proliferation: attenuation of ICOS, IL-4, and IL-21, but not CD28, IL-7, and IL-15 responses. J Immunol 2003;170:711–8.
35. Zhong X, Bai C, Gao W, Strom TB, Rothstein TL. Suppression of expression and function of negative immune regulator PD-1 by certain pattern recognition and cytokine receptor signals associated with immune system danger. Int Immunol 2004;16:1181–8.
36. Hatachi S, Iwai Y, Kawano S, Morinobu S, Kobayashi M, Koshiba M, et al. CD4+ PD-1+ T cells accumulate as unique anergic cells

in rheumatoid arthritis synovial fluid. J Rheumatol 2003;30: 1410–9.
37. Wan B, Nie H, Liu A, Feng G, He D, Xu R, et al. Aberrant regulation of synovial T cell activation by soluble costimulatory molecules in rheumatoid arthritis. J Immunol 2006;177:8844–50.
38. Panayi GS, Lanchbury JS, Kingsley GH. The importance of the T cell in initiating and maintaining the chronic synovitis of rheumatoid arthritis [editorial]. Arthritis Rheum 1992;35:729–35.
39. Liu MF, Kohsaka H, Sakurai H, Azuma M, Okumura K, Saito I, et al. The presence of costimulatory molecules CD86 and CD28 in rheumatoid arthritis synovium. Arthritis Rheum 1996;39:110–4.
40. Namekawa T, Wagner UG, Goronzy JJ, Weyand CM. Functional subsets of CD4 T cells in rheumatoid synovitis. Arthritis Rheum 1998;41:2108–16.
41. Shimoyama Y, Nagafuchi H, Suzuki N, Ochi T, Sakane T. Synovium infiltrating T cells induce excessive synovial cell function through CD28/B7 pathway in patients with rheumatoid arthritis. J Rheumatol 1999;26:2094–101.
42. Berner B, Wolf G, Hummel KM, Muller GA, Reuss-Borst MA. Increased expression of CD40 ligand (CD154) on CD4+ T cells as a marker of disease activity in rheumatoid arthritis. Ann Rheum Dis 2000;59:190–5.
43. Okamoto T, Saito S, Yamanaka H, Tomatsu T, Kamatani N, Ogiuchi H, et al. Expression and function of the co-stimulator H4/ICOS on activated T cells of patients with rheumatoid arthritis. J Rheumatol 2003;30:1157–63.
44. Ruth JH, Rottman JB, Kingsbury GA, Coyle AJ, Haines GK III, Pope RM, et al. ICOS and B7 costimulatory molecule expression identifies activated cellular subsets in rheumatoid arthritis. Cytometry A 2007;71:317–26.
45. Carter LL, Leach MW, Azoitei ML, Cui J, Pelker JW, Jussif J, et al. PD-1/PD-L1, but not PD-1/PD-L2, interactions regulate the severity of experimental autoimmune encephalomyelitis. J Neuroimmunol 2007;182:124–34.
46. Goeb V, Buch MH, Vital EM, Emery P. Costimulation blockade in rheumatic diseases: where we are? Curr Opin Rheumatol 2009;21:244–50.
47. Vincenti F, Luggen M. T cell costimulation: a rational target in the therapeutic armamentarium for autoimmune diseases and transplantation. Annu Rev Med 2007;58:347–58.
48. Dudler J, Li J, Pagnotta M, Pascual M, von Segesser LK, Vassalli G. Gene transfer of programmed death ligand-1.Ig prolongs cardiac allograft survival. Transplantation 2006;82:1733–7.
49. Ozkaynak E, Wang L, Goodearl A, McDonald K, Qin S, O'Keefe T, et al. Programmed death-1 targeting can promote allograft survival. J Immunol 2002;169:6546–53.