A. Research Proposal *(9 pages)*

INTRODUCTION

The intestine is a unique microenvironment. In addition to the uptake of nutrients and water, the intestine must remain hyporesponsive to commensal organisms, but maintain the ability to respond rapidly and specifically to infections through the activation of a wide variety of immune cells leading to an acute inflammatory response. However, failure to resolve inflammatory processes leads to chronic inflammation. In the intestine, chronic inflammation is manifested as the inflammatory bowel diseases (IBDs), a group of diseases that include ulcerative colitis and Crohn's disease and affect millions worldwide. Dysregulated T cell responses are a characteristic of the IBDs[1,2] and a better understanding of the factors that underlie the development and function of T cells would aid the development of novel treatments. In recent years, it has become increasingly clear that the Vitamin A-derived metabolite retinoic acid (RA) plays an important role in regulating intestinal T cell responses during homeostatic and inflammatory conditions. *Although RA is critical for intestinal T cell responses, the molecular mechanisms downstream of RA signalling that control T cell function are unclear.* We have previously shown that metabolism of RA in T cells is an important aspect of T cell function[3] and have recently identified the transcriptional repressor Hypermethylated in cancer-1 (HIC1, ZBTB29) as a critical RA-dependent regulator of intestinal T cell function[4]. In this proposal, we provide new preliminary data that identifies the RA-HIC1 axis as a central control point in T cell function. Based on our extensive preliminary data, *we hypothesize that HIC1 is a critical regulator of T cell function and will prove to be a novel therapeutic target to treat inflammatory disease*. The **overall aim** is to define the role of RA and HIC1 in T cells during immunity and inflammation.

The **specific aims** are:

1. To define how HIC1 regulates T_H17 cell differentiation.
2. To determine the roles of RA and HIC1 in tissue-resident memory T cell development.
3. To delineate the roles of RA and HIC1 in intestinal T cell homeostasis and disease.

Significance and Innovation. A wide variety of chronic inflammatory diseases are associated with dysfunctional T cell responses. These diseases affect millions of individuals in Australia alone and therapies are limited and costly. Although RA is a critical regulator oif T cells, the molecular mechanisms of RA-dependent regulation of T cell responses are still unclear. Based on our preliminary data, we have proposed experiments that will define how RA and HIC1 regulate T cell differentiation and function by combining powerful molecular genetic tools with unique in vivo models that are all available in our lab. These studies have the potential to identify novel targets for the development of therapeutic interventions to treat these inflammatory diseases.

BACKGROUND

Intestinal immune system: A fine balance. The intestinal immune system has evolved to tolerate commensal organisms and food antigens while maintaining the ability to rapidly respond to diverse infectious organisms. To regulate this balance, specialised subpopulations of tissue-resident immune cells populate the intestinal lamina propria (LP) and intraepithelial compartment. The intestinal microenvironment is enriched for $CD4^+$ T helper 17 (T_H17) and regulatory T (T_{reg}) cells, group 3 innate lymphoid cells (ILC3s) and multiple macrophage and DC subsets in the LP, as well as long-lived, $CD8^+$ intraepithelial lymphocytes (IELs), that together promote intestinal immune homeostasis. However, when this balance is disrupted, chronic inflammation can develop, leading to inflammatory bowel diseases (IBDs). IBDs are a group of chronic intestinal inflammatory diseases that include ulcerative colitis (UC) and Crohn's disease (CD). Australia has one of the world's highest rates with approximately 80,000 people suffering from IBD (~0.4% of the population)[5]. IBDs are thought to occur as a result of a complex interplay between host genetics and environmental factors such as infection or the composition of the intestinal microbiota, which then leads to chronically dysregulated intestinal T cell responses[1].

RA and immune homeostasis. RA is a vitamin A metabolite that is enriched in the intestine and is produced primarily by a subset of dendritic cells (DCs) that express the marker CD103 ($CD103^+$ DCs). In T cells, RA acts via the RA receptor-α (RARα) to induce expression of intestinal homing molecules CCR9 and α4β7 integrin, inhibit T_H17 cell responses, enhance T_{reg} cell development, promote IgA-producing B cells and control T cell homeostasis in the intestine[6]. However, the function of RA in immunity and inflammation is controversial. In addition to its role in the steady state regulation of intestinal immunity, several studies have shown that inflammatory cues including infection, adjuvants and inflammatory cytokines synergize with localized peripheral production of RA to promote proinflammatory responses and RARα-dependent effector T cell differentiation[7–9]. Indeed, vitamin A deficiency results in impaired vaccine-induced intestinal immune responses in mice and humans[10]. Further, RA is also important for lineage integrity of effector T_H1 cells by regulating expression of the master regulatory transcription factors TBX21 and RORγt[11]. Thus, RA has distinct and opposing roles in T cell homeostasis and activation. However, despite these important functions, the molecular mechanisms regulating the effects of RA on T cells remain unclear.

