Protein Kinase A regulates GATA-3 dependent Activation of IL-5 Gene Expression in T Helper Lymphocytes

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The hereby submitted thesis was completed from March 2000 until January 2003 at the Department of Molekulare Pathologie, Institute für Pathologie, Bayerische Julius-Maximilians Universität, Würzburg under the supervision of Professor Dr. Edgar Serfling (Faculty of Medicine) and Professor Dr. George Krohne (Faculty of Biology).

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I hereby declare that the submitted dissertation was completed by myself and no other. I have not used any sources or materials other than those enclosed.

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Würzburg, den 04.02.2003 **Mithilesh Kumar Jha**

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1. Introduction

Overview

The immunological protection from infection in a world of pathogens that is forever evolving new variants is largely achieved by adaptive immune responses which involve the activation of naïve lymphocytes upon recognition of foreign antigens. There are two major branches of the adaptive immune responses: humoral immunity and cell-mediated immunity. Humoral immunity involves the production of antibody molecules in response to an antigen and is mediated by B lymphocytes and helper T lymphocytes. Cell-mediated immunity involves the cytotoxic T lymphocytes, macrophages, NK cells, and initiated by the production of cytokines in response to an antigen. The induction of an adaptive immune response begins when a pathogen is ingested by an immature dendritic cell (DC) in the infected tissue. The primary function of DCs is to carry pathogen antigens to peripheral lymphoid organs and present these foreign antigens to T lymphocytes, which are the central regulatory cells of the adaptive immune system. The two major subsets of T lymphocytes had been described: T helper cells, which express the surface protein CD4, and T cytotoxic cells, which express the CD8 marker. Whereas $CD8^+$ T cytotoxic lymphocytes were known to mediate lysis of autologous cells infected by intracellular pathogens, primarily $CD4^+$ T helper cells were known to induce B cell production of antibodies. As $CD4^+$ T cells control the activation of B cells, macrophages, and in some cases, $CD8^+$ T cells, the activation of naïve $CD4^+$ T cells by antigen may be the most important event in the initiation of adaptive immunity. Much of their function is mediated by a set of small proteins, designated as cytokines, whose expression, secretion, or both is induced as a result of antigen-stimulated cell activation.

Generally speaking, the adaptive immune response to a pathogen can develop towards a more strongly cellular type (type 1), or towards a more allergic type of response (type 2). When well developed, these gear the immune response for the effective elimination of different types of pathogens, with a type 1 response being more potent against intracellular pathogens, for example *Listeria monocytogenes* or mycobacteria. Type 2 allergic responses, in contrast, favor the elimination of parasites such as helminthes (Fig 1.1). The basis for this selective action resides in the cytokines made by the distinct $CD4^+$ T helper cell subsets in these responses. T helper type 1 (Th1) cells produce several characteristic cytokines, most notably IL-2 and IFN-γ,

whereas Th2 cells produce a set of cytokines, most notably IL-4, IL-5, and IL-13. In turn, IL-2 and IFN-γ promote the development of strong cell-mediated immunity, whereas the type 2 cytokines promote allergic responses effective in eliminating parasites.

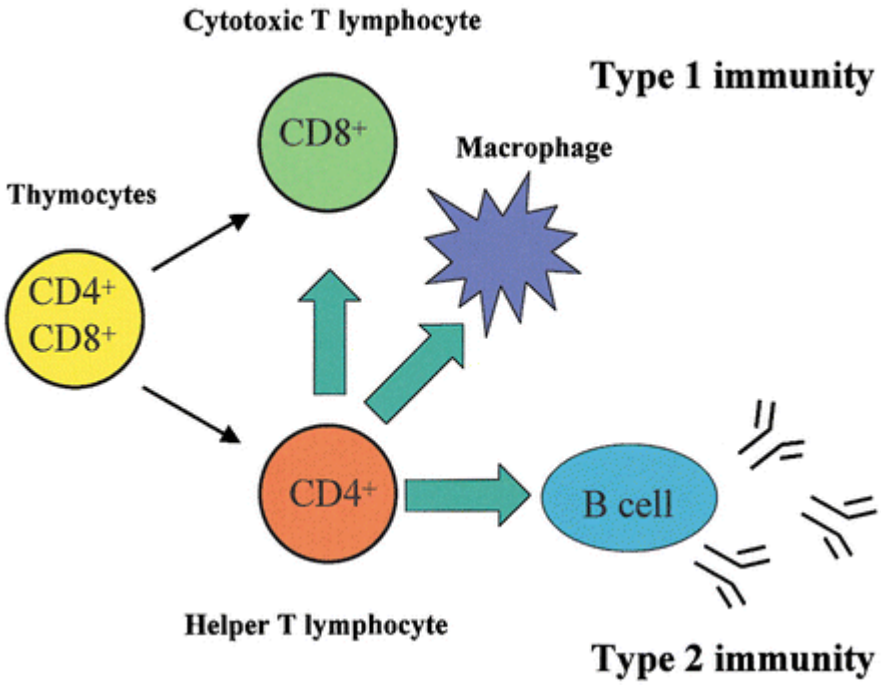


Fig. 1.1: T lymphocyte differentiation. $CD4^+$ and $CD8^+$ cells arise from a common lymphoid progenitor cell in the thymus. Type 1 immunity relies on differentiation of one major subset of T lymphocytes, the T helper cell, that induces both inflammatory and cytotoxic responses essential for destruction of intracellular pathogens such as *Mycobacterium tuberculosis* and *Leishmania major*. The Th lymphocyte activates macrophages and also activates a second major type of T cell, the $CD8^+$ cytotoxic T cell, which is critical for the effective handling of microbial agents such as the human immunodeficiency virus, and bacteria such as *Listeria monocytogenes*. In addition to this cell-mediated series of immune responses, Th lymphocytes generate type 2 immunity, humoral immunity, in which they signal to B lymphocytes to produce antibodies. Type 2 immunity is particularly important to neutralize certain viruses and to ward off parasites. It is now clear that type 1 and type 2 immunity are actually directed by two distinct subsets of T helper lymphocytes [adapted from (Ho and Glimcher, 2002)].

In this part of my dissertation, I have endeavored to review the in depth analysis and up to date research progress achieved so far in understanding the differentiation pathways of $CD4^+$ T cells into Th1- and Th2-lineages, various transcription factors and signaling molecules involved in Th2 effector functions and especially in the regulation of IL-5 gene expression.

1.1. Differentiation of T Helper Lymphocytes

Naïve T helper (T_Hp) cells exist in our peripheral lymphoid organs as immunocompetent precursors which represent a set of relatively recent thymic emigrants that have not yet encountered antigen. These cells are usually small and dense. They express LECAM-1, a selectin involved in migration through endothelial venules, and relatively large amounts of CD45RB. When stimulated by receptor engagement in the context of a co-stimulatory signal, naïve Th cells produce IL-2 but little or no IL-4 or IFN-γ (Swain, 1991). Upon priming, T_Hp lymphocytes can develop into at least two distinct subsets with different cytokine production profiles and functional capabilities in immune regulation, namely Th1 and Th2 cells [Fig.1.2; (Mosmann and Coffman, 1989)]. Generally, Th1 cells produce IFN-γ and lymphotoxin, and retain the capacity to produce IL-2, whereas Th2 cells produce IL-4, IL-5, and IL-13 and do not retain the capacity to produce IL-2.

Aside from Th1 and Th2 cells, several other subtypes of T helper cells have been described. Mature T cells secreting both IFN-γ and IL-4 are known as Th0 cells. These Th0 cells are lymphocytes that do not polarize during maturation and thus retain the attributes of both Th1 and Th2 cells (Street et al., 1990). Although the physiological significance is not known, the Th0 cytokine patterns are most noticeable early after lymphocyte activation (Kelso, 1995). Additional populations of $CD4^+$ T helper lymphocytes called Th3 cells and T-regulatory 1 (Tr1) cells have been described. Th3 cells secrete transforming growth factor (TGF)-β and are thought to regulate mucosal immunity in mammals (Weiner, 2001). Tr1 cells, which appear to be similar to Th3 cells, secrete unusually high levels of IL-10 and lower levels of TGF-β, and they have been implicated in general suppression of immunity (Buer et al., 1998; Groux et al., 1997).

IFN-γ producing $CD4^+$ cells (Th1) confer cell-mediated immunity against intracellular pathogens, and mediate delayed type hypersensitivity (DTH) and organ-specific autoimmune diseases (Abbas et al., 1996; Mosmann and Coffman, 1989). A Th1 response is often accompanied by the production of complement-fixing antibodies of the IgG2a isotype, as well as the activation of natural killer cells (Kelso, 1995) and cytotoxic $CD8^+$ T cells expressing IFN-γ and perforin (Abbas et al., 1996; Mosmann and Sad, 1996; Sher and Coffman, 1992). In contrast, Th2 cells produce IL-4, IL-5, and IL-13, can activate mast cells and eosinophils, thereby control the eradication of

extracellular helminthic pathogens, and are implicated in atopic and allergic manifestations (Romagnani, 1994).

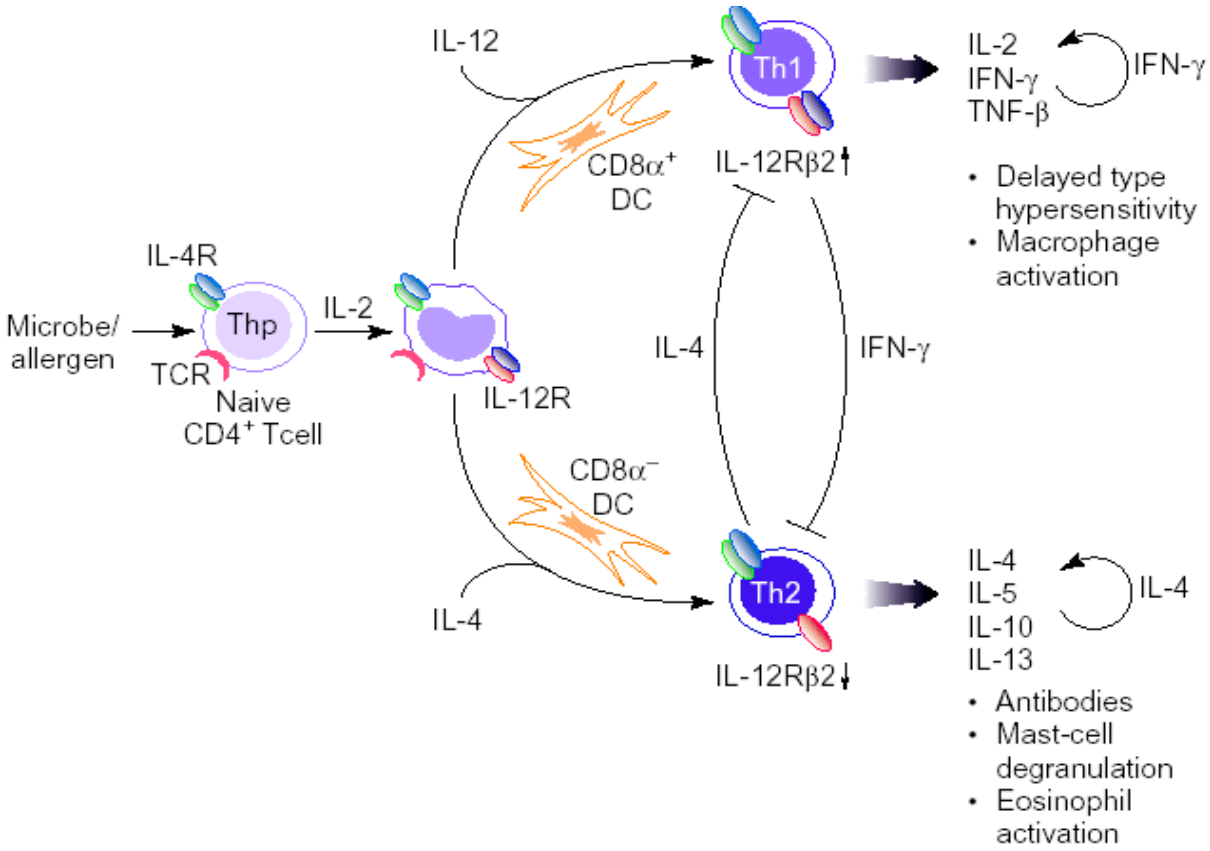


Fig. 1.2: Overview of cellular-based differentiation of T helper lymphocytes. A naive $CD4^+$ T cell is activated via the TCR when it encounters antigen (for example, derived from a microbe or allergen) presented by an antigen presenting cell. Once activated, the Th cell starts to proliferate and secrete IL-2, and expresses the IL-12 receptor (Wu et al., 1998). On encountering IL-12 secreted by macrophages and/or in contact with $CD8\alpha^+$ DCs, a Th1 differentiation program is initiated. The IL-12R β2 chain is upregulated in the developing Th1 cell and ligation of the IL-12R by IL-12 leads to the activation of Stat4 and initiation of the Th1 differentiation program. IL-4 produced intrinsically by the Thp and extrinsically by several cell types as well as contact with $CD8\alpha^-$ DCs induces differentiation into the Th2 subset and downregulation of IL-12R β2 expression. Ligation of the IL-4R (expressed on the naive $CD4^+$ T cell surface) by IL-4 activates Stat6 and initiates the Th2 differentiation program [adapted from (Rengarajan et al., 2000)].

The development of these discrete subsets of Th cells is determined by a number of factors, including cytokines, dose and form of antigens, the affinity of the peptide antigen–T cell receptor (TCR) interaction and co-stimulatory interactions between cell-surface molecules such as B7 and CD28; ICAM-1 and LFA-1; CD40 and

CD40L, stage of antigen resenting cells, and the genetic background of the responding host (Abbas et al., 1996; Constant and Bottomly, 1997; O'Garra, 1998). The role of B7 and CD28 interactions in Th cell development are unclear since they might or might not favor Th2 cell development, depending on the system under study (Brown et al., 1996; Rulifson et al., 1997). Interestingly, ICAM-1 and LFA-1 interactions inhibit the expression of Th2 cytokines (Luksch et al., 1999; Salomon and Bluestone, 1998). In the mouse, $CD8\alpha^+$ lymphoid-like DCs produce IL-12 and preferentially stimulate Th1 differentiation. A second subset of myeloid-like $CD8\alpha^-$ DCs has been shown to stimulate Th2 differentiation (Rengarajan et al., 2000). The mechanism by which this occurs remains unclear. DCs from Payer's patches preferentially stimulate Th0 clones to produce large amounts of IL-4, whereas DCs from spleen induces high IFN-γ production (Everson et al., 1997).

Although these factors might offer some explanation with respect to the development of distinct Th cell subsets, cytokines are major inducers of Th cell differentiation (Abbas et al., 1996; O'Garra, 1998; Paul and Seder, 1994). Cytokines, such as IL-12 and IL-4, play a dominant role in driving the development of Th1 and Th2 cells, respectively (Hsieh et al., 1993; Nelms et al., 1999; Swain et al., 1990). In addition to the expression of distinct cytokine genes, the molecular basis for commitment to a Th1 or Th2 phenotype can be explained by multiple mechanisms, including differential cytokine signaling, exclusive cytokine receptor expression, differential expression of transcription factors and/or differential chromatin remodeling of Th1- and Th2-specific genes (Agarwal and Rao, 1998; Murphy et al., 1999; O'Garra, 1998; Rincon and Flavell, 1997b; Szabo et al., 1997b). Both Th1- and Th2-specific cytokines can promote growth or differentiation of their own respective T cell subset, but additionally might inhibit the development of the opposing subset.

T helper cell differentiation can be observed not only during *in vivo* immune reactions, but also can be recapitulated *in vitro* by two methods. These are the polyclonal stimulation of T cells, e.g, by triggering the TCR with antibody against CD3 together with the co-stimulator anti-CD28, or stimulation of T cells derived from TCR transgenic mice with specific antigen and APC (Abbas et al., 1996; O'Garra, 1998; Paul and Seder, 1994). Under these conditions, addition of IL-12, normally produced by activated APCs *in vivo*, can strongly induce Th1-polarized differentiation, whereas addition of IL-4 skews Th cells to become Th2 effectors. The differentiation of naive Th

cells into homogeneous populations of committed Th1 or Th2 cells displaying an exclusive cytokine pattern is achieved largely after repeated antigenic stimulation under the appropriate conditions (Mocci and Coffman, 1995; Murphy et al., 1996; Perez et al., 1995; Szabo et al., 1995).

Th1 and Th2 populations at an early stage of development are heterogeneous, contain relatively low frequencies of cytokine producing cells and are reversible, suggesting that a number of molecular events must occur in order to establish a committed profile of cytokine gene expression. Progressive polarization of $CD4^+$ T cells under Th1- or Th2- inducing conditions ultimately leads to the commitment of mutually exclusive Th phenotypes (Murphy et al., 1996; Nakamura et al., 1997), as observed after chronic antigenic stimulation such as in parasitic diseases or allergic manifestations (Romagnani, 1994). Cell surface molecules that reliably mark Th1 or Th2 cells were also sought, and to date the chemokine receptors are probably the most convenient markers of functional subsets. Th1 cells preferentially express CC chemokine receptor 5 (CCR5) and CXC chemokine receptor 3 (CXCR3), and migrate in response to a selected set of chemokines induced by cytokines such as IFN-γ or IL-1, whereas Th2 cells preferentially express CCR3 and CCR4 and migrate in response to chemokines induced by IL-4 or IL-13 (Bonecchi et al., 1998; Gerber et al., 1997; Loetscher et al., 1998; Sallusto et al., 1998; Sallusto et al., 1997; von Andrian and Mackay, 2000).

The molecular basis for the commitment of Th phenotypes can be explained, in part, by specific loss of cytokine receptors such as the IL-12R2 chain, which is lost in Th2 cells but maintained in Th1 cells (Rogge et al., 1997; Rogge and Sinigaglia, 1997; Szabo et al., 1997a). Furthermore, IL-4 upregulates the IL-4R chain (Kotanides and Reich, 1996) and downregulates the IL-12R2 chain (Szabo et al., 1997a), whereas IFN-γ upregulates the IL-12R2 chain (Szabo et al., 1997a). Moreover, IL-4R-mediated activation of STAT6 and IRS-2 was shown to be blocked in Th1 cells (Huang et al., 1997b; Kubo et al., 1997). Facilitated by the *in vitro* culture systems, powerful molecular cloning and mouse genetics techniques, several key transcription factors and signaling molecules that govern the Th differentiation program have been identified over the past years (Fig. 1.3). The transcription factors GATA-3 and c-Maf are selectively expressed in Th2 but not in Th1 cells (Ho et al., 1996; Zhang et al., 1997; Zheng and Flavell, 1997). Although GATA-3 strongly transactivates the IL-5 promoter and weakly activates the IL-4 promoter, it strongly activates both IL-4 and IL-5 expression (Lee et al., 1998;

Ranganath et al., 1998; Zhang et al., 1997; Zhang et al., 1998). Furthermore, its ectopic expression in developing Th1 cells leads to upregulation of IL-4 and IL-5 and inhibition of IFN-γ partly by downregulating the IL-12Rβ2 chain (Ferber et al., 1999; Ouyang et al., 1998; Zheng and Flavell, 1997). c-Maf appears to act as a synergistic factor in IL-4 production and downregulates IFN-γ production in Th cells cultured under non-skewing conditions (Ho et al., 1996; Ho et al., 1998).

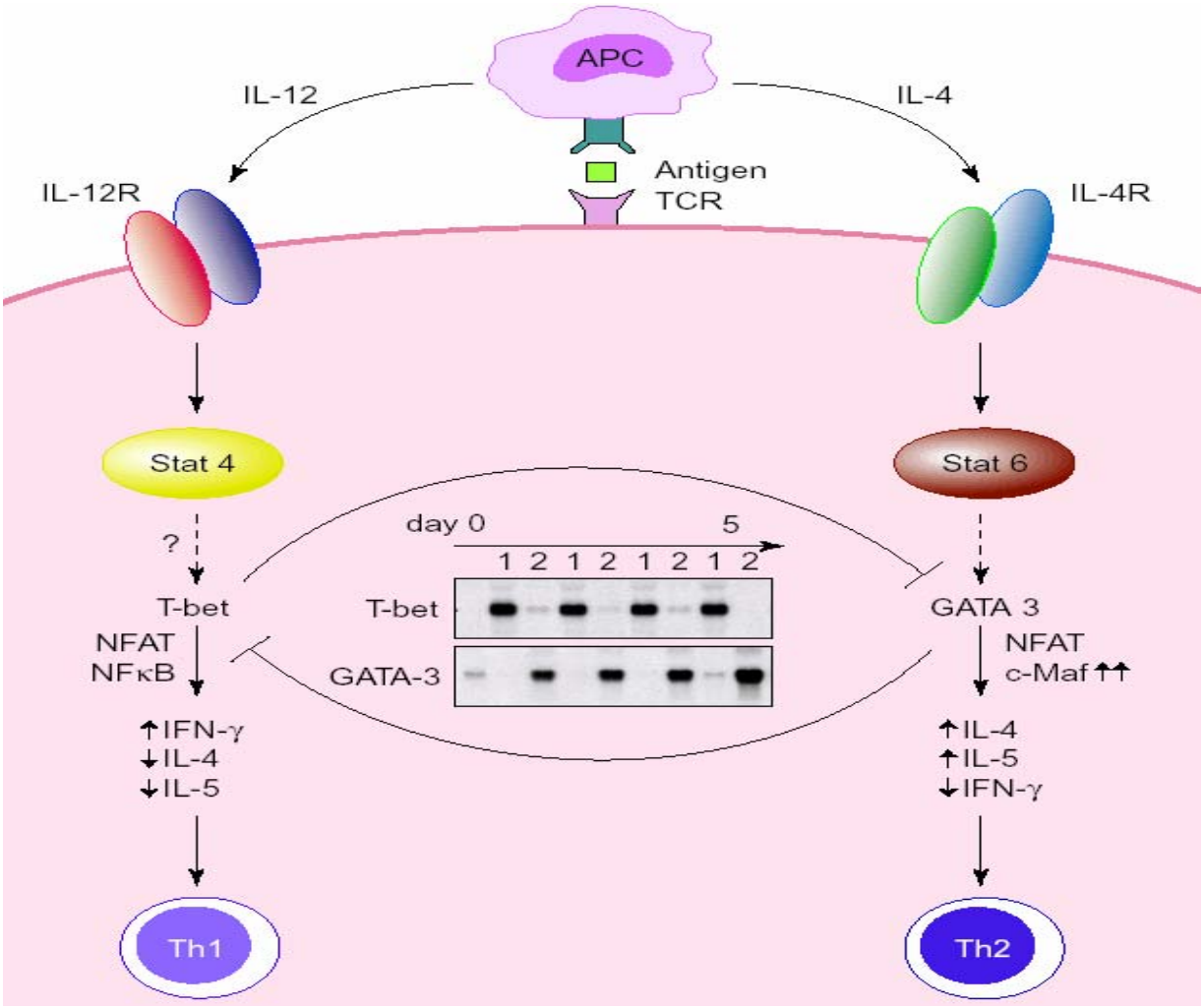


Fig. 1.3: Schematic diagram of signaling and transcriptional events that lead to the differentiation of Th1 and Th2 cells. Signals through the TCR and cytokine receptors can lead to the initiation of a Th1 program (via STAT-4 activation) and the induction of T-bet, which promotes Th1 lineage commitment. Signals that favor the activation of STAT-6 induce GATA3 leading to Th2 differentiation and upregulation of c-Maf. These Th2-specific factors increase IL-4 production and Th2 polarization. The middle panel shows the mutually exclusive expression pattern of T-bet and GATA3 in Th subtypes. The relative predominance of T-bet and GATA3 may thus determine Th1/Th2 polarization [adapted from (Rengarajan et al., 2000)].

1.2. Signaling Pathways in Th2 Lymphocytes

Production of IL-4 early in an immune response directs the development of a Th2 response, accompanied by the production of IL-4, IL-5 and IL-13, and activation of mast cells and eosinophils (Paul and Seder, 1994; Swain et al., 1990). Substantial progress has been made in identifying the transcription factors and signaling molecules responsible for the tissue-specific expression of the IL-4 and IL-5 genes in Th2 cells (Fig. 1.4; (Glimcher and Singh, 1999; Szabo et al., 1997b). Two Th2-specific factors, the c-Maf proto-oncogene and the GATA-3 zinc finger factor, have been identified (Ho et al., 1996; Zhang et al., 1997; Zheng and Flavell, 1997). Transcription factors which are also expressed in cells other than Th2 cells such as AP-1, NFAT proteins (Northrop et al., 1994; Rao et al., 1997; Rooney et al., 1995), and a novel nuclear antigen, NFAT-interacting protein 45 KD (NIP45) (Hodge et al., 1996a), also help to regulate Th2 cytokine production. Activation of the T cell receptor through interaction with antigen on antigen presenting cells activates NFAT and other transcription factors that cooperate with GATA-3 in inducing Th2 cell differentiation.

cAMP is an important second messenger with immunomodulatory properties. The role of cAMP has been well documented in the regulation of lymphokine gene expression. Elevation of intracellular cAMP in T cells, induced by agents such as IL-1α or PGE_2, inhibits T cell activation. There are strong evidences that cAMP exerts differential effects on Th1 and Th2 in TCR-mediated activation and proliferation ((Betz and Fox, 1991; Munoz et al., 1990b; Novak and Rothenberg, 1990). cAMP inhibits the production of IL-2 and IFN-γ in Th1 cells, whereas Th2-specific lymphokines IL-4, IL-5, IL-6, and IL-10 are all resistant to elevated cAMP level by PGE_2 or forskolin treatment. In particular, PGE_2 augmented IL-5 production four to five fold. There is also a report that Th2 cells contain higher level of cAMP than Th1 cells (Novak and Rothenberg, 1990). In addition, IL-3, which is produced by both cell types, is inhibited by PGE_2 treatment in Th1 cells but not in Th2 cells (Li and Fox, 1993). These observations suggest that the differences in the signaling system of both types of $CD4^+$ T cells may account for the differential effect of cAMP on the expression of Th1- and Th2-specific lymphokines.

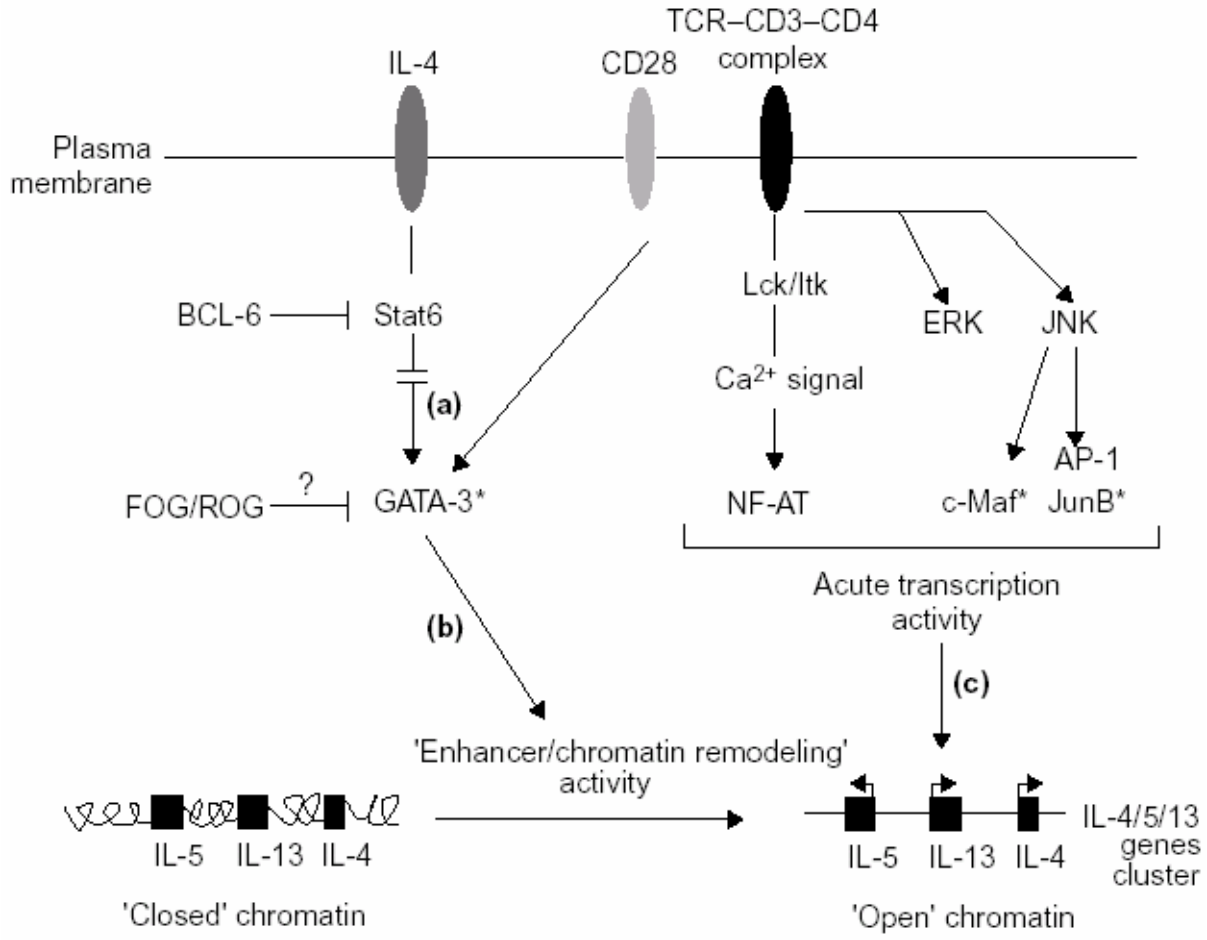


Fig. 1.4: Model of signaling pathways leading to Th2 differentiation. In the presence of IL-4 and TCR–CD3–CD4 triggering, activation of naïve $CD4^+$ T cells leads to the accumulation of Th2-specific transcription factors (indicated by asterisks). **(a)** Expression of GATA-3 may be regulated by both a Stat6-dependent pathway and by signals derived from CD28. **(b)** GATA-3 may act directly or indirectly to promote epigenetic modifications of the IL-4/5/13 genes locus, favoring Th2 development. **(c)** After 'remodeling' of Th2 cytokine genes, activation of acute transcription factor can achieve transcription of accessible promoters independently of IL-4 signaling. CD4 preferentially associates with Lck, which may feed into activation of Itk, Ca^{2+} signaling and NFAT activation, thereby augmenting acute transcriptional activity [adapted from (Asnagli and Murphy, 2001)].

1.2.1. JAK-STAT Pathway

STAT proteins play an important role in the selective response of cells to particular cytokines (Kaplan and Grusby, 1998). Ligation of IL-4 with its receptor (IL-4R) activates at least two signaling, JAK-STAT and IRS2, pathways (Ryan et al., 1996). Ligand binding to the IL-4R activates Jak1 and Jak3, leading to recruitment and phosphorylation of STAT-6, IRS-1/2, Shc, and SHP-1 (Ryan et al., 1996). STAT-6 is an

848–amino acid protein and shares homologous domains with other STAT proteins, including an N-terminal domain, a DNA-binding domain (DBD), SH2 and SH3 domains, and a C-terminal transactivation domain (TAD) (Moriggl et al., 1997; Quelle et al., 1995). Upon phosphorylation of a specific C-terminal tyrosine residue, STAT-6 homodimerizes, translocates into the nucleus, and binds to specific sequence motifs located in the promoters of IL-4-responsive genes (Hou et al., 1994; Quelle et al., 1995).

Although IL-4$^{-/-}$ mice have residual Th2 responses, it has been convincingly demonstrated that STAT-6 is essential for maximal Th2 differentiation *in vitro* (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996). STAT-6$^{-/-}$ Th cells, generated under Th2 skewing conditions, produced very small amounts of Th2 cytokines. The STAT-6-mediated signaling cascade was further studied by ectopic expression of conditional version of STAT-6 into naïve T cells driven under Th1 conditions, where activation of STAT-6 induced the production of IL-4, IL-5 and IL-10 and repressed Th1 development and production of IFN-γ at least in part through inhibition of IL-12Rβ2 (Kurata et al., 1999). In addition to a change in cytokine profile, the induction of mRNA for the Th2-specific transcription factors GATA-3 and c-maf was also observed upon the activation of STAT-6, suggesting that GATA-3 and c-maf are downstream from STAT-6 (Kurata et al., 1999).

The i*n vivo* Th2 immune responses, however, can still be elicited in STAT-6$^{-/-}$ animals. Th2 cells derived from STAT-6$^{-/-}$ mice express high levels of GATA-3 and produce normal levels of Th2 cytokines (Finkelman et al., 2000; Jankovic et al., 2000; Ouyang et al., 2000). These results imply the presence of STAT-6-independent, non-autonomous pathways for Th2 cell differentiation, albeit the nature and composition of these pathways remain unknown so far. One clue came from studies on Bcl-6$^{-/-}$ mice. Bcl-6 is a zinc finger protein that belongs to the POZ family of transcriptional repressors, and mutation or translocation of Bcl-6 is commonly found in diffuse large cell B lymphoma (Staudt et al., 1999). Strikingly, Bcl-6$^{-/-}$ mice spontaneously develop multiorgan inflammation characterized by marked infiltration of Th2 cells, eosinophils, and IgE-bearing B cells (Dent et al., 1997; Ye et al., 1997). Bcl-6$^{-/-}$ Th cells produce very high levels of Th2 cytokines in a cell-autonomous fashion, however, the enhanced *in vitro* Th2 differentiation was completely reversed in the absence of STAT-6 (Harris et al., 1999). Therefore, Bcl-6 negatively regulates the differentiation of Th2 cells at least partly via a STAT-6-dependent mechanism. Surprisingly, Bcl-6$^{-/-}$STAT-6$^{-/-}$ mice still develop the lethal Th2 inflammatory response *in vivo*, and Th cells directly harvested

from the inflamed organs continued to produce Th2 cytokines at levels comparable to those of Bcl-6 $^{-/-}$ animals (Dent et al., 1998). This surprising phenotype might well be explained by the STAT-6-independent, non-autonomous Th2 pathway, which is also augmented by the absence of Bcl-6 *in vivo*. Earlier work demonstrated that the chemokine MCP-1 promoted Th2 differentiation as MCP-1$^{-/-}$ mice displayed defects in Th2 immune responses *in vivo* (Gu et al., 2000) and the expression of the chemokine MCP-1 in macrophages was negatively regulated by Bcl-6 at the level of transcription (Toney et al., 2000). Taken together, these results suggest a model in which Bcl-6 attenuates the differentiation of Th2 cells by inhibiting the production of MCP-1 by non-B, non-T cells.

1.2.2. GATA-3

GATA-3, a zinc finger protein, was first described as a transcription factor that interacted with the TCR-α gene enhancer (Ho et al., 1991). GATA-3 belongs to the GATA family of transcription factors, which bind to the WGATAR (W = A/T; *R* = A/G) DNA sequence through a highly conserved C_4 zinc finger domain. Six members (GATA-1 to GATA-6) of this family have been identified in avians, with homologues in mammals. Based on their expression profile and structure, the GATA proteins may be classified as hematopoietic (GATA-1 to GATA-3) or non-hematopoietic (GATA-4 to GATA-6). Targeted disruption of the GATA-3 gene in mice results in embryonic death on day 12, with a failure of fetal liver hematopoiesis and defects in the central nervous system (Pandolfi et al., 1995). The lethal effects of GATA-3-deficiency were bypassed by generating *GATA-3*$^{-/-}$ chimeras, which lacks GATA-3 in the lymphoid system. These mice do not generate thymocytes or mature peripheral T cells because GATA-3$^{-/-}$ thymocytes arrest at or before the earliest double-negative (DN) stage of thymocytes development, establishing an essential role for GATA-3 in the earliest steps of T cell development (Hendriks et al., 1999; Ting et al., 1996).

