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The role of PIM kinases in human and mouse CD4+ T cell activation and inflammatory bowel disease

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ABSTRACT

PIM kinases are a family of three serine/threonine kinases expressed following T cell activation. Using potent selective small molecule antagonists of PIM-1/3 kinases, we demonstrate a potential role for these enzymes in naïve and effector CD4+ T cell activation. PIM-1/3 inhibition prevented CD4+ T cell proliferation by inducing a G0/G1 cell cycle arrest without affecting cellular survival. In the absence of PIM-1/3 kinase activity, naïve CD4+ T cells failed to fully differentiate into effector cells both *in vitro* and *in vivo*. Therapeutic dosing of a PIM-1/3 inhibitor was efficacious in a CD4+ T cell-mediated model of inflammatory bowel disease suggesting that PIM-1 and PIM-3 kinase activity contributes to sustained disease severity. These results demonstrate that PIM-1/3 kinases have an important role in CD4+ T cell responses and inhibition of this activity may provide a therapeutic benefit in T cell-mediated diseases.

1. Introduction

In addition to their physiological roles in host protection, T lymphocytes play an important role in transplant rejection, autoimmune and inflammatory diseases. Therapeutics preventing T cell activation, trafficking or cytokine production have shown efficacy in a variety of autoimmune and inflammatory diseases pre-clinically and clinically. Thus, it is of interest to identify signal transduction pathways involved in T cell activation which could be targeted by small molecule drugs.

PIM kinases are a family of 3 highly conserved serine/threonine kinases expressed in immunological organs, including the bone marrow, fetal liver, thymus and spleen, as well as non-hematopoietic tissues such as oral epithelia, prostate, and hippocampus [1]. Unlike many kinases whose activity is regulated by phosphorylation, activity of PIM kinases is regulated at the level of expression by transcriptional, post-transcriptional, translational, and post-translational mechanisms [2–7]. PIM-1 expression has been reported to be induced by a variety of Jak/Stat-utilizing cytokines, hormones (prolactin, and erythropoietin) and mitogens (PMA and ConA) [8]. In addition, PIM-1 has been reported to be expressed upon T and B cell antigen receptor engagement [9,10]. The over-expression of PIM-1 and 2 kinases has been reported in various human lymphomas, leukemias, and prostate cancers [11–13], resulting in phosphorylation of proteins mediating cellular survival, differentiation, proliferation, and oncogenic transformation [14,15]. In addition, an anti-apoptotic function of PIM-1 has been described in IL-3- and IL-5-induced survival of basophils and eosinophils respectively [2,16,17]. The expression and functionality of PIM-2 and PIM-3 is less extensively characterized.

Studies of T cell-specific over-expression of PIM-1 revealed a role for this kinase in T cell development. Transgenic expression of the PIM-1 protein is sufficient to bypass a cell cycle arrest that occurs between the double negative (DN) to double positive (DP) transition in the thymus resulting in an increased percentage of double positive T cells in wild-type and Rag-deficient thymocytes suggesting that expression of PIM-1 during thymic development can compensate for defective pre-TCR CD3 signaling [18–20]. Mature T cells from PIM-1,2,3 triple-deficient mice exhibited reduced proliferative responses to anti-CD3 and IL-2 stimulation as a result of a failure to enter S phase, without an increase in cell death [21]. Although it has been reported that CD4+ T cells from PIM deficient animals have defects in proliferation, it is unclear if this effect is driven by potential abnormalities in thymic development since blockade of PIM kinase activity may affect the ability of the TCR to properly signal during T cell maturation.

To investigate the role of PIM kinases in mature CD4+ T cells independent of their role in thymic development, selective small

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molecule antagonists of PIM-1/3 kinases were utilized *in vitro* and *in vivo* revealing a role for these kinases in the activation and differentiation of CD4+ T cells. Specifically, PIM-1/3 kinase activity was required for naïve CD4+ T cell proliferation and differentiation, as inhibition of these kinases in naïve T cells induced a G0/G1 cell cycle arrest resulting in the inability to generate effector T cells. In addition, PIM-1/3 kinases have a role in proliferation of effector T cells, but are differentially involved in cytokine production from Th1, Th2, and Th17 effectors. The importance of PIM-1/3 kinases for effector CD4+ T cell function was confirmed by showing that therapeutic administration of a PIM-1/3 inhibitor decreased colon inflammation in the CD4+ CD45RBhi adoptive transfer model of inflammatory bowel disease. These results support a role for PIM-1/3 kinases in CD4+ T cell responses and suggest that inhibition of this activity may provide a therapeutic benefit in T cell-mediated diseases.

2. Materials and methods

2.1. Characterization of PIM inhibitors

Cellular IC50's for PIM inhibition were determined by assessing Ser112 phosphorylation of transiently transfected BAD substrate in HEK cell lines engineered to express PIM-1, PIM-2 or PIM-3. Cellular IC50's (in nM) for AR448254 and AR452530 are reported in the table below:

	PIM-1	PIM-2	PIM-3
AR448254	150	>10000	500
AR452530	1000	>20000	2000

With minor exceptions (~50% inhibition of Haspin, CAMKII) these compounds were selective when tested at 1 μM against 230 kinases in enzymatic assays (Millipore panel). Due to superior pharmacokinetics of AR452530 over AR448254, all *in vivo* experiments were performed using AR452530.