HIC1 is a ZBTB transcriptional repressor. The POZ and Kruppel/Zinc Finger and Broad complex, Tramtrack, Bric-a-brac (or ZBTB) proteins are part of a large family of over 40 transcription factors that play critical roles in development, differentiation and cancer[12]. ZBTB proteins function primarily as transcriptional repressors by recruiting a variety of co-repressors including histone deacetylases, nuclear corepressors (N-CoR1 and -2), C-terminal binding protein (CtBP), as well as the E3 ubiquitin ligase Cullin 3 via the BTB/POZ domain. In the immune system, ZBTB proteins are key regulators of cellular differentiation and function[12] (**Fig. 1**). For example, B-Cell CLL/Lymphoma 6 (BCL6, ZBTB27) is expressed at high levels in germinal centre B cells[13] and T follicular helper (T_{FH}) cells[14] that together control the germinal centre response. Promyelocytic leukaemia zinc finger (PLZF, ZBTB16) is required for the development of several innate lymphoid cell populations[15,16] and T helper-inducing POZ/Kruppel-Like factor (ThPOK, ZBTB7B) is a master regulator of T_H cell development in the thymus[17,18]. **Hypermethylated in cancer 1** (**HIC1, ZBTB29**) is a member of this family and has been shown to regulate proliferation and p53-dependent survival in a wide range of tumours by repressing expression of target genes. As its name suggests, the *Hic1* gene is epigenetically silenced through DNA methylation in various human cancers[19,20] and it has been proposed that HIC1 function was critical for the function of p53[19]. However, the role of HIC1 in immune cells has not been examined. We have now identified a cell-intrinsic role for HIC1 in the regulation of intestinal T cell responses[4]. This proposal is focused on defining the molecular mechanisms of HIC1-dependent regulation of T cell differentiation and function in the steady-state, as well as during immunity and inflammation.

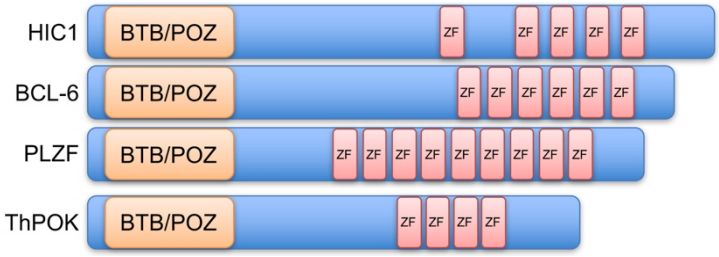

Fig. 1. ZBTB family members are critical regulators of immune cell development and function.

Chronic infection and inflammation are associated with long-lived, tissue-resident T cells. Following infection, $CD4^+$ and $CD8^+$ T cells acquire effector functions including production of cytokines and cytotoxic effector proteins that lead to the killing of pathogens or infected cells. Following clearance of infection, pathogen-specific T cells differentiate into long-lived, quiescent memory cells that respond more quickly and more vigorously to secondary challenge by proliferating and producing effector cytokines. In addition to circulating memory T cells, a subset of memory T cells called tissue-resident memory T (T_{RM}) cells have been described. T_{RM} cells are non-circulating, long-lived, reside primarily in non-lymphoid tissues, express surface markers including CD69 and CD103 and rapidly respond to infection[21]. Both $CD4^+$ and $CD8^+$ T_{RM} cells have been identified in skin, lungs, liver and intestine[21]. Indeed, $CD8^+$ IELs that are specific for

intestinal antigens from commensal organisms and mediate tolerance are uniformly $CD69^+$ $CD103^+$, suggesting naturally occurring T_{RM} cells are important for intestinal homeostasis. However, it is clear that tissue-resident T_{RM} cells are associated with many forms of chronic disease including inflammation of the intestines and lungs[22–24]. In humans, diseases such as psoriasis and mycosis fungoides have been shown to be directly caused by T_{RM} cells and the clinical characteristics of many other chronic diseases including IBDs and asthma suggest a pathogenic role for T_{RM} cells[24]. Thus, T_{RM} cells are critical regulators of not only long-term immunity to infection but also chronic inflammatory diseases and may offer a novel therapeutic target for T cell-mediated inflammation.

Metabolic control of T cell effector and memory development. T cell activation and differentiation is dependent upon cellular proliferation that requires the consumption of considerable amounts of energy. It is becoming clear that metabolic reprogramming during cellular lineage differentiation is a central and critical control point[25]. Quiescent T cells including naive and memory T cells, consume lipids through oxidative phosphorylation for energy. In contrast, activation of T cells leads to the upregulation of glucose transporters and glycolysis, as well as glutamine uptake and oxidation. Certain T cell subsets also employ specific metabolic pathways, including T_{reg} cells (lipid oxidation>glycolysis) and T_H17 cells (glycolysis>lipid oxidation). Mechanistically, nutrient sensors mTOR and AMPK regulate the balance of anabolic and catabolic pathways through transcription factors such as c-Myc and HIF1α[26]. Memory T cells are highly dependent upon fatty acid oxidation for their survival and function. Modulation of fatty acid oxidation by metformin (an AMPK activator and inhibitor of glycolysis) leads to enhanced memory T cell responses following infection[27]. In addition, a recent study has shown that T_{RM} cells are critically dependent upon fatty acid uptake and metabolism for survival[28]. Thus, defining the molecular mechanisms of metabolic programming in T cells is fundamental to understanding the intersection between metabolism and function. In this proposal, we provide new preliminary data placing HIC1 at the intersection of RA signaling, T_{RM} cell generation and metabolic regulation of T cell responses, and describe experiments to define the role of RA and HIC1 in T cell function during health and disease.