In addition to its essential role in T cell development, GATA-3 has also been identified as a Th2 differentiation factor. Naive $CD4^+$ T cells express low levels of GATA-3 mRNA (Zhang et al., 1997; Zheng and Flavell, 1997). The expression of GATA-3 is, however, markedly upregulated in cells differentiating along the Th2 lineage, and is downregulated in cells differentiating along the Th1 pathway. Ectopic expression of a GATA-3 transgene led to increased levels of Th2 cytokines, whereas a dominant negative mutant of GATA-3 transgene inhibited Th2 differentiation (Zhang et al., 1999;

Zheng and Flavell, 1997). Th2 cells derived from these animals expressed significantly lower levels of Th2 cytokines upon rechallenge with antigens. GATA-3 directly controls IL-5 gene expression through binding to and transactivating elements in the region −70 to −59 (Lee et al., 1998; Siegel et al., 1995; Zhang et al., 1997; Zhang et al., 1998). GATA-3 probably does not directly bind to and transactivate the IL-4 promoter (Zhang et al., 1997; Zhang et al., 1998). Rather, several genomic regions within the IL-4/IL-13 locus have GATA-3-dependent enhancer activity (Ouyang et al., 1998) suggesting GATA-3 may augment expression of IL-4 or IL-13 via interactions at sites distant from the proximal promoter. In addition to increasing expression of Th2-selective cytokines, GATA-3 inhibits Th1 development independently of IL-4, perhaps in part by inhibition of IL-12Rβ2 expression (Ouyang et al., 1998).

Although GATA-3 is a STAT-6-inducible gene, the developmental program initiated by GATA-3 can operate independently of STAT-6 (Ouyang et al., 2000). When GATA-3 was expressed by retrovirus in STAT-$6^{-/-}$ T cells, all components of Th2 development were observed, including induction of Th2 cytokines, inhibition of Th1 cytokines, induction of c-Maf, and the formation of DNase I hypersensitive sites in the IL-4 locus. In addition, the endogenous GATA-3 gene was observed to be induced in these cells, despite the addition of IL-12 to the culture, normally a signal that inhibits GATA-3 expression (Ouyang et al., 1998). These results suggest a pathway of GATA-3 autoactivation, which could provide a mechanism for acquisition of the IL-4 independence that develops in Th2 cells as described before (Huang et al., 1997b).

The potential importance of this STAT-6-independent pathway has been brought out by two studies. First, Th2 cells can develop both *in vivo* in response to *S. mansoni* infection even in T cells lacking STAT-6 or IL-4 receptor, suggesting the existence of an unrecognized pathway stabilizing Th2 development once these cells have emerged (Jankovic et al., 2000). Second, in the primary response to pathogens, STAT-$6^{-/-}$ mice generated wild type IL-4 responses, delineating a requirement for STAT-6 in only the secondary response (Finkelman et al., 2000). These findings are all consistent with a model in which the initial expression of GATA-3 can be independent of IL-4 and STAT-6 activation but is repressible by IL-12. In the absence of such repression, GATA-3 can be induced through STAT-6-independent autoactivation to induce the development of the Th2 phenotype. Addition of GATA-3 antisense oligonucleotides during *in vitro* differentiation of Th2 cells significantly reduced the levels of GATA-3 protein and subsequent production of IL-4 (Finotto et al., 2001). In addition, local administration of

GATA-3 antisense oligonucleotides markedly attenuated airway hyperresponsiveness, mucus production, and infiltration of eosinophils in an animal model of allergic asthma. These results strongly indicate that GATA-3 is essential for the development of Th2 cells and might also be essential to maintain Th2 phenotype and function.

The mechanism by which GATA-3 controls Th2 cytokine gene expression is still unclear and may differ for each of the Th2 cytokines. Thus, GATA-3 can directly transactivate the IL-5 and IL-13 promoters, but has only a minimal effect on the IL-4 promoter (Kishikawa et al., 2001; Lee et al., 1998; Siegel et al., 1995; Zhang et al., 1997). The expression of T-bet and GATA-3 is mutually exclusive during the priming of Th cells. This observation raises the question whether T-bet and GATA-3 counter regulate each other. The activity of the GATA-3 protein can also be modulated by post-translational mechanisms. For example, it has been known for many years that an increase in intracellular cyclic AMP enhances cytokine production by Th2 cells but has opposite effects on Th1 cytokines (Munoz et al., 1990b). This effect has recently been suggested to be due to a specific activation of p38 MAP kinase in Th2 cells by increased intracellular cAMP that results in phosphorylation of GATA-3 (Chen et al., 2000). In addition to phosphorylation, mature GATA-3 proteins appear to be heavily acetylated (Yamagata et al., 2000). This may be the basis for the dominant negative phenotype of the KRR mutant of GATA-3, where the substitution of the lysine-arginine-arginine (KRR) residues at amino acid 305–307 with alanines (AAA) significantly diminishes GATA-3 acetylation.

Despite these observations, it remains unclear whether post-translational modifications of GATA-3 are functionally relevant *in vivo*. In addition to post-translational modification, the activities of GATA-3 can be modulated by physical interaction with other nuclear proteins. A cDNA encoding a lymphoid-specific GATA-3-interacting protein, repressor of GATA (ROG), was shown to attenuate the activities of GATA-3 and repress the production of cytokines by Th2 clones *in vitro* (Miaw et al., 2000). Moreover, Friend of GATA-1 (FOG-1), a GATA interacting zinc finger protein enriched in hematopoietic cells, was found to be critical for the development of the T cell lineage. Forced expression of FOG-1 significantly repressed the transcriptional activity of GATA-3, the production of Th2 cytokines, and the differentiation of Th2 cells *in vitro* (Zhou et al., 2001). However, the roles of ROG and FOG-1 in modulating the activity of GATA-3 and, subsequently, the differentiation of Th cells *in vivo* remain unclear.

Very little is known about the transcriptional regulation of GATA-3. As mentioned above, GATA-3 is a T cell-specific transcription factor that is rapidly induced by IL-4/STAT-6. Once induced, GATA-3 can undergo autoactivation, a STAT-6-independent process, to augment its expression. The *cis*-acting elements that are required for T or Th2 cell-specific expression of the *gata-3* gene remain elusive. A YAC encompassing approximately 625 kb of the murine *gata-3* locus failed to support the expression of a reporter gene in thymi or lymphoid organs in transgenic animals, indicating that *cis*-acting elements outside the 625 kb YAC are required to achieve cell type-specific expression of GATA-3 (Lakshmanan et al., 1999). Even less is known about the transcription factors, other than STAT-6, that regulate the expression of GATA-3.

Two reports provide some clues to the regulation of GATA-3. The transcription factor NF-κB regulates the expression of many cytokine genes. Interestingly, mice rendered deficient in the p50 subunit of NF-κB were resistant to allergic airway inflammation, and were unable to mount normal Th2 immune responses in both *in vivo* and *in vitro* systems, whereas their Th1 responses were intact (Das et al., 2001). In addition, developing p50-deficient Th cells expressed substantially lower levels of GATA-3 even under Th2-skewing conditions. Furthermore, inhibition of NF-κB translocation by a synthetic peptide, containing a signal sequence of Kaposi's fibroblast growth factor and the nuclear localization sequence of the p50 subunit, during the differentiation of Th2 cells almost completely abolished the induction of GATA-3 and the production of Th2 cytokines.

Somewhat unexpectedly, blockade of NF-κB translocation had no effect on the expression of GATA-3 and the production of cytokines in mature effector Th2 cells. These results imply that GATA-3 might be a downstream target gene of p50 in developing Th2 cells and that the transcriptional regulation of GATA-3 might vary at different stages of Th cell development. Recently another report emphasizes the crucial role of JunB in the differentiation and effector functions of $CD4^+$ T cells. The loss of JunB in *in vitro* polarized Th2 cells led to an unregulated expression of the Th2-specific cytokines IL-4 and IL-5, but not IL-13 (Hartenstein et al., 2002). JunB-deficient $CD4^+$ T cell population that was strictly kept under Th2-polarizing conditions expressed somehow reduced levels of GATA-3.

The Polycomb group of genes was originally identified in *Drosophila* as transcriptional repressors. Many Polycomb members have mammalian homologs, which

are involved in the regulation of Hox gene expression (van Lohuizen, 1999): mel-18 is one of the mammalian homologs. Unexpectedly, mel-18$^{-/-}$ mice had moderate reductions in numbers of thymocytes and peripheral T cells, and exhibited a striking impairment in Th2 immune responses (Kimura et al., 2001). The *in vitro* differentiation of mel-18$^{-/-}$ Th2 cells produced significantly lower levels of Th2 cytokines. In addition, antigen-induced IgG1 production and Nippostrongylus-induced eosinophilia were attenuated in mel-18 mice. Interestingly, mel-18$^{-/-}$ Th2 cells expressed GATA-3 at much lower levels than wild type Th2 cells despite normal IL-4/ STAT-6 signaling (Kimura et al., 2001). It remains unanswered whether the lower levels of GATA-3 in mel-18$^{-/-}$ Th2 cells are the cause or the result of defective Th2 cell differentiation.

1.2.3. c-Maf

c-Maf was isolated using a yeast two hybrid screen with NFATc1 as bait and cDNAs from activated Th2 cells as library (Ho et al., 1996). c-Maf, the cellular homolog of the avian viral oncogene v-maf, is a member of the AP-1 family of basic region/leucine zipper factors and binds to a consensus site (MARE) in the proximal IL-4 promoter. The expression of c-Maf is limited to Th2 cells, and its own expression is induced by signals transmitted via the TCR. Ectopic expression of c-Maf in all cells tested including yeast potently activates the IL-4 promoter. In CD4$^+$ T cell lines prepared from patients with atopic disease, c-Maf is expressed at very high levels in Th2 clones but minimally in Th0 clones that produce IFN-γ and very little IL-4. Extremely strong evidence for the critical role of c-Maf in controlling IL-4 production has been gathered *in vivo* with the production of c-Maf overexpressing transgenics (Ho et al., 1998) and most convincingly with mice that lack c-Maf (Kim et al., 1999). c-Maf transgenic mice have an increased Th2 immune response *in vivo* and *in vitro* that can be ablated by backcrossing onto an IL-4-deficient background (Ho et al., 1998).

Targeting of the c-maf locus revealed severely impaired IL-4 production in the absence of c-Maf, although interestingly other Th2-specific cytokines were unaffected (Kim et al., 1999). These data provide very strong evidences that c-Maf is required for the production of IL-4 *in vivo*. However, the provision of c-Maf to mature effector Th1 cells cannot allow them to produce IL-4, suggesting that other factors such as STAT-6 or GATA-3 may also be required. An attractive overall scheme suggests that signals through the TCR activate c-Maf and GATA-3 as well as NFATc1 transcription factors, whereas IL-4 signaling induces GATA-3 and STAT-6 activities. GATA-3 is

autoregulatory (Ouyang et al., 2000), and it is possible that GATA-3 and c-Maf regulate each other in an autocrine loop, resulting in IL-4 production. GATA-3 downregulates expression of the IL-12Rβ2 chain, whereas both GATA-3 and c-Maf repress IFN-γ production in Thp cells thus inhibiting the Th1 pathway (Ho et al., 1998; Ouyang et al., 2000).

1.2.4. NFAT

The transcription factor NFAT (Nuclear Factor of Activated T cells) was first identified as a TCR-inducible factor that regulated the IL-2 gene (Rao et al., 1997; Serfling et al., 2000; Shaw et al., 1988). NFAT proteins also regulate the promoters of multiple other cytokine genes expressed in T cells, including IL-4, GM-CSF, IL-3 and TNFα (Chuvpilo et al., 1993; Goldfeld et al., 1993; Masuda et al., 1993; Miyatake et al., 1991; Rooney et al., 1994; Rooney et al., 1995). Isolation of the genes encoding these proteins has yielded four NFAT family members called NFATc1 (also called NFATc, NFAT2), NFATc2 (also called NFATp, NFAT1), NFATc3 (NFAT4 and NFATx), and the non-lymphoid NFATc4 (NFAT3) that are highly homologous within a region distantly related to the Rel domain (Hoey et al., 1995; Masuda et al., 1995; McCaffrey et al., 1993; Northrop et al., 1994).

Although the three lymphoid NFAT proteins are expressed in both Th1 and Th2 cells (Ranger et al., 1998a), the phenotype of single NFATc1- and NFATc2 deficient mice reveals that NFAT proteins might reciprocally regulate Th cytokines and consequently the Th1/Th2 balance. This turned out to be the case for NFAT-regulated Th2 cytokine production. T cells from mice lacking NFATc1 in the lymphoid system have reduced IL-4 production (Ranger et al., 1998b; Yoshida et al., 1998), consistent with a function of NFATc1 as a direct transcriptional activator of the IL-4 gene. Conversely, NFATc2 and NFATc3 negatively control proliferative responses, Th2 cell formation, and lymphocyte activation as revealed by the phenotype of mice lacking these genes (Hodge et al., 1996b; Kiani et al., 1997; Xanthoudakis et al., 1996). Mice lacking NFATc2 display a moderate increase in Th2-type cytokines (Hodge et al., 1996a; Xanthoudakis et al., 1996). Although mice lacking NFATc3 have normal cytokine production (Oukka et al., 1998), the very dramatic phenotype of mice lacking both NFATc2 and NFATc3 (DKO mice) proved that these two members had redundant function (Ranger et al., 1998b). These DKO mice had massive overproduction of Th2 cytokines with concomitant extreme elevations in levels of IgG1 and IgE,

immunoglobulins whose production is IL-4-dependent. The constitutive localization of NFATc1 to the nucleus in the DKO T cells is consistent with unopposed transactivation of IL-4 by NFATc1 (Ranger et al., 1998a; Yoshida et al., 1998) which likely acts in concert with the Th2-specific transcription factors c-Maf and GATA-3 (Ho et al., 1996; Zheng and Flavell, 1997) to drive IL-4 gene transcription.

1.2.5. cAMP signaling

Reversible protein phosphorylation is a key regulatory mechanism in eukaryotic cells. Protein phosphorylation was first demonstrated to regulate the activity of glycogen phosphorylase in response to glucagons. A heat stable factor mediating the effect of glucagon on the phosphorylation status of glycogen phosphorylase was next examined as 3', 5' adenosine cyclic monophosphate (cAMP), and the concept of cAMP as an intracellular second messenger to a wider range of hormones, neurotransmitters, and other signaling substances was developed. Since its discovery, cAMP has been implicated as the paradigm for the concept of an intracellular second messenger. The cAMP signaling pathway involves seven helix transmembrane receptors that, upon binding of ligands, transduce their signal across the cell membrane to G proteins that interact with membrane-bound adenylyl cyclase either to increase or decrease the production of cAMP, which in turn, regulates a number of different cellular processes such as metabolism, gene transcription, cell growth & differentiation, ion channel conductivity and neurotransmitter release.

In most cell types cAMP serves to inhibit cell growth. However, to confound this, in certain cell types, such as those from the thyroid and pituitary, it can actually stimulate cell growth. Stimulation of a transmembrane receptor coupled to the stimulatory trimeric G protein activates adenylyl cyclase to produces cAMP. This small molecule diffuses into the cell cytosol and activates PKA. With few exceptions where cAMP is reported to directly regulate certain ion channels (DiFrancesco and Tortora, 1991; Nakamura and Gold, 1987), all known cAMP-dependent events in eukaryotic cells are mediated through PKA-dependent phosphorylation (Krebs and Beavo, 1979). Therefore, activated PKA is envisaged as an effector system that responds functionally to changes in intracellular cAMP. This activated system returns to its basal state by the concerted actions of protein phosphatases, the hydrolysis of cAMP effected by phosphodiesterases (PDEs), receptor agonist removal and receptor desensitization.

1.2.5.1. Protein Kinase A (PKA)

PKA is a holoenzyme which consists of a regulatory (R) subunit dimer and two catalytic (C) subunits [Fig. 1.5; (Corbin et al., 1978)]. Activation of PKA occurs upon binding of four cAMP molecules, two molecules to each R subunit, releasing two free active C subunits that phosphorylate specific serine and threonine residues on target proteins [Fig 1.5; (Corbin et al., 1975b; Doskeland, 1978)]. Two major isozymes of PKA in eukaryotic cells are described, and designated as type I and type II (PKAI and PKAII), respectively (Corbin et al., 1975a). The distinction between PKAI and PKAII is due to differences in the regulatory subunits, termed RI and RII. A greater heterogeneity of PKA subunits has been described, and isoforms of RI, RII, and C are reported [RIα, RIβ, RIIα, RIIβ, Cα, Cβ, and Cγ] (Tasken et al., 1997). Differential isozyme expression, holoenzyme composition and subcellular localization contribute specificity to the PKA signaling pathway. In addition to different biochemical properties of the various isozymes, the different subunits reveal cell-specific expression, differential regulation, and distinct subcellular localization primarily due to interaction with A kinase anchoring proteins [AKAPs; (Clegg et al., 1989; Rosenmund et al., 1994; Rubin, 1994; Scott, 1991; Solberg et al., 1991; Tasken et al., 1997)].

Several reports demonstrate that the PKA holoenzyme has a very long half life in contrast to the free RIα and C subunits which are degraded very rapidly (Lorimer and Sanwal, 1989; Richardson et al., 1990; Tasken et al., 1993). Genes for R and C subunits are widely dispersed in the genome and are all located at different chromosomes (Tasken et al., 1997). Investigation on the expression of PKA, R, and C subunits, the level of endogenous cAMP as well as cell growth in lymphoid cell lines and normal peripheral blood T and B lymphocytes demonstrated cell-specific differences in the expression and levels of R and C subunits (Skalhegg et al., 1998). Certain T cell lines (RPMI 8402, Molt-4, and Jurkat) and one B cell line (Reh) are practically devoid of PKAII, thus making PKAI the predominant isoenzyme in these cells. Furthermore, certain cell lines demonstrated high R/C ratios that appeared to be associated with high levels of endogenous cAMP, whereas low R/C ratios appeared to some extent to be associated with low levels of endogenous cAMP, although this correlation was not significant. Finally, levels of endogenous cAMP were inversely correlated with growth rate of lymphoid cells, i.e., cells with high levels of endogenous cAMP were growing slowly. The R/C ratios in the various cells investigated were mainly between 1.3 and

1.8. The exceptions were normal quiescent B and T cells which displayed R/C ratios closer to 1 and the B cell lines Nalm-6, Daudi, and U-266, which all displayed R/C ratios above 2.0 (Skalhegg et al., 1998).

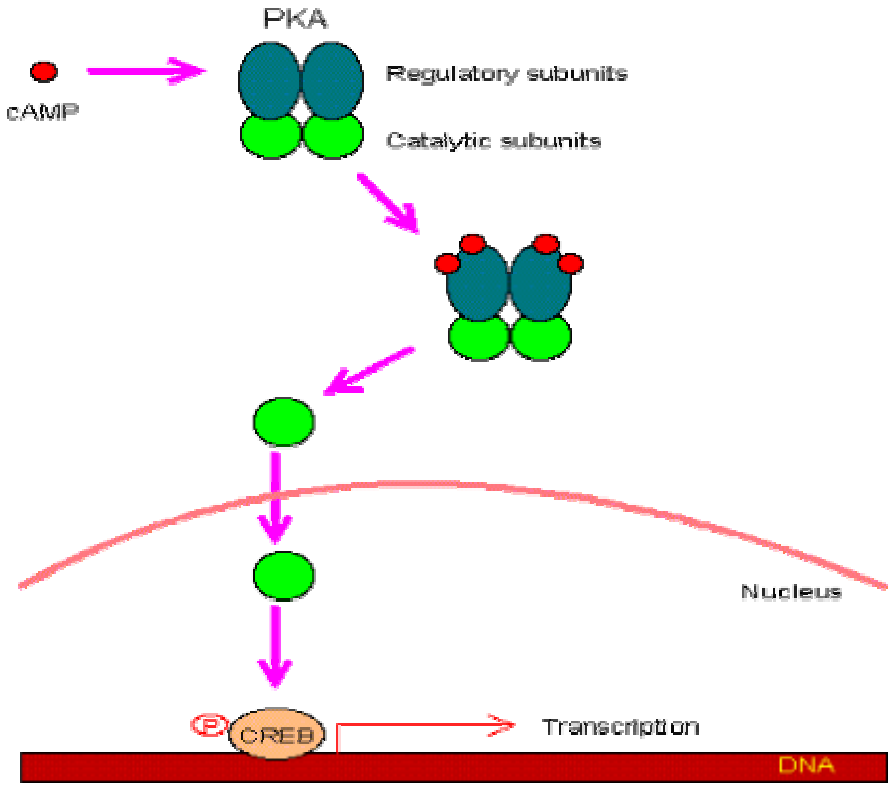


Fig 1.5: A schematic representation of PKA pathway in mammalian cell systems. The catalytic subunits of PKA become free upon binding of cAMP to regulatory subunit dimer. These enzymatically active catalytic subunits phosphorylate several target proteins leading to the modulation of gene expression.

In lymphoid cells, a transient increase in intracellular cAMP has been reported after antigen receptor stimulation. Cyclic AMP through PKA-dependent phosphorylation regulates a number of cellular events, including NK cell cytotoxicity, production of lymphokines, and lymphokine receptor expression as well as proliferation (Blomhoff et al., 1987; Kammer, 1988; Torgersen et al., 1997). Furthermore, activation of PKAI, but not PKAII, is sufficient to inhibit antigen-receptor complex (TCR/CD3) stimulated proliferation of T cells (Skalhegg et al., 1992). Moreover, PKAI redistribute and colocalizes with the TCR/CD3 complex during activation and capping and serves to negatively regulate signals induced through the antigen receptor (Skalhegg et al., 1994). PKA substrates involved in immune activation include transcription factors, members of the MAP kinase pathway and phospholipases.

As a result of TCR engagement, activation specific genes are induced by binding of multiple transcription factors including members of the NF-κB/Rel/NFAT, AP-1 and CREB/ATF families to their promoters. Several of these transcription factors can be modulated by PKA activity. The transcriptional activity of CREB (cAMP response element binding protein) is regulated by a single phosphorylation on Ser-133 that leads to complex formation with the coactivator, CBP and binding to CRE elements (Brindle et al., 1993; Chrivia et al., 1993; Kwok et al., 1994). In T cells, CRE elements can be found in the TCR and CD3 genes (Anderson et al., 1989; Gupta and Terhorst, 1994) as well as in other genes involved in lymphocyte activation.

Biochemical characterization shows that 70–80% of the total PKA in lymphocytes consist of a soluble PKA type I isozyme (RIα2C2), whereas approximately 20–25% is constituted by a PKA type II enzyme (RIIα2C2) mainly anchored in the Golgi-centrosomal region of the cell (Dell'Acqua and Scott, 1997). In contrast to PKA type II, PKA type I colocalizes with the capped TCR (Skalhegg et al., 1994). Furthermore, specific activation of PKA type I is sufficient for cAMP-mediated inhibition of T and B cell proliferation (Skalhegg et al., 1992), as well as NK cell cytotoxicity (Torgersen et al., 1997). In addition, pretreatment of normal T cells or Jurkat cells with cAMP elevating agents prior to TCR stimulation, inhibits tyrosine phosphorylation of proteins involved in proximal TCR signaling (Chen and Rothenberg, 1994; Klausner et al., 1987; Patel et al., 1987), pointing towards a target for PKA in the early T cell activation process. In the search for a proximal target for PKA type I, a novel inhibitory pathway was identified that involves activation of Csk. PKA phosphorylates Ser-364 in Csk both *in vitro* and *in vivo* leading to an up to four fold increase in Csk activity (Vang et al., 2001). Previous reports have established that a doubling in Csk expression completely blocks TCR signaling (Chow et al., 1993), suggesting that a two- to four fold increase in Csk activity is physiologically significant *in vivo*.

Lymphokine gene regulation by PKA: The inhibitory effect of PKA on IL-2 expression has been reported by several groups (Chen and Rothenberg, 1994; Neumann et al., 1995). Transfection of PKA catalytic subunit inhibits IL-2 promotor activity, while overexpression of NFAT counteracts the inhibitory effect of PKA (Tsuruta et al., 1995). Phosphorylation of NFAT by PKA occurs at conserved sites adjacent to a nuclear localization sequence (NLS) and that this phosphorylation regulates the association between NFAT and the 14-3-3 protein (Chow and Davis, 2000). Mutations at these sites

abolished the inhibitory effect of PKA on NFAT. This introduces a model where calcineurin and PKA act as opposing regulators of the NFAT–14-3-3 complex. Dephosphorylation by calcineurin results in the release of NFAT from 14-3-3 and nuclear translocation, while phosphorylation by PKA creates a binding site for 14-3-3 and results in reduced NFAT activity. The observation that overexpression of 14-3-3 inhibits IL-2 but not IL-4 (Meller et al., 1996), supports this hypothesis and might provide a useful model to explain the differential effect of PKA on IL-2 and IL-4 expression.

Regulation of NF-κB activity provides another way by which PKA can modulate the transcription of genes involved in immune activation. NF-κB consists of homodimers or heterodimers belonging to the Rel family of proteins. Most of these contain a PKA consensus phosphorylation site (RRXS) localized close to the NLS, and several groups have reported activation of NF-κB as a result of PKA phosphorylation (Blank et al., 1992; Verma et al., 1995). Neumann et al has shown that in $CD4^+$ T cells, stimulation of PKA leads to suppression of IL-2 production, while induction of the genes encoding for the IL-4 and IL-5 is enhanced. This differential effect of PKA on the induction of IL-2 and IL-4 genes is mediated through their promoters. The suppressive effect of PKA on the IL-2 promoter induction is mediated by κB site present in IL-2 promoter (Neumann et al., 1995). Stimulation of the PKA pathway in Jurkat T cells with the PKA activator, forskolin, leads to an increase in the synthesis of c-Rel and p105/p50, while synthesis of p65/RelA remains unchanged (Neumann et al., 1995). Activation of PKA signaling pathway inhibits nuclear translocation of p65 and generation of nuclear κB complexes in peripheral murine T lymphocytes. These observations led to the conclusion that PKA-mediated suppression of NF-κB activity plays an important role in the control of activation of peripheral T lymphocytes (Neumann et al., 1995).

The cAMP signal proved to be essential for the expression of IL-5 gene in EL-4 cells and action of cAMP IL-5 promoter was mimicked by co-transfection of the catalytic subunit of PKA cDNA (Lee et al., 1993). In fact, the IL-5 promoter was activated more effectively by PKA cDNA than by Bt_2cAMP. The physiological target(s) of PKA in EL-4 cells is unknown so far.

1.3. Biology of Interleukin-5 (IL-5)

1.3.1. Genomics and Biochemistry of the IL-5 System

IL-5 is a member of the cytokine family of inducers of hemopoiesis and immune response, which is shared by IL-3, IL-4, IL-13 and the granulocyte-macrophage colony-stimulating factor (GM-CSF). IL-5 is a homodimeric glycoprotein. In both humans and mice, the production of IL-5 is restricted to a few cell types, which include T cells (Robinson et al., 1992), mast cells (Plaut et al., 1989), and eosinophils (Broide et al., 1992), the predominant source being T cells of the Th2-type (Robinson et al., 1992). The cDNA that encodes murine IL-5 was cloned in 1986 from a T cell line, followed by the isolation of IL-5 cDNA from a human T cell leukemia line (Azuma et al., 1986; Kinashi et al., 1986) using a murine IL-5 cDNA as a probe. No overall significant amino acid sequence homology was found to exist with other cytokines, except for short stretches in the murine IL-3, murine GM-CSF, and murine IFN-γ proteins (Kinashi et al., 1986). Furthermore, in the IL-5 promoter region there are short stretches of conserved sequence motifs, designated as CLE0, CLE1 and CLE2, which are also found in the 5' flanking regions of the IL-3, IL-4, and GM-CSF genes (Miyatake et al., 1991; van Leeuwen et al., 1989).

Biologically active IL-5 is a disulfide-linked homodimer that is held together by the highly conserved cysteine residues that orient the monomers in an anti-parallel arrangement (Minamitake et al., 1990). The higher homology of mouse and human IL-5 found in the carboxyl terminal compared with the amino terminal half is consistent with the binding site for the IL-5 receptor that resides between helices C and D at an arginine-rich region that comprises residues 89 through 92 (Dickason et al., 1996; Kodama et al., 1991). The broad range of apparent molecular weights (45–60 kDa) of recombinant murine IL-5 and human IL-5 results from differential glycosylation, but deglycosylated IL-5 retains full biological activity (Tominaga et al., 1990). Crystal structure shows that human IL-5 acquires a novel two domain configuration with each domain requiring the participation of two chains, with a high degree of similarity to the cytokine fold found in GM-CSF, IL-3, and IL-4 (Milburn et al., 1993).

1.3.2. Physiological significance of IL-5

IL-5 was originally described as an eosinophil differentiation factor (EDF) (Sanderson et al., 1988; Sanderson et al., 1985). EDF is identical to the murine B cell growth factor. However, despite the fact that the *in vitro* activity on murine B cells is well characterized, there is little evidence to suggest a biological role for IL-5 in the B cell system *in vivo*. In contrast, the central role of IL-5 in the control of production, activation and differentiation of eosinophils *in vitro* as well as *in vivo* is well documented (Kopf et al., 1996; Lee et al., 1997; Sanderson, 1992). Eosinophils are important to the host in mediating parasite rejection. However, they are also involved in the pathogenesis of a number of allergic diseases, most notably asthma (De Monchy et al., 1985). Asthma is a chronic obstructive disease of the small airways. Evolving evidence indicates that asthma is the result of an inflammatory process interacting with a susceptible airway best defined at present as airway hyperresponsiveness (Martin et al., 1996). In asthma, the most striking and consistent pathophysiology is damage to the bronchial epithelium caused by cytotoxic cationic proteins released by infiltrating eosinophils (Martin et al., 1996). Increasing evidences place IL-5 in a key role in the development of eosinophilia in asthma (Hamid et al., 1991). IL-5 mRNA was significantly enhanced in bronchoalveolar lavage cells obtained from asthmatics challenged with ragweed antigen (Krishnaswamy et al., 1993). Again, peripheral blood T cells from asthmatics were found to secrete IL-5 in response to the common house dust mite (*Dermatophagoides farinae*) antigen (Kamei et al., 1993). Most striking, in ovalbumin-sensitized guinea pigs and mice, monoclonal antibody to IL-5 decreased pulmonary eosinophilia and prevented the development of airway hyperresponsiveness (Nakajima et al., 1992).

The contribution of IL-5 to allergic disease has been greatly understood by the generation of mice deficient in the IL-5 gene. In contrast to normal mice, allergic IL-5 deficient mice do not generate eosinophilia in the bone marrow, blood, or lung in response to respiratory allergen provocation (Foster et al., 1996). The production of IL-5 by recombinant vaccinia viruses instilled into the airways of allergic IL-5 deficient mice completely restores aeroallergen-induced eosinophilia in the bone marrow, blood, and airways to levels normally observed in allergic wild type mice (Foster et al., 1996). Thus, in response to allergic stimulation in the lung, IL-5 critically regulates expansion of eosinophils in the bone marrow and potentiates their mobilization from this compartment to the blood. These effects indirectly amplify eosinophil recruitment into lung tissues in

response to locally derived chemotactic signals. IL-5, therefore, may be the most relevant Th2 cytokine in eosinophil biology, but it must work coordinately with other cytokines in this regard (Clutterbuck and Sanderson, 1990). Eotaxin, a member of the C-C branch of chemokines, is also a potent and rapid inducer of pulmonary eosinophil recruitment (Jose et al., 1994; Rothenberg, 1999). Investigations in guinea pigs and mice suggest that eotaxin and IL-5 act cooperatively to promote the recruitment of blood eosinophils into tissues (Mould et al., 1997).

Both IL-5 and eosinophils likely play a complex role in the curative responses to parasites and diseases such as asthma, although, in many experimental parasitic infections, protective roles for IL-5 and eosinophils have been difficult to demonstrate (Finkelman et al., 1997). However, larvae, but not adults, of Schistosoma and Onchocerca spp. are moderately susceptible to the cytotoxic effects of eosinophils (Folkard et al., 1996; Gounni et al., 1994). Interestingly, adult strongyloides may be susceptible to IL-5 independently of eosinophils through an undefined mechanism (Korenaga et al., 1994). In experimental asthma, eosinophils promote AHR in some (Foster et al., 1996) but not all (Hogan et al., 1998) strains of mice. Ultimately, however, it is the coordinated expression of IL-5 in conjunction with other cytokines, especially IL-4, IL-13, and eotaxin, which determines maximum lung pathology (Webb et al., 2000). Mast cells and eosinophils also synthesize IL-5, indicating that autocrine production of IL-5 may contribute to the chronicity of inflammation (Bradding et al., 1994). The significance of IL-5 in the production of eosinophils suggests a unique and tight control of IL-5 gene expression

1.3.3. IL-5 Gene Regulation

The genes encoding IL-3, IL-4, IL-13, GM-CSF and IL-5 cytokines are located in a cluster on the human chromosome 5q31 and in the mouse on chromosome 11q, indicating a common evolutionary origin [Fig. 1.6; (van Leeuwen et al., 1989)]. In general, IL-5 is not produced constitutively by Th2 cells. IL-5 gene expression has been shown to be stimulated by antigen, mitogens (concanavalin A), eicosanoid compounds (leukotriene B4 and prostaglandins), and cytokines (Bohjanen et al., 1990; Lee et al., 1993). The peculiarity of IL-5 gene expression is its dependence on *de novo* protein synthesis. The protein synthesis inhibitors CHX and anisomycin completely blocked IL-5 mRNA synthesis in murine Th2 cell clone D10.G4.1 and primary T cells, though these

inhibitors did not inhibit the expression of IL-2, IL-3, IL-4, IL-10, and GM-CSF mRNAs in these cells (Naora and Young, 1995; Stranick et al., 1995; Van Straaten et al., 1994).

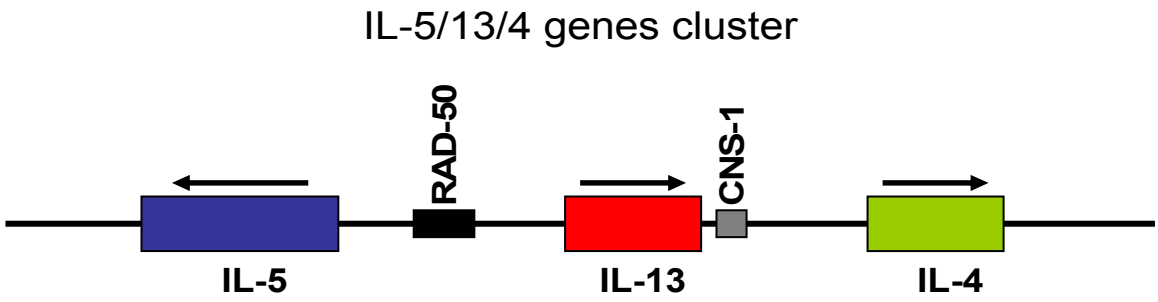


Fig. 1.6: Organisation of the IL-5/13/4 genes locus.

Agents which increase intracellular cAMP levels, such as IL-1α, prostaglandin E2, and the cAMP-analogue dibutyryl cyclic AMP (Bt_2cAMP) have been shown to differentially regulate cytokine production by Th1 and Th2 cells. Whereas the production of the Th1 response inducing cytokine IL-12 and that of the Th1 cytokines IL-2 and IFN-γ are inhibited by cAMP-increasing agents, the production of IL-5 is strongly induced by these agents, suggesting a possible immunoregulatory role for this second messenger (Lee et al., 1993; Munoz et al., 1990a; Snijdewint et al., 1993). As with other cytokines, regulation of IL-5 production is thought to result from the activation of gene transcription (Van Straaten et al., 1994). IL-5 synthesis is also regulated at the level of mRNA stability (Umland et al., 1998). Regulation of IL-5 expression is likely to involve calcium- and protein kinase C-dependent signaling pathways, as calcium ionophore and phorbol ester synergistically increase IL-5 expression and IL-5 promoter activity (Gruart-Gouilleux et al., 1995; Tominaga et al., 1988). Activation of Th2 cells is accompanied by increased activities of phorbol ester responsive AP1 and calcium responsive NFAT as well as of GATA-3 (Rincon and Flavell, 1997a). All three transcription factors have been found to be able to activate the IL-5 promoter [Fig. 1.7; (Stranick et al., 1995; Zhang et al., 1997)], suggesting that they may play an important role in controlling IL-5 expression.