2.2. Characterization of other pharmacological inhibitors

Mechanistic and functional IC50's for PI-103 and Everolimus were determined by measuring the ability of the compounds to inhibit phosphorylation of S6 or proliferation respectively in PC3 cells. The maximal inhibition of PC3 proliferation in the presence of Everolimus was 50%. Mechanistic IC50's for the Incyte pan JAK inhibitor was determined by measuring the ability of the compound to inhibit TPO, IFNα, or IL-15 induced phosphorylation of STAT5 in human whole blood. The functional IC50 for the Incyte pan JAK compound was determined by measuring its ability to inhibit interferon induced upregulation of MHC Class I on CD4+ PBMC's. Mechanistic IC50's for the p38 and MEK inhibitor was determined by assessing the ability of these compounds to block phosphorylation of HSP27 in Hela cells or ERK in MALME-3 M cells respectively. Functional IC50's for p38 and MEK inhibitors was determined by the ability of the compounds to inhibit LPS or TPA induced TNF in human whole blood 24 h post activation.

Compound	Target	Mechanistic cell IC50 (nM)	Functional cell IC50 (nM)
PI-103	PI3 kinase	24	58
Everolimus	mTORc1	0.5	5
INCB18424	Pan JAK	30–50	65
AR447	p38	40	83
AR509	MEK	2	24

2.3. Mice

All mice were used in accordance with IACUC and Array BioPharma guidelines. C57BL/6 (#000664), DO.11.10 (#003303), $p27^{-/-}$ mice (#002781) and age matched wild-type controls (#000664) were obtained from Jackson Laboratories and used between 8 and 12 weeks of age. BALB/c and Fox Chase SCID mice were obtained from Charles River Laboratories.

2.4. Reagents

2.4.1. Human and murine T cell activation

Murine CD4+ T cell isolation kit (Miltenyi #130-090-860), CD90.2 depletion kit (Miltenyi #130-049-101), anti-CD3 (BD Biosciences #553057), anti-CD28 (BD Biosciences #553294), anti-IL-4 (R&D Systems #AB-404-NA), anti-IFNγ (R&D Systems #AB-485-NA) Human T cell activation beads (Miltenyi #130-091-441), Human CD4+ T cell isolation kit (Miltenyi #130-091-155), Cell-titre glo (Promega #G7570).

2.4.2. Cytokines

All cytokines were obtained from R&D Systems. IL-12 (#419-ML-050/CF), IL-4 (#404-ML-010/CF), IL-6 (#406-ML-025/CF), TGFβ (#303-B2-002), IL-23 (#1887-ML-010/CF).

2.4.3. Measurement of murine cytokines

IL-2 ELISA (R&D Systems #DY402), IFNγ ELISA (R&D Systems #D4485), IL-22 ELISA (R&D Systems #M2200).

Th1/Th2 cytokine kit (Mesoscale Discovery #K15013B-2)

2.4.4. Measurement of human cytokines

IL-2 (Mesoscale Discovery #K151AHC-2) IFNγ (Mesoscale Discovery #K151AEB-2)

2.4.5. Flow cytometry

anti-IL-2 (eBioscience #12-7021), anti-CD25 (R&D Systems #FAB2438A), anti-CD62L (R&D Systems #FAB5761F), anti-CD71 (Miltenyi #130-091-727), anti-CD98 (BD Biosciences #556077), Aposcreen Annexin V Apoptosis Kit-FITC (Southern Biotech #10010-02), Propidium Iodide (BD Biosciences #556463), CellTrace™ CFSE Cell Proliferation Kit (Invitrogen #C34554)

2.4.6. Western blots

anti-IL-2 (BD Biosciences #554375), PI3 kinase inhibitor PI-103 (Calbiochem #528100), mTORc1 inhibitor Everolimus (Sigma Aldrich #07741), Incyte JAK-1,2,3 inhibitor (INCB18424), MEK inhibitor (AR-509) and p38 inhibitor (AR-447) (see http://www.arraybiopharma.com/_documents/Publication/PubAttachment350.pdf), anti-PIM-1 (Santa Cruz #sc13513), anti-PIM-2 (Santa Cruz #sc13514), anti-PIM-3 (Santa Cruz #sc49488), anti-$p21^{Cip1/WAF1}$ (BD Biosciences #556430), anti-$p27^{kip1}$ (Cell Signaling #2552), anti-$p57^{Kip2}$ (Abcam #ab75974), anti-$p16^{INK4a}$ (Abbiotec #250804), anti-ERK (Santa Cruz #sc94), anti-rabbit 800 (Rockland #611-732-127), anti-goat 680 (Invitrogen #A21084), anti-mouse 680 (Invitrogen #A10038).

2.5. Murine CD4+ T cell experiments

2.5.1. Western blot analysis

Purified splenic DO.11.10 CD4+ T cells (5×10^5 cells in 100 ul complete medium in a 96 well plate) were activated with 1 μg/ml immobilized anti-CD3 and 1 μg/ml soluble anti-CD28 in the presence or absence of neutralizing anti-IL-2, or activated in the absence (Th0) or presence of polarizing cytokines: 20 ng/ml IL-12, and 2 μg/ml anti-IL-4 (Th1); 10 ng/ml IL-4 and 2 μg/ml anti-IFNγ (Th2) or