AIMS

Aim 1. To define how HIC1 regulates T_H17 cell differentiation.

1.1. What are the molecular interactions of HIC1 in T_H17 cells? HIC1 was first identified as an RA-responsive gene in 2 independent unbiased expression analyses examining the response of T_H cells to RA[11,29], however, no further analysis on the function of HIC1 was examined. To determine the role of HIC1 in T cells, we generated mice with a T cell-specific deletion of HIC1 (*Hic1*$^{\Delta T}$ mice; by crossing *Cd4*-Cre and *Hic1*$^{fl/fl}$ mice). In the absence of HIC1, we found that T cell-intrinsic deletion of HIC1 resulted in an increased frequency of T_H17 cells in vitro and in vivo[4], suggesting that *HIC1 is a negative regulator of T_H17 cell differentiation.* RA has been shown to repress T_H17 cell differentiation[30,31]. However, we found that HIC1 was dispensable for the RA-dependent reduction of T_H17 cell responses in vitro[4]. *Thus, although HIC1 is required to limit T_H17 cell development, this effect is independent of RA, suggesting a novel HIC1-dependent pathway that controls T_H17 cell responses.*

Mechanistically, we and others have found that HIC1 directly interacts with STAT3, a transcription factor that is central to T_H17 cell differentiation[4,32,33]. In the absence of HIC1, we found increased STAT3 binding to the *Il17a* promoter in T_H17 cells, identifying a role for HIC1 in limiting STAT3 binding and thus IL-17A production[4]. We now extend these findings to show that HIC1 interacts with a component of the epigenetic regulatory machinery. We have previously shown that the epigenetic regulator G9a also has a T cell-intrinsic role in limiting T_H17 cell responses[34,35]. G9a is a lysine methyltransferase that mono- and dimethylates lysine residues in histones (H3K9) and other non-histone proteins[36] and has been associated primarily with gene repression[37]. G9a also contains an ankyrin repeat domain that can bind to dimethylated lysine residues, identifying it as one of the few epigenetic modifiers that can bind to its own enzymatic product[38]. Consistent with the similar

phenotypes observed in *Hic1*$^{\Delta T}$ and *G9a*$^{\Delta T}$ mice, we find that HIC1 can directly interact with G9a (**Fig. 2, (1)**). Strikingly, we also find that mutation of the active site (G9aΔSET (**2**) and G9aH/K (**3**)) or the dimethyllysine-binding ankyrin repeats (**not shown**) abolishes the interaction between HIC1 and G9a in transfected 293T cells. Based on these results ***we hypothesize that G9a dimethylates HIC1, binds via ankyrin repeats and generates a transcriptional complex that can regulate T_H17 cell differentiation, linking HIC1-dependent transcriptional repression with epigenetic silencing.***

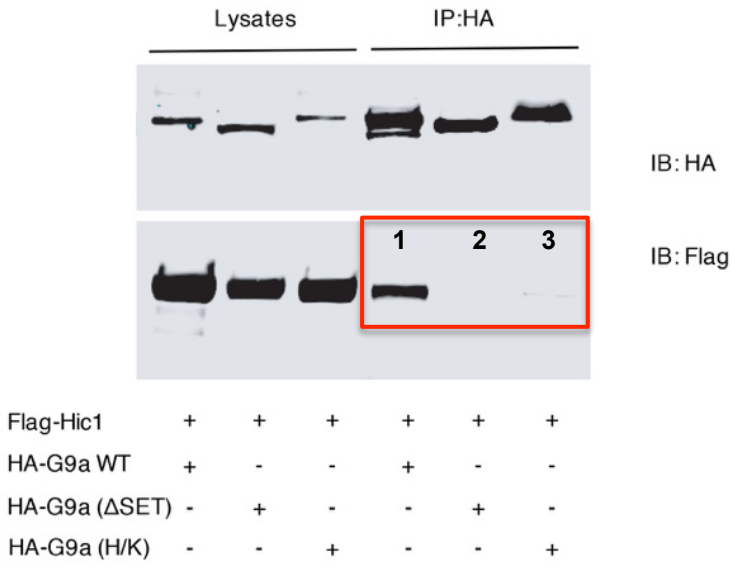

Fig. 2. HIC1 interacts with G9a in a methyltransferase-dependent manner.