The IL-5 promoter contains potential binding sites for multiple transcription factors including NFAT, AP-1, Oct, and Elf-1 (Campbell et al., 1988; Mizuta et al., 1988). The AP-1- and Elf-1 binding sites together constitute an element called the consensus lymphokine element 0 (CLE0) found in cytokine genes including IL-3, IL-4, IL-5, and GM-CSF (Arai et al., 1990; Miyatake et al., 1991; Nimer et al., 1990). It has been suggested that the TCATTT element, which overlaps with the AP-1-like element within

the CLE0 element in the IL-5 promoter, is important for the induction of IL-5 gene expression in response to mitogens and PMA (Naora et al., 1994). The TCA sequence in the TCATTT element overlaps with the AP-1 site, while the TTT sequence overlaps with the Elf-1 binding site within the CLE0 element. In EMSAs reported by Naora et al. (1994), mutation of the TCATTT element to cgAaTT (which also mutated the AP-1 element) abolished protein binding. Blumenthal et al. found that Ets1 and Ets2, but not Elf-1, were able to activate the IL-5 promoter in Jurkat cells in the presence of either PMA plus ionomycin or PMA plus Tax1 (Blumenthal et al., 1999). Using Kasumi cells, they showed that Ets1 and Ets2 are needed to cooperate with GATA3 for their stimulatory activities on the IL-5 promoter. These synergisms were enhanced in the presence of ionomycin or HTLV-I Tax1. These data suggested an important role for Ets proteins in the regulation of the IL-5 expression in Th2 lymphocytes and HTLV-I transformed leukemic cells (Blumenthal et al., 1999).

Fig.1.7: *cis*-acting elements of IL-5 gene. Within the promoter region, IL-5 gene contains sequence motifs for Th2 - specific as well as non-specific transcription factors. Coordinated and specific expression of the IL-5 gene in Th2 cells may be regulated through these elements.

A new regulatory element located in the 3´ flanking region of the mIL-5 gene was identified (Salerno et al., 2000). This murine downstream regulatory element-1 (mDRE1) is 40 bp long, consists of four direct repeats of ATGAATGA distributed in a symmetrical manner, and was shown to be protected in DNase I footprinting assays. Deletion of this sequence from a 9.5 kb construct containing the whole mIL-5 gene decreased the promoter activity upon PMA/cAMP stimulation by 3.5 fold, suggesting that this part of the 3´ untranslated region is involved in the positive regulation of the gene. Using nuclear extracts from the thymoma derived EL4 cell line and primary T cell, it was shown that transcription factors Oct-1 and Oct-2 bind to the mDRE1 sequence (Salerno et al., 2000). In transient transfection assays, dibutyryl cAMP-induced activation of the IL-5 promoter was mimicked by transfection of an expression plasmid encoding the catalytic subunit of protein kinase A, suggesting that cAMP stimulates IL-5 transcription via the protein kinase A signaling pathway (Lee et al., 1993).

1.4. Aim and Experimental Design of the Project

The cytokine production profiles of Th1 and Th2 cells parallel their effector functions during immune responses, in that Th1 cells act as mediators of cellular immune responses and Th2 cells, by virtue of their synthesis of IL-4 and IL-5, induce allergic immune responses involving elevated IgE levels and selective differentiation of eosinophils (Mosmann and Coffman, 1989). Thus, a better understanding of the regulation of IL-5 gene expression in T cells could provide important insights into the possible dysregulation of this cytokine in diseases characterized by eosinophilia, such as asthma. The differential role of cAMP on the effector function of T helper cytokine gene expression has been largely analyzed in several transformed T cell lines and clones. Unlike other cytokines, little is known regarding IL-5 gene regulation *in vitro*, particularly in untransformed, mature T cells. The studies presented in this thesis investigated the *in vitro* gene regulation of IL-5 by cAMP in murine T helper cells obtained from ova transgenic mice model, which offered the tremendous advantage of non-transformed nature of cells, in contrast to previous studies using transformed cell types.

In different mammalian cell systems, the physiological target of cAMP is PKA and therefore, in this work we have tried to gain evidence of the role of PKA in the IL-5 production in primary T lymphocytes. Three primary murine $CD4^+$ T cell systems were used in addition to mouse thymoma cell line, EL-4. Usually lymph node naïve $CD4^+CD62L^{high}$ T lymphocytes were activated in the presence of cognate antigen plus irradiated APC and skewed either in the presence of Th1 or Th2 or Th0 conditions for 5-7 days and then analyzed for lymphokine expression pattern under desired stimulus. The *in vitro* activation of the Th cells was achieved by using either plate-bound αCD3 plus soluble αCD28 to activate TCR and co-stimulatory pathways, respectively or phorbol 12-myristate 13-acetate (PMA) in combination with ionomycin (A23187). The intracellular level of cAMP was elevated by forskolin, which rapidly activates adenylyl cyclase, the enzyme responsible for cAMP generation in the cell. The retroviral gene transfer was usually achieved after one day of primary stimulation and separation of transduced cell was done by FACsorting after two days of transduction. The research progress achieved in understanding the cAMP/adenylyl cyclase pathway in primary lymphocytes will be presented and discussed in detail in the forthcoming sections of this dissertation.

2. Results

2.1. Elevated cAMP level augments T/I-mediated induction of IL-4 and IL-5 expression in EL-4 thymoma cells.

A growing body of evidence shows that an increase in cAMP levels results in differential effects on Th2-type vs Th1-type cytokine gene expression. Thus, while cAMP inhibits Th1-type cytokine gene expression, it upregulates Th2-type cytokine gene expression [reviewed in (Haraguchi et al., 1995)]. During the start of my doctoral work, I wished to establish the T cell - based experimental model to delineate the cAMP/adenylyl cyclase pathway in T lymphocyte.

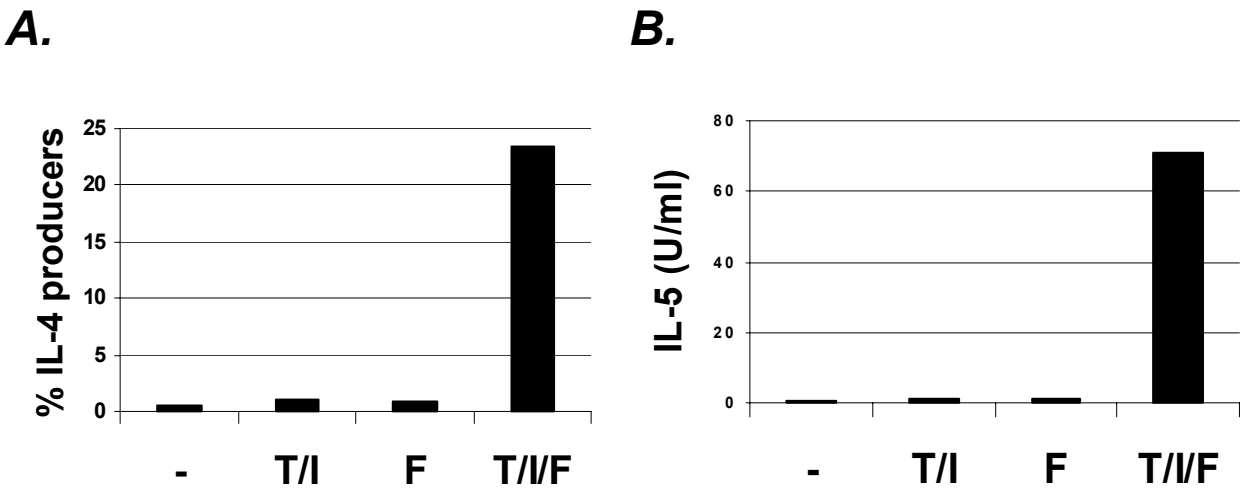


Fig 2.1: Forskolin enhances T/I-mediated induction of IL-4 and IL-5 expression in EL-4 thymoma cells. ***A*****,** EL-4 cells were induced for 12 h with TPA (10 ng/ml) and ionomyin (0.5 µM) (T/I), forskolin (50 µM) (F), T/I/F or left untreated (-). Half of the cultures were treated with brefeldin (10 µg/ml), and 4 h later IL-4 producing cells were determined by intracellular staining. ***B*****,** Measurement of IL-5 concentration was done 48 h after activation by ELISA in the cell-free supernatant of the other half of the cultures.

I selected the mouse thymoma cell line EL-4 cells, which is widely used in IL-5 research, as cell line model because using EL-4 cells, previous studies from different laboratories demonstrated the differential effect of cAMP on Th lymphokine expression. Forskolin is an activator of adenylyl cyclase enzyme, which catalyzes the synthesis of cAMP from ATP in different cell systems. In these preliminary assays, EL-4 cells were treated either with T/I, which activates T lymphocytes; or with forskolin, which

instantaneously elevates cAMP level in the cell; or with T/I/F for 48 hours. Expression of IL-4 and IL-5 was analyzed by intracellular staining (for methodical details, see section 5.2.4.3) and ELISA in the cell-free supernatant (for technical details, see section 5.2.4.3), respectively. In agreement with previous findings, it is highly evident in figure 2.1, that along with the activation of TCR signaling pathways mimicked by T/I, elevation of cAMP is an absolute requirement for the full expression of both Th2-type cytokine genes, namely IL-4 and IL-5, in EL-4 cells.

2.2. Activation or Inhibition of IL-5 promoter activity in EL-4 cells by modulation of PKA activity.

Since the immediate target of cAMP in mammalian cells is PKA, initial attempts were made to analyze the effect of transient activation or inhibition of PKA on the IL-5 promoter activities in T cells. The importance of PKA in executing the effect of cAMP was tested by co-transfection of catalytic and regulatory subunits of PKA into EL-4 cells. PKA exists as a holoenzyme consisting of two catalytic subunits and one regulatory subunit dimer, which harbors four cAMP binding sites. By binding to regulatory subunit dimer, cAMP dissociates the regulatory subunit dimer from catalytic subunits, which then phosphorylate specific target proteins (for details, see section 1.2.5.1). Ectopic expression of only catalytic subunit results in increased PKA activity, whereas, expression of engineered regulatory subunit, which contains mutations in cAMP binding sites and thereby worked like dominant negative version of PKA, allows the constitutive physiological inhibition of PKA. Using these two expression plasmids, the activity of PKA was modulated in transient transfection assays (for methodical details, see section 5.2.6.1) to determine promoter activity of IL-5. For this, EL-4 cells were co-transfected with the –507 IL-5 promoter luciferase plasmid and expression vector either for murine PKA catalytic subunit α (PKA_C) or mutant PKA regulatory subunit (PKA_{RM}). Luciferase activity was measured in cultures after stimulation for 20 hours

As expected, the luciferase activity was significantly increased in T/I/F - treated control transfected cells compared to T/I induction of these cells (Fig. 2.2). Cells transfected with the PKA catalytic subunit produced very high levels of luciferase activity after T/I stimulation and further treatment with forskolin did not augment reporter activity. On the other hand, transfection of PKA_{RM} strongly inhibited the promoter

activity. These data demonstrate that PKA is required for the full activation of IL-5 promoter activity in EL-4 cells.

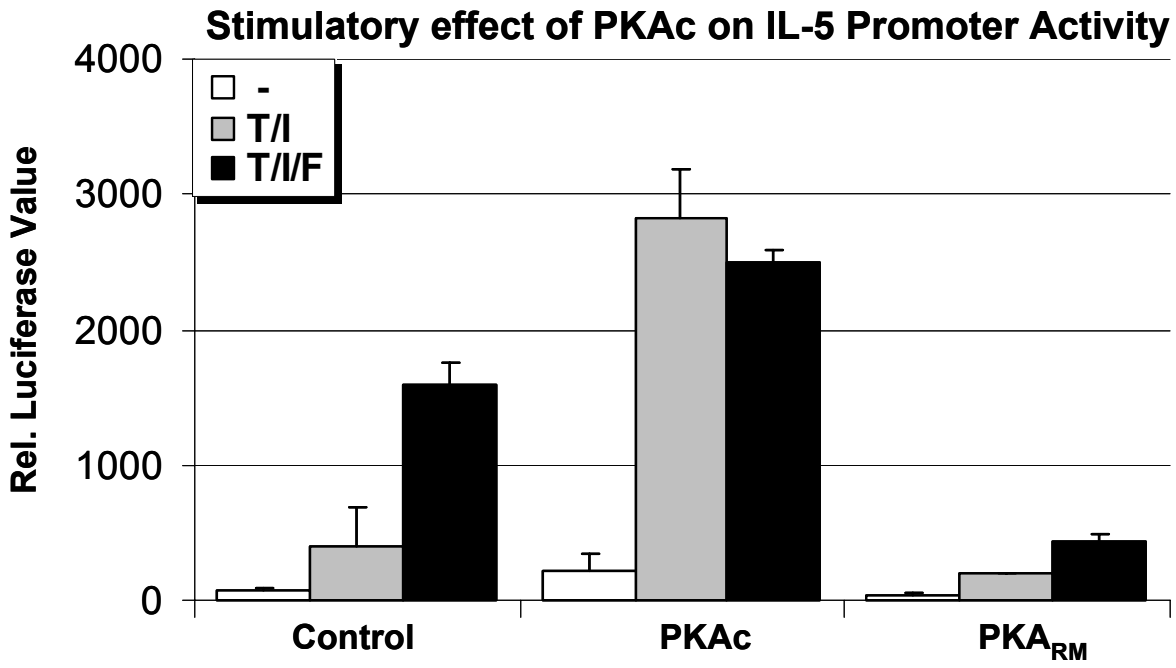


Fig 2.2: Active PKA enhances IL-5 promoter activity in EL-4 cells. EL-4 cells were co-transfected with a –507 IL-5 promoter driven luciferase plasmid and vectors expressing PKA_C or mutant PKA_{RM}. Luciferase activity was measured from cells which were either left untreated (-), or induced with T/I or T/I/F for 20 h. Mean values of three independent experiments are shown.

2.3. Active PKA induces IL-4 and IL-5 mRNA synthesis in EL-4 cells.

We wished to determine whether activation of PKA results in the induction of Th2-type lymphokine, especially IL-5, gene expression in a similar fashion to the forskolin effect (Fig. 2.1) and transient activation of IL-5 promoter (Fig. 2.2) in these EL-4 cells. In this regard, we used the highly efficient retroviral gene transfer in lymphocytes. We constructed a PKA_c (PKA catalytic subunit) encoding retroviral vector that, due to the presence of an internal ribosomal entry site (IRES), expresses also enhanced green fluorescence protein (eGFP) and zeocin for the selection of positively transduced cells. Using this retroviral vector, we generated highly infectious retroviruses containing the gene of interest and transduced them into EL-4 cells using a standard infection protocol (see section 5.2.2.5). Owing to the presence of zeocin, a selection marker for mammalian cells, positively transduced cells expressing the PKA catalytic subunit

α (PKA_C) or control retrovirus (retroviral vector without gene of interest) were selected by zeocin treatment. The lymphokine mRNA level in response to different signals was analyzed by RNase protection assay (for methodical details, see section 5.2.3.4) after restimulation with different stimuli for 16 hours.

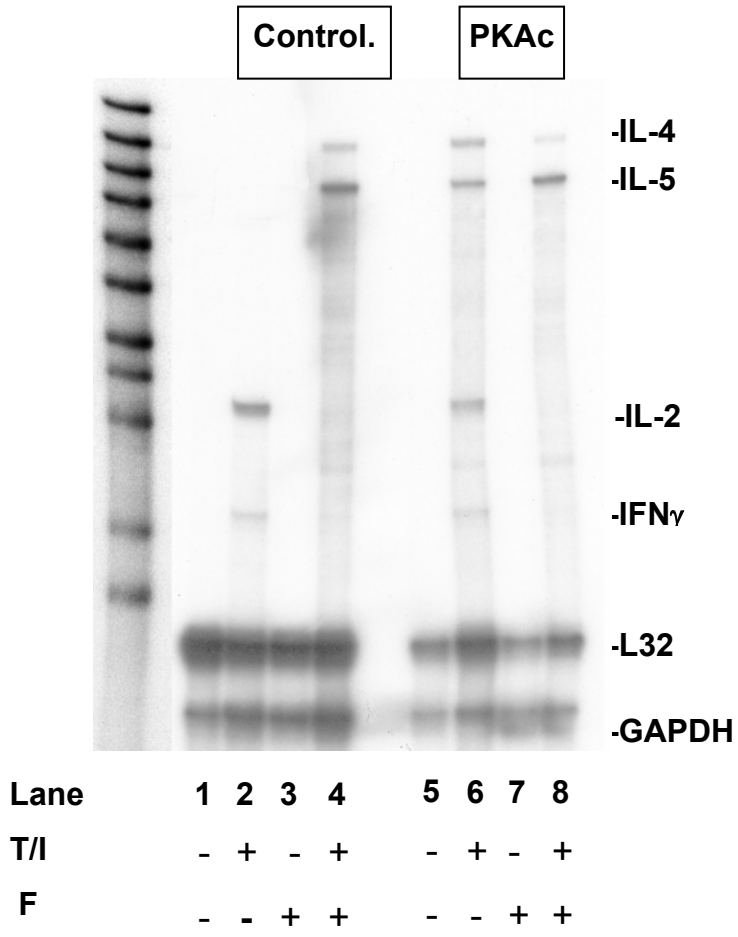


Fig 2.3: PKA activity can replace forskolin in the induction of IL-4 and IL-5 expression in EL-4 cells. RNAs from EL-4 cells infected with the control retrovirus pEGZ (lanes 1-4) or with PKA_C virus (lanes 5-8) were isolated 16 h after stimulation and assayed using the RiboQuant RNase mCK-1 protection kit (BD Biosciences). The cells were either left untreated (-) or treated as indicated with T/I, F, or T/I/F.

As shown in figure 2.3, T/I treatment of control EL-4 cells alone fails to induce Th2-specific lymphokine gene expression, however strongly induces IL-2 and IFN-γ expression. The additional treatment with forskolin dramatically induces transcription of IL-4 and IL-5 genes, and remarkably downregulates IL-2 and IFN-γ mRNA production. The constitutive expression of PKA_C is sufficient to replace the forskolin signal in the T/I-

mediated induction of IL-4 and IL-5 mRNA synthesis (Fig. 2.3, compare lanes 4 and 6). Interestingly, in contrast to forskolin treatment, the overexpression of PKA_C did not impair IL-2 and IFN-γ mRNA synthesis in EL-4 cells (Fig. 2.3, compare lanes 4 and 6) suggesting that this immunomodulatory effect of cAMP by the synthesis of different lymphokines is regulated by different effector molecules in the cell.

2.4. Forskolin selectively enhances IL-5 production in primary T cells.

Several investigations by different laboratories provided ample evidences in favor of differential gene regulation by cAMP using different clones and thymoma cell lines like EL-4. However these clones and EL4 cells possess a highly confused molecular traffic in general and therefore do not represent the real *in vivo* scenario of lymphokine gene regulation. The conspicuous lack of research in the field of cAMP-mediated regulation of lymphokine gene expression in primary T lymphocytes inspired us to investigate the stimulatory effect of elevated cAMP level on Th2 cytokine production in murine $CD4^+$ T cells. We isolated $CD4^+$ T cells by negative selection from LN of DO11.10 TCR tg mice and further enriched on CD62L column as naïve $CD4^+CD62L^{high}$ T cells (see section 5.2.2.3). These naïve $CD4^+CD62L^{high}$ T cells were activated in the presence of ova antigen and irradiated splenic dendritic cells, and cultured for five days under Th0, Th1 or Th2 priming conditions. On day 6, cells were thoroughly washed with BSS/BSA to remove any trace of cytokines and they were restimulated for 48 hours by T/I, F or T/I/F or left untreated (-) as indicated. Cytokine production was measured by ELISA in the cell-free supernatant of unstimulated (-) and restimulated cells.

As shown in figure 2.4, Th1 cells are unable to synthesize IL-5 despite of any treatment. However Th2 cells produce large amounts of IL-5 after T/I treatment, suggesting that all essential factors for optimal IL-5 secretion are present in these effector Th2 cells and therefore these cells are highly competent to produce optimal level of IL-5 after TCR stimulation, mimicked by T/I treatment. Further treatment with forskolin marginally enhanced IL-5 secretion by Th2 cells. Forskolin alone never induced any IL-5 expression in any type of cells, suggesting the essential requirements of TCR signaling pathways for the IL-5 gene expression. Interestingly, the Th0 cells, which represent an intermediate phenotype, synthesize minor amounts of IL-5 upon TCR stimulation; however, they become bonafide IL-5 producers like Th2 cells upon

additional provision of forskolin. This clearly indicates that these Th0 cells lack cAMP signal, which otherwise is sufficient in Th2 cells to drive the IL-5 expression.

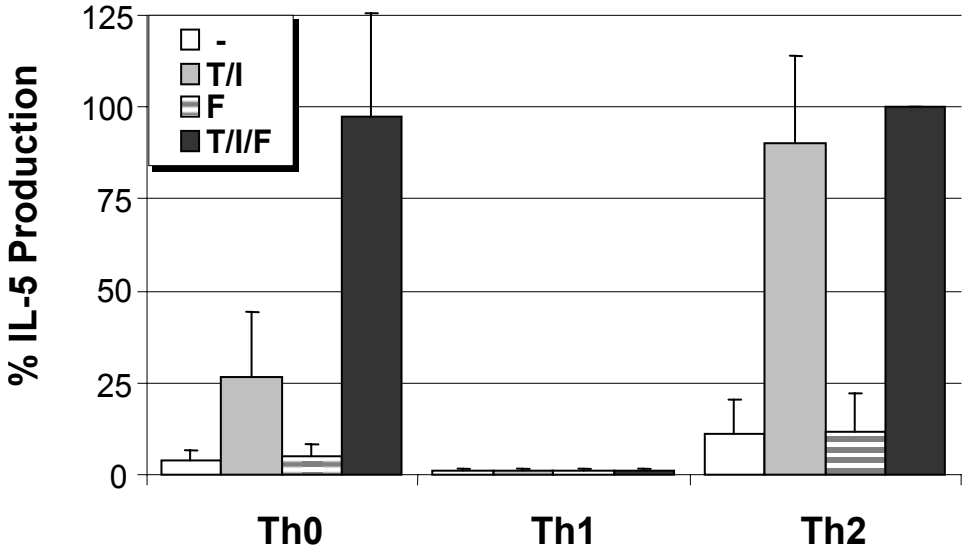


Fig 2.4: Induction conditions for IL-5 secretion by primary Th cells. $CD4^+CD62L^{high}$ T cells from LN of DO11.10 TCR tg mice were maintained after primary stimulation for 5-7 days under Th0, Th1 or Th2 priming conditions. IL-5 production by unstimulated (-) and cells restimulated for 48 h by T/I, F or T/I/F was determined by ELISA. Relative values to T/I/F-treated Th2 cells (100%) of two independent experiments are shown.

2.5. The induction of IL-5 expression by forskolin in primary Th0 cells is a transcriptional event.

Since elevation in intracellular cAMP level drives the IL-5 production in Th0 cells, we wished to determine whether cAMP induces transcription of the IL-5 gene in these cells. For this, $CD4^+CD62L^{high}$ T cells were activated and cultured for 5 days in the presence of Th0 conditions. Total RNAs were isolated from cells either non-restimulated or restimulated for 16 hours with indicated stimuli. RNase protection assay for cytokine genes reveals that cAMP enhances IL-5 production, as observed in Fig. 2.5, by activating transcription of IL-5 gene, which is in agreement with our previous data (Fig. 2.4). However other Th2-specific cytokines like IL-4, IL-9 and IL-13 were found to be resistant to the cAMP-mediated gene regulation as shown by normalized signal intensities (for IL-5 and IL-4 band intensities, see Fig. 2.5B; data not shown for IL-9 and IL-13).

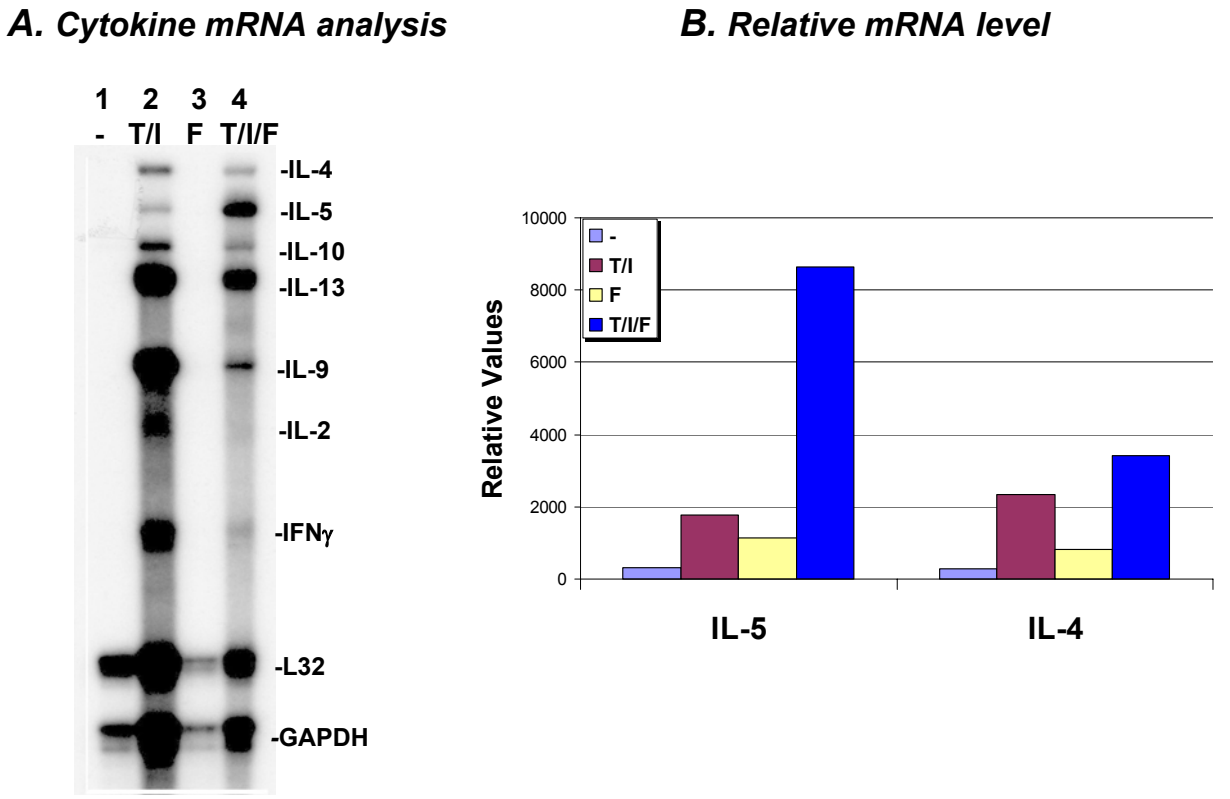


Fig 2.5: PKA activation by forskolin treatment increases IL-5 mRNA level in primary CD4+ T cells. ***A,*** *Cytokine mRNA analysis in primary Th0 cells.* RNAs from $CD4^+$ T cells, cultured under Th0 conditions for 5 days, were isolated 16 h after restimulation on day 6 after primary stimulation and assayed using the RiboQuant RNase mCK-1 protection kit (BD Bioscience). The cells were either left untreated (-) or treated as indicated with T/I, F, or both. ***B,*** *Phosphorimager analysis of signals.* The intensity of each band was measured by Phosphorimager and relative intensity of each band is graphically shown with reference to that of the GAPDH house keeping gene.

2.6. Proper IL-5 gene expression in EL-4 and primary Th0 cells requires combined signals of TPA, Ionomycin and cAMP.

Several studies indicate that signal requirements for optimal IL-5 production depend on the type of cell system examined. For example, RT-PCR analysis revealed that Jurkat cells fail to express IL-5 mRNA, with any combinations of the compounds examined. In contrast, ATL16 cells express IL-5 mRNA constitutively and neither PMA alone nor a combination of PMA and A23187 enhanced the level of IL-5 mRNA. One study (Lee et al., 1993) shows that EL-4 cells, which express no detectable levels of IL-5 mRNA in the absence of stimuli, produced a low level of IL-5 mRNA in response to PMA stimulation and exposure of these cells to both PMA plus Bt_2cAMP substantially increased the IL-5

mRNA levels. Another study (Chen et al., 2000) also claims that cAMP synergizes with PMA alone for IL-5 induction in EL-4 cells and these two signals are important and sufficient for full IL-5 production. We wished to determine the optimal conditions for IL-5 production in both EL-4 and primary T lymphocytes. In our study as shown in figure 2.6, ionomycin treatment along with TPA plus cAMP was crucial for high level of expression in both EL-4 as well as primary Th0 cells.

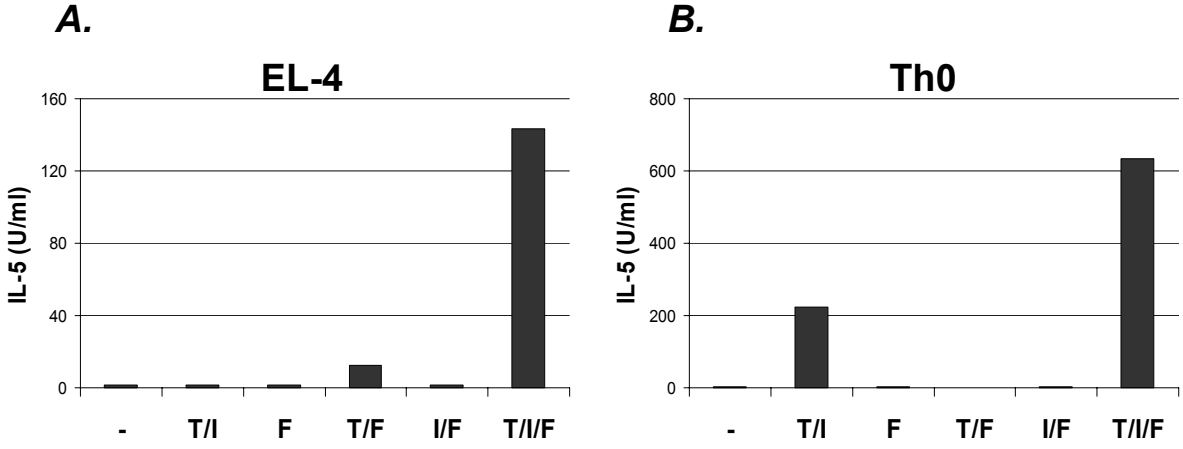


Fig 2.6: Induction conditions for IL-5 secretion by EL-4 and primary Th0 cells. ***A,*** Murine EL-4 thymoma cells were induced for 48 h with TPA (T), ionomycin (I), forskolin (F), TPA and ionomyin (T/I), TPA and forskolin (T/F), Ionomycin and forskolin (I/F) or by TPA, ionomycin and forskolin (T/I/F) or left untreated (-). ***B,*** Naïve $CD4^{+}CD62L^{high}$ T cells were cultured under Th0 conditions for five days. On day 6, cells were washed and equal number of cells were either restimulated for 48 hours or left non-restimulated as that of EL-4 cells. IL-5 production was measured by ELISA in the cell-free supernatant of non-restimulated and different restimulated cells. One representative experiment out of three is shown here.

2.7. Ectopic expression of constitutive active PKA selectively induces IL-5 expression in murine Th0 cells.

As shown in Fig. 2.3, ectopic expression of PKA_C in EL-4 cells is sufficient to replace the forskolin signal in the T/I-mediated induction of IL-4 and IL-5 mRNA synthesis. In order to investigate whether active PKA_C plays a similar stimulatory role in IL-5 synthesis in primary T cells, $CD4^{+}$ T cells from DO11.10 tg mice were infected with either recombinant retroviruses containing PKAc or empty retroviruses. Forskolin enhanced the T/I-mediated increase in IL-5 secretion by at least 3 fold in both control populations, i.e., EGZ or PKA (-), and ectopic expression of PKA_C was sufficient to

enhance the T/I-mediated IL-5 induction to a very similar level (Fig. 2.7A). Interestingly, PKA_C could not inhibit the IFN-γ expression in these cells, suggesting that two distinct pathways regulate the differential effects of cAMP on lymphokine gene expression.

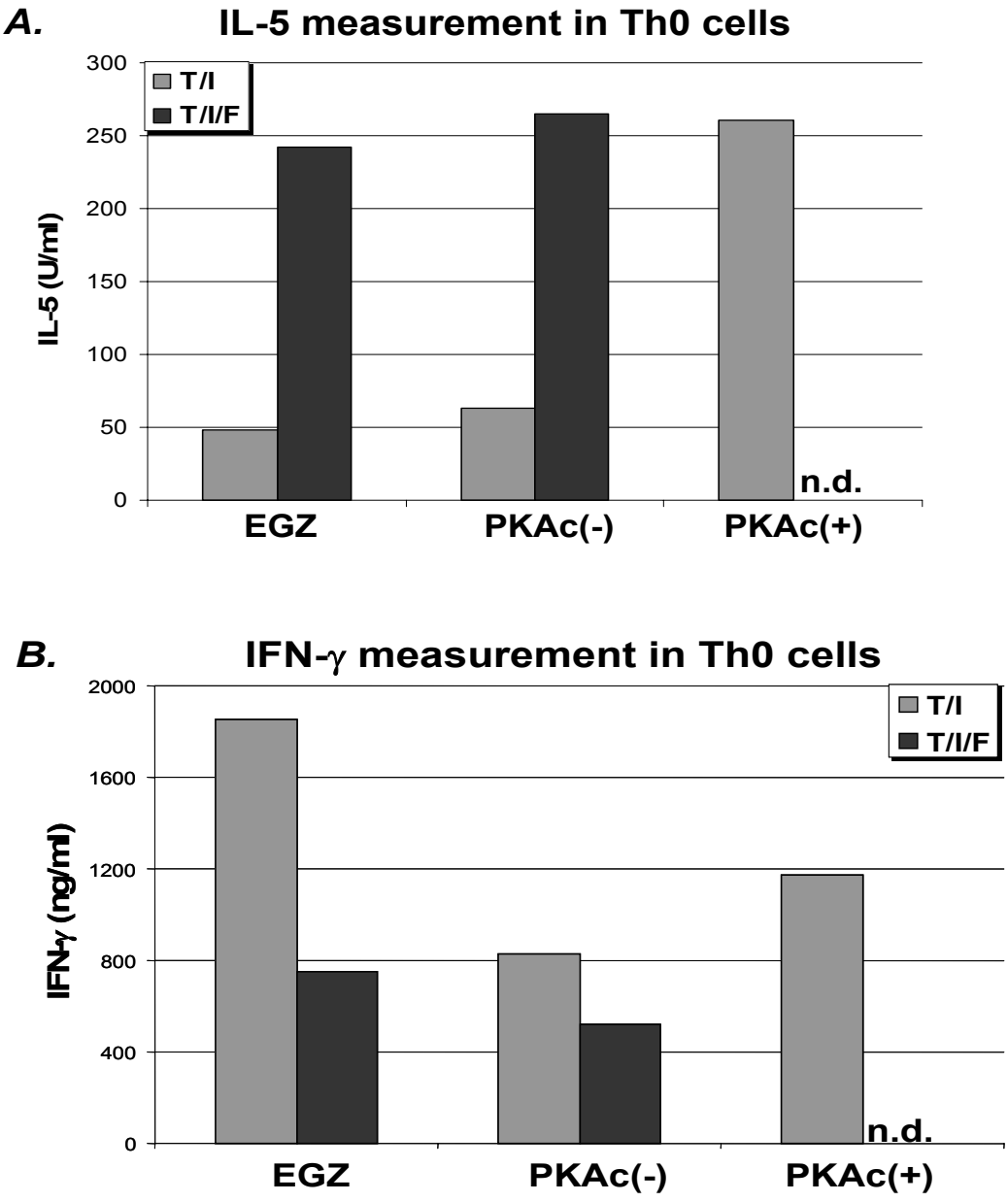


Fig 2.7: PKAc selectively induces IL-5 expression in primary Th0 cells. After 24 hours of primary stimulation $CD4^+$ T cells from LN of DO11.10 TCR tg mice were infected with recombinant retroviruses containing the PKA catalytic subunit (EGZ-PKAc) or with control virus (EGZ-HA) and maintained under Th0 (IL-2, anti-IFNγ and anti-IL-4) conditions. Two days post-infection, cells were sorted according to marker fluorescence by FACS into EGZ-HA-infected [EGZ] and PKAc-infected [PKAc(+)] or PKAc-noninfected [PKAc(-)] populations. After 24 hours the cells were restimulated for 48 hours with TPA and ionomycin (T/I) or TPA, ionomycin and forskolin (T/I/F) to measure IL-5 ***(A)*** and IFN-γ level ***(B)*** in the cell-free supernatant by ELISA.

2.8. Forskolin or active PKA synergizes with physiological signals in the induction of IL-5 expression in murine Th0 cells.

Since the recognition of a cognate antigen associated with the MHC on the APC by the TCR triggers a series of biochemical events, including elevation of intracellular calcium ion concentration and activation of PKC which can be bypassed by stimulating T cells with TPA and ionomycin, we wished to confirm whether forskolin or PKA is able to synergize signals emanating from TCR in mature Th0 cells for the cAMP-mediated effect on IL-5 production.

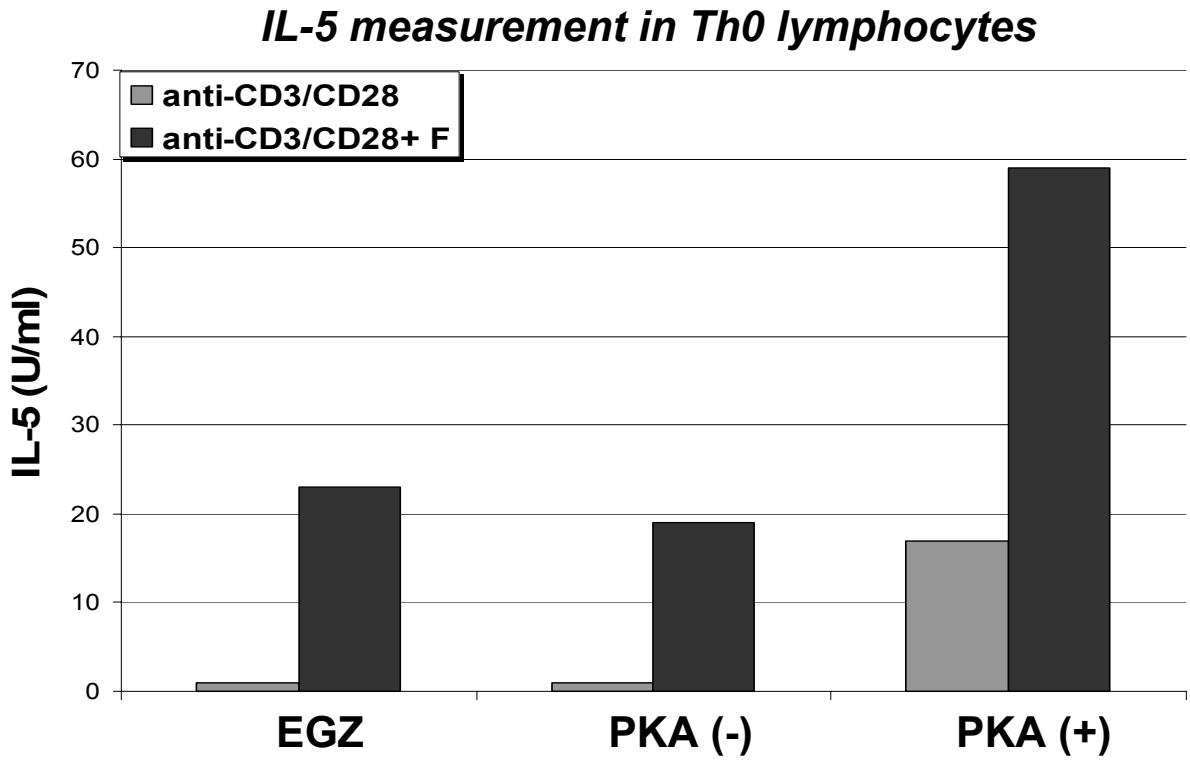


Fig 2.8: PKAc synergizes with TCR/CD28-mediated signals in the induction of IL-5 expression in murine Th0 cells. After 24 hours of primary stimulation $CD4^+$ T cells from LN of DO11.10 TCR tg mice were infected with recombinant retroviruses containing the PKA catalytic subunit (EGZ-PKAc) or control (EGZ-HA) and maintained under Th0 (IL-2, αIFN-γ and αIL-4) conditions. Two days post infection, cells were sorted according to marker fluorescence by FACS into EGZ-HA-infected [EGZ] and PKAc-infected [PKAc(+)] or PKAc-non-infected [PKAc(-)] populations. After 24 hours the cells were restimulated for 48 hours with either plate bound αCD3 Ab (10 µg/ml) and αCD28 (5 µg/ml, BD Biosciences) or plate bound αCD3 Ab, soluble αCD28 and forskolin to measure IL-5 in the cell-free supernatant by ELISA.

To do this, $CD4^+$ T cells, activated and cultured in Th0 conditions, were infected after 24 hours of primary stimulation with either recombinant retroviruses containing PKAc or control retroviruses and sorted into pure (≥95%) populations. Sorted cells were restimulated for 48 hours by plate - bound αCD3 antibody for TCR activation and αCD28 antibody for costimulation. The production of IL-5 upon αCD3 plus αCD28 treatment was negligible in both Th0 control populations, i.e., EGZ as well as PKAc-non-infected, however, additional forskolin treatment markedly enhanced the IL-5 secretion by these cells, similar to T/I/F treatment (Fig. 2.8). The ectopic expression of PKAc in PKAc-infected Th0 population, PKAc(+) was sufficient to replace this forskolin effect, clearly demonstrating that PKAc synergizes the cAMP-induced signal with physiological TCR activation pathways (Fig. 2.8).

This result deserves clearly high importance from the physiological point of view, since *in vivo* the activation of PKC and calcium pathways by TCR activation originate from the T cell membrane and not by our *in vitro* pharmacological activators TPA and ionomycin, which escape the control mechanisms of T cell activation downstream to TCR activation. Interestingly, additional treatment of PKAc-infected Th0 lymphocytes with forskolin enhanced the IL-5 production to more than 3 fold compared to PKAc-expressing Th0 cells or forskolin - treated control Th0 cells. This observation gains significance with respect to the possible additional pathway(s) acting in synergy with PKA pathway in regulating IL-5 gene expression.

2.9. Inhibition of PKA activity by H-89 downregulates IL-5 synthesis in primary Th2 cells.

IL-5 is predominantly produced by effector Th2 lymphocytes after i*n vitro* activation by phorbol 12-myristate 13-acetate (PMA) in combination with ionomycin or by αCD3 plus αCD28 treatment. In contrast to undifferentiated Th0 cells, these Th2 lymphocytes do not require an additional treatment of cAMP elevating agents for optimal IL-5 secretion. This observation is corroborated by results of one study in which Th2 cells were shown to contain higher level of cAMP than Th1 cells (Novak and Rothenberg, 1990). Therefore, we hypothesized that Th2 lymphocytes have higher level of endogenous PKA activity, which would augment IL-5 gene expression. In order to inhibit this endogenous PKA activity, we used the highly specific PKA inhibitor, H-89, at low concentrations (Davies et al., 2000).

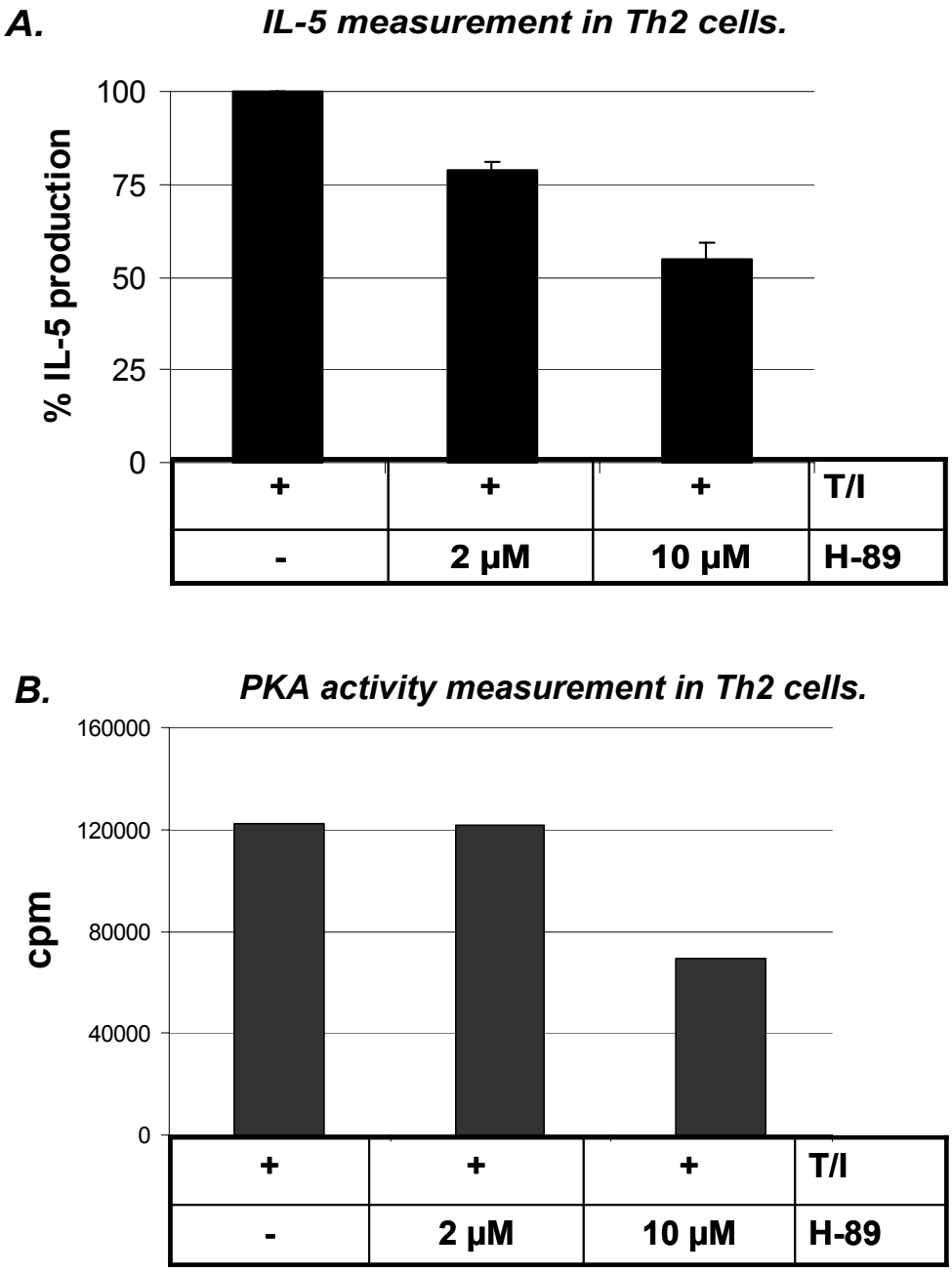


Fig 2.9: Inhibition of PKA activity by H89 impairs IL-5 expression in primary Th2 cells. ***A****, IL-5 production is inhibited in Th2 cells by H89 treatment.* $CD4^+CD62L^{high}$T cells from LN of DO11.10 TCR tg were cultured under Th2-skewing conditions for 5 days and restimulated by T/I in the absence or presence of 2 μM or 10 μM H89 (these cells were pre-treated for 30 minutes, followed by T/I) for 48 h and IL-5 secretion was measured by ELISA. Relative values to control cells (100%) of two independent experiments are shown. ***B,*** *H-89 treatment suppresses PKA activity.* $CD4^+CD62L^{high}$ T cells were activated and propagated in Th2 priming conditions for 5 days and then restimulated by T/I in the absence or presence of 2 μM or 10 μM H89 (these cells were pre-treated with H-89 accordingly for 30 minutes) for 1 h. Whole cell extracts were used to detect the activity of PKA by PKA detection kit (Upstate) according to manufacturer's instructions.

The $CD4^+$ T cells were cultured under Th2-skewing conditions for 5-6 days, followed by restimulation under desired conditions for 48 hours and an IL-5 assay was performed. As shown in figure 2.9A, H-89 inhibited the secretion of IL-5 in a dose-dependent manner, validating our earlier results that the active PKA is crucial for proper IL-5 production by activated Th2 lymphocytes.

To control experiments, we measured actual PKA activity in Th2 cells after treatment with PKA inhibitor, H-89. The $CD4^+$ T cells were cultured under Th2 skewing condition for 5-6 days and then restimulated in the presence or absence of H-89 for 1 hour and then measured the PKA activity. As shown in figure 2.9B, the activity of PKA is suppressed to almost half in Th2 cells treated with 10 μM H-89, the concentration which impaired the IL-5 expression to a similar level. This strengthened our assumption that the suppression of IL-5 expression in response to optimal dose of H-89 is due to the lower PKA activity.

2.10. Inhibition of PKA activity by retroviral overexpression of PKA_{RM} leads to downregulation of IL-5 synthesis in primary Th2 cells.

We were prompted to examine the effect of retroviral-mediated overexpression of PKA_{RM} on IL-5 production in primary Th2 cells because of our two prominent observations: (1) IL-5 promoter activity was completely suppressed by the expression of PKA_{RM} in a transient transfection assay (Fig. 2.2) and (2) IL-5 production was downregulated up to 50 % by the treatment with H-89 in Th2 lymphocytes (Fig. 2.9). Under low cAMP concentration, the natural mechanism of the inhibition of PKA activity lies in binding of regulatory subunit to the catalytic subunit of PKA. Therefore the inhibition of PKA activity by ectopic expression in primary Th2 cells of mutated version of regulatory subunit of PKA (PKA_{RM}) has physiological advantage over the pharmacological PKA inhibitor like H-89. We constructed a PKA_{RM}-encoding retroviral vector that, due to the presence of an internal ribosomal entry site (IRES), expresses also a green fluorescence protein (GFP) for FACsorting of positively transduced cells. When we infected activated $CD4^+$ T cells cultured in the Th2 skewing conditions by retroviruses expressing PKA_{RM} and sorted into pure populations by FACS on the basis of GFP expression, the IL-5 production was inhibited up to 50 % in PKA_{RM}-infected Th2 cells and not in EGZ-infected or PKA_{RM}-uninfected Th2 populations (Fig. 2.10A). This

strongly demonstrates that higher endogenous PKA activity in Th2 cells is required for optimal IL-5 production.

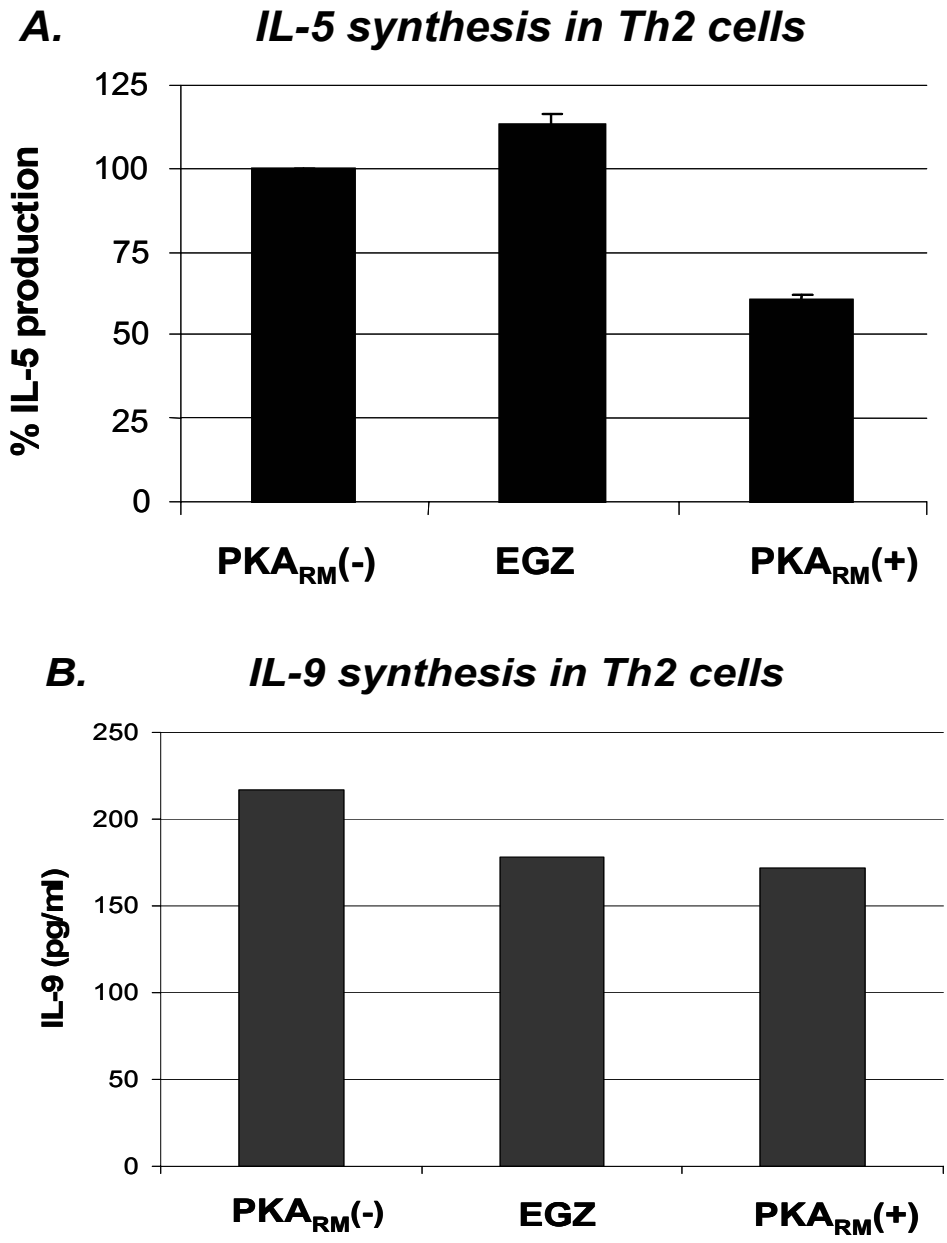


Fig 2.10: Inhibition of PKA activity by a mutant of the PKA regulatory subunit I (PKA$_{RM}$) impairs IL- 5 synthesis in primary Th2 cells. ***A,*** *IL-5 production in Th2 cells is inhibited by the ectopic expression of PKA$_{RM}$.* One day after primary stimulation, CD4$^+$ T cells from LN of DO11.10 TCR tg mice were infected with recombinant retroviruses transducing PKA$_{RM}$ or a control virus and maintained under Th2 conditions. Two days post infection, cells were sorted by FACS into EGZ-infected cells [EGZ] and PKA$_{RM}$-infected cells [PKA$_{RM}$(+)] as well as PKA$_{RM}$-non-infected cells [PKA$_{RM}$(-)]. Three days later, cells were restimulated for 48 h by T/I and IL-5 secretion was determined by ELISA. Relative values to uninfected cells (100%) of two independent experiments are shown. ***B,*** *Inhibition of PKA does not impair IL-9 expression in Th2 cells.* CD4$^+$ T cells stimulated and infected as in (A) were assayed for IL-9 by ELISA.

Furthermore, we also investigated whether the inhibition of active PKA in Th2 cells has some general effect on other Th2-specific cytokine expression or this inhibitory effect is specific to IL-5. For this, we quantified the production of IL-9 in the same supernatants by ELISA. Indeed we did not observe any difference in the level of IL-9 production among different control Th2 cells and PKA_{RM}-expressing Th2 cells (Fig. 2.10B). This result indicates that activation of PKA consequent to rise in intracellular cAMP level is selective for the induction of IL-5 synthesis.

2.11. The level of PKA activity does not differ in different effector Th lymphocytes.

The observation in Fig. 2.10 advocated that higher levels of endogenously activated PKA after T/I activation in Th2 cells contribute to IL-5 production. This hypothesis gained significance with respect to earlier observation that Th2 cells contain relatively higher levels of cAMP compare to Th1 cells (Novak and Rothenberg, 1990). Thus we examined this possibility in all three subtypes of effector $CD4^+$ T cells i.e. Th0, Th1 and Th2, which were cultured for 5 days under skewing conditions after primary stimulation with antigen. These mature cells were restimulated for 1 hour by T/I, F or T/I/F or left untreated (-) as indicated in Fig. 2.11. The intracellular PKA activities were analyzed in whole cell protein extracts using the PKA assay kit (Upstate biotechnology), which measures the phosphotransferase activity of PKA. The assay utilized the phosphorylation of a specific substrate, KEMPTIDE, using the transfer of the γ-phosphate of adenosine-5-[^{32}P] triphosphate ([γ-^{32}P] ATP) by active PKA (see section 5.2.4.4).

However, contrary to our assumption that Th2 cells contain higher levels of active PKA, we could not observe any difference in PKA activity in whole cell protein extracts (Fig. 2.11) or nuclear extracts (data not shown) of these cells upon T/I restimulation. Irrespective of cell type, forskolin induced PKA activity in all these cells suggesting that assay is functional. Furthermore we also checked the possibility of higher PKA activity in Th2 cells at other time points like 10 minutes, 30 minutes, 3 hours and 12 hours after restimulation and did not find any difference in the activity at these time points (data not shown). Despite our inability to observe higher PKA activity in Th2 lymphocytes, we assume that active PKA might be localized in discrete microdomains in cytoplasm to

constitute highly ordered signaling complex with other molecules like AKAPs (see discussion 3.2).

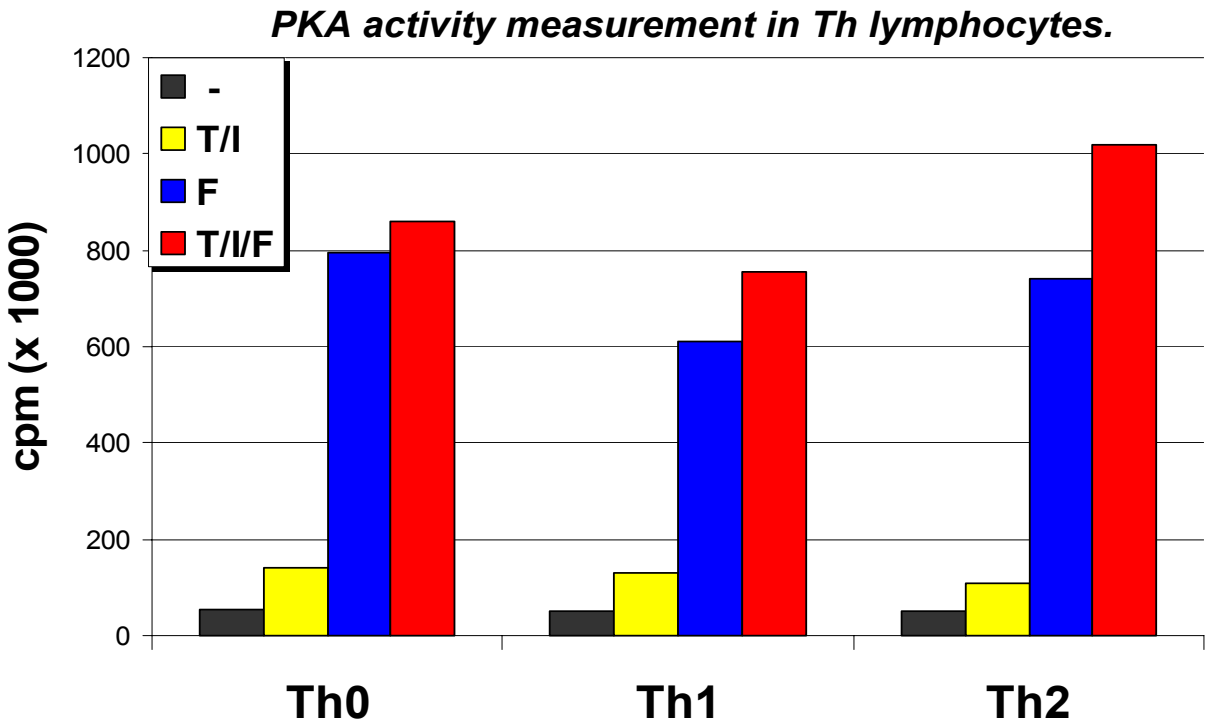


Fig 2.11: There is no difference in PKA activity in whole cell protein extracts of Th cells. $CD4^{+}CD62L^{high}$ T cells from LN of DO11.10 TCR tg mice were maintained after primary stimulation for 5 days under Th0, Th1 or Th2 priming conditions. PKA activity in 5 µg of whole cell protein extracts of unstimulated (-) and cells restimulated for 1 h by T/I, F or T/I/F was determined using PKA assay kit (Upstate biotechnology) according to supplier's instructions. One representative experiment out of three is shown here.

2.12. Catalytic active PKA enhances GATA-3-mediated IL-5 and IL-4 expression in primary Th1 cells.

We observed that forskolin treatment or ectopic expression of PKA catalytic subunit in Th1 effector cells could not induce Th2-specific lymphokines suggesting that under no circumstances cAMP or PKA alone induces Th2-specific cytokines in Th1 cells. However, forcible expression of GATA-3 in committed Th1 cells induces Th2-specific cytokines, which was markedly enhanced by cAMP (Lee et al., 2000) suggesting an active collaboration between GATA-3 and cAMP pathways in the induction of Th2-specific cytokines. A role for GATA-3 in the expression of a Th2 cytokine gene was first established by one study, which demonstrated the critical importance of a GATA-3

binding site in the IL-5 promoter (Siegel et al., 1995). Therefore we hypothesized that the threshold level of GATA-3 is required for the stimulatory effect of PKA on the expression of Th2-specific lymphokines, particularly on IL-5.

To explore this functional cooperation between PKA and GATA-3 in primary cells, we took the advantage of Th1 lymphocytes, which express only minor amounts of GATA-3. Retroviruses expressing the PKA_C or GATA-3 were double-transduced in Th cells, maintained under Th1 conditions. In this experiment, a GATA-3 expressing retrovirus was constructed which upon transduction, expressed a yellow fluorescence protein (YFP) instead of the GFP. This GATA-3+YFP virus was cotransduced with the PKA_C+GFP-expressing virus into $CD4^+$ T cells from DO11.10 TCR tg mice one day after primary activation. Two days after infection, the Th1 cells were sorted according to their fluorescence and 6 days after the first antigen stimulation they were restimulated for 48 hours by T/I or T/I and dibutyryl-cAMP (cAMP), a cAMP analogue.

As shown in Fig. 2.12A&B, expression of GATA-3 in Th1 cells led to a strong increase in the T/I-mediated induction of IL-5 and IL-4 secretion. In addition, it reduced IFN-γ synthesis (Fig. 2.12C), as observed by other authors (Ouyang et al., 1998). Moreover, addition of dibutyryl-cAMP enhanced IL-5 and IL-4 secretion in the GATA-3 expressing, but not in uninfected Th1 cells, and ectopic expression of PKA_C was able to mimic this effect. This indicates that provision of PKA provides complete signal for optimal expression of IL-5 and IL-4 in primary lymphocytes and GATA-3 is absolutely required for the stimulatory effect of PKA on Th2-type lymphokine expression. The usage of different signaling pathways by cAMP in regulating Th lymphokine expression and thereby executing its immunomodulatory function, is reflected in the inability of PKA to downregulate the expression of IFN-γ in primary Th1 lymphocytes (Fig. 2.12C) as observed earlier in EL-4 cells (Fig. 2.3)

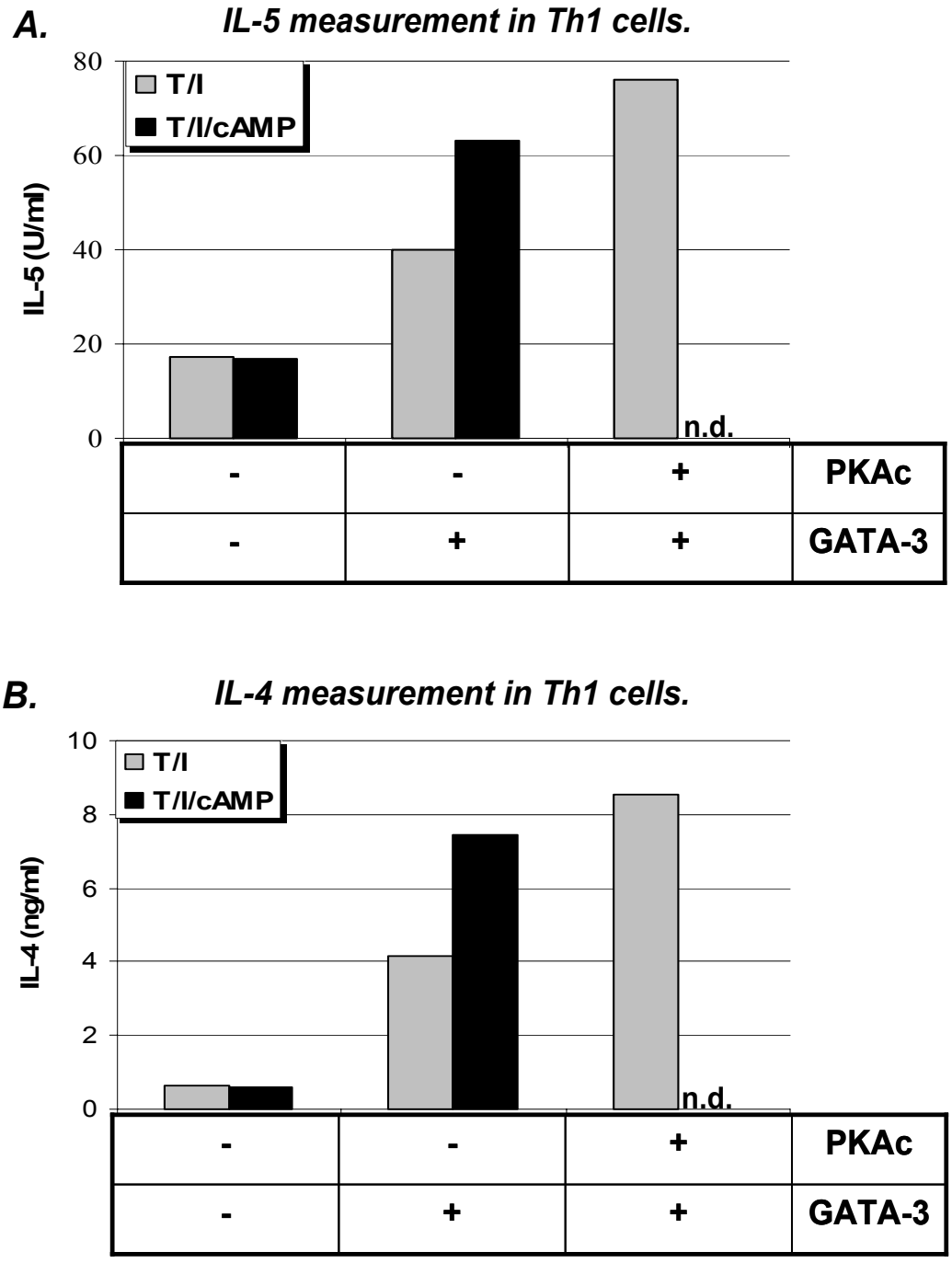


Fig 2.12A&B: PKAC and GATA-3 functionally cooperate in the activation of IL-5 and IL-4 expression in Th1 cells. ***A,*** *Production of IL-5 in GATA-3-expressing Th1 cells is enhanced by PKA_C.* One day after primary stimulation $CD4^+$ T cells from LN of DO11.10 TCR tg mice were infected with recombinant retroviruses transducing EYZ-GATA-3 (GATA-3), EGZ-PKA_C (PKAc) or both, and maintained under Th1 conditions. Two days post infection cells were sorted into infected and non-infected (-) populations. Two days later, the cells were restimulated for 48 h with T/I or T/I+dibutyryl-cAMP and production of IL-5 was determined by ELISA. ***B,*** *Activation of IL-4 synthesis in Th1 cells by PKA_C and GATA-3.* $CD4^+$ T cells were activated, infected and restimulated as in (A) and IL-4 secretion was assayed by ELISA.

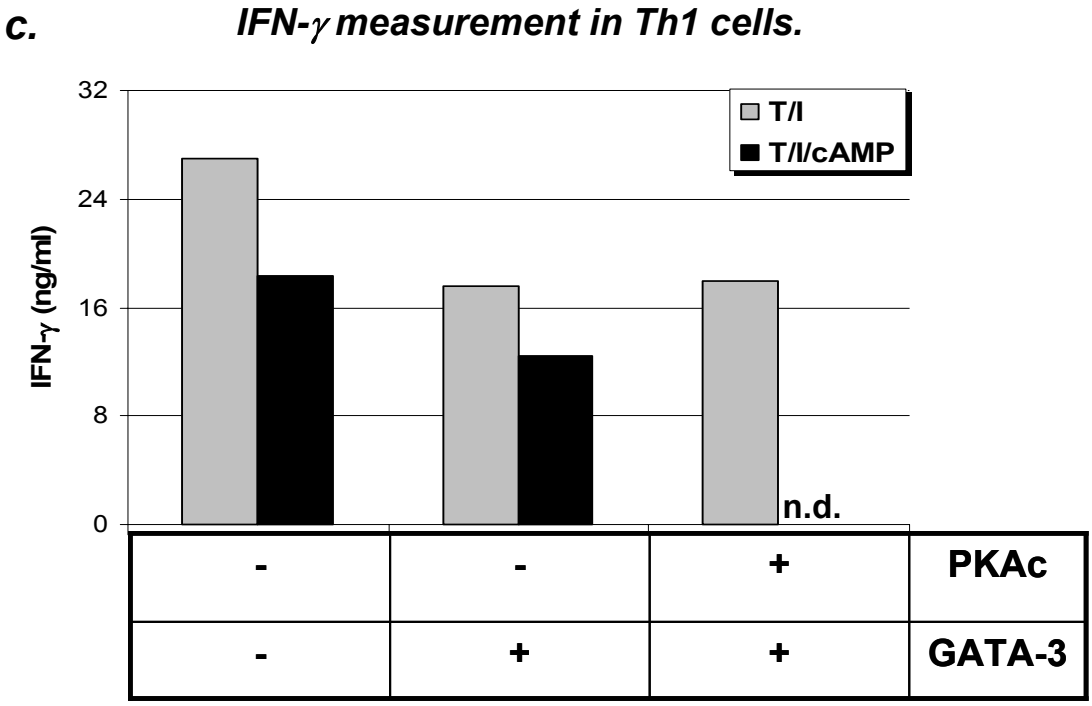


Fig 2.12 C: PKAc does not mimic the cAMP-mediated inhibition of IFN-γ secretion. *Effect of PKA_C and GATA-3 expression on IFN-γ secretion in Th1 cells.* $CD4^+$ T cells stimulated and infected as in (A) were assayed by ELISA. The experiment shown is representative of two similar experiments.

2.13. PKAc and GATA-3 cooperate in the activation of IL-5 promoter in 293 HEK cells.

Like most cytokine genes, the regulation of IL-5 message is primarily at the level of transcription and is likely to be controlled to a large extent by regulatory elements in the promoter region that can influence the transcriptional activity of the gene (Crabtree, 1989). We found that PKA alone was unable to augment the secretion of Th2-type lymphokines in Th1 cells, which are considered as a GATA-3-defecient environment, and only could mediate the enhancing effect of cAMP on IL-5 and IL-4 secretion in a GATA-3-dependent manner (Fig. 2.12A &B). This finding encouraged us to investigate if the functional cooperation between PKAc and GATA-3 induces a Th2-specific promoter. Since the critical importance of a GATA-3 binding site in the IL-5 promoter was established earlier (Siegel et al., 1995) and activation of the IL-5 promoter by Bt_2cAMP and PMA in EL-4 cells requires a region located between -70 and -59 that binds GATA-

3, we chose the IL-5 promoter to analyze the potential requirement of GATA-3 in IL-5 gene induction by PKA in 293 HEK cell, a non-T cell environment.