To directly test this, we will first examine whether we can detect HIC1 in a complex with both G9a and STAT3 by co-immunoprecipitation studies using commercially available antibodies in T_H17 cells as we have done for HIC1 and STAT3[4]. As our results show that the interaction between G9a and HIC1 is dependent upon the methyltransferase activity and dimethyllysine binding activities of G9a, we hypothesize that G9a dimethylates HIC1 and/or STAT3, which creates a binding site for itself. To directly address this, we will examine if HIC1 and STAT3 are methylated by G9a. We have previously shown using mass spectrometry that a related methyltransferase SETD7 methylates the Hippo pathway member YAP1[39]. We will use our established protocols in collaboration with the Monash Biomedical Proteomics Facility (MBPF) to determine the methylation status of HIC1 and STAT3 in the presence or absence of G9a. We will generate T_H17 cells in vitro from wild-type and G9a-deficient mice, immunoprecipitate HIC1 and STAT3 using commercially-available antibodies, digest with proteases and analyse peptides by mass spectrometry using multiple reaction monitoring (MRM) to look for methylated lysine residues. For STAT3, several potential methylation sites have previously been described[40,41], with lysine residues K49, K140 and K180 identified as potential di- and/or trimethylation sites. In addition to trypsin, we will use a variety of other proteases (Arg-C, Glu-C) to optimize peptide generation to obtain complete coverage of all lysine residues for analysis. We will try to carry out these studies in primary T_H17 cells using native proteins, but if necessary, we will transfect 293T cells with plasmids expressing tagged versions of HIC1, G9a and STAT3 in order to obtain sufficient amounts of immunoprecipitated proteins for MS analysis. Thse studies will all be carried out at the MBPF and will provide fundamental insight into the molecular interactions of HIC1, G9a and STAT3.

Next, we will examine whether interactions between HIC1 and STAT3 are dependent upon G9a and whether HIC1-G9a interactions are STAT3-dependent. To do this, we will activate naive $CD4^+$ T cells isolated from wild type or G9a-deficient or STAT3-deficient mice under T_H17 cell-polarizing conditions for 5 days, immunoprecipitate HIC1 (or STAT3) and immunoblot for STAT3 (or HIC1). Together, these studies will define whether a HIC1-STAT3-G9a complex forms in T_H17 cells, whether G9a-dependent methylation is involved and whether STAT3 is required for the interaction between HIC1 and G9a. As above, we can transfect cell lines deficient in G9a or STAT3 with tagged proteins for analysis. These studies will be carried out in collaboration with **AIA Daniel Gough (Hudson Institute of Medical Research)**, an expert on STAT3 signaling and function. **AIA Gough** will provide STAT3-deficient T cells (isolated from *Stat3*$^{fl/fl}$ mice crossed with *Rag1*-

Cre mice), expertise in experimental design, STAT3-deficient cell lines and can generate plasmids with mutations in the specific lysine residues of STAT3 to allow us to test whether STAT3 methylation is critical for interactions with HIC1 and/or G9a. Together, experiments proposed here will establish a picture of the interactions between HIC1, G9a and STAT3 and will provide the basis for future examination of the role of post-translational methylation of non-histone proteins in cellular signalling and function.

1.2. How does HIC1 regulate the Myc-dependent metabolic gene program? To begin to address how HIC1 regulates T_H17 cell differentiation, we carried out unbiased expression analysis on control and HIC1-deficient T_H17 cells using RNA-Seq. As HIC1 is primarily associated with gene repression, we focused on the genes that were upregulated in the absence of HIC1. Gene set enrichment analysis of the top 325 upregulated genes (Z-score>1.5) identified with high confidence gene sets associated with Myc-dependent regulation of metabolism and cellular proliferation including *Myc targets* (P=1.29 x 10^{-124}), *mTORC1 signaling* (P=1.80 x 10^{-52}), *E2F targets* (P=6.18 x 10^{-46}) and *G2M checkpoint* (P=1.34 x 10^{-33}). Thus, in T_H17 cells, loss of HIC1 is associated with dysregulated gene expression that regulates metabolic reprogramming and proliferation. As several studies have shown that T_H17 cells are highly dependent upon glycolysis for their differentiation[26], and Myc regulates glycolysis and proliferation in effector T cells[42], we next examined if HIC1 deficiency has any effect on metabolic reprogramming during T cell activation. We found that HIC1-deficient T_H17 cells have a severely dysregulated metabolic profile. Analysis of glycolytic activity of T_H17 cells (**Fig. 3**) using a Seahorse metabolic analyzer showed that the absence of HIC1 resulted in heightened basal glycolysis (**1**), as well as increased glycolytic activity (**2**) and glycolytic capacity (**3**). Based on these findings, ***we hypothesize that HIC1 expression is associated with metabolic control of T_H17 cell differentiation and function.*** One obvious possibility is that in the absence of HIC1, Myc levels are increased. However, we failed to detect any changes in the levels of *Myc* mRNA in our RNA-Seq data, suggesting that HIC1 is regulating Myc-dependent pathways post-transcriptionally. To directly address this, we will first examine levels of Myc protein in wild-type and HIC1-deficient T_H17 cells by intracellular staining and immunoblotting. If we detect reduced Myc protein levels, we will next test measure Myc stability using cycloheximide-treated cells and following Myc degradation over time in the absence or presence of HIC1, as well as in the presence or absence of protesome ihibitors (MG132). If we fail to detect changes in Myc protein levels, it is possible that HIC1 is directly regulating Myc-dependent target genes by interacting directly with Myc as we have observed with STAT3. We will carry out co-IP experiments to test whether HIC1 and Myc interact in T_H17 cells.