We performed transient transfections of vectors expressing PKA_C or GATA-3 with a luciferase construct driven by the proximal IL-5 promoter into 293 cells. Co-transfection of GATA-3 and PKA_C-expressing vectors resulted in a synergistic activation of the proximal IL-5 promoter, whereas none or very weak activation was observed for a mutated proximal IL-5 promoter (Gm) which is unable to bind GATA-3 (Fig. 2.13). This observation provides evidence that the stimulatory effect that active PKA exerts on the IL-5 promoter requires GATA-3 activity.

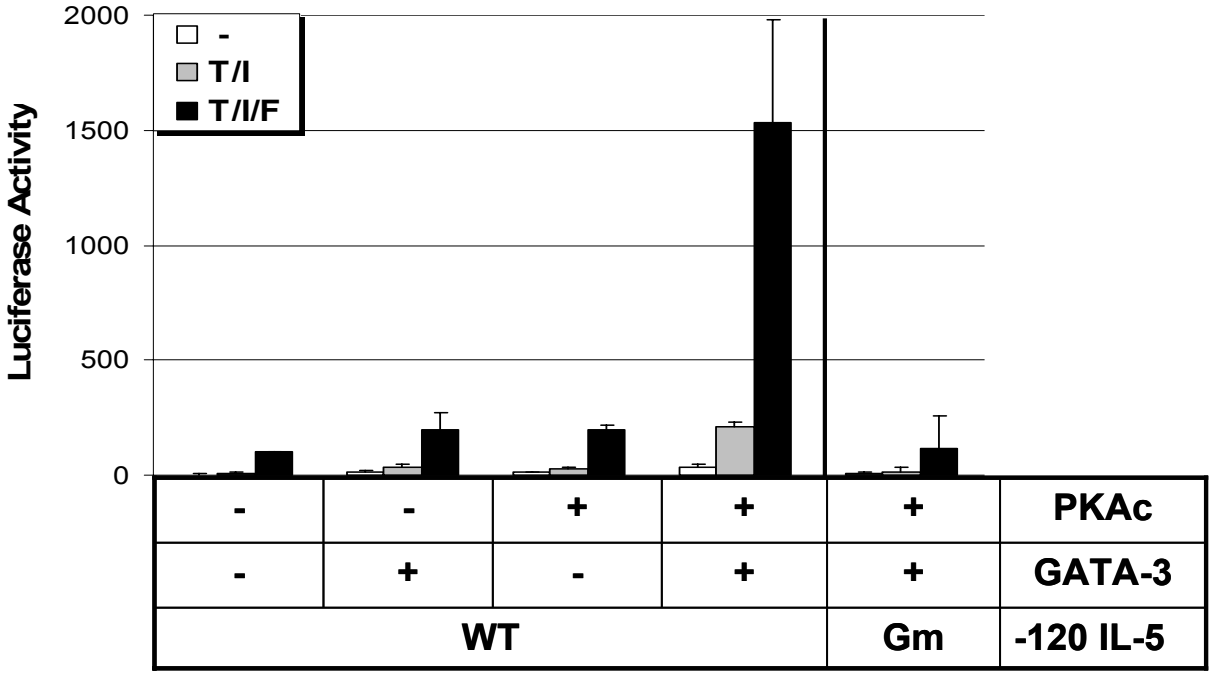
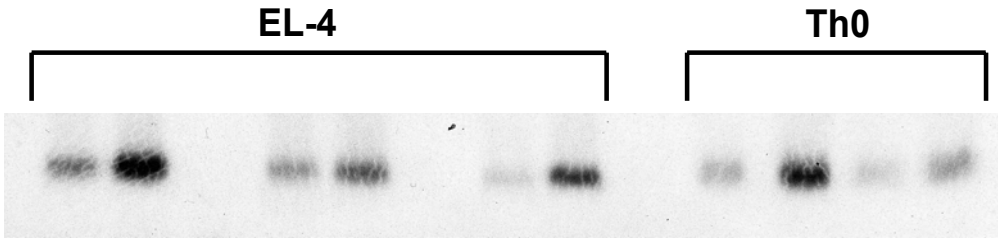


Fig 2.13: Synergistic activation of proximal IL-5 promoter activity by PKA_C and GATA-3 in 293 cells. For the read out of –120 IL-5 promoter activities, 293 human embryonic kidney (HEK) cells were cotransfected with the –120 IL-5 promoter luciferase plasmid (or a –120 IL-5 promoter with mutation in the GATA-3 binding site (Gm)) and expression vectors for murine PKA catalytic subunit α (PKA_c), murine GATA-3 or both. Luciferase activity was measured in cultures that were left untreated (-), induced with T/I or T/I/F for 20 hours.

2.14. Elevation in cAMP level induces GATA-3 mRNA synthesis in EL-4 T cells, not in primary Th0 cells.

The importance of GATA-3 in IL-5 production has been well documented by several studies (see section 1.2.2). The Th2-specific transcription factor GATA-3, in cooperation with a factor(s) induced upon TCR signaling, governs Th2 - specific expression of the IL-5 gene by directly interacting with a critical regulatory element of its promoter (Lee et al., 1998; Zhang et al., 1997). Threshold level of GATA-3 is required for Th2 cytokine production. Since cAMP levels were found to be higher in Th2 cells than in Th1 cells (Novak and Rothenberg, 1990), It is possible that cAMP may also dictate GATA-3 gene expression in Th2 cells and bring the threshold level required for full IL-5 gene expression in Th2 cells.



1	2		3	4		5	6		7	8	9	10	Lane
+	+		+	+		+	+		-	+	-	+	T/I
-	+		-	+		-	+		-	-	+	+	Bt_2cAMP
3			7			28			16				Duration (hours)

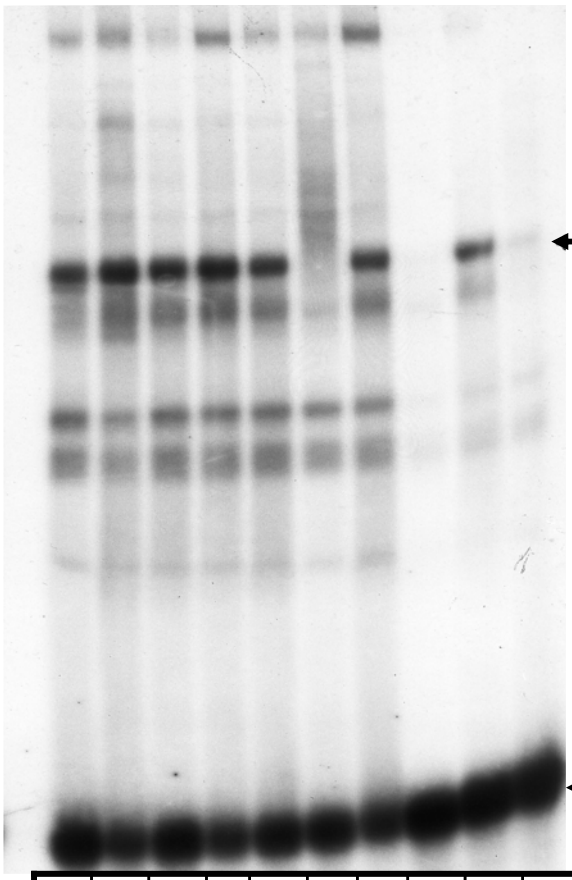
Fig 2.14: GATA-3 mRNA is induced in EL-4 cells, not in Th0 lymphocytes, upon cAMP treatment. Total RNAs from EL-4 cells were isolated at different time points after stimulation with either T/I or T/I and Bt_2cAMP (1 mM) and subjected to Northern hybridization with radiolabelled GATA-3 probe (lane 1-6). $CD4^+CD62L^{high}$ T cells from LN of DO11.10 TCR tg mice were maintained after primary stimulation for 5 days under Th0 priming conditions. Total RNAs were isolated from these cells after 16 hours of restimulation with indicated stimuli and GATA-3 mRNA level in these cells was analyzed by Northern hybridization using radiolabelled GATA-3 probe (lane 7-10).

Therefore we investigated the level of GATA-3 message in T lymphocytes in response to elevated cAMP levels. To address this point, we selected EL-4 and mature Th0 cells, because both cell types produce minimal IL-5 upon T/I treatment and further provision of cAMP signal augments full IL-5 production. Total cellular RNAs were

isolated from EL-4 cells, stimulated by either T/I or T/I and Bt_2cAMP for different time points as indicated (Fig. 2.14, lane 1-6) and primary effector Th0 cells, stimulated for 16 hours as indicated. The presence of GATA-3 RNA was detected in these RNA samples by Northern hybridization against radiolabelled GATA-3 probe (for methodical details, see section 5.2.3.3). Interestingly, GATA-3 mRNA level in EL-4 cells was enhanced after 3 hours of Bt_2cAMP treatment and this induction persisted for longer period of time (Fig. 2.14). In contrary, mature Th0 expressed considerably high levels of GATA-3 only after T/I stimulation and additional treatment with Bt_2cAMP did not induce GATA-3 RNA level further in these cells.

2.15. Cyclic AMP does not induce GATA-3 binding in the proximal regulatory region of IL-5 promoter in T cells.

Our observation that cAMP induces GATA-3 transcription (Fig. 2.14) encouraged us to dissect the significance of this cAMP-mediated induction of GATA-3 message. Previous studies have shown that transfection and expression of GATA-3 was sufficient for transcription of the IL-5 but not the IL-4 promoter in spite of the presence of putative GATA-3 sites in the IL-4 promoter (Lee et al., 2000; Ranganath et al., 1998; Zhang et al., 1998). Therefore, we analyzed the effect of cAMP on the binding ability of GATA-3 in the upstream regulatory regions of IL-5 by electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared, according to the protocol described in section 5.2.4.1, from non-stimulated EL-4 cells and cells that were stimulated for 6 hours by T/I or T/I+Bt_2cAMP. The probes in the EMSA were prepared by radioactive labeling of complementary oligonucleotides corresponding to GATA consensus sequence, CACTTGATAACAGAAAGTGATAACTCT (SC-2531; Santa Cruz Biotechnology, Santa Cruz, CA) (for methodical details, see section 5.2.5.1). Protein-DNA binding specificity was tested by competition assays in which the binding reactions were pre-incubated with different GATA antibodies or excess unlabeled specific or nonspecific competitor prior to the addition of the labeled probe.



1	2	3	4	5	6	7	8	9	10	Lane
-	+	+	+	+	+	+	+	+	+	T/I
-	-	+	+	+	+	+	+	+	+	cAMP
-	-	-	αGATA-1 Ab	αGATA-2 Ab	αGATA-3 Ab		-120 WT IL-5P	-120 Gm IL-5P	GATA cons.	Competitor

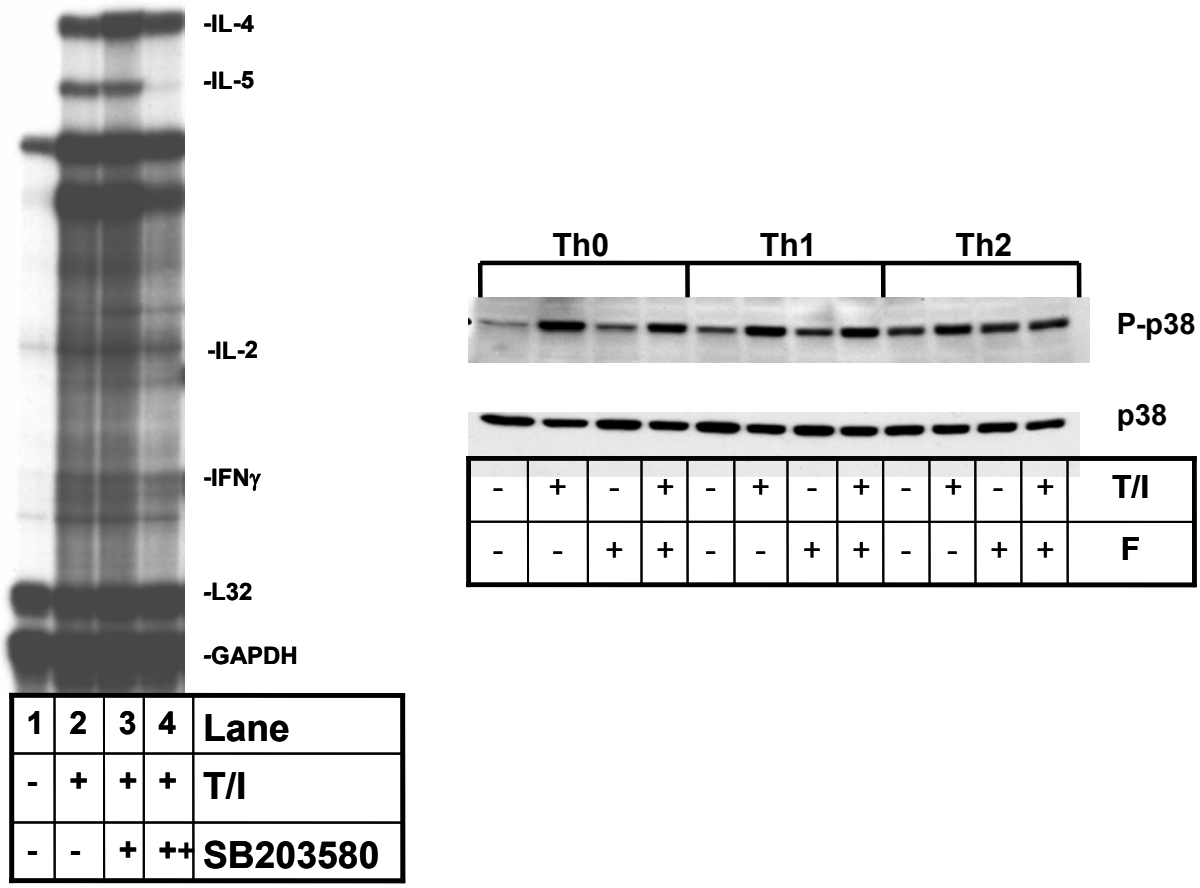
Fig 2.15: cAMP does not induce GATA-3 binding in electrophoretic mobility shift assay with EL-4 nuclear extracts and GATA consensus oligonucleotide probe within the IL-5 proximal region. Nuclear extracts from non-stimulated EL-4 cells and cells that were stimulated for 6 hours by T/I or T/I+Bt_2cAMP were prepared and subjected to EMSA with ^{32}P-labelled oligo probe. Supershift EMSA involved addition of 1 µg polyclonal antibodies against either GATA-1, GATA-2 or GATA-3 (Santa Cruz) prior to addition of the probe and incubated for 1 h on ice. For specificity control, unlabelled competitor oligonucleotides (at 100-fold molar excess) were included in the binding reactions as indicated (lane 8-10).

When EMSA was done with nuclear extracts of EL-4 cells, the GATA-3 complex was already observed in non-stimulated cells and this complex was induced by T/I-treatment (lanes 1 and 2 in Fig. 2.15). Contrary to our expectation, T/I+cAMP-treatment did not appear to intensify this complex any more (lane 3; Fig. 2.15). The specificity of the GATA-3 band was determined by supershift EMSA using antibodies against GATA proteins. Since the binding complex is supershifted by GATA-3 antibody and not by GATA-1 or GATA-2 antibody (compare lanes 4-6), we conclude that the complex is GATA-3-specific. The oligonucleotide competition assays suggest that the GATA-3 binds to the -120 WT IL-5 promoter with intact GATA-3 binding site and not to the -120 Gm IL-5 promoter, mutated in the GATA binding site from CTATC to GCATT within its unique GATA-3 binding site (around position - 72) rendering it unable to bind GATA-3 (lanes 8-9). In contrary to enhanced GATA-3 transcription by T/I+cAMP as observed in Fig. 2.14, this experiment indicates that GATA-3 binding to the IL-5 promoter remains unaffected by additional treatment with cAMP.

2.16. Measurement of p38 activity in effector Th cells and the regulation of IL-5 production by p38 in Th2 lymphocytes.

In a previous study, it was reported that cAMP stimulates the activation of p38 MAPK, and not PKA pathway, in Th2 cells and promotes the production of IL-5 in PKA-independent manner (Chen et al., 2000). We also observed the involvement of p38 pathway in the regulation of IL-5 gene expression in Th2 cells, where inhibition of downstream p38 signaling by a relatively high dose of SB203580, a p38 MAPK inhibitor, resulted in abrogation of IL-5 production (Fig. 2.16A). Since T/I/F treatment augments IL-5 expression in Th0 lymphocytes and potentiates the same in Th2 cells, we were prompted to investigate whether similar treatment leads to the enhanced activity of p38 MAPK in these primary cell types. For this, we restimulated effector Th0, Th1 and Th2 cells for 30 minutes under different conditions and whole cell extracts were subjected to immunodetection using phospho-p38 antibody. We found that the p38 activation is not increased in response to additional forskolin treatment in either Th0 lymphocytes or Th2 lymphocytes (Fig. 2.16B). This finding contradicts the previous report by Chen et al., (2000), and suggests that increased cAMP levels do not induce p38 MAPK in either Th0 or Th2 cells.

A. Lymphokine mRNA Level ***B. p38 activity measurement in Th cells***



1	2	3	4	Lane
-	+	+	+	T/I
-	-	+	++	SB203580

-	+	-	+	-	+	-	+	-	+	-	+	T/I
-	-	+	+	-	-	+	+	-	-	+	+	F

Fig 2.16A&B: The p38 pathway regulates IL-5 production in Th2 lymphocytes. ***A,*** *Inhibition of p38 pathway abrogates IL-5 synthesis in Th2 lymphocytes.* Total cellular RNAs were isolated from $CD4^+$ T cells maintained under Th2 conditions for 5 days followed by restimulation for 18 hours by T/I in the absence or presence of 2 μM (lane 3) or 10 μM (lane 4) SB203580 (these cells were pretreated for 30 minutes with the inhibitor at corresponding concentration, followed by T/I activation) or left untreated. RNAs were subjected to RNase protection assay using RiboQuant RNase mCK-1 protection kit (BD Biosciences) for the detection of cytokine RNA level. ***B,*** *Elevated cAMP level fails to stimulate the activation of p38.* Whole cell extracts from 1 x 10^6 $CD4^+$ T cells, maintained under Th0, Th1 and Th2 conditions for 5 *days* and restimulated as indicated or left untreated, were prepared and analyzed for p38 phosphorylation status in response to different stimuli by Western hybridization using phopho-p38 antibody raised against phospho-(Thr180/Tyr182) p38 (Cell Signaling Technology).

C. Cross talk between p38 and PKA pathways.

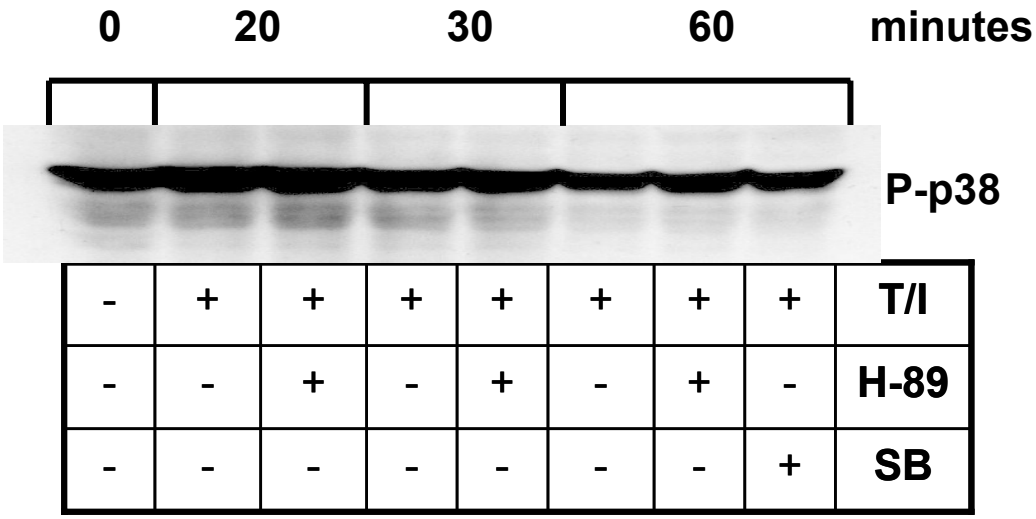


Fig 2.16C: Inhibition of PKA activity by H89 does not impair the p38 activity in Th2 lymphocytes. $CD4^{+}CD62L^{high}$T cells from LN of DO11.10 TCR tg were cultured under Th2 skewing conditions for 5 days and restimulated by T/I in the absence or presence of 10 μM H89 (these cells were pretreated for 30 minutes, followed by T/I) for different time points as indicated. Whole cell protein extracts were prepared and subjected to Western hybridization using phopho-p38 antibody raised against phospho-(Thr180/Tyr182) p38 (Cell Signaling Technology) for the analysis of p38 phosphorylation status.

We also investigated the possibility of PKA - mediated regulation of p38 activation in Th2 lymphocytes with the assumption that blockade of PKA activation by 10 μM of H-89 could result in decreased phosphorylation of p38 compared to non-treated cells. With this objective, we restimulated effector Th2 cells for 20, 30 or 60 minutes by T/I in the presence or absence of H-89 and whole cell protein extracts were analyzed in SDS-PAGE for the detection of activated p38 by standard protocol. As shown in figure 2.16C, there is no perceptible change in the level of activated p38 by the treatment of H-89, suggesting that these two pathways might operate independently of each other in the regulation of IL-5 expression.

3. Discussion

On the basis of their lymphokine secretion patterns, $CD4^+$ Th cells are divided into at least three effector subsets. The polarized Th1 (producing IL-2, IFN-γ and lymphotoxin) and Th2 (secreting IL-4, IL-5, IL-10, and IL-13) subsets are often mutually exclusive in situations of chronic infection or hyper immunization. A third subset, called Th0, displays a mixed lymphokine pattern and is mostly found in the early phase of the immune response (Firestein et al., 1989). This subset exhibits an unrestricted cytokine profile and represents a very high proportion in short term $CD4^+$ T cell clones.

Similar to Th2 cells, these Th0 cells are found to be IL-1-responsive and therefore it is possible that accessory cells presenting membrane IL-1 may be required for activation of Th0 cells as they are for Th2 cells (Weaver et al., 1988). Interestingly, Th0 cells have been identified at high frequencies in panels of $CD4^+$ human T cell clones (Maggi et al., 1988; Swain et al., 1988). In contrast, human cells belonging to the Th1 or Th2 subsets as defined in the mouse have been described at much lower frequencies. The physiological role of Th0 cells is undefined. However, Th0 cells may represent a precursor to the Th1 and Th2 subsets through either terminal differentiation or differential activation.

The activation of T cells requires the interaction of the T cell receptor (TCR) with antigen in association with the MHC, which leads to an increase in intracellular calcium concentration, activation of protein kinase C (PKC) and other protein kinases (Perlmutter et al., 1993). However, stimulation of the TCR alone is insufficient to fully activate T cells and a second signal provided by APC is also required (Rincon and Flavell, 1994). This co-stimulatory signal is generated by the CD28 ligand (Jenkins and Johnson, 1993). CD28 was reported to increase the stability of several cytokine mRNAs (Thompson et al., 1989), and was also observed to participate directly in the transcription of some interleukin genes (Fraser et al., 1991). APC-stimulated T cells produce a wide range of lymphokines. IL-5 is often, but not always, co-expressed with Th2-type cytokines such as IL-4 and IL-13. It is unclear why certain antigens induce IL-5 and others do not. However, this indicates that the IL-5 gene is individually regulated and control mechanisms for IL-5 expression can be based on the antigen-specific T cell activation.

The activation of the major TCR pathways can be achieved *in vitro* by using phorbol 12-myristate 13-acetate (PMA) in combination with ionomycin (or A23187). Many, if not all, lymphokine genes are induced by PMA and ionomycin. However, detailed studies revealed that the signals for optimal induction of the various lymphokine genes are not exactly the same for each gene. PMA/anti-CD28 stimulation activates the expression of a variety of cytokine genes, including IL-2, IL-3, IL-4, IL-10 and GM-CSF.

Several observations strongly suggest a diverse cell-type specific regulatory role for cAMP in lymphocytes. The elevation of intracellular cAMP levels in T cells, induced by treatment with various agents such as IL-1α, PGE_2, cholera toxin, forskolin or by direct stimulation of cAMP pathways by dibutyryl cAMP inhibits IL-2 and IL-2 receptor expression thereby blocking cell cycle progression and cell proliferation (Anastassiou et al., 1992; Betz and Fox, 1991; Chen and Rothenberg, 1994; Mary et al., 1987). Beside proliferation, cAMP has been found to exert an inhibitory effect on the expression of other cytokines such as IL-3, IL-10, GM-CSF (Lee et al., 1993) and IL-12 (van der Pouw Kraan et al., 1995). On the other hand, previous studies indicate that the elevation of intracellular cAMP is associated with inhibition of Th1-type cytokine production but augmentation of Th2-type cytokine gene expression (Betz and Fox, 1991; Munoz et al., 1990b; Neumann et al., 1995). The higher cAMP level in Th2 cells compare to Th1 cells provided further clue to the possible role of cAMP in Th effector functions (Novak and Rothenberg, 1990). However, the importance of cAMP signaling in Th effector function has been mostly studied in tumor cell lines and clones.

The conspicuous lack of these studies in primary cells inspired me to investigate the effect of cAMP signaling on Th cytokine expression pattern in primary murine effector Th subsets. Although EL-4 cell line was used as starting T cell model for lymphokine expression as well as in transient transfection assays, I mostly investigated the lymphokine gene regulation in mature effector T helper cells, generated by *in vitro* activation of naïve $CD4^+$ T cells which were isolated directly from murine lymphnodes. Furthermore attempts were made to dissect the downstream signaling pathways activated by cAMP in lymphocytes.

3.1. Activation of cAMP pathway is critical for the Th2-type effector functions in murine T lymphocytes.

In different experimental assays, it was found that elevation of cAMP levels in T cells by stimulation of adenylyl cyclase with forskolin exerts a strong stimulatory effect on the induction of Th2 responses, particularly IL-5. This is demonstrated in Fig. 2.1 for EL-4 thymoma cells and in Fig. 2.4 and 2.5 for primary effector Th cells. The treatment of EL-4 cells with the phorbol ester TPA and the ionomycin (T/I), being potent inducers of IL-2 and IFN-γ mRNA synthesis (Fig. 2.3), hardly affected IL-4 and IL-5 expression, whereas triple treatment of cells by T/I and forskolin exerted a strong synergistic effect on the expression of both IL-4 and IL-5 (Fig. 2.1 and 2.3). A similar strong effect of T/I/F treatment on IL-5 synthesis was detected in Th0 cells which, otherwise, synthesize only low amounts of IL-5 (Fig. 2.4 and 2.5). As expected, Th1 cells did not secrete any IL-5, irrespective of their treatment (Fig. 2.4). In contrast, Th2 cells secrete IL-5 upon T/I stimulation alone, and additional forskolin treatment did not further enhance IL-5 production (Fig. 2.4).

Whereas, IL-5 gene expression can be induced by the treatment of additional forskolin, another prominent Th2-type cytokine, IL-4, was not affected by this combined treatment in primary Th0 cells, in contrast to mouse thymoma cell line EL-4 cells, where T/I/F treatment augmented the expression of both types of cytokines. These data prompted us to conclude two things: First, in contrast to effector Th2 lymphocytes, effector Th0 lymphocyte, the cell type which produces mixed pattern of Th lymphokines, lack cAMP signal. The external provision of cAMP along with T/I in these Th0 cells augmented efficient IL-5 production at par with T/I-stimulated Th2 cells. Additional forskolin treatment did not enhance the IL-5 production capability of Th2 cells, where probably high saturating levels of cAMP facilitate the T/I-mediated IL-5 induction to an optimal level. Second, cAMP regulates the expression of only IL-5 gene *in vivo* and IL-4 is resistant to the effect of elevated cAMP in primary Th lymphocytes, in contrast to EL-4 cells, where in a similar fashion to IL-5, triple treatment of T/I/F augmented IL-4 production as well. The discrepancy between tumor cell line and primary cells in IL-4 gene regulation by cAMP is a vivid example of difference in control mechanisms between lymphoma cell lines and primary cells.

Several studies indicate that efficient production of IL-5 requires activation of both the TCR and a second signaling pathway. Using EL-4 cells, anti-CD28 antibody in combination with PMA was shown to be activator for the IL-5 gene expression (Karlen et al., 1996) and cAMP in combination with PMA was shown to be necessary for the optimal induction of IL-5 synthesis (Lee et al., 1993). However, the combination of PMA and anti-CD28 was less efficient than the combination of PMA and cAMP. It was suggested that the signaling pathways used by CD28 are independent of changes in cAMP concentrations (Pages et al., 1994).

Stimulation for 12 hours in the presence of PMA and cAMP was optimal for expression from the IL-5 promoter in EL-4 cells. Whereas, PMA and cAMP individually were shown to have almost no effect on IL-5 promoter activity, combination of both activators caused a marked increase in the production of IL-5 in EL-4 cells, although incubation for 48 hours with PMA alone was sufficient to stimulate some IL-5 production. Interestingly, incubation with PMA alone led to detectable IL-5 promoter activity only 24 hours after the beginning of treatment (Karlen et al., 1996). In contrast, expression from the IL-5 promoter was considerably increased 12 hours after addition of both PMA and cAMP, but after 48 hours promoter activity had decreased to the level obtained with PMA alone. These observations confirm that cAMP in combination with PMA is required for early promoter activity in EL-4 cells.

To identify the signal requirements in primary $CD4^+$ T cells for optimal production of IL-5, EL-4 and Th0 cells were chosen because of their dependence on forskolin for optimal IL-5 expression. For this, $CD4^+$ T cells were activated and cultured in the presence of Th0 conditions for 5-7 days to obtain mature Th0 phenotype. Different signal requirements for IL-5 production were analyzed in these Th0 and EL-4 cell upon restimulation with desired stimuli for 48 hours followed by IL-5 assay in the cell-free supernatants. As shown in Fig. 2.6, in the absence of ionomycin signal, TPA and forskolin augmented some IL-5 production in EL-4 cells and completely failed to augment IL-5 production in Th0 cells. However, triple treatment T/I/F induced full IL-5 production in these cell types suggesting that in addition to T/I, cAMP signaling was absolutely required for optimal IL-5 production in both EL-4 and primary $CD4^+$ T cells. This suggests that activation of NFAT plays very important role in IL-5 expression.

3.2. Modulation of PKA activity regulates IL-5 production in T lymphocytes.

In lymphoid cells and numerous other cells, cAMP is an activator of Protein Kinase A (PKA) (Kammer, 1988), which plays a major role in cAMP-induced responses in many cell types. Several isozymes of PKA have been described and it has shown that the type I isozyme is the major form present in T cells (Skalhegg et al., 1992). Expression of the PKA catalytic subunit was shown to efficiently activate the IL-5 promoter in EL-4 cells in a PKA-dependent fashion (Lee et al., 1993). Thus, in EL-4 cells, the PKA pathway and, most likely, the phorbol ester-induced PKC pathways synergistically activate the IL-5 promoter.

Initial attempts were made to analyze the effect of transient activation or inhibition of PKA on the IL-5 promoter activities in T cells. PKA exists as a holoenzyme consisting of two catalytic subunits and one regulatory subunit dimer, which harbors four cAMP binding sites. By binding to regulatory subunit dimer, cAMP dissociates catalytic subunits, which then phosphorylate specific target proteins (for details see section 1.2.5.1). Ectopic expression of only catalytic subunit results in increased PKA activity, on the other hand, expression of engineered regulatory subunit, which harbor mutations in cAMP binding sites and thereby worked like dominant-negative version of PKA, allows the constitutive physiological inhibition of PKA.

Using these two expression plasmids, the activity of PKA was modulated in transient transfection assays to determine the regulation of IL-5 promoter by PKA. For this, EL-4 cells were co-transfected with the –507 IL-5 promoter luciferase plasmid and expression vector either for murine PKA catalytic subunit α (PKA_C) or mutant PKA regulatory subunit. As expected and in line with previous studies (Lee et al., 1993) the promoter activity was significantly induced in T/I/F-treated untransfected cells compared to T/I induction of these cells. Cells transfected with the PKA catalytic subunit produced very high levels of luciferase activity after T/I stimulation and further treatment of these cells with forskolin did not augment any increased reporter activity (Fig. 2.2). On the other hand, transfection of PKA_{RM} completely abolished the promoter activity (Fig. 2.2). These data demonstrate that PKA is required for full activation of IL-5 promoter activity in EL-4 cells.

In line with these promoter studies, it was important to test whether the activation of IL-5 promoter by cAMP/PKA pathway results in the IL-5 production in EL-4 T cells. Since additional forskolin treatment is required in EL-4 T cells for the induction of Th2-type lymphokine production (Fig. 2.1), it was checked if the missing signal is delivered by active PKA. To this end, we constructed a PKA_C-encoding retroviral vector that, due to the presence of an internal ribosomal entry site (IRES), expresses also a green fluorescence protein (GFP) and zeocin for the selection of positively transduced cells. EL-4 cells were stably transduced with retroviruses expressing the PKA catalytic subunit α (PKA_C) or control retrovirus (control). RNase protection assay of mRNA level of different lymphokines in these transduced cells is shown in Fig. 2.3. The expression of PKA_C is sufficient to replace the forskolin signal in the T/I-mediated induction of IL-4 and IL-5 RNA synthesis (compare lanes 4 and 6 in Fig. 2.3). Interestingly, in contrast to forskolin treatment, the overexpression of PKA_C did not impair IL-2 and IFN-γ RNA synthesis in EL-4 cells (compare lanes 4 and 6 in Fig. 2.3). Collectively, these results indicate that the immediate effector molecule of cAMP for the induction of Th2-type lymphokines is PKA in EL-4 T cells.

Armed with these observations in EL-4 T cells (Fig. 2.1, 2.2 and 2.3) and $CD4^+$ Th0 lymphocytes (Fig. 2.4 and 2.5), further investigations were performed whether active PKA_C plays a similar stimulatory role in IL-5 synthesis in primary T cells. $CD4^+$ Th0 cells were infected with EGZ (control) and EGZ-PKA_C recombinant retroviruses and sorted into green cells transduced with EGZ virus [EGZ] and EGZ-PKA_C virus [PKAc(+)] or into EGZ-PKA_C-non-transduced cells [PKAc(-)] and five days after primary activation, restimulated by T/I for 48 h (for detail see section 2.7). The sorted EGZ-infected [EGZ] and PKA_C-non-infected [PKAc(-)] populations were used as control cells. As shown in Fig. 2.7, forskolin enhanced the T/I-mediated increase in IL-5 secretion by at least 3 fold in control cells, and ectopic expression of PKA catalytic subunit in Th0 lymphocytes mimicked the stimulatory effect of cAMP on the T/I-mediated IL-5 induction to a very similar level, suggesting that the potential target of cAMP in primary lymphocytes is PKA in augmentation of efficient IL-5 expression.

As stated before, the recognition by the TCR of a cognate antigen associated with the MHC on the APC triggers a series of biochemical events, including elevation of intracellular calcium ion concentration and activation of Protein Kinase cascades and these processes can be bypassed by stimulating T cells with TPA and ionomycin. To examine whether forskolin or active PKA is able to synergize signals emanating from

TCR in Th0 cells, $CD4^+$ T cells, maintained under Th0 conditions, were infected with EGZ (control) and EGZ-PKA_C recombinant retroviruses after one day of primary stimulation. Infected populations were FACsorted on day 3 as described just before in the previous paragraph. The controls and PKAc-expressing populations were restimulated five days after primary activation by plate-bound αCD3 Ab and soluble αCD28 Ab with or without forskolin. The analysis of IL-5 expression in these populations shows that stimulation of these populations by αCD3 and αCD28 Ab could not induce any IL-5 production, whereas additional forskolin markedly induced the IL-5 secretion by these cells (Fig 2.8). The ectopic expression of PKAc was sufficient to replace this forskolin effect, clearly demonstrating that forskolin as well as PKAc synergizes the cAMP-induced signal with physiological TCR activation pathways (Fig. 2.8). Interestingly, treatment of PKAc-expressing Th0 lymphocytes with forskolin dramatically enhanced the IL-5 production to more than 3 fold compared to PKAc-expressing Th0 cells or forskolin-treated control Th0 populations (Fig. 2.8). This observation gains significance with respect to the possible additional pathway(s) acting in synergy with PKA pathway in regulating IL-5 gene expression.

Results obtained with these cells were confirmed and extended in a second cell type, *in vitro* differentiated murine Th2 cells, which represent the typical *in vivo* IL-5-producing cell type. To determine whether active PKA plays similar important stimulatory role in the activation of IL-5 in Th2 lymphocytes, effector Th2 cells were treated with 2-10 μM of the protein kinase inhibitor H89, which inhibits specifically PKA activity at these low concentrations (Davies et al., 2000). These treatments led to a significant decrease in IL-5 production up to almost 50% (Fig. 2.9A). To control experiments, actual PKA activity in these Th2 cells was measured according the standard protocol (Graves et al., 1996). As shown in Fig. 2.9B, the activity of PKA was suppressed to almost half in Th2 cells treated with 10 μM H-89, the concentration which impaired the IL-5 expression to a similar level. This strengthened the assumption that the suppression of IL-5 expression in response to optimal dose of H-89 is due to the lower PKA activity.

To rule out the artifacts associated with chemical inhibitor like H-89, the inhibition of PKA activity was achieved in primary Th2 cells by more physiological approach, i.e. by ectopically expressing the mutant version of the regulatory type I subunit of PKA (PKA_{RM}) which is unable to bind cAMP (Clegg et al., 1987). For this, a PKA_{RM}-encoding

retroviral vector was constructed and $CD4^+$ T cells were infected with EGZ (control) and EGZ-PKA_{RM} recombinant retroviruses one day after primary stimulation and maintained under Th2 conditions. It is highly evident in Fig. 2.10 that stimulation of PKA_{RM}(+) cells led to almost 50% reduction in IL-5 secretion compare to both types of control populations, EGZ and PKA_{RM}(-). This observation provides a direct clue that high levels of endogenously activated PKA in Th2 cells contribute to IL-5 production.

The level of PKA activity in different subsets of $CD4^+$ T helper cells is unknown. However, the differential level of cAMP in Th1 and Th2 cells as reported earlier could result in different levels of activated PKA in these cells. Thus this possibility was examined in all three effector $CD4^+$ T cells i.e. Th0, Th1 and Th2, with the assumption that Th2 cells naturally contain higher level of activated PKA. To test this hypothesis, intracellular PKA activities were measured in whole cell protein extracts obtained from all three subsets of mature Th cells at several time points of restimulation on day 5 using the standard protocol (Graves et al., 1996). However none of these experiments using KEMPTIDE substrate and several protein kinase inhibitors for the specificity control gave the result which was expected, such as a high constitutive PKA activity in Th2 cells (Fig. 2.11). Instead in all three cell types, forskolin treatment alone resulted in a distinct increase in PKA activity.

Besides of the rise in cAMP level, there is now a growing body of evidences suggesting that a group of proteins, known as A-kinase anchor proteins (AKAPs), plays an important role in regulating the activity of PKA by targeting the enzyme to different subcellular compartments (Feliciello et al., 2001). Thus, all of the AKAPs possess not only a subcellular targeting motif but also a motif that binds the type II regulatory subunit (RII) of the PKA holoenzyme, thereby enabling compartmentalization of PKA (Michel and Scott, 2002). This compartmentalization of PKA by AKAP has two potentially important consequences. First, by anchoring PKA close to the site of cAMP generation, AKAPs may increase sensitivity to incoming cAMP signals. Second, by targeting PKA to different subcellular organelles, AKAPs may influence the enzyme substrate specificity of PKA (Michel and Scott, 2002). Therefore, a model has been proposed in which AKAPs play crucial roles in cAMP signaling by integrating upstream activators and downstream targets of PKA (Dodge and Scott, 2000). The cellular function of PKA has been attributed to the compartmentalization of PKA within the three dimensional matrix of the cell via anchoring to AKAPS (Schillace and Scott, 1999).

Analyses of lipid raft purifications from normal resting T cells for the presence of different subunits of PKA reveal both catalytic subunit and the regulatory subunit RIα (but no RII subunits) constitutively associated with rafts (Vang et al., 2001). This suggests that the observed colocalization of PKA type I and TCR in capped T cells occurs in lipid rafts and that there are mechanisms for specific targeting of PKA type I to these areas. Based on results from other systems, this would most probably involve anchoring of the PKA type I holoenzyme to an unknown AKAP in lipid rafts. However, other possibilities include anchoring of the PKA catalytic subunit, e.g., via the N-terminal myristyl group into rafts, or via interactions to a caveolin-like protein in T cell rafts, similar to the PKA - Ca interaction with caveolin in other cell types (Razani et al., 1999).

Studies of the organization of G proteins in the plasma membrane revealed that in addition to G proteins, the low density membrane fraction (that most probably represents lipid rafts) contains adenylyl cyclase activity (Huang et al., 1997a). In fact, a substantial fraction of the total isoproterenol or forskolin stimulated adenylyl cyclase in S49 lymphoma cells was present in these fractions, strongly suggesting that the receptor-G protein and G protein-adenylyl cyclase coupling occur in lipid rafts. This implies targeting of the molecular machinery necessary for generation of cAMP and activation of PKA type I after engagement of G-protein coupled receptors to lipid rafts.

Therefore, we assume that the active PKA in a cell represents only to a minor subset of PKA molecules, which by binding to specific AKAPS (A-kinase anchoring proteins) are recruited to structurally ordered and functionally active microdomains, which were shown to consist of adenylyl cyclase, cAMP, phosphodiesterases, and several other signaling components of the cascade. This assumption gains significance in the light of several recently published data on the organization of adenylyl cyclase/cAMP/PKA signaling pathways in other cell types, e.g., cardiac myocytes, where cAMP specificity is guaranteed by tight localization of signaling events.

3.3. Cyclic AMP/PKA pathway augments Th2 effector functions in a GATA-3 - dependent manner.

The loss of Th2 cytokine gene expression in Th1 effector cells is attributed to the diminished expression of GATA-3, indicating that a relatively high level of GATA-3 is required for Th2 cytokine gene expression. The expression of different Th2 cytokine genes requires different threshold of GATA-3. In this context, Th1 effector cells, which express only minor amounts of GATA-3, T/I/F treatment did not induce IL-5 production (Fig. 2.4) suggesting an important role of GATA-3 in the PKA-mediated increase in IL-5 synthesis.

In order to investigate the functional cooperation between PKA and GATA-3 for Th2 effector functions, PKA_C or GATA-3 were co-expressed in primary Th1 cells by retroviral double transduction method as described in the section 2.12. As shown in Figs. 2.12A&B, expression of GATA-3 in Th1 cells led to a strong increase in the T/I-mediated induction of IL-5 and IL-4 secretion. In addition, it reduced IFN-γ synthesis (Fig. 2.12C), as observed by other authors (Ouyang et al., 1998). Moreover, addition of dibutyryl-cAMP enhanced IL-5 and IL-4 secretion in the GATA-3 expressing, but not in uninfected, Th1 cells and ectopic expression of PKA_C was able to replace these effects. This indicates that GATA-3 is required for the stimulatory effect of PKA on Th2-type lymphokine expression.

It was important to determine whether this functional cooperation between PKA and GATA-3 originates at the transcription level. Hence several transient transfection assays were done to understand whether the stimulatory effect that active PKA exerts on the IL-5 promoter (Fig. 2.2) requires GATA-3 activity. This is shown in Fig. 2.13 by expression of vectors expressing PKA_C or GATA-3 along with a luciferase construct driven by the proximal IL-5 promoter into 293 HEK cells. Co-transfection of GATA-3 and PKA_C expressing vectors resulted in a synergistic activation of the proximal IL-5 promoter (Fig. 2.13), whereas none or very weak activation was observed for a mutated proximal IL-5 promoter (Gm) which is unable to bind GATA-3. This suggests that this cooperation is strongly required for the full activation of IL-5 promoter and thereby enhancing IL-5 production.

Several studies have examined the regulatory sequences and associated transcription factors that regulate the T-cell specific expression of IL-5 (for details see section 1.3.3). Although the importance of GATA-3 in IL-5 production has been well documented by numerous studies (see section 1.2.2), the mechanism underlying the GATA-3 gene expression remains largely elusive. Threshold level of GATA-3 is required for Th2 cytokine production. Since cAMP levels were found to be higher in Th2 cells than in Th1 cells and cAMP enhanced the GATA-3 effects, one may assume that cAMP induces the Th2-type cytokine, especially IL-5, expression by regulating GATA-3 gene expression in Th2 cells. Therefore the level of GATA-3 was analyzed by Northern hybridization upon cAMP-treatment in both EL-4 and primary Th0 cells, the cell types which required external provision of cAMP for full IL-5 gene expression. Interestingly, GATA-3 mRNA level in EL-4 cells was enhanced after 3 hours of Bt_2cAMP treatment and this induction persisted for longer period of time (Fig.2.14). In contrary, mature Th0 cells after T/I treatment expressed considerably high levels of GATA-3 and additional treatment with Bt_2cAMP did not enhance GATA-3 transcription further in these cells. This suggests, although not convincingly, that GATA-3 transcription might be positively regulated by cAMP signaling at least in EL-4 cells.

The amplification of GATA-3 transcription by cAMP as shown in Fig. 2.14 prompted us to investigate the mechanistic significance of this induction in the context of Th2 effector functions. Studies of the gene promoter for IL-5 have established independently the important roles of GATA-3 and cAMP in specific expression of the IL-5 gene in a transformed thymoma cell line EL-4 as well as Th2 clones (Lee et al., 1998; Zhang et al., 1997). It has been convincingly demonstrated that GATA-3, in cooperation with a factor(s) induced upon TCR signaling, governs Th2-specific expression of the IL-5 gene by directly interacting with a critical regulatory element of its promoter (Lee et al., 1998; Zhang et al., 1997). In one study, it is shown that the GATA site located between -70 and -60 in the IL-5 promoter is important for IL-5 gene expression (Prieschl et al., 1995). A GATA-3-binding site was identified in the regulatory sequences that lie within 1 kbp upstream of the IL-5 gene; four *cis*-acting elements including the GATA-3 binding site have been mapped here (Lee et al., 1998; Siegel et al., 1995; Zhang et al., 1997). By contrast, a role for GATA-3 in the activation of the IL-4 promoter has been elusive.

These observations argued in favor of the hypothesis that induction of IL-5 promoter by cAMP is possibly due to the enhanced GATA-3 transcription and therefore increased binding of GATA-3 in the IL-5 regulatory regions. This assumption was tested by electrophoretic mobility shift assay (EMSA) to detect the binding ability of GATA-3 in the upstream regulatory regions of IL-5. Using GATA consensus oligo probe, the EMSA with nuclear extracts prepared from non-stimulated EL-4 cells and cells that were stimulated for 6 hours by T/I or T/I+Bt_2cAMP revealed that the GATA-3 complex was already observed in non-stimulated cells and this complex was induced further by T/I treatment (lanes 1 and 2 in Fig. 2.15). Contrary to our assumption, T/I+cAMP treatment did not induce this complex any more (lane 3; Fig. 2.15). The specificity of the GATA-3 complex was determined by supershift EMSA using antibodies against different GATA proteins as well as competition with 100-fold excess cold oligos. This experiment suggests that cAMP does not induce IL-5 promoter activation by enhancing GATA-3 binding in the regulatory regions of IL-5. The molecular mimicry involved in cAMP-mediated regulation of GATA-3 and IL-5 genes are under intense investigation.

The discrepancy regarding the involvement of PKA pathway in cAMP-mediated IL-5 gene expression in Th2 cells has arisen from one study, which claimed that cAMP-induced effects in Th2 cells are independent of the PKA pathway but instead are mediated by p38 MAPK (Chen et al., 2000). Using the Th2 clone D10, this study concluded that the PKA pathway is not critical for cAMP-mediated IL-5 production in Th2 effector cells. Whereas this conclusion does not hold true in our different experimental primary lymphocyte models, we also observed involvement of p38 pathway in the regulation of IL-5 gene expression in Th2 cells, where inhibition of downstream p38 signaling by a relatively high dose of SB203580, a p38 MAPK inhibitor, resulted in abrogation of IL-5 production (Fig. 2.16A).

Since T/I/F treatment augments IL-5 expression in Th0 lymphocytes and potentiates the same in Th2 cells, we were prompted to investigate whether similar treatment leads to the enhanced activity of p38 MAPK in these cell types. For this, effector Th0, Th1 and Th2 cells, cultured in the respective skewing conditions for 5-7 days after primary stimulation, were restimulated for 30 minutes under different conditions and whole cell protein extracts from these restimulated or unstimulated cells were subjected to immunodetection using phospho-p38 antibody. As shown in Fig. 2.16B, the p38 activity is not increased in response to additional forskolin treatment in either Th0 lymphocytes or Th2 lymphocytes. This finding, in contrast to previous report

(Chen et al., 2000), suggested that increased cAMP levels do not appear to induce p38 MAPK in either Th0 or Th2 cells.

To determine, whether there is any cross talk between these two pathways, effector Th0, Th1 and Th2 cells, cultured in the respective skewing conditions for 5-7 days after primary stimulation, were restimulated by different stimuli for additional 30 minutes either in the absence or presence of H-89. Whole cell protein extracts from these restimulated or unstimulated cells were subjected to immunodetection by phospho-p38 antibody to detect the activation status of p38. However, no alteration in the pattern of p38 activity was observed by the inhibition of PKA, suggesting that these two pathways are independent of each other in the regulation of IL-5 gene expression (Fig. 2.16C). The significant inhibition of PKA activity in Th2 cells was observed at optimal concentration of H-89, 10 μM and not by lower dose of H-89, 2 μM (Fig. 2.9).

On the basis of these experiments, we conclude that the observation by Chen et al. (2000) implying the non-importance of PKA pathway in cAMP-mediated IL-5 production in Th2 effector cells which was gathered using PKA inhibitor, H-89 at extraordinarily low concentrations i.e. 0.5 μM and 1.0 μM, which probably could not have blocked the PKA activity in these Th2 clones, does not hold true. Although it is highly evident from our different assays that activation of PKA is the necessary component of cAMP signaling cascade in Th2 lymphocytes and this activation of PKA positively regulates the IL-5 promoter activity in GATA-3 dependent manner, suggesting that activated PKA mediates its function by the induction of IL-5 gene transcription. On the other hand, investigations into the p38 pathway revealed that the p38 MAPK inhibitor SB203580 resulted in a dose-dependent decrease in IL-5 promoter activity, and cAMP can induce GATA-3 phosphorylation via activation of p38 (Chen et al., 2000). However it is not clear from these experiments if these two pathways originating from cAMP ultimately converge at downstream signaling events to regulate synergistically the IL-5 expression or act independently by entirely different mechanisms.

4. Summary

Elevation of intracellular cAMP in T lymphocytes, induced by agents such as IL-1α, prostaglandins or forskolin, inhibits Th1-type cytokine production but stimulates Th2-type cytokine production. The signaling pathway engaged in cAMP-mediated induction of Th2 lymphokines remains obscure and therefore my doctoral work was focused on the elucidation of cAMP pathway in primary Th lymphocytes. While forskolin treatment of EL-4 cells led both to an activation of Th2 lymphokines and inhibition of Th1 lymphokines, ectopic expression of catalytically active PKA stimulated Th2 lymphokines but failed to inhibit Th1 lymphokine expression. Thus, the PKA activity is selectively involved in the stimulation of Th2 lymphokine expression whereas other cAMP-dependent pathway(s) appears to downregulate Th1 lymphokines. By investigating different types of primary murine Th cells, it was found that active PKA enhanced IL-5 expression only in Th0 and Th2 but not in Th1 cells. This is likely due to the different levels of GATA-3 whose expression is high in Th2, moderate in Th0 and very low in Th1 cells. Ectopic expression of GATA-3 in Th1 cells induced Th2 lymphokine expression which could be further enhanced by increased cAMP levels or PKA activity.

Investigations on the role of increased cAMP levels on Th2 lymphokines in D10 cells, a Th2-type cell line, led to the conclusion that elevated cAMP concentrations do not stimulate PKA but p38 activity which, through phosphorylation of GATA-3, appeared to induce IL-5 and IL-13 expression (Chen et al., 2000). While focusing on primary Th lymphocytes, it was observed that expression of the catalytic subunit α of PKA is sufficient for optimal IL-5 expression in primary Th0 cells. In addition, downregulation of IL-5 production in primary Th2 cells by the treatment with low concentrations of H-89, a PKA specific inhibitor, as well as by the ectopic expression of a negatively acting version of regulatory PKA subunit I demonstrates that active PKA plays an important role in IL-5 gene regulation. These findings using different types of primary $CD4^+$ T lymphocytes, including Th2 cells, the one likely to represent the native IL-5 producers *in vivo*, demonstrates that the adenylyl cyclase/cAMP/PKA signaling pathway plays an important role in IL-5 gene expression in primary Th2 cells. Thus the importance of cAMP/PKA signaling pathway in Th2 effector function was established during this doctoral research work.

Zusammenfassung

Die durch Agentien wie IL-1α, Prostaglandine oder Forskolin induzierte Erhöhung von intrazellulärem zyklischem Adenosin-Monophosphat (cAMP) in T-Lymphozyten inhibiert die Synthese Th1-typischer Zytokine und stimuliert die Synthese Th2-typischer Zytokine. Die für die cAMP-vermittelte Induktion von Th2-Zytokinen verantwortlichen Signaltransduktionskaskaden sind bisher nur unvollständig aufgeklärt. Deshalb konzentrierte sich meine Dissertation auf die Erforschung des cAMP-Signalweges in primären T-Helferzellen. Während die Induktion muriner EL-4 T-Zellen mit Forskolin sowohl zur Aktivierung Th2-typischer als auch zur Inhibierung Th1-typischer Lymphokine führt, kann die ektopische Expression einer katalytisch aktiven Proteinkinase A (PKA) zwar die Synthese von Th2-typischen Lymphokinen stimulieren, nicht jeder die Expression Th1-typischer Lymphokine inhibieren. Dies bedeutet, dass die Aktivierung von PKA selektiv an der Stimulation der Th2-Lymphokinexpression beteiligt ist, während andere, cAMP-abhängige Signaltransduktionswege zur Inhibierung Th1-typischer Lymphokine führen. Durch vergleichende Analysen verschiedener Th-Zellen konnte im Rahmen dieser Arbeit gezeigt werden, dass durch aktive PKA in Th0- und Th2, nicht jeder in Th1-Zellen die Expression von IL-5 erhöht wird. Dieses Phänomen ist wahrscheinlich auf die unterschiedliche Konzentration des Transkriptionsfaktors GATA-3 zurückzuführen. So kommt GATA-3 in Th2-Zellen in hoher, in Th0-Zellen in geringerer und in Th1-Zellen in sehr geringer Konzentration vor. Die ektopische Expression von GATA-3 in Th1-Zellen induziert die Synthese Th2-typischer Lymphokine, die durch erhöhte cAMP-Konzentration oder durch aktive PKA noch verstärkt werden kann.

Untersuchungen bezüglich des Einflusses erhöhter cAMP-Spiegel auf Th2-Lymphokine in der Th2-Zelllinie D10 zeigten, dass erhöhte cAMP-Konzentrationen nicht die PKA-Aktivität, sondern vielmehr die Aktivität der p38-Kinase stimuliert. Diese Aktivierung führt zur Phosphorylierung von GATA-3 und dadurch zur Induktion der IL-5- und IL-13-Expression (Chen *et al.*, 2000). In primären T-Helferzellen, die im Mittelpunkt der hier vorgelegten Arbeit standen, konnte beobachtet werden, dass bereits die Expression der katalytischen Untereinheit α der PKA ausreichend für eine optimale IL-5-Expression in Th0-Zellen ist. Die Beobachtung, dass primäre Th2-Zellen sowohl auf die Behandlung mit dem spezifischen PKA-Inhibitor H-89 als auch auf die ektopische

Expression der negativ wirkenden Untereinheit 1 der PKA mit signifikant verminderter IL-5-Produktion reagierten, unterstreicht die wichtige Rolle aktiver PKA bei der Regulation des IL-5 Gens.

Zusammenfassend konnte in dieser Arbeit durch die Untersuchung verschiedener primärer $CD4^+$ T-Lymphozyten, einschließlich der auch *in vivo* IL-5 produzierenden Th2-Zellen, gezeigt werden, dass der Adenylzyklase/cAMP/PKA-Signaltransduktionsweg bedeutend für die IL-5 Genexpression in primären Th2-Zellen und somit auch wichtig für deren Effektorfunktion ist.

5. Materials and Methods

5.1. Materials

5.1.1. General Materials

Cell strainer (70 µM)	Falcon
2 ml cryotubes	Greiner bio-one
Disposable needles, Cuvettes & Syringes	Hartenstein
FACS tubes	Hartenstein
Glasswares	Schott
Nitrocellulose Membrane	Schleicher & Schuell
Polypropylene tubes	Greiner bio-one, Nunc
Parafilm	Hartenstein
Pipette tips	Eppendorf
Pipettes	Sarstedt
Röntgen film (13x18 cm, BioMax)	Kodak
Sterile filters (0.2 µM/ 0.45 µM)	Schleicher & Schuell
Tissue culture plates	Greiner bio-one, Falcon
Tissue culture flask (50, 250, 500 ml))	Greiner bio-one
Tissue culture dish (60 mm, 90 mm)	Falcon, Greiner bio-one
Tubes (1.5 & 2 ml)	Sarstedt, Eppendorf
Whatmann paper	Schleicher & Schuell

5.1.2. Chemical Materials

Acetic Acid [$C_2H_4O_2$]	Carl Roth
Acrylamid solution	Carl Roth
AEBSF (Pefabloc SC)	Roche
Agar-Agar	Carl Roth
Agarose	Sigma-Aldrich
Ampicillin	Hoechst
APS	Merck Eurolab
ATP-disodiumsalt [$C_{10}H_{14}N_5O_{13}P_3Na_2$]	Sigma-Aldrich
ß-glycerophosphate [$C_3H_7O_6PNa_2$]	Carl Roth

ß-mercaptoethanol	Carl Roth
BioRad protein assay (5x Bradford reagent)	BioRad
Bisoprolol	Tocris
Boric Acid	Merck Eurolab
Bromophenol blue	Merck Eurolab
BSA Fraction V	Carl Roth
Butanol [$C_4H_{10}O$]	Carl Roth
Calcium chloride [$CaCl_2$]	Carl Roth
CD62L MACS beads	Miltenyl Biotech
Chloroform [$CHCl_3$]	Carl Roth
Citric acid [$C_6H_8O_7 \cdot H_2O$]	Carl Roth
Column for murine $CD4^+$ cell	CEDARLANE®
Coomassie brilliant blue R-250	Roche Applied Science
Cyclosporin A [CsA]	Novartis Pharma
Cyclic AMP	Sigma
DEPC	Carl Roth
Diethanolamine	Roth
Disodiumhydrogenphosphate [$Na_2HPO_4 \cdot 7H_2O$]	Merck Eurolab
D-Luciferin [$C_{11}H_8N_2O_3S_2$]	AppliChem
DMEM	Gibco BRL
DMSO	Carl Roth
dNTPs	MBI-Fermentas
DTT	Carl Roth
ECL Chemiluminiscence Kit	Amersham, Roche
EDTA [Na_2 EDTA$\cdot 2H_2O$]	Carl Roth
EGTA	Sigma-Aldrich
ELISA Reagents	
• Phosphatase substrate (Disodium 4-nitrophenylphosphate hexahydrate)	Sigma
• StreptABComplex/AP	DAKO
Ethanol [C_2H_5OH]	Carl Roth
Ethidium Bromide [EtBr]	Sigma-Aldrich
Ferrous(II)sulfat [$FeSO_4$]	Carl Roth

Ferric(III)chloride Hexahydrat [$FeCl_3 \cdot 6H_2O$]	Carl Roth
FCS	Gibco BRL
Ficoll	Amersham Pharmacia
Formaldehyde [CH_2O]	Carl Roth
Forskolin	Calbiochem,
Gel extraction kit (Jetsorb)	Genomed
Glutathione sepharose	Sigma-Aldrich
Glycerin (87%)	Carl Roth
Glycin [$C_2H_5NO_2$]	Merck Eurolab
H-89	Sigma
HBSS	Gibco BRL
Hepes	Carl Roth, Gibco BRL
Hydrochloric Acid [HCl]	Merck Eurolab
Hydrogen Peroxide [H_2O_2]	Carl Roth
4-Hydroxytamoxifen [$C_{26}H_{29}NO_2$, Z-isomer]	Sigma-Aldrich
ICI-118 551	Tocris
IL-12, Murine, Recombinant, *E.coli*	Dr. E. Schmitt, Mainz
IL-2, Murine, Recombinant, *E.coli*	Prof. A. Schimpl, Wuerzburg
IL-4, Murine, Recombinant, *E.coli*	Dr. E. Schmitt, Mainz
Ionomycin	Sigma-Aldrich
IPTG	Boehringer-Ingelheim
Isoamylalcohol	Carl Roth
Isopropanol [2-Propanol, C_3H_8O]	Carl Roth
Isoproternol	Sigma
Leupeptin hydrochloride	Roche Applied Science
L-Glutamine	Gibco BRL
L-Glutathione [M_r: 307·3]	Serva
Lithium chloride [LiCl]	Sigma-Aldrich
Milk powder	Saliter
Magnesium acetate [$Mg(C_2H_3O_2)_2 \cdot 4H_2O$]	Sigma-Aldrich
Magnesium chloride [$MgCl_2$]	Carl Roth
Magnesium sulfate [$MgSO_4 \cdot 7H_2O$]	Carl Roth
Manganese chloride [$MnCl_2$]	Fluka

MEM [100x]	Gibco BRL
MES [$C_6H_{13}NO_4S$]	Sigma-Aldrich
Methanol [CH_4O]	Carl Roth
Metrizamide [$C_{18}H_{22}I_3N_3O_8$]	Serva
MOPS	Carl Roth
Paraformaldehyde	Merck Eurolab
PCR purification kit	Qiagen
PEG 4000	NEB
Penicillin (10,000 IU/ml)	Hoechst
Phenol [C_6H_6O, TE equilibrated]	Carl Roth
PIPES	Serva
PKA assay kit	Upstate biotechnology
Plasmid-DNA Isolation kit (Maxi)	Macherey-Nagel, Qiagen
Plasmid DNA Isolation kit (Mini)	Genomed
PMSF	Serva
Poly dI/dC	Boehringer Ingelheim
Polybrene	Sigma Aldrich
Ponceau Red	Sigma Aldrich
p-ONPG	Sigma Aldrich
Potassium acetate [$C_2H_3KO_2$]	Carl Roth
Potassium chloride [KCl]	Sigma-Aldrich
Potassium dihydrogen phosphate [KH_2PO_4]	Sigma-Aldrich
Potassium hydrogen phosphate [$KHPO_4$]	Sigma-Aldrich
Potassium hydroxide [KOH]	Carl Roth
Propidiumiodide (PI 1 mg/ ml ddH_2O)	Sigma Aldrich
Protease inhibitor tablet (complete mini)	Roche Applied Science
Protein-A/G sepharose	Santa Cruz
Radioactive nucleotides [γ^{32}P-ATP, α^{32}P-dCTP, α^{32}P-UTP]	Amersham Pharmacia
RNase Protection Assay Kit (RiboQuant)	BD Pharmingen
RPMI 1640 (DUTCH modified)	Gibco BRL
RPMI 1640	Gibco BRL
Rubidium chloride [RbCl]	Carl Roth
Saponin	Sigma

SB 203580	Sigma
Sodium acetate [$CH_3COONa \cdot 3H_2O$]	Merck Eurolab
Sodium azide [NaN_3]	Merck Eurolab
Sodium carbonate [Na_2CO_3]	Carl Roth
Sodium chloride [NaCl]	Carl Roth
Sodium fluoride [NaF]	Sigma-Aldrich
Sodium hydrogen phosphate [$NaH_2PO_4 \cdot H_2O$]	Merck Eurolab
Sodium hydroxide [NaOH]	Carl Roth
Sodium orthovanadate [Na_3VO_4]	Fluka
Sodium pyruvate [$C_3O_3H_3Na$]	Gibco BRL (100 mM)
Sodium citrate [$C_6H_5Na_3O_7 \cdot 2H_2O$]	Carl Roth
SDS	Carl Roth
Sephadex G50	Amersham Pharmacia
Streptomycinsulfat (10 mg/ml)	Hoechst
Sulfosalicylic acid	Sigma Aldrich
Tamoxifan	Sigma
TaqDyePrimer sequencing Kit	Perkin Elmer
TEMED	Carl Roth
TPA	Sigma Aldrich
Transfection reagents (SuperFect™, PolyFect™)	Qiagen
Trichloroacetic acid [$C_2HCl_3O_2$]	Sigma Aldrich
Tris	Carl Roth
Triton X-100	Sigma Aldrich
Trizol reagent	Gibco BRL
Trypan blue 0.1%	Gibco BRL
Trypsin EDTA	Gibco BRL
Tween 20	Carl Roth
Western blotting substrate (Lumi light)	Roche
X-VIVO 15	BioWhittaker
Xylene cyanol FF	Serva
2-YT Broth	GibcoBRL
Zeocin (100 mg/ml)	Invitrogen

5.1.3. Instruments

Autoclave	Stiefenhofer
Balance machine	Sartorius, Hartenstein
Cold centrifuge	Heraeus
DNA sequencer 373A	Perkin Elmer
ELISA plate reader	BioRad
FACScan™	Becton Dickinson
Fluorescence microscope (Dialux 20)	Leica
Gel documentation system	Herolab
Gel camera	Stratagene, Hoefer
Gel dryer	H.Hölzel
Haemocytometer	Brand
Heating blocks	Hartenstein
Humidified tissue culture incubators	Nuaire US, Heraeus
Hybridization Oven	Bachofer
Ice machines	Genheimer
Intensifying screen	DuPont
Laminar hoods	Heraeus, Gelaire
Light microscope	Olympus, Leica
Liquid nitrogen tank	Tec-lab
Luminometer	Berthold
Microliter pipettes	Eppendorf, Brand
Microcentrifuge	Eppendorf
Multichannel pipette	Eppendorf
Multi dispencer pipette	Eppendorf
PCR machine	Perkin Elmer, MWG
pH meter	Ingold, Hartenstein
Phosphorimager	Molecular Dynamics
Quartz cuvettes	Hellma
Refrigerators (-20°C; -70°C)	Privileg, Bosch, Heraeus
Rotors (JA-10, JA-14)	Beckman
Scintillation counter	Canberra Packard
Shaking incubator	Hartenstein

Power supplier	Amersham Pharmacia
SDS-PAGE apparatus	BioRad
Spectrophotometer	Amersham Pharmacia
Ultracentrifuge	Beckman
UV lamp (UVT-20M)	Herolab
Vortexer	Hartenstein
Waterbath	Hartenstein
Water filtration unit (MilliQ Plus)	Millipore
Western blot apparatus	Hoefer

5.1.4. Reagents

5.1.4.1. DNA size markers

The GeneRuler 100 bp and 1 kb DNA size markers were procured from MBI-Fermentas. The size of fragments in markers was as following:

100 bp Marker 1,031 / 900 / 800 / 700 / 600 / 500 / 400 / 300 / 200 / 100 / 80

1 kb Marker 10,000 / 8,000 / 6,000 / 5,000 / 4,000 / 3,500 / 3,000 / 2,500 / 2,000 / 1,500 / 1,000 / 750 / 500 / 250

5.1.4.2. Protein standards

The protein size marker BENCHMARK™ was procured from Gibco BRL.

5.1.4.3. Enzymes

All restriction endonucleases and modifying enzymes	MBI-Fermentas
SAWADY PWO DNA polymerase	Peqlab
Proteinase K and RNase [Ribonuclease] Type I-A	Sigma-Aldrich

5.1.4.4. Monoclonal antibodies for ELISA

Ag	Ab	Format	Clone	Manufacturer
IL-5	Anti-mouse rat IL-5	Purified	TRFK5	BD Pharmingen
IL-5	Anti-mouse rat IL-5	Biotinylated	TRFK4	BD Pharmingen
IL-4	Anti-mouse rat IL-4	Purified	BVD4-1D11	BD Pharmingen
IL-4	Anti-mouse rat IL-4	Biotinylated	BVD6-24G2	BD Pharmingen
IFNγ	Anti-mouse rat IFNγ	Purified	R4-6A2	BD Pharmingen
IFNγ	Anti-mouse rat IFNγ	Biotinylated	XMG1.2	BD Pharmingen

5.1.4.5. Antibodies for flow cytometry

Ag	Ab	Format	Clone	Source
Fc receptor	Anti-mouse rat FcR	Purified	2.4G2	Hybridoma
CD4	Anti-mouse rat CD4	PE	RM4-5	BD Pharmingen
CD62L	Anti-mouse rat CD62L	Magnetic beads	MEL 14	Miltenyi Biotech
CD62L	Anti-mouse rat CD62L	Biotinylated	MEL 14	BD Pharmingen
IFN-γ	Anti-mouse rat IFN-γ	PE	XMG1.2	BD Pharmingen
IL-4	Anti-mouse rat IL-4	PE	BVD4-1D11	BD Pharmingen
Isotype control	Anti-mouse rat IgG1	PE		BD Pharmingen

5.1.4.6. Primary monoclonal antibodies

Antigen	Source	Clone	Manufacturer
Murine CD3ε	Armen. Hamster	145-2C11	BD Pharmingen
Murine CD28	Syrian Hamster	37.51	BD Pharmingen
HA Epitope	Mouse	HA.11	BabCO
FLAG Epitope	Mouse	M2	Sigma-Aldrich
FLAG Epitope	Mouse	M5	Sigma-Aldrich
β-Actin	Mouse	AC-15	Sigma-Aldrich
phospho-Tyr	Mouse	p-Y99	SantaCruz
p-JNK1	Mouse	G7	SantaCruz

5.1.4.7. Primary polyclonal antibodies for immunodetection

Antigen	Source	Manufacturer
Phospho-PKA substrate	Rabbit	Cell Signaling
ERK1/2	Rabbit	Santa Cruz
Phospho-ERK1/2 (Thr202/Tyr204)	Rabbit	Cell Signaling
JNK1	Rabbit	Santa Cruz
Phospho- JNK	Rabbit	Cell Signaling
p38	Rabbit	Santa Cruz
p-p38 (Thr180/Tyr182)	Rabbit	Cell Signaling

5.1.4.8. Secondary polyclonal antibodies

Antigen	Source	Manufacturer
Armenian Hamster IgG	Goat	Dianova

5.1.4.9. Secondary coupled antibodies for immunodetection

Antigen	Source	Coupled to	Manufacturer
Mouse IgG	Sheep	HRPO	Amersham Pharmacia
Goat IgG	Donkey	HRPO	Santa Cruz
Rabbit IgG	Donkey	HRPO	Amersham Pharmacia

5.1.4.10. Antibodies for $CD4^+$ lymphocyte preparation

Antigen	Origin	Clone	Source
Anti-CD8	Rat IgM	AK 3.168.1	Rob Mc Donald
Anti-HSA (J11D)		J11D	ATCC

5.1.4.11. Cytokines and antibodies for Th differentiation

Cytokine/Ab	Clone	Source
IL-2	P-30	Prof. A. Schimpl (Würzburg)
IL-4	CHO	Prof. E. Schmitt (Mainz)
Anti-IFNγ	XMG1.2.9	Prof. E. Schmitt (Mainz)
IL-12		Prof. E. Schmitt (Mainz)
Anti-IL-4	11B11	Prof. E. Schmitt (Mainz)

5.1.4.12. Ova peptide

For the primary stimulation of $CD4^+$ T cells purified from lymph nodes of DO11.10 TCR transgenic (tg) Balb/c mice (Murphy et al., 1990), the OVA-albumin peptide 323-339 (OVA) from Jerini corp. was used. The amino acid sequence of this antigenic epitope is: ISQAVHAAHAEINEAGR.