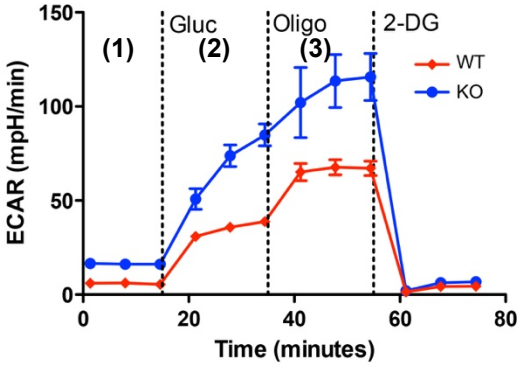

Fig. 3. HIC1-deficient T_H17 cells have dysregulated glycolytic metabolism.

In collaboration with **AIB Zane Andrews (Monash University)**, an expert in metabolic control of cellular function, we will examine expression of the upstream factors that control metabolic reprogramming such as AMPK, HIF1α, LKB1 and mTORC1. We will use established protocols for measuring metabolic function in T cells including analysis of critical signal transduction pathways (*e.g.* AMPK and S6K phosphorylation), measurement of oxygen consumption and extracellular acidification rates (using the Seahorse analyser) and gene and protein expression levels of critical metabolic enzymes and regulators. We will examine the role of HIC1 in T cells following glucose/glutamine withdrawal, metformin or rapamycin treatment, and growth in alternative carbon sources[25,43]. **AIB Andrews** will provide the specific reagents to carry out these studies as well as expertise in experimental design of assays to examine metabolic function. In addition, **AIA Gough** has previously identified a critical role for STAT3 in mitochondrial function[44,45], with STAT3 playing a central role in metabolic reprogramming[46]. Thus, in addition to examining the pathways

that regulate oxidative phosphorylation in HIC1-deficient T cells, we will also examine if HIC1 affects the ability of STAT3 to localize and function at the mitochondria. Together, these studies will determine whether HIC1 regulates metabolic reprogramming in T_H17 cells.

Future studies. Together these studies will begin to dissect the molecular interactions between HIC1, STAT3 and G9a and how they affect Myc-dependent metabolism in T cells. Importantly, a recent study has shown that Myc is a critical regulator of the 'division destiny' of T cells, a measure of the proliferative capacity of activated T cells[47]. Whether Myc is regulating metabolism in T cells to control division destiny, or if RA or HIC1 are potential regulators of the proliferative capacity is unknown. However, our data showing that HIC1 is linked to Myc-dependent metabolism raises the distinct possibility that RA-induced HIC1 may cause Myc-dependent changes in metabolism that could affect division destiny, further linking RA, HIC1 and cellular homeostasis.

Aim 2. To determine the roles of RA and HIC1 in tissue-resident memory T cell development.

2.1. Does HIC1 regulate T_{RM} cell development and homeostasis? We have recently shown that T cell-intrinsic deletion of HIC1 resulted in a significant reduction in the frequency of T cells in the intestine (**Fig. 4**), including a loss of $CD69^+$ $CD103^+$ tissue-resident T_{RM} cells from the LP and intraepithelial compartment[4]. Several studies have used genome-wide expression analyses to identify a genetic signature that is conserved amongst tissue resident cells from diverse sites and cellular origins[49–51]. Interestingly, the majority of the genes in the signature are expressed at lower levels in tissue resident cells than naive or activated circulating immune cells. As HIC1 is a transcriptional repressor, we tested whether RA was driving this gene repression program through HIC1. We activated wild-type or HIC1-deficient T cells in vitro in the absence or presence of RA and analysed global gene expression by RNA-Seq. We focused on 57 genes that have been shown to be significantly downregulated in tissue-resident T_{RM} cells compared to lymphoid resident or circulating T cells[49–51]. Strikingly, expression of 52 of these genes (91%) were down-regulated by RA treatment (**Fig. 5**). Importantly, in the absence of HIC1, the effects of RA were minimal on these genes, demonstrating that RA signaling through HIC1 may be a central regulator of the tissue residency gene program. Based on these results, ***we hypothesize that HIC1-dependent RA***

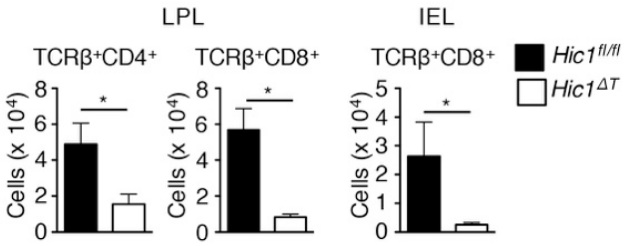

Fig. 4. T cell-specific deletion of HIC1 results in a severe reduction in the number of tissue-resident T cells in the intestine.

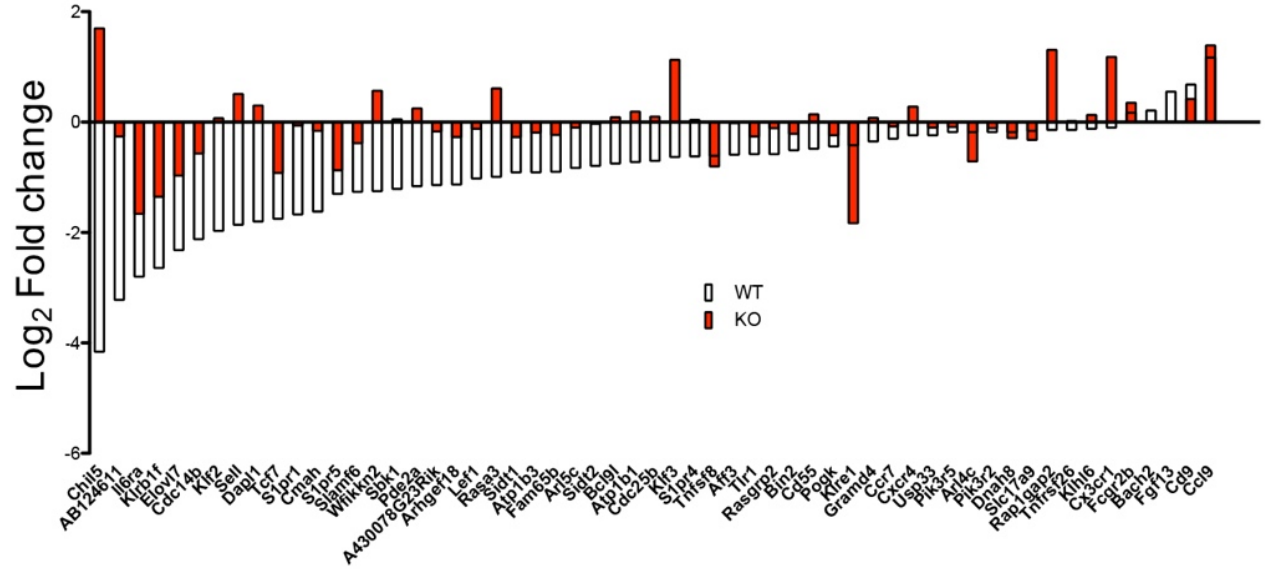

Fig. 5. RA promotes a HIC1-dependent T_{RM} cell gene program. Data represent log2 fold change of RA-untreated/ RA-treated WT or KO T cells.

signaling is critical for the establishment of the tissue-residency gene program in T cells. To directly assess this, we will first use in vitro-derived T_{RM} cells to test whether HIC1 is required for T_{RM} cell differentiation. $CD8^+$ T cells are activated in vitro with antibodies against CD3, CD8, LFA-1 and CD28 for 48 hours, followed by expansion in the presence of IL-2 for 3 additional days. Expanded cells are then transferred intradermally into congenic hosts and T_{RM} cells in the skin are enumerated 14 days later by flow cytometric and immunofluorescent analyses[49,51,52]. In parallel, we will test whether HIC1 is required for the development of T_{RM} cells in vivo. To do this, we will use a viral infection model that induces T_{RM} cells in a wide variety of tissues. Lymphocytic choriomeningitis virus (LCMV) is a viral infection that generates potent acute effector $CD4^+$ and $CD8^+$ T cell responses, resulting in a strong T cell memory response including LCMV-specific $CD8^+$ T_{RM} cells that reside in the intestine, skin and liver. Control *Hic1*$^{fl/fl}$ or *Hic1*$^{\Delta T}$ mice will be infected intraperitoneally with 2 x 10^5 pfu of the Armstrong strain (acute) of LCMV and antigen-specific T cell responses will be analysed in various tissues (spleen, intestine, liver) at days 8 (acute) and 30 (memory) using MHCI and MHCII tetramers (gp_{33-41} and $NP_{396-404}$) and intracellular cytokine staining. We will use intravascular staining (intravenous injection of antibody 3 minutes prior to killing) with fluorescently labelled anti-CD8β (or CD45.2 or Thy1.2 depending on the experiment) to identify T cells that are circulating and not tissue-resident[53]. These studies will define whether HIC1 is required for T_{RM} cell generation.

We may find that the loss of HIC1 will lead to defective primary T cell responses, which will affect the quality and quantity of the memory T cell response and not allow us to accurately compare wild type and knockout mice. Further, it is possible that in addition to affecting the generation of the T_{RM} cell genetic program, HIC1 is critical for maintaining the repression of genes in tissue-resident T_{RM} cells. To address these points, we have generated inducible HIC1-deficient mice (*Hic1*$^{\Delta Ti}$ mice) by crossing *Hic1*$^{fl/fl}$ mice with mice that express a tamoxifen-inducible form of Cre driven by the *Cd4* promoter/enhancer[54] (*Cd4*-Cre^{ERT2} mice; from Dr Thorsten Buch, Munich). Using these mice, we can allow the normal generation of an acute and memory response prior to deletion. We will generate T_{RM} cells following either in vitro activation or LCMV infection and delete HIC1 at a later time point to define whether HIC1 is required for the generation of T_{RM} cells or the maintenance, or both. These studies (and the ones described below) will take place in collaboration with **AIC Laura Mackay (Doherty Institute, University of Melbourne)**, a leader in the T_{RM} cell field. **AIC Mackay** has pioneered studies on T_{RM} cells, defining the developmental requirements and the molecular gene signature of T_{RM} cells, and is the ideal collaborator for this aspect of the proposal.

2.2. What is the role of RA in T_{RM} cell development? Based on our preliminary data showing that RA treatment of T cells in vitro results in upregulation of HIC1 and establishment of the tissue residency gene program, ***we hypothesize that RA will be critical for the induction of the T_{RM} cell gene program in vivo.*** To directly test this hypothesis, we will also examine the role of RA/RARα signalling in T_{RM} cell generation during LCMV infection. We have obtained mice that carry a 'floxed' allele of RARα (*Rara*$^{fl/fl}$ mice) from Dr Yasmine Belkaid (NIAID, NIH, Bethesda, USA) and have begun crossing them to both *Cd4*-Cre mice (*Rara*$^{\Delta T}$ mice) and *Cd4*-Cre^{ERT2} mice (*Rara*$^{\Delta Ti}$ mice). We will infect mice with LCMV as above and analyse both the acute effector stage (d8) and memory stage (d30). We expect that the absence of RARα in T cells will cause a reduction in the effector T cell generation, which will likely impact on memory generation. If this is the case, we will use the inducible *Rara*$^{\Delta Ti}$ mice to not only determine whether RA is required for T_{RM} cell generation (delete post-day 8), but also if RA signalling is required in the tissues to maintain the T_{RM} cell program (delete post-day 30). In parallel studies, we will also block RA signalling using the selective RARα antagonist BMS493[55]. Intraperitoneal injection of BMS493 (up to 14 daily injections of 220 ug/mouse are tolerated well[55]) at different time points will complement our genetic studies and define the role of RA in T_{RM} cell development and maintenance. Together, these experiments will delineate the role of RA and HIC1 in T_{RM} cell development.

Future studies. These exciting studies have the potential to redefine the signals that drive the acquisition of tissue residency in immune cells. As acute and chronic infections have differential requirements for T_{RM} cell development and survival[56], we will also infect *Hic1*$^{\Delta T}$ and *Rara*$^{\Delta T}$ mice with LCMV Clone 13, a well-characterized chronic viral infection. In addition, a recent study has shown that uptake of lipids and lipid metabolism is critical for T_{RM} cell survival[28], and our results linking HIC1 to metabolic reprogramming provide a rationale to examine the role of HIC1 in T_{RM} cell metabolism. Finally, as we found that HIC1 interacts with G9a, we will test if G9a is involved in the epigenetic maintenance of the T_{RM} cell gene program by carrying out the same analyses as above in *G9a*$^{\Delta T}$ mice and *G9a*$^{\Delta Ti}$ mice. These studies will potentially link RA, HIC1 and G9a-dependent epigenetic regulation in the control of T_{RM} cell development and survival.

Aim 3. To delineate the roles of RA and HIC1 in intestinal T cell homeostasis and disease.

3.1. Does HIC1 regulate persistence of T cells in the intestine? Our preliminary data showed that T cell-intrinsic expression of HIC1 was required for T cell homeostasis in the intestine (**Fig. 4**). As our recent study showed that CCR9 or α4β7-dependent migration to the intestine is not impaired in the absence of HIC1[4], ***we hypothesize that T cell migration to the intestine is independent of HIC1 but that retention or survival of T cells in the intestine is HIC1-dependent.*** To address this, we will first treat *Hic1*$^{\Delta Ti}$ mice with tamoxifen (200 mg/kg, orally by gavage daily for 5 days) and analyse the peripheral and intestinal T cell compartments at 1, 2, 4 and 6 weeks post-final tamoxifen treatment. Based on our preliminary results, we envision that we will observe a decline in the frequency and number of T cells specifically in the intestine after deletion of HIC1. To begin to dissect the mechanisms of intestinal T cell loss, we will examine both markers of apoptosis (Annexin-V and PI staining), tissue retention (CD103, CD69, S1PR1 by flow cytometry and qPCR), as well as T cell proliferation (by measuring 8 and 24 hour EdU incorporation rates) at the various time points. As STAT3 has been implicated in inhibiting apoptosis[57,58] and promoting cell proliferation[59,60], it is possible that the loss of HIC1 could lead to heightened STAT3-dependent cell proliferation and increased survival resulting in T cell exhaustion, intestinal egress or activation-induced cell death. However, it is possible that we will find a recovery of T cells in the intestine over time as new T cells replace the lost cells. The turnover rate of intestinal T cells is not clearly defined and these studies will not only provide information about the role of HIC1 in T cell homeostasis but also will help determine the rates of T cell turnover in the intestinal microenvironment.

3.2. How does HIC1 control the development of intestinal inflammation? In addition to regulating T cell homeostasis under non-inflammatory conditions, we have also shown that HIC1 is required for the development of pathogenic T cells in multiple models of intestinal inflammation, including T cell transfer colitis (**Fig. 6**)[4]. Transfer of HIC1-deficient CD4$^+$ CD45RB^{high} CD25^{neg} T_H cells into *Rag1*$^{-/-}$ mice failed to induce inflammation, as measured by weight loss (**Fig. 6A**). As naive T_H cells in spleen and peripheral lymph nodes (the source of the naive donor cells) do not express measurable levels of HIC1, these results suggest that acquisition of HIC1 expression is required for the development of pathogenic T_H cells. Consistent with this, transfer of naive T_H cells isolated from *Hic1*^{Citrine} mice demonstrated that T_H cells found in the inflamed tissues acquire HIC1 expression, while T_H cells in lymphoid tissues do not upregulate HIC1 expression (**Fig. 6B**). This is consistent with recent studies proposing that RA is

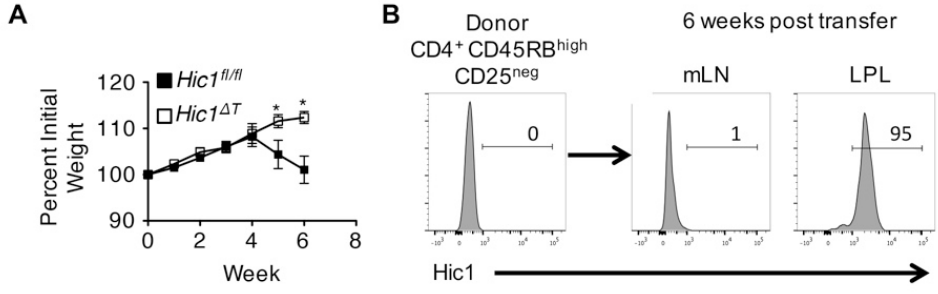

Fig. 6. HIC1 is expressed in pathogenic T cells and is required for the development of intestinal inflammation.

required for optimal effector T cell responses in vivo[7,8,11] and that RA can synergize with pro-inflammatory cytokines to promote intestinal inflammation[63]. To test this, we will transfer naive $CD4^+$ T cells from *Hic1*^{Citrine} mice into vitamin A-deficient mice *Rag1*$^{-/-}$ mice to examine whether host derived RA is required for the upregulation of HIC1 in donor T cells and whether RA is required for the development of disease. Transfer of RARα-deficient T cells as well as *Rag1*$^{-/-}$ mice treated with BMS493 will be used to define the role of RA signalling on HIC1 expression and the development of intestinal inflammation. Finally, we will use naïve T_H cells from the *Hic1*$^{\Delta Ti}$ mice in the T cell transfer colitis model. We will induce HIC1 deletion in mice that have established intestinal inflammation (approximately 4-5 weeks post-transfer) and examine if the pathogenic intestinal T cells are also lost in the absence of HIC1. It is possible that HIC1 deletion will lead to a loss of T cells from the intestine and amelioration of intestinal inflammation. These results would identify HIC1 as a potential therapeutic target to treat intestinal inflammation and suggest that chemical inhibition of HIC1 may provide a novel drug for inflammation in the intestine.

3.3. Is HIC1 expressed in human intestinal T cells? Our preclinical data suggests that HIC1 is an attractive target to modulate intestinal inflammation. However, whether HIC1 is expressed intestinal T cells from humans is unknown. We are collaborating with **AID Edward Giles (Hudson Institute of Medical Research, Monash University)**, a gastroenterologist with extensive experience in isolating T cells from human intestinal biopsies to begin to analyse whether HIC1 is a valid target in human patients. First, we will isolate $CD4^+$ and $CD8^+$ T cells from intestinal biopsies from inflamed and uninflamed tissues of IBD patients. Briefly, 6-8 intestinal biopsies obtained endoscopically will be digested with collagenase type II, separated by Ficoll and $CD4^+$ or $CD8^+$ cells purified by FACS. From experience[61], approximately 50,000 $CD4^+$ T cells per biopsy can be recovered (300,000-400,000 $CD4^+$ T cells per patient). We will examine the T cells from both ulcerative colitis and Crohn's disease patients (10 of each to start), as well as healthy controls, and subjects selected will be chosen to control for disease activity, medication use and other disease and demographic parameters such as disease location, age and gender. **AID Giles** has extensive experience in patient recruitment in IBD, will readily be able to recruit this cohort size, and has the ethics approval for these studies. We will also compare with T cells isolated from peripheral blood. We expect that HIC1 will be specifically expressed in intestinal T cells and will be upregulated in human T cells by RA. These studies will provide the rationale to follow up with HIC1 as a potential target in IBD.

Future studies. These studies will define the role of HIC1 in T cell homeostasis and inflammation in the intestine. We will extend these studies to other models of T cell mediated chronic inflammation at distinct sites such as the lungs. We have extensive experience in house dust mite antigen induced lung inflammation models, which will allow us to test if the role of RA and HIC1 in T cells is a global regulatory mechanism or if it is restricted to the intestine. Further, if we find that HIC1 expression correlates with inflammation in human IBD patients, we will move forward to try and identify small molecules that may prevent HIC1 activity in T cells. The Centre for Drug Candidate Optimisation (CDCO) at the Monash Institute of Pharmaceutical Science is a platform service that aids in the design of screens, provides libraries and high-throughput screens to find candidate molecules for novel targets.

Timeline. This five-year proposal takes advantage of the strengths and expertise of the CI and AIs to ask important and fundamental questions that will generate new information and potentially lead to new ways to limit inflammatory disease or promote immunological memory. The three related yet distinct Aims can be carried out in parallel and the results gained from each Aim will inform the planning and implementation of the other Aims. Although it is impossible to predict exactly when experiments will be completed, all of the mouse strains are in-house and breeding, the technical expertise and equipment is accessible, collaborations and ethics are in place and all the personnel (except potential honours or PhD students to work on specific aspects of this proposal) are identified, allowing us to proceed immediately.

B. References

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