5.1.4.13. Oligonucleotides and primers

All oligonucleotides and primers were procured from MWG Biotech. The lyophilized oligos were dissolved in 10 mM Tris, pH 7.5 to a final concentration of 100 pmol/µl. For the sequencing reaction, lyophilized oligos were dissolved in 10 mM Tris, pH 7.5 to a

final concentration of 10 pmol/µl. Primers were stored in –20°C. For the construction of retroviral expression plasmids, following oligonucleotides were used:

HA-tag1 upper: 5´-AATTGCACCATGGCCTACCCATACGACGTGCCTGACTACGCCTCC-3´

HA-tag1 lower: 5´CGATGGAGGCGTAGTCAGGCACGTCGTATGGGTAGGCCATGGTGC-3´

HA-tag2 upper:

5´ TACGCCTCCATCGATGGCGCGCCCGTACGCGGACCGGTTAACCCGCGG-3´

HA-tag2 lower: 5´-AATTCCGCGGGTTAACCGGTCCGCGTACGGGCGCGCCAT-3´

For the sequencing reactions, following primers were used:

pEGZ forward	5´-CACGTGAAGGCTGCCGAC-3´
pEGZ reverse	5´-CTTCGGCCAGTAACGTTAGG-3´
T3 promoter	5´-ATTAACCCTCACTAAAGGGA-3´
T7 promoter	5´-AAGGCTAGAGTACTTAATACGA-3´
T7 promoter	5´-AAGGCTAGAGTACTTAATACGA-3´
KRSPA forward	5´-GTATTTAAGTGCCTAGCTCGATA-3´

5.1.4.14. Antibiotics

Ampicillin (100 mg/ ml); final Conc. 100 µg/ ml	Hoechst
Penicillin (10,000 IU/ml)/ Streptomycinsulphate (10 mg/ml)	Hoechst
Zeocin (100 mg/ml)	InvitroGen

5.1.5. Solutions and Buffers

All chemicals of molecular biology research grade were procured from respective manufacturers and all solutions were prepared using pure distilled (Milli-Q grade) autoclaved water. Wherever necessary, solutions were sterile filtered or autoclaved.

APS stock solution (10 %, 10 ml)

APS	1 g

Blocking buffer for Western hybridization

Milk powder	5 g
Dissolved in 100 ml of 1x TBS-T.	

Blocking buffer for Western hybridization using phospho-specific antibodies

BSA fraction V	5 g
Dissolved in 100 ml of 1x TBS-T.	

Brefeldin A: Dissolved in absolute ethanol at a concentration of 2 mg/ml.

BSS (Balanced Salt Solution)

Working solution: BSS I & BSS II (1:1) + dd H_20 [8:10]

BSS I (10x, 1000 ml, Sterile filtered)

Glucose	10.0 g
KH_2PO_4	0.6 g
$Na_2H\ PO_4.2H_20$	2.3 g
Phenol Red	0.1g

BSS II (10x, 1000 ml, Sterile filtered)

$CaCl_2.\ 2H_20$	1.86 g
KCl	4.0 g
NaCl	80.0 g
$MgCl_2.6H_20$	2.0 g
$MgSO_4.7H_20$	2.0 g

Calcium chloride stock solution (1 M, 1000 ml)

$CaCl_2$	110.98 g

Coating buffer for anti-CD3 antibody 0.05 M Tris of pH 9.5

Colony hybridization solutions

CH solution I (always prepared fresh)

NaOH	0.5 N

CH solution II

Tris- HCl (pH 7.5)	0.2 M
NaCl	1.0 M

CH solution III

SDS	1%
EDTA	1 mM
Na_2HPO_4 (pH 6.8)	40 mM

CH pre-hybridization solution

SSC	5x
SDS	0.2%
Denhardt solution	2x
Salmon sperm DNA	100 μg/ml

CH stop buffer

Loading dye	6x
SDS	0.5%
EDTA	50 mM

CH washing buffer

SSC	2x
SDS	0.2%

Coomassie blue solution (1000 ml)

Coomassie Brilliant Blue R-250	2.5 g
Methanol	450 ml
Acetic Acid	100 ml

DEPC-treated ddH_2O (RNase-free, 1000 ml)

DEPC	1 ml

DEPC was mixed thoroughly, incubated overnight (~ 16 h) under hood at RT, autoclaved and stored at RT; all solutions and buffers for RNA work were prepared in DEPC-treated ddH_2O.

Denaturing PAA-Gel for RNase Protection Assay (1000 ml; 6% gel solution)

Urea	280.0 g (8M)
30% Acryl-Bisacrylamide solution	240 ml
10x TBE buffer	100 ml

Composition of one gel (Polymerization takes approx. 1 -2 hours at RT)

6% gel solution	30 ml
10% APS	300 µl
TEMED	60 µl

DNA Electrophoresis Buffer (1000 ml)

TAE (50x)	20 ml

The solution was supplemented with 0.25 ml EtBr stock solution per liter of TAE and boiled for dissolving the agarose.

DNA gel composition

	0.7%	1.0 %	2.0 %
Agarose	1.05 g	1.5 g	3.0 g
20x TAE	7.5 ml	7.5 ml	7.5 ml
ddH_2O	142.5 ml	142.5 ml	142.5 ml
EtBr (5mg/ml)	25 µl	25 µl	25 µl

DTT stock solution (1 M, 20 ml)

DTT	3.09 g

DTT powder was dissolved in 10 mM sodium acetate (pH 5.2), aliquoted and frozen in -20°C.

EDTA stock solution (0.5 M, 1000 ml)

Na_2 EDTA·$2H_2O$	186.1 g

pH of the solution was adjusted to 8.0 with 10 M NaOH (~ 50 ml); EDTA gets dissolved only in correct pH.

EGTA stock solution (0.25 M, 1000 ml)

EGTA	95 g

pH of the solution was adjusted to 8.0 with KOH; EGTA gets dissolved only in correct pH.

ELISA blocking buffer

FCS	10 %
1x PBS	90%

ELISA coating buffer

Na_2HPO_4	0.1 M

pH of the solution was adjusted to 9.2.

ELISA sample dilution buffer

FCS	10 %
1x PBS	90%
Tween 20	0.05 %

ELISA washing buffer (5000 ml)

10x PBS	500 ml
Tween 20	2.5 ml

ELISA substrate solution (stored at 4°C, 500 ml)

$MgCl_2·6H_2O$	40 mg
H_2O	400 ml
pH of the solution was adjusted to 9.8 and then added	
Diethanolamine	48.5 ml

ELISA color development solution (always fresh; for one 96-well plate)

ELISA substrate solution	10 ml
AP-Phosphatase substrate	10 mg

EMSA Solutions

PAA gel for the preparation of radioactive DNA probes (50 ml, 12%)

ddH_2O	30 ml
30% acryl-bisacrylamide solution	15 ml
10x TBE buffer	5 ml
10% APS	300 µl
TEMED	60 µl

Gel polymerization takes approx. 1 hour.

EMSA gel (6%, 100 ml)

dd H_2O	70 ml
30% acryl-bisacrylamide solution	20 ml
10x TBE buffer	10 ml
10% APS	500 µl
TEMED	50 µl

EMSA binding buffer (3x, 50 ml)

1M Hepes/KOH (pH 7.9)	3 ml
1 M KCl	7.5 ml
0.5 M $Na_2EDTA{\cdot}2H_2O$ (pH 8.0)	300 µl
1 M DTT	150 µl
Ficoll	6 g

Aliquotes were stored at -20°C and thawed before use on ice.

EMSA running buffer (1x, 1000 ml)

TBE (10x)	100 ml

EMSA stop buffer

SDS	0.5 %
EDTA	20 mM
Bromophenol blue	

Ethidium bromide stock solution (100 ml)

EtBr	1 g

The solution was stored at 4°C in a dark bottle.

FACS buffer (stored at 4°C)

10x PBS, pH 7.4	50 ml
Cell culture grade H_20	450 ml
1.0 M Sodium azide	0.5 ml

2.5 g of BSA was layered on top of the liquid mixture, dissolved at RT without stirring, sterile filtered the mixture and stored at 4°C.

4% Formaldehyde (100 ml)

10x PBS	14 ml
37.5 % Formaldehyde	10.8 ml

The solution was filtered to remove any particulates.

Freezing medium

DMSO	10 %
FCS	20 %
RPMI 1640 / 2mM L-Glutamin	70 %

Gel loading sample buffer, 6x (MBI Fermentas)

Glycerine	60%
EDTA	60 mM
Bromophenol blue	0.09%
Xylene Cyanol FF	0.09%

2x HBS

Hepes/KOH (pH 7.05)	50 mM
KCl	10 mM
Dextrose	12 mM
NaCl	280 mM
Na_2HPO_4	1.5 mM

The solution was sterile filtered through 0.45 µ filter, aliquoted and stored in -20°C.

HEPES/ KOH stock solution (1 M, 1000 ml)

HEPES	238.33 g

pH of the solution was adjusted to 7.2/ 7.4/ 7.9 with KOH.

Luciferase harvesting buffer (50 ml)

1.5 M Tris/HCl (pH 7.8)	1.7 ml
1 M MES	2.5 ml
Triton X-100	50 µl

The solution was freshly prepared and 50 µl DTT stock solution (1M) was added just before use.

Luciferase assay buffer (50 ml)

1.5 M Tris/HCl (pH 7.8)	4.17 ml
1 M MES	6.25 ml
1 M $Mg(C_2H_3O_2)_2.4H_2O$	1.25 ml

The solution was freshly prepared and supplemented with very little ATP just before use.

Luciferin solution for luciferase assay (100 ml)

Luciferin	28 mg
1 M $KHPO_4$ (pH 7.8)	0.5 ml

The solution was aliquoted and stored at -20°C.

Metrizamide solution

Metrizamide	14.5 g
Dendritic cell medium	100 ml

The solution was sterile filtered by passing through 20 µ filter, aliquoted and stored at -20°C.

Northern blot agarose gel solution

Agarose	1.5 g
H_20	70 ml
10x MOPS	10 ml
Formaldehyde	18 ml

Northern blot pre-pre hybridization solution 2x SSC +1% SDS

Northern blot pre-hybridization solution

ULTRAhyb™ (Ambion): ULTRAhyb contains 50% formamide. Exact composition of this commercial hybridization solution is not disclosed. Ambion claims that ULTRAhyb contains a unique blend of hybridization accelerators and blocking agents that greatly enhance the levels of hybridization so that signals that once took days to visualize become apparent in hours.

Northern blot denaturation solution 0.01 N NaOH + 3M NaCl

Northern blot washing solution I (RT) 2x SSC + 0.1% SDS

Northern blot washing solution II (68°C) 0.2x SSC + 0.1% SDS

Nuclear and cytoplasmic extract preparation buffers

Extraction buffer A (Hypotonic, 1000 ml)

1 M Hepes/KOH (pH 7.9)	10 ml
1 M KCl	10 ml
0.5 M $Na_2EDTA{\cdot}2H_2O$ (pH 8.0)	200 µl
0.25 M EGTA (pH 8.0)	400 µl

Solution was stored at 4°C. For 10 ml Buffer A, following inhibitors were added before experiment: 10 µl DTT stock solution (1M) and 50 µl AEBSF (0.2 M).

Extraction buffer C (High salt, 1000 ml)

1 M Hepes/KOH (pH 7.9)	20 ml
1 M KCl	400 ml
0.5 M $Na_2EDTA.2H_2O$ (pH 8.0)	2 ml
0.25 M EGTA (pH 8.0)	4 ml

Solution was stored at 4°C. For 10 ml Buffer C, following inhibitors were added before experiment: 10 µl DTT-stock solution (1M), protease inhibitors [100 µl AEBSF (0.2 M), 10 µl leupeptin (2 mM) and 10 µl aprotinin (0.3 M)].

PBS (10x, 1000 ml)

NaCl	80 g
KCl	2 g
$CaCl_2$	1 g
$MgCl_2$	1 g
$Na_2HPO_4 \cdot 7H_2O$	26.8 g
KH_2PO_4	2.4 g

pH of the solution was adjusted to 7.4 with 1 N HCl.

Phosphatase inhibitor (stock solution, final working concentration is indicated)

Sodium Orthovanadate [Na_3VO_4 stock solution (0.2 M): 4 mg/ml in H_2O]:1 mM

pH of the solution was adjusted to 10.0 with 1 N NaOH or 1 N HCl (solution becomes yellow), boiled for 10 min at 100°C (solution becomes colorless), cooled to RT and subsequently pH was again adjusted to 10.0; this was repeated till solution becomes colorless at RT and pH gets stabilized at 10.0. Aliquots were stored at -20°C and just before use boiled for 5 min at 100°C and left at RT to cool down.

Ponceau red solution (100 ml)

Ponceau red	2.0 g
Trichloroacetic acid	30.0 g
Sulfosalicylic acid	30.0 g

Potassium chloride stock solution (1 M, 1000 ml)

KCl	74.6 g

Potassium hydrogen phosphate stock solution (1 M, 1000 ml)

$KHPO_4$	135.1 g

pH of the solution was adjusted to 7.8 with KOH.

Potassium phosphate buffer (0.2 M, 1000 ml)

KH_2PO_4	27.2 g

pH was adjusted to 7.0 with 1 M KOH.

Protease inhibitors (final working concentration is indicated)

Aprotinin [Stock solution (0.3 M): 2 mg/ml H_2O]	0.3 µM
Leupeptin [Stock solution (2 mM): 1 mg/ml H_2O]	2 µM
AEBSF [Stock solution (0.2 M): 50 mg/ml H_2O]	1 mM

RNA loading buffer (5x, 10 ml, stored at 4°C)

Saturated bromophenol blue solution	16 µl
500 mM EDTA, pH 8.0	80 µl
37% (12.3 M) formaldehyde	720 µl
100% glycerol	2 ml
Formamide	3.084 ml
10x RNA gel buffer	4 ml

RNA gel buffer (10X, Stored in dark bottle at RT, 1000 ml)

MOPS (0.2M)	41.85 g
NaOAc (0.051M)	6.8 g
EDTA (0.01M)	2.92 g

Adjust pH to 7.0 with NaOH (~15 ml)

RNA gel running buffer (1x, 1000 ml in DEPC - treated H_2O)

10x RNA gel buffer	100 ml
37% (12.3 M) formaldehyde	20 ml

Saponin buffer (stored at 4°C)

Saponin	1 ml
FCS	10 ml
Na azide	100 µl
PBS	989 ml

SDS stock solution (10%, 1000 ml)

SDS	100 g

The solution was warmed to 70°C to dissolve and pH was set to 7.2 with 1 N HCl.

SDS-PAGE sample buffer (4 x, 100 ml)

1.5 M Tris/HCl (pH 6.8)	20 ml
SDS	2.4 g
Glycerine (87 %)	50 ml
ß-mercaptoethanol	25 ml
Bromphenol blue	0.04%

The solution was warmed to 70°C and stored at -20°C.

SDS-PAGE running buffer (10x, 1000 ml)

Tris	30.3 g
Glycin	144.1 g
10% SDS	100 ml

pH of the solution was adjusted to 8.5 with 1 N HCl.

Sodium acetate stock solution (3 M, 1000 ml)

$CH_3COONa \cdot 3H_2O$	408.24 g

pH of the solution was adjusted to 5.2 with concentrated acetic acid.

Sodium chloride stock solution (5 M, 1000 ml)

NaCl	292.22 g

The solution was dissolved by heating to 60°C.

Sodium hydroxide stock solution (10 M, 1000 ml)

NaOH	400 g

Sodium phosphate buffer (0.2 M, 1000 ml)

$NaH_2PO_4 \cdot H_2O$	27.6 g

Stripping buffer for nitrocellulose membrane (1000 ml)

1.5 M Tris/HCl (pH 6.8)	41.7 ml
10% SDS	200 ml

Before use, 100 ml buffer was supplemented with 700 µl ß-mercaptoethanol.

TAE buffer (Tris/Acetate/EDTA, 50x, 1000 ml)

Tris	242 g
0.5 M $Na_2EDTA \cdot 2H_2O$ (pH 8.0)	100 ml
Concentrated acetic acid	57.1 ml

TBE buffer (Tris/Borate/EDTA, 10x, 1000 ml)

Tris	108 g
Boric Acid	55 g
0.5 M $Na_2EDTA \cdot 2H_2O$ (pH 8.0)	40 ml

TBS (20x, 1000 ml)

Tris	121.0 g
NaCl	175.2 g
KCl	7.5 g

pH of the solution was adjusted to 7.6 with 1M HCl (~ 10.2 ml).

TBS/Tween (TBS-T, 1x, 1000 ml)

TBS (20x)	50 ml
Tween 20	1 ml

TE buffer (Tris/EDTA, pH 8.0, 1000 ml)

1.5 M Tris/HCl (pH 8.0)	6.7 ml
0.5 M EDTA (pH 8.0)	0.2 ml

Transfer buffer for Western blot (1000 ml)

Glycine	2.9 g
Tris	5.8 g
10% SDS	3.7 ml
Methanol	200 ml

Tris/ HCl stock solution (1.5 M, 1000 ml)

Tris	181.7 g

pH of the solution was adjusted to 6.8/ 7.5/ 7.8/ 8.0/ 8.8 with 1 N HCl.

Whole cell extract preparation buffer (Kyriakis Lysis Buffer Modified, 1000 ml)

1 M Hepes/KOH (pH 7.4)	20 ml
0.25 M EGTA (pH 8.0)	8 ml
NaF	2.1 g
ß-Glycerophosphate	10.8 g
Glycerine (87%)	115 ml
Triton X-100	10 ml
NaN_3-solution (10%)	4 ml

The solution was stored at 4°C. For 10 ml $KLBM^+$ buffer, following inhibitors were added before experiment: 10 µl DTT-stock solution (1M), protease inhibitors [50 µl AEBSF (0.2 M), 10 µl leupeptin (2 mM) and 10 µl aprotinin (0.3 M)] and phosphatase inhibitor [50 µl Sodium orthovanadate (0.2 M)].

5.1.6. DNA Vectors

5.1.6.1. Eukaryotic expression plasmid and reporter constructs

pBS KRSPA: This eukaryotic expression plasmid is a derivative of Bluescript-KS^+ from Stratagene.

-120 IL-5 TATA-luc WT: The mouse IL-5 promoter region (spanning -120 to +44) was cloned in the Hind III and Xho I site in pTATA-Luc plasmid. The structure is as following:

-120 IL-5 TATA-luc Gm: The mouse IL-5 promoter region (spanning -120 to +44) was mutated in the GATA binding site from CTATC to GCATT within its unique GATA-3 binding site (around position - 72) rendering it unable to bind GATA-3.and this mutated version was cloned in the Hind III and Xho I site in pTATA-Luc plasmid.

5.1.6.2. Retroviral packaging- and expression vector

The retroviral packaging vectors pHIT 60, pHIT 123, pHIT 456 and the expression vectors pEGZ/MCS, pEYZ/MCS were kindly provided by Dr. Ingolf Berberich (Institute for Virology and Immunobiology, University of Wuerzburg, Germany). They were constructed as a derivative of the Murine Leukemia Virus (MLV) (Soneoka et al., 1995) wherein the different cDNAs were cloned under the control of the Cytomegalovirus (CMV) promoter. Moreover all plasmids contain a SV40 poly A adenylation signal and the ampicillin resistant gene.

pHIT 60: This packaging vector encodes for retroviral structural protein, gag and reverse transcriptase, pol. It harbours no retroviral packaging signal ψ+.

pHIT 123: This packaging vector encodes for the ecotropic envelope (env), which allows virus to transduce murine cells. It harbours no retroviral packaging signal ψ+.

pHIT 456: This packaging vector encodes for the amphotropic envelope protein (env), which allows virus to transduce cells of murine as well as different species. It harbours no retroviral packaging signal ψ+.

pEGZ/MCS: This retroviral expression vector is derived from bicistronic vectors pczCFG2 hCD8 IEYZ (Berberich-Siebelt et al., 2000; Lindemann et al., 1997) in which the CMV enhancer replaces the U3 region of the 5´ LTR of murine leukemia virus (MuLV). The original viral vector contained a human CD8 cDNA (hCD8) as gene of interest. It harbours an Encephalomyocarditis viral Internal Ribosomal Entry Site (IRES) which allows the coordinate expression of a desired cDNA with an Enhanced Green Fluorescence Protein (EGFP). The pEGZ/MCS was constructed by replacing the hCD8 cDNA by an oligonucleotide encompassing restriction sites for EcoRI, SmaI and BamHI as multiple cloning site (MCS). Owing to the retroviral packaging signal ψ+, the cDNA of interest is packaged in viruses which can be used for gene transfer to target cells by transduction. This vector also contains a zeocin resistant gene which allows selection of positively transduced cells.

pEGZ-HA: This retroviral expression vector was derived from pEGZ/MCS. Oligonucleotide, comprising recognition sequence from *Haemophilus aureus* (HA-tag) followed by restriction enzyme sites ClaI, BsiWI, RsrII, HpaI, SacII, was cloned in EcoRI site of pEGZ/MCS. Cloning of cDNA in these sites allowed the HA-tagged version of protein, which is extremely helpful in the identification of the protein as well as in different assays.

pEGZ-HA-PKAc: This vector, contains its PKA cDNA of 1.3 kb (Uhler and McKnight, 1987). PKA cDNA was PCR-cloned as a Cla I/Bam HI fragment in frame with a HA-tag sequence at its N-terminus into the Eco RI site of pEGZ/MCS.

pEGZ-HA-PKAc-ER^{TM}: BamH I fragment of ER was cloned in BamH I site of pEGZ-HA-PKAc.

pEGZ-PKA$_{RM}$: The retroviral vector expressed a mutated version of the regulatory PKA subunit Iα, PKA$_{RM}$, which is unable to bind cAMP and, therefore, acted as a repressor of PKA activity (Clegg et al., 1987). It contains a PCR-cloned Cla I/Bam HI cDNA fragment of 1.2 kb inserted into pEGZ/MCS.

pEYZ-FLAG-GATA-3: The retroviral vector expressing murine GATA-3 (Weiss and Orkin, 1995) was constructed by cloning a PCR Eco RI/Bam HI fragment of 1.2 kb with a 5' FLAG encoding sequence into the EYZ/MCS vector.

5.1.7. Growth Medium

5.1.7.1. Liquid medium and agar plates for bacterial culture.

2xYT Medium

2-YT Broth	31 g

Powder was dissolved in 1000 ml ddH_2O and autoclaved at 121°C, 15 psi for 30 minutes. After autoclaving, the medium was cooled down to 60°C and then respective antibiotic (ampicillin, final conc.100 µg/ml) was added.

2xYT Agar Plate

2YT Broth	31 g
Agar-Agar	12 g

These were dissolved in 1000 ml ddH2O and autoclaved at 121°C, 15 psi for 20 minutes. After autoclaving, the medium was cooled down to 60°C to add respective antibiotic (ampicillin, final conc.100 µg/ml), immediately distributed in bacterial dishes under the laminar hood and allowed to solidify at RT overnight. The plates were stored at 4°C.

LB Medium

Bacto tryptan	10 g
Yeast extract	5 g
NaCl	10 g

The pH of the medium was adjusted to 7.0 with 10N NaOH (~ 200 µl), the total volume was adjusted to 1000 ml with ddH_2O and autoclaved at 121°C, 15 psi for 20 minutes. The medium was stored at 4°C. Before use, respective antibiotic was added to the medium.

LB Agar plate

Bacto tryptan	10 g
Yeast extract	5 g
NaCl	10 g
Agar-Agar	12 g
ddH_2O	800 ml

The pH of the medium was adjusted to 7.0 with 10 N NaOH, the total volume was adjusted to 1000 ml with ddH_2O and autoclaved at 121°C, 15 psi for 20 minutes. After autoclaving, the medium was cooled down to 60°C to add antibiotic, immediately distributed in bacterial dishes under the laminar hood and allowed to solidify at RT overnight. The plates were stored at 4°C.

5.1.7.2. Mammalian cell culture media

Suspension Cell line (Jurkat, EL-4, WEHI-231, and other lymphoid cells)

RPMI 1640	1000 ml
FCS (Jurkat, EL-4)	5 %
FCS (U-937, WEHI-231)	10 %
L-Glutamin (200 mM)	10 ml
Penicillin (10.000 IU/ ml), Streptomycinsulphate (10 mg/ ml)	6 ml
β-Mercaptoethanol (50 mM)	1 ml

Adherent cell line (293T)

DMEM	500 ml
FCS	10%
L-Glutamine (200 mM)	5 ml
Penicillin (10,000 IU/ ml), Streptomycinsulphate (10 mg/ ml)	3 ml
β-Mercaptoethanol (50 mM)	500 µl
Sodium pyruvate (100 mM)	5 ml
HEPES (1 M)	5 ml

Primary murine splenic dendritic cell

RPMI 1640 DUTCH modified (without L-Glutamine)	1000 ml
FCS	10%
Penicillin (10.000 IU/ ml), Streptomycinsulphate (10 mg/ ml)	6 ml

Primary murine CD4$^+$ T lymphocytes

X-VIVO 15	1000 ml
FCS	10%
L-Glutamin (200 mM)	10 ml
Penicillin (10.000 IU/ ml), Streptomycinsulphate (10 mg/ ml)	6 ml
β-mercaptoethanol (50 mM)	1 ml
Sodium pyruvate (100 mM)	10 ml
Hepes (1 M)	10 ml
MEM (Non-essential amino acids, 100x)	10 ml

5.1.8. Biological Materials

5.1.8.1. Bacteria

All bacterial strains were *E. coli*, procured from Stratagene. DH5α was used for the usual transformation of plasmid DNAs and gene cloning purposes. For the expression of retroviral expression vectors and accessory vectors, XL-10 Gold strain was used.

5.1.8.2. Mammalian cells and culture

All mammalian cell lines were procured from either German collection of microorganisms and cell cultures (DSMZ), or European collection of cell cultures (ECACC) or American Type Culture Collection (ATCC). **293** cell line is established by transformation of primary human embryonic kidney cells with Adenovirus Type 5 (Ad 5). This is an adherent cell line (DSMZ Nr. ACC 305). **EL-4** is a murine thymoma cell line established from 9,10-dimethyl-1, 2-benzanthrazen - induced T cell lymphoma. These cells express constitutively active calcineurin, a phosphatase, which activates NFAT by dephosphorylation. Upon phorbol ester (plus ionomycin) stimulation, this cell line secretes several cytokines like IL-2, IFN-γ, IL-10 and GM-CSF (ATCC Nr. TIB-39). **WEHI-231** is murine B cell line established from mineral oil induced B cell lymphoma in (BALB/cxNZB) F1 progeny. Upon LPS stimulation, these cells secrete IgM (ATCC Nr. CRL 1702; ECACC 85022107). **Primary murine CD4$^+$ T lymphocytes** were isolated from lymph nodes of DO11.10 TCR transgenic (tg) Balb/c mouse (Murphy et al., 1990) according to the method described in 5.2.2.3. **Dendritic cells** were prepared from spleen of DO11.10 TCR transgenic or wild type BALB/C mouse. Spleens were homogenized and washed in BSS/BSA and cultured overnight in RPMI 1640 DUTCH modified (without L-Glutamine).

5.1.8.3. Mice

DO.11.10. (H-2^d): These mice carry the MHC class II restricted rearranged T cell receptor transgene, which react to OVA peptide antigen. Intraperitoneal administration of OVA to Tg mice results in a rapid deletion of the immature $CD4^+CD8^+$ TCR^{lo} thymocytes with progression to mature thymocytes (Murphy et al., 1990). Use of this TCR transgene requires H-2^d background.

5.2. Methodology

5.2.1. Recombinant DNA Methods

5.2.1.1. Amplification of plasmid DNA

Culture and preservation of E. coli: For miniprep of plasmid DNA, *E. coli* colonies or 5-10 µl of previous culture were inoculated in 3 ml liquid medium (LB or 2YT) supplemented with selective antibiotic and were incubated overnight (8-16 h) at 37°C, 220 U/min in a shaking incubator. Bacterial cells were generally preserved in -80°C. In cryotube, 800 µl of overnight culture was mixed with 200 µl of 75% glycerol and immediately stored in -80°C.

Transformation of competent bacteria: The transforming DNA was pipetted into 100 µl suspension of competent bacterial cells, swirled gently to mix and left on ice for 30min. Cells were heat shocked at 42°C for 90 seconds, placed on ice for 2 minute, 1 ml of sterile LB medium was added and incubated at 37°C for 45 min with gentle shaking. Cells were centrifuged at 3000 rpm for 2 minute and supernatant was removed. The pellet was resuspended, seeded on to an LB ampicillin agar plate and incubated overnight at 37°C incubator.

Screening of transformants by colony hybridization (Grunstein and Hogness, 1975): Bacterial colonies growing on agar plates were transferred en masse to nitrocellulose filters. The spatial arrangement of colonies on the plates was preserved on the filters. After transfer, the filters were processed for hybridization to an appropriate radiolabeled probe while the original (master) plate is incubated for a few hours to allow the bacterial colonies to re-grow in their original positions. The success of colony hybridization depends on high specificity of synthetic oligonucleotide probe used.

The transformed E. coli culture was plated onto 90-mm LB agar plates, at dilutions roughly to generate up to 2500 transformed colonies. A nitrocellulose filter (disc, 0.45 micron) was placed, numbered side down, on the surface of the LB agar plate, in contact with the bacterial colonies, until it was completely wet. Once the filter was in place, the filter was marked to the underlying agar medium by stabbing in three or more asymmetric locations through the filter with a 23G needle. The edge of the filter was gripped with blunt-ended forceps and, in a single smooth movement, the filter was peeled from the surface of the agar plate and placed on the Whatmann filter paper pre-soaked with fresh CH solution I, with colonies on the membrane facing up, and left for 10 minutes to lyse bacterial colonies. After this treatment, membrane was placed on Whatmann filter papers pre-soaked with CH solution II and left for 10 minutes. This step was repeated once again. Now disc was air dried on Whatmann filter paper and further incubated for 10 minutes with shaking in CH solution III. Again the membrane was dried on Whatmann paper and baked at 80°C for 30 min. Now membrane was ready for hybridization process.

The CH prehybridization solution was pre-warmed at 42°C and membrane was prehybridized for 30 minutes to 1 hour at 42°C in this solution. In the meanwhile, radiolabeled probe was prepared by labeling of oligonucleotides by Polynucleotide kinase (PNK). Synthetic oligonucleotides lacking phosphate groups at their 5' termini could easily be radiolabeled by transfer of the -^{32}P from [-^{32}P]ATP in a reaction catalyzed by bacteriophage T4 polynucleotide kinase as following:

DNA (forward and reverse primers)	2 µg
10x PNK buffer	2.0 µl
^{32}P-γATP	3.0 µl
H_20	12.0 µl
PNK	1.0 µl

The reaction mixture was incubated at 37°C for 30 minutes and reaction was stopped by adding 5 µl of CH stop buffer. The probe was not purified and directly used for hybridization by mixing in CH prehybridization solution. The hybridization was performed at 60°C for 90 minutes in this solution. To remove unbound probe, membrane was washed 4-5 times at 55°C with CH washing solution. Radioactivity on disc was monitored after each washing to avoid excessive washing. The membrane was air dried, exposed overnight with intensifying screen at -70°C and autoradiogram was developed to detect positive clones.

5.2.1.2. Plasmid DNA Isolation

Mini preparation of plasmid DNA (Analytical): One colony from the bacterial transformed plate was inoculated in 3 ml LB ampicillin medium and cultured overnight at 37°C. Mini preparation of plasmid DNA was done according to the instructions of Genomed.

Maxi preparation of plasmid DNA (Preparative): 100 µl of overnight fresh grown culture was inoculated into 100 ml of LB or 2YT medium supplemented with antibiotics and cultured overnight at 37°C, 220 rpm. Plasmid DNA was isolated using Genomed kit. Depending on the construct, usually 200 - 1000 µg of DNA was obtained from 100 ml culture. Plasmid DNA was dissolved in 10 mM Tris and stored at -20°C.

Measurement of DNA concentration: The DNA concentration was determined by using an UV spectrophotometer at wavelength of 260 nm. The absorption of 1 at 260 nm corresponds to a concentration of 50 mg/ml double-stranded DNA. Identity, integrity and purity of the DNA were subsequently analyzed on an agarose gel.

Agarose gel electrophoresis: Agarose gel electrophoresis was employed to check the progression of a restriction enzyme digestion, to quickly determine the yield and purity of a DNA isolation or PCR reaction, and to size fractionate DNA molecules, which then could be eluted from the gel. An agarose gel was prepared according to the recipes listed in section 4.1.5 and DNA samples were electrophoresed in DNA electrophoresis buffer.

5.2.1.3. Elution of DNA fragment from agarose gel

DNA fragment of interest was eluted from agarose gel using JETSORB kit. Following electrophoresis, DNA fragment of interest was excised under UV-light and collected in pre-weighed eppendorf tube and isolated according to the instructions of JETSORB kit.

5.2.1.4. Enzymatic manipulation of DNA

The foreign DNA was introduced in any vector backbone by standard cloning methods, in which both foreign DNA and vector DNA were specifically digested with restriction enzymes, then target DNA was ligated to the plasmid DNA, and the products of the ligation reaction were used to transform competent E. coli. In principle, the maximum number of "correct" clones can generally be obtained from ligation reactions containing equimolar amounts of plasmid and target DNAs, with the total DNA concentration being <100 µg/ml. Blunt-end ligation catalyzed by bacteriophage T4 DNA ligase is suppressed

by high concentrations (5 mM) of ATP and polyamines such as spermidine. During ligation *in vitro*, T4 DNA ligase catalyzes the formation of a phosphodiester bond between adjacent nucleotides only if one nucleotide carries a 5´-phosphate residue and the other carries a 3´-hydroxyl terminus. Recircularization of vector DNA can therefore be minimized by removing the 5´-phosphate residues from both termini of the linear, double-stranded plasmid DNA with alkaline phosphatase.

5.2.1.5. DNA amplification by Polymerase Chain Reaction (PCR)

PCR is an enzymatic method for the *in vitro* synthesis of multiple copies of specific sequences of DNA. The cocktail for PCR contained the following components:

DNA-template (plasmid ~ 9 Kb)	100-250 ng
Forward primer (100 pmol/µl)	0.7 µl
Reverse primer (100 pmol/µl)	0.7 µl
MgSO4 (25 mM),	6-7 µl (end conc. 3 - 3.5 mM)
10 x buffer without MgSO4	5 µl
dNTP-mix (10 mM)	4.5 µl
PWO-polymerase (1 U/µl)	4 µl
ddH_2O	up to 50 µl

The PCR cocktail was immediately incubated in PCR machine for amplification using the cycling program as following:

	2 min	95°C
7 cycles:	15 s	94°C
	30 s	55°C
	6 min	72°C
28 cycles:	20 s	94°C
	40 s	65°C
	7 min	72°C
	∞	4°C

5.2.1.6. DNA sequencing

DNA sequencing was done using automatic sequencer based on a method which is a variant of dideoxynucleotide method. Sequencing of DNA was achieved by generating fragments through the controlled interruption of enzymatic replication (Sanger et al., 1977). DNA polymerase I is used to copy a particular sequence of a single-stranded DNA. The synthesis is primed by complementary fragment, which may be obtained from a restriction enzyme digest or synthesized chemically. In addition to the four deoxyribonucleoside triphosphates (ddNTP), the incubation mixture contained a 2', 3'-dideoxy analogue of one of them. The incorporation of this analogue blocked further

growth of the new chain because it lacked the 3'-hydroxyl terminus needed to form the next phosphodiester bond. A fluorescent tag was attached to the oligonucleotide primer, a differently colored one in each of the four chain-terminating reaction mixtures. The reaction mixtures were combined and electrophoresed together. The separated bands of DNA were then detected by their fluorescence as they pass out the bottom of the tube, and the sequence of their colors directly yielded the base sequence. A high sensitivity fluoroscent detector measures the amount of each fluorophore as a function of time and hence sequence is determined from the order of peaks of four different dyes.

PCR for sequencing: In sterile PCR microfuge sequencing reaction was set up as following:

Reaction mix:	5 µl plasmid DNA (from miniprep, approx. 1-2 µg), 2 µl primer (20 pM), 7 µl sequencing mix*.
PCR program:	25 cycle: 96°C/15 s; 52°C (or 55°C)/15 s; 60°C/4 min

*A-dye: Terminator labeled with dichloro[R6G]; C-dye: Terminator labeled with dichloro[TAMRA]; G-Dye: Terminator labeled with dichloro[R110]; T-Dye: Terminator labeled with dichloro[ROX].

***Purification of sequencing products*:** 200 µl of Sepharose G-50 was packed in PCR filter tips and mounted onto microfuge tube with the help of an adapter. About one-tenth (1.4 µl) volume of sequencing reaction mix (14 µl), 3 M sodium acetate, pH 5.2, was added to fresh microfuge tube mounted with G-50 column. Sequencing mixture was loaded onto the G-50 column and centrifuged at 3000 rpm for 5 minutes. To each tube, 70 µl of 96% ethanol was added, followed by centrifugation at 15,000 rpm, for 10 minutes at RT. The supernatant was aspirated completely taking care of DNA pellet. Another quick spin was given to collect and aspirate traces of ethanol, if any, and the pellet of DNA was air dried at 37°C for 5 minutes and submitted for automated sequencing.

5.2.2. Mammalian Cell Culture

5.2.2.1. Maintenance of cell lines

All cell lines were handled in highly sterile condition under laminar hood and cultivated in appropriate media supplemented with antibiotics. Cells were incubated at 37°C, 5 % CO_2 in humidified incubator. The 293 HEK cell line, being adherent cell line, was split by trypsin-EDTA treatment every 3-4 days at 80% confluency. Suspension cell lines like EL-4 and Jurkat T cells were split every 3-4 days just by refreshing with complete RPMI medium to a small aliquot of cell suspension.

5.2.2.2. Induction of cells

T lymphocytes were induced either by plate bound αCD3 Ab plus soluble αCD28 Ab or pharmacological agents TPA and ionomycin that activate Ras/Protein Kinase and calcium-dependent pathways, respectively, to mimic signal through the TCR.

αCD3 Ab :5 µg/ml for primary stimulation of naïve T cells, 10 µg/ml for restimulation of primary effector T cells. Coating of 24 well plate (1000 µl/well) or 48 well plate (500 µl/well) for O/N at 4°C. Afterwards, coated wells were washed with BSS/BSA as following: 2x immediate and 1x for 5 minutes.

αCD28 Ab :1:100 dilution of clone 37.51 hybridoma culture supernatant obtained from Prof. A. Schimpl, or 5 µg/ml soluble Ab (BD Biosciences) for costimulation

TPA :20 ng/ml for stimulation of stable cell line, 10 ng/ml for primary cells and EL-4 cells

Ionomycin :1 µM for stimulation of stable cell line, 0.5 µM for primary cells and EL-4 cells

5.2.2.3. Preparation and activation of naïve CD4+ T lymphocytes

Isolation of peripheral CD4$^+$ T lymphocytes from lymphnodes (LNs): $CD4^+$ T lymphocytes were isolated from LNs of 5-6 weeks old healthy mice which were sacrificed by cervical dislocation. Before dissecting, mice were sprayed with disinfectant to minimize contamination. LNs were collected in cold BSS/BSA buffer and $CD4^+$ T lymphocytes were isolated by negative selection procedure. LN cells were incubated with antibodies against CD8 and HSA which are surface markers for $CD8^+$ T lymphocytes and B lymphocytes, respectively. The antibody coupling to the cell surface

receptor of unwanted cells allows the retention of these cells on the glass matrix of the column, whereas $CD4^+$ T cells pass through the column unhindered.

Preparation of the column: The column was installed onto the stand and washed with 15-20 ml of cold BSS/BSA buffer. In the meantime, the lyophilized Ab supplied with column was solubilized in 1.5 ml of BSS/BSA buffer, loaded onto the column, and left for one hour at RT.

Preparation of the lymphnode cells: All LNs were collected in cold BSS/BSA buffer from sacrificed healthy mice, squeezed on 70 micron cell strainer and collected in 50 ml tube. LN cells were washed twice with BSS/BSA buffer. To the pellet, 2.0 ml of antibodies mixture cocktail (Column Mixture) plus 3.0 ml of BSS/BSA were added and incubated on ice for 30 minutes. After incubation, cells were washed two times with BSS/BSA buffer and finally resuspended in 10 ml of BSS/BSA buffer. LN cells were now ready to load on the column.

Purification of $CD4^+$ T lymphocytes: Before loading cells, each column was washed again with 20 ml of BSS/BSA buffer and flow rate was adjusted to 8-10 drops per minute. Now 10 ml of resuspended cells were loaded onto the column and the flow through was collected in a strerile tube. The flow through contained enriched $CD4^+$ population which was centrifuged, resuspended the pellet in 10-12 ml of X-VIVO medium supplemented with 5% FBS and kept on ice in cold room O/N.

Enrichment of $CD62L^{high}CD4^+$ T lymphocytes: Mouse CD62L (L-selectin) MACS microbeads were used for the positive selection of naïve $CD4^+$ T cell population. CD62L is expressed on B cells, monocytes, neutrophils, eosinophils, thymocytes and T cells. The level of CD62L expression is high on naive T cells, but low on T cells pre-activated to effector or memory Th cells. The enrichment of naive T cells from $CD4^+$ T cell population was achieved by removing pre-activated $CD62L^{low}$ T cells on MACS column.

Magnetic Labeling of $CD4^+$ Cells: $CD4^+$ Cells were pelleted down, and for every $1x10^7$ cells, mixed with 10 µl of CD62L MACS beads plus 90 µl of BSS/BSA and incuabted in dark for 15 minutes at RT/4-8°C. (Optional, for purity analysis in flow cytometry: added CD62L-biotinylated antibody [usually 1:200 dilution] and incubated for an additional 10 minutes at RT/4-8°C in dark. Cells were washed with BSS/BSA, resuspended in 100 µl of BSS/BSA, added 1µl of streptavidin-Cytochrome [usually 1:100 dilution] and incubated for further 5-10 minutes.) Now cells were washed with BSS/BSA and resuspended in 1 ml of BSS/BSA. Cells are ready for loading on the MACS column.

Preparation of LS column: The midiMACS Separation Unit was attached to the MACS Multistand and placed the LS separation column into the magnet and one sterile tube under the column to collect the flow through. In all further steps special care was taken to prevent the entry of air bubble into the column. In this regard, the buffer was degassed for 3 hours. The column was washed with 5 ml of fresh BSS/BSA and let the buffer run through. Magnetically labeled cell suspension containing up to 10^8 positive cells in $2x10^9$ total cells was loaded onto the LS column and allowed the cell suspension to run through in a very strong magnetic field and collected the effluent as negative fraction. The column was detached from separator (very strong magnetic field) and placed on a new collection tube under no magnetic field. Now 5 ml of buffer was applied to the column and firmly flushed out cells using the plunger supplied with the column. The flushed out cells belonged to $CD62L^{high}$ $CD4^+$ T cell population, which was further washed once with BSS/BSA buffer.

Preparation of dendritic cells from spleens of Ova-tg mice: Spleens of sacrificed healthy mice were homogenized in BSS/BSA buffer and centrifuged at 1200 rpm for 10 minutes. The cell pellet was resuspended in 4 ml/spleen of dendritic cell culture medium and cultured overnight. Following day, splenic cells were layered carefully on top of the metrizamide solution (3 ml/ two spleens) in 15 ml tube. Tubes were balanced exactly and centrifuged at 1800 rpm for 10 min at 25°C without brake**.** Dendritic cells form a ring at the interface of aqueous and metrizamide phases**.** This interphase ring was carefully pipetted out with the help of sterile glass Pasteur pipette, washed twice with BSS/BSA buffer and resuspended in 1.5 ml of X-VIVO medium. Cells were X-ray irradiated at 2000 rad, washed and resuspended in X-VIVO medium at a density of $2x10^5$ cells/ml. DCs were then used as antigen presenting cells for $CD4^+$ T cells.

$CD4^+$ T cell activation and differentiation: $CD4^+$ T lymphocytes were induced to proliferate and differentiate *in vitro* by antigen stimulation in the presence of respective cytokines and neutralizing antibodies. The establishment of stable phenotype was achieved after 5-7 days of culture under skewing conditions. The default or undifferentiated Th (Th0) cells were obtained usually by activating naïve $CD4^+CD62L^{high}$ cells in the presence of IL-2 and neutralizing antibodies to IFN-γ and IL-4 (to block Th1 and Th2 differentiation, respectively). The culture of $CD4^+$ or $CD4^+CD62L^{high}$ T cell was set up at a density of $5x10^5$ cells per ml with a maximum volume of 2 ml per well in 24 well plate. The day of activation was considered as day 0. Usually cells were confluent at day 3 and therefore transferred to 12 well plates with additional 2 ml of fresh X-VIVO

medium supplemented with 50 U/ml IL-2 for further culture. The following cocktail was used to activate $CD4^+$ T lymphocytes isolated from mice of Ova-Tg background:

$CD4^+$ $CD62^{high}$ T lymphocytes	$1x10^6$
Irradiated DC	$1x10^5$
Ova	0.5 μg/ml
IL cocktail	100 μl
X-VIVO	up to 2 ml.

Table 5.1 : IL cocktail for default or differentiation (final concentration is indicated):

T_h0-type		**T_h1-type**		**T_h2-type**	
IL-2	50 U/ml	IL-2	50 U/ml	IL-4	1000 U/ml
αIL-4 Ab	10 μg/ml	IL-12	10 ng/ml	αIFN-γ	10 μg/ml
αIFN-γ Ab	10 μg/ml	αIL-4 Ab	10 μg/ml	-	

5.2.2.4. Production of helper-free recombinant retroviruses

Helper-free recombinant virus was produced by calcium phosphate method in 293 cells according to the standard protocol described below. Briefly, viral mRNA is transcribed from retroviral plasmid template transfected in 293T cells along with two helper plasmids, namely pHIT 60 (which codes for gag and pol) and pHIT 123/456 (which codes for ecotropic or amphotropic envelope of retrovirus). The viral mRNA was encapsulated and shed non-lytically into the cell culture medium, collected by filtration and added to the target cell for transduction. Standard biosafety level 2 precautions were used in working with the producer cells or materials derived from them. This was done in collaboration with Dr. Ingolf Berberich at the Institut for Virology and Immunbiology, Universität Würzburg.

Production of viral supernatant: Calcium phosphate forms an insoluble precipitate with DNA, which attaches to the cell surface and is taken into the cells by endocytosis. 293 HEK cells were plated at a density of $12.5x10^5$ cells in 60 mm cell culture dish one day before transfection (upto a confluency of 50-60 %) in DMEM medium supplemented with HEPES (growth medium). Two to three hours before transfection, the growth medium was changed to DMEM without HEPES (transfection medium) and again cells

were incubated. Just before transfection, following components were brought in 1.5 ml tube, mixed by vortexing and briefly centrifuged:

H_2O	425 µl
pHIT 60	2.5 µg
pHIT 123 (for infection of primary T cells or Wehi cells) or pHIT 456 (for infection of Jurkat, EL-4, U-937)	2.5 µg
Retroviral expression vector for gene of interest	7 µg
2M $CaCl_2$	65 µl.

This transfection cocktail was mixed with 500 µl of HBS, pipetted vigorously till fine precipitate like structures were visible and transferred the mixture drop wise onto the cells. The transfected plates were returned immediately to the incubator. After 24 hours of transfection, fresh growth medium was added to the cells, and the plates were returned to incubator. The transfection efficiency was checked in fluorescent microscopy after 48 hours of transfection. These cells were cultured for three consecutive days post-transfection to collect viral supernatant.

Storage of retroviral supernatant: Efficient transfection of 293T HEK cell line with retroviral expression plasmid (gene of interest) and packaging plasmids (pHIT 60 and pHIT123/456) results in the generation and secretion of ampho- or ecotropic viruses after 48 hours of transfection. Therefore supernatants were collected at 48 h, 72 h and 96 h of post-transfection, filtered through 0.45 µM sterile filter to remove contaminating cells and polybrene was added in the supernatant to a final concentration of 10 µg/ml. After harvest, supernatants were either used fresh or stored at -70°C. The packaging cells were replenished with fresh complete medium and again incubated till next harvest of supernatant.

5.2.2.5. Transduction of target cells

WEHI-231/EL-4 cells: Usually WEHI-231 cells were infected with retroviruses to determine the quality of virus. 5 x 10^5 WEHI-231 or EL-4 cells were mixed with 1 ml of viral supernatant (containing polybrene) generated with pHIT 123 or 456 respectively, incubated at RT for 10 minutes and centrifuged at 2200 rpm for 180 min at 32°C. After centrifugation, cells were resuspended in 2 ml RPMI 1640 medium and incubated for 48 hours. EGFP expression in infected cells was checked by flow cytometry. Infection of more than 80% in Wehi cells was considered efficient for primary cell. Infected EL-4 cells were treated with 250 µg/ml of zeocin for enrichment of infected pure population.

CD4$^+$ T lymphocytes: After 24 hours of primary stimulation, 1x10^6 CD4$^+$ T cells were mixed with 2 ml of viral supernatant generated with pHIT123, incubated at RT for 10 minutes and centrifuged at 2200 rpm for 180 min at 32°C. After centrifugation, cells were resuspended in the old medium and further incubated for 48 hours. Infected cells were enriched up to 95-98 % by FACsorting and further cultured for the required time.

5.2.3. RNA Methods

5.2.3.1. RNA isolation from mammalian cells

RNA isolation was done in a clean, ribonuclease-free environment. All glasswares were baked overnight at 180°C. Double distilled water and all solutions except Tris-containing solutions were treated with 0.1% DEPC solution, followed by autoclaving. Disposable plastic wares were used. Chloroform resistant plastic wares were rinsed in chloroform. Cuvette was washed every time with 0.1M NaOH and 1 mM EDTA, followed by washing with RNase-free water. Disposable gloves were worn all times during the experiment. Total cellular RNA was isolated using TRIZOL reagent and the procedure was followed according to the instructions of manufacturer.

5.2.3.2. RNA electrophoresis in formaldehyde agarose gels

Electrophoresis tank was cleaned with detergent solution (0.5% SDS), thoroughly rinsed with RNase-free water followed by ethanol and allowed to dry. The edges of the gel-tray were sealed with tape and appropriate comb cleaned with 70% ethanol was placed. For RNA gel preparation, 1.2 – 1.5 g agarose was dissolved in 70 ml of DEPC - treated water by heating in a micro oven and cooled to approx. 60°C. RNA gel was prepared, poured in the gel tray and allowed to polymerize at RT. In the meanwhile, one volume of 5x RNA loading buffer was mixed with 4 volumes of RNA sample, briefly spinned down, incubated for 3-5 minutes at 65°C and chilled on ice. Samples were loaded into the wells of the polymerized agarose gel and electrophoresed at 5-7 V/cm in 1x RNA gel running buffer.

5.2.3.3. Northern blot hybridization

The level of RNA in cell was detected by Northern blot hybridization. This is a sensitive technique to identify the change in level of particular RNA. Total RNAs from different cell samples were isolated by TRIZOL reagent and electrophoresed in formaldehyde agarose gel.

Preparation of the gel for transfer to charged nylon membranes: The gel was rinsed with DEPC-treated water and soaked for 20 minutes in 5 gel volumes of Northern blot denaturation solution. A small triangular piece was cut from the upper left corner of the gel to simplify the orientation. In the meanwhile, two pieces of longer and wider 3MM Whatmann papers were placed as wicks on the glass plate support kept inside a large baking dish, which was filled with transfer buffer. When the Whatmann papers were thoroughly wet, all air bubbles were smoothed out with a pipette.

Preparation of the charged nylon membranes for transfer: The charged nylon membrane filter was floated in deionized water until it wetted completely from beneath, and then immersed in 10x SSC for at least 5 minutes. The membrane was cut in the upper left corner for orientation.

Assembly of the transfer system and transfer of the RNA: The gel was placed in an inverted position on the support, surrounded with saran wrap to avoid short-circuiting and the top of the gel was wet with the transfer buffer. Now the wet membrane was placed on top of the gel, being careful not to trap any air bubble under the membrane (Caution: The membrane was not moved once it has been applied to the surface of the gel.). The wet nylon membrane was covered with 2 layers of pre-wet (with transfer buffer) 3MM paper (cut to exactly the same size as the gel). Now trapped air bubble was removed by rolling over the paper with a pipette and a thick stack of paper towels (5-8 cm, just smaller than the blooting papers) was put on the blotting papers. A glass plate was put on top of the stack and weighed down with a 400 g weight. The upward transfer of RNA was allowed to occur for overnight. The capillary system was dismantled and the positions of the slots were marked on the membrane. The membrane was transferred to a tray containing ~300 ml of 6x SSC and agitated the membrane very slowly for 5 minutes. Partially the membrane was air dried and irradiated at 254 nm for 1 minute 45 seconds at 1.5 J/cm^2.

Radioactive probe synthesis: Probe for Northern hybridization was synthesized by PCR amplification of cDNA in the presence of α^{32}P-dCTP, whose incorporation in PCR amplified products results in highly radioactive probe. The labeled probe was purified on sephadex G-50 column. The cocktail of PCR reaction contained as following:

DNA-template (cDNA of interest)	200 ng
Forward primer (100 pmol/µl)	1.0 µl
Reverse primer (100 pmol/µl)	1.0 µl
Mg^{++} (25 mM),	8.0 µl (end conc. 4.0 mM)
10 x buffer without Mg^{++}	5.0 µl
dNTP mix minus dCTP (10 mM)	1.0 µl
$^{\alpha 32}$P-dCTP	10.0 µl
Taq-polymerase (10 U/µl)	0.5 µl
ddH_2O	up to 50 µl

The PCR cocktail was immediately incubated in PCR machine for amplification using the cycling program as following:

	2 min	95°C
5 cycles:	30 s	95°C
	30 s	55°C
	1.30 min	72°C
28 cycles:	30 s	94°C
	30 s	65°C
	1.30 min	72°C
	10 min	72°C
	∞	4°C

Hybridization: The membrane was incubated initially for 2 hours at 68°C in 10-20 ml of pre-pre-hybridization solution, followed by pre-hybridization solution for 4-6 hours at 68°C. The ^{32}P-labelled double-stranded DNA probe was denatured by heating at 100°C for 5 minutes and chilled rapidly on ice. The denatured probe was added directly to the pre-hybridization solution and continued incubation for 16-24 hours at 42°C. After hybridization, the membrane was transferred quickly to a plastic box containing ~200 ml of Northern blot washing solution I and agitated for 15 minutes at RT. The membrane was transferred to another plastic box containing ~200 ml of Northern blot washing solution II (at 68°C) and agitated for 30 minutes at 68°C. This step was repeated once more for a total of two washes at 68°C. The membrane was dried on a blotting paper and autoradiography was established by exposing the membrane for 24-48 hours to X-ray film at -70°C with an intensifying screen.

5.2.3.4. Ribonuclease protection assay

The method can be used to quantitate RNAs, to map the positions of introns, and to identify the locations of 5´ and 3´ ends of mRNAs on cloned DNA templates. Preparations of RNA containing an mRNA of interest were hybridized to a radiolabeled single-stranded RNA probe. At the end of the reaction, a mixture of RNase A and

RNase T1 was used to degrade unhybridized regions of the probe, and the surviving molecules are then separated by denaturing gel electrophoresis and visualized by autoradiography.

Probe synthesis: Before the start of probe synthesis, the heating block was set at 37°C and following reagents were brought to room temp: α-^{32}P-UTP, GACU nucleotide pool, DTT, 5X transcription buffer and RPA template set. For each probe synthesis, following reagents from the kit were added (in order) to a 1.5 ml eppendorf tube, mixed by gentle pipetting or flicking, quickly spinned in a microfuge and incubated at 37°C for 1-2 hour:

1 µl RNasin®
1 µl GACU pool
2 µl DTT
4 µl 5X transcription buffer
1 µl RPA Template Set
10 µl [α-^{32}P]UTP
1 µl T7 RNA polymerase

The reaction was terminated by adding 2 µl of DNase, mixed by gentle flicking, quickly spinned in a microfuge and incubated at 37°C for 30 minutes. To get rid of contaminating proteins**,** following reagents (in order) were added to each 1.5 ml eppendorf tube, mixed by vortexing into an emulsion and spinned in a microfuge for 5 minutes at RT:

75µl of 20mM EDTA
50 µl of Tris-saturated phenol
50 µl of chloroform:isoamyl alcohol (50:1)
2 µl yeast tRNA

The upper aqueous phase containing RNA was transferred to a new 1.5 ml eppendorf tube, added 100 µl chloroform:isoamyl alcohol (50:1), mixed by vortexing, then spinned in a microfuge for 2 minutes at RT and transferred the upper aqueous phase to a new 1.5 ml tube. To precipitate RNA, 100 µl of 4 M ammonium acetate and 500 µl ice cold 100% ethanol were added, mixed by inverting the tube(s), incubated for 2-3 minutes on dry ice (or 30 minutes at -70°C) and centrifuged for 30 minutes at RT (or 15 minutes at 4°C). The supernatant was removed carefully and pellet was dried for 5 to 10 minutes at RT. The RNA pellet was solubilized in 50-100 µl of hybridization buffer and 1 µl was taken for quantification in the scintillation counter. Usually maximum yield was expected in the range of 1-3 x 106 Cherenkov counts/µl (measurement of cpm/µl without the presence of scintillation fluid) with an acceptable lower limit of 3 x 105 Cherenkov

counts/µl. After quantification, this 1 µl probe was used as undigested probe upon dilution to 1:50 in the blue buffer. The probe was stored at -20°C no longer than 2-3 days.

RNA hybridization: Following things were arranged before the start of hybridization: heating block at 90°C, precipitated RNA of desired amount and hybridization oven at 56°C. For hybridization, 5 µg of target RNA was mixed with 10 µl of diluted probe in a 1.5 ml tube. The RNA was solubilized by gentle vortexing for 3-4 minutes followed by quick spin in the microfuge. Samples were placed in a heating block pre-warmed to 90°C for 5-10 minutes (up to 30 minutes). The tubes were transferred to hybridization oven set at 56°C and incubated for 12-16 hours.

RNase treatment: The heating block was turned to 30°C for 15 minutes prior to the RNase treatment. For 20 samples, RNase cocktail was prepared by mixing 2.5 ml RNase buffer and 6 µl of RNase A + T1 mix. Overnight samples were mixed with 100 µl of RNase cocktail, briefly centrifuged, and incubated for at least 45 minutes at 30°C.

Proteinase K treatment: The heating block was turned to 37°C. For 20 samples, proteinase K cocktail was prepared by mixing 390 µl of proteinase K buffer, 30 µl of proteinase K and 30 µl of yeast tRNA. After RNase treatment, 18 µl of proteinase K cocktail was added, mixed and incubated for 30 minutes at 37°C.

Precipitation and electrophoresis of RNA: For the precipitation of dsRNA, 100 µl of ammonium acetate and 600 µl of isopropanol were mixed to each sample, incubated for 15 minutes at -20°C and centrifuged at RT for 15-30 minutes. The supernatant was removed and the pellet was dried at 95°C for 2-3 minutes. The pellet was dissolved in 4 µl loading buffer, denatured at 90°C for 3 minutes and transferred on ice. In the meanwhile, gel solution was prepared (for composition see 5.1.5). The polymerized gel was pre-run at 30W for 1 hour in 0.5x TBE. Samples were loaded on the gel along with undigested probe. After the blue dye reached 2/3rd of the length of gel, electrophoresis was stopped, gel was dried in vacuum for 1 hour at 95°C and autoradiogram was established by exposing the gel for 24-48 hours to X-ray film with an intensifying screen at -70°C.

5.2.4. Protein Methods

5.2.4.1. Preparation of protein extracts

Preparation of whole protein extract from mammalian cells: Cells were centrifuged (1200 rpm, 5 minutes, 4°C), washed with cold PBS (Without Ca^{++} and Mg^{++}), resuspended in 1 ml of PBS (without Ca/Mg), transferred to 1.5 ml tubes and again pelleted down (2000 rpm, 2 minutes, RT). The cell pellet was resuspended in cold whole cell extract preparation buffer supplemented with protease and phosphatase inhibitors ($KLBM^{+}$) (100 µl per 1 x 10^7 cells). Cells were disrupted by two times freezing and thawing on dry ice or passing the cell suspension through 26G needle 10 times and incubated for further 10 minutes. The cell suspension was centrifuged for 30 minutes at 14,000 rpm, 4°C and supernatant was saved as whole cell extract, which was stored for future use at -70°C. The protein concentration of the supernatant was determined by Bio-Rad protein assay.

Preparation of nuclear and cytoplasmic protein extracts from mammalian cells: Cells were centrifuged at 1200 rpm for 5 min at RT and the cell pellet was resuspended in 1 ml cold PBS buffer (without Ca^{++} and Mg^{++}), transferred to a 1.5 ml tube and again centrifuged at 2000 rpm for 2 min at RT to remove the supernatant. The pellet was resuspended in 200 µl to 1 ml of extraction buffer A^{+} (100 µl per 1x10^7 cells) and incubated for 20-30 min at 4°C. Extraction buffer A^{+} is a low salt buffer ($^{+}$ indicates that DTT and PMSF were added to buffer A), which allowed the cells to swell. To destroy the swollen cells, the solution was passed 10 times through 1 ml syringe with 26G needle and centrifuged at 7,000 rpm for 2 minutes in the cold room. The supernatant contained cytosolic fraction and the pellet, which appeared transparent, contained nuclear fraction. The supernatant was transferred to a fresh tube and kept on ice. The pellet was washed with 800 µl extraction buffer A^{+}, centrifuged at 7,000 rpm for 2 minutes in the cold room and the pellet was resuspended in extraction buffer C^{+} (leupeptin was added in addition to DTT and PMSF) by pipetting and vigorously mixing with brutal force, followed by vortexing the nuclear extract vigorously for 30 minutes in the cold room. Now the suspension was centrifuged at 14,000 rpm for 30 minutes in the cold room and supernatant containing nuclear proteins was frozen in –70°C. The protein concentration of the supernatant was determined by Bio-Rad protein assay.

Measurement of protein concentration (Bio-Rad protein assay): The Bio-Rad protein assay is based on the observation that when Coomassie Brilliant Blue G-250 binds to the protein, the absorbency maximum shifts from 450 nm to 595 nm. Equal volumes of cell lysate containing 1 - 20 µg of protein was added to 1 ml of diluted dye reagent (1:5 dilution of dye reagent concentrate in ddH_2O), mixed well and O.D value was measured at 595 nm. KLBM buffer was always included in the control as following: 2 µl of KLBM buffer + 998 µl of Bradford reagent. The O.D value of the sample was divided by 0.178 to determine the protein concentration.

5.2.4.2. Immunodetection

SDS-polyacrylamide gel preparation and electrophoresis: SDS-polyacrylamide gels were prepared in 8 cm x 10 cm x 1.5 mm mini gel format according to the standard Laemmli method (Laemmli, 1970). Separating or lower gel mix was prepared according to the volume required, poured in the gel apparatus, overlaid gently with 0.1 % SDS and allowed to polymerize at room temperature.

Table 5.2: Composition of protein gels (all numerical figures are in ml)

	Stacking gel, pH 6.8	**Separating gel, pH 8.8**			
Percentage of the gel	**4%**	**8%**	**10%**	**12%**	**15%**
Distilled water	6.8	5.8	5.0	4.1	2.85
1.5 M Tris-HCl, pH 8.8	-	3.125	3.125	3.125	3.125
1.5 M Tris-HCl, pH 6.8	1.25	-	-	-	-
Acryl-/ Bisacrylamide (29% / 1% w/v)	1.7	3.35	4.15	5.0	6.25
10% (w/v) SDS	0.1	0.125	0.125	0.125	0.125
10% APS	0.1	0.125	0.125	0.125	0.125
TEMED	0.01	0.01	0.005	0.005	0.005
Total Volume	10.0	12.5	12.5	12.5	12.5

After the separating gel was polymerized, the overlay was decanted, thoroughly and gently washed with distilled water. The stacking gel was poured, the comb was inserted and allowed to polymerize at RT. Requisite concentration of protein samples were mixed with 4x Laemmli buffer and denatured by heating at 95°C for 5 min, loaded in the wells of polymerized gel and electrophoresed at constant current, 25-30 mA per gel, in 1x SDS-PAGE running buffer.

Western blotting and hybridization: SDS-PAGE gel was electrotransferred onto nitrocellulose membrane at 40 mA overnight at 4°C. The air dried membrane was incubated in a blocking solution (5% fat free milk in 1X TBS-T) for 30 minutes to 1 hour at RT. Membrane was directly incubated in primary antibody solution (1:2000 in blocking solution) for 2 to 3 hours at RT. After incubation, membrane was washed in 1X TBS-T for 1 x 20 minutes and 3 x 5 minutes each. Now membrane was incubated in secondary antibody conjugate solution (1:2000 in blocking solution) for 1 to 2 hours at room temperature and washed in 1X TBS-T for 1 x 20 minutes and 3 x 5 minutes each. Colour was developed with ECL developing solution according to the instructions of the manufacturer (Amersham).

Stripping of nitrocellulose membrane: Nitrocellulose membrane was stripped of the first antibody to detect the level of another protein by hybridization with another antibody. This was done by incubating the membrane in stripping buffer (pre-warmed to 60°C) and placing in a water bath set at 60°C for 30 min with shaking. Now the membrane was washed one time with ddH_2O and three times with TBS/Tween for 5 min each. The membrane was ready for staining with another primary antibody.

5.2.4.3. Cytokine analysis

Detection of intracellular cytokine level by flow cytometry: This protocol was adapted from the instruction manuals supplied by BD Biosciences.

Stimulation of Cells: Restimulation of $CD4^+$ T lymphocytes was either done by plate bound αCD3 Ab and soluble αCD28 antibody or TPA and ionomycin for 5-6 hours, whereas EL-4 cells were stimulated always by TPA and ionomycin for 16 hours. $CD4^+$ T cells were properly washed to remove any residual cytokine, resuspended in culture medium at a density of $1x10^6$/ml, plated in desired size wells or plates, and restimulated according to the protocol. After two hours of incubation, Brefeldin A (10 µg/ml) was added to the culture to inhibit the secretion of cytokines and further incubated for 3-4 hours at 37 at -70°C. EL4 cells were stimulated by T/I for 12 hours, added Brefeldin A (10 µg/ml) and further incubated for 4 hours.

Fixation and Intracellular Staining of Cells: Cells were harvested in FACS tubes and centrifuged at 1600 rpm for 5 minutes. The supernatant was flicked out and the cell pellet was washed in FACS buffer. To block the Fc receptor, anti-Fc antibodies (24G2 clone) was added to the resuspended cell pellet at a dilution of 1:100 and left at RT for 5 min. Cells were fixed by adding 200 µl of 4% paraformaldehyde and incubating for 20

min at RT followed by washing in FACS buffer (at this stage cells could be left overnight at 4°C). Cells were permeabilized by incubating in 1 ml of saponin buffer for 10 minutes at RT followed by centrifugation at 1600 rpm for 5 minutes. The supernatant was flicked out and the cell pellet was resuspended in 250 µl of saponin buffer (after flicking supernatant, cell pellet consisted of nearly 70 µl of suspension) making the total volume of cell suspension approx. ≥300 µl, which was aliquoted into three FACS tubes (100 µl per tube) for the staining of IL-4, IFN-γ and isotype control. Intracellular staining of IL-4, IFN-γ and isotype control was performed by addition of 1.0-2.0 µl of PE-conjugated anti-IL4, PE-conjugated anti-IFN-γ, PE-conjugated anti-IgG1 respectively and incubation for 15 minutes at RT in the dark. Cells were diluted with 2 ml of saponin buffer, centrifuged at 1600 rpm for 5 minutes, flicked out supernatant and again washed in FACS buffer. Cells were analyzed in FACS using Cell quest software (BD Biosciences).

Cytokine ELISA

Day 1: The purified anti-cytokine antibody was diluted to 1 - 4 µg/ml (preferrably 2 µg/ml) in ELISA coating buffer, 100 µl of diluted antibody was added to each well of 96 well plate and incubated overnight at 4°C.

Day 2: The plate was brought to RT and washed 1x with ELISA washing buffer. Blocking of non-specific binding was done by adding 200 µl of ELISA blocking buffer to each well and incubated at RT for 2 - 3 hours, followed by washing 1x with ELISA washing buffer. Standard and samples were diluted in ELISA sample dilution buffer, 100 µl per well was added and incubated O/N at 4°C.

Day 3: The plate was brought to RT and washed 4x with ELISA washing buffer. The biotinylated anti-cytokine antibody was diluted to 1 - 4 µg/ml (preferrably 1 µg/ml) in ELISA sample dilution buffer, 100 µl was added to each well and incubated 1-2 hour at RT. In the meanwhile, 11 µl streptavidin and 11 µl AP solutions (for 22 ml end volume in ELISA sample dilution buffer) were mixed in 300 µl of ELISA sample dilution buffer and left at RT. The plate was washed 6x with ELISA washing buffer. 100 µl of the diluted streptavidin - AP complex solution was added to each well and incubated 30 minutes - 1 hour at RT followed by washing for 8x with ELISA washing buffer. The color was developed by adding 100 µl of the ELISA color development solution to each well and measured the O.D at 405 nm (490 nm as reference). The exact concentration of cytokine was calculated against the standard curve in Microsoft EXCEL program.

5.2.4.4. Determination of PKA activity (Graves et al., 1996)

The intracellular PKA activities were analyzed in whole cell protein extracts using the PKA assay kit (Upstate biotechnology) and followed the instructions of manufacturer. This assay kit is designed to measure the phosphotransferase activity of PKA in protein extracts and immunoprecipitates. The assay is based on the phosphorylation of a specific substrate, Kemptide, using the transfer of the γ-phosphate of adenosine-5-[^{32}P] triphosphate ([γ-^{32}P] ATP) by active PKA. The phosphorylated substrate is separated from the residual ([γ-^{32}P] ATP and quantified by using a scintillation counter.

$$\text{Kemptide} + [\gamma\text{-}^{32}\text{P}]\ \text{ATP} \xrightarrow[\text{PKA, } Mg^{2+}]{30^{o}\text{C, 10 minutes}} \text{Kemptide } [^{32}\text{P}] + \text{ADP}$$

5.2.5. DNA/Protein Interaction Assay

The Electrophoretic Mobility Shift Assay (EMSA) provides a simple and rapid method for detecting DNA-binding proteins. This method has been used widely in the study of sequence-specific DNA-binding proteins such as transcription factors. The assay is based on the observation that complexes of protein and DNA migrate through a nondenaturing polyacrylamide gel more slowly than free DNA fragments or double-stranded oligonucleotides. The gel shift assay is performed by incubating a purified protein, or a complex mixture of proteins (such as nuclear or cell protein extract preparations), with a ^{32}P end-labeled DNA fragment containing the putative protein binding site. The reaction products are then analyzed on a non-denaturing polyacrylamide gel. The specificity of the DNA-binding protein for the putative binding site is established by competition experiments using unlabeled DNA fragments or oligonucleotides containing a binding site for the protein of interest or other unrelated DNA sequences.

5.2.5.1. Radioactive labeling and purification of DNA probe

Oligonucleotides were dissolved in ddH_2O to a final concentration, 100 pmol/µl. For each probe, 20 µl of sense and antisense oligonucleotides were mixed, vortexed, spinned them down briefly, and incubated in a thermoblock at 65°C until they reached this temperature. Now DNA was taken out from 65°C and let it cool down to RT for hybridization. Now the final volume obtained was 40 µl of 100 pmol/µl double stranded

DNA. This was diluted to 20 ng/µl (for dsDNA of 25 nucleotides in length, 1pmol corresponds to 9ng, so 100 pmol/µl = 900 ng/µl and therefore dilution factor was 45x). All reactions were setup in tightly fitting screw cap tubes as following:

dd H_2O	7.5 µl
10x PNK buffer	1.5 µl
ds-Oligo	2.0 µl (40 ng)
^{32}P-γATP	3.0 µl (40 mCi)
PNK	1.0 µl

The reaction mix was vortexed, spinned down and tubes were placed behind a radioactive shield for incubation at 37°C for 30 min (The incubation time of 30 minutes was strictly followed since the PNK enzyme has the property of removing phosphate group upon longer incubation). In the meanwhile, 12 % acrylamide gel was prepared for purification of labeled probe. After incubation, 5 µl of EMSA stop buffer was added to stop the reaction. Now the samples were loaded on the polymerized gel, run in 1x TBE at 200V for 2 hours and the desired band was cut after exposing the gel to a film. The gel slice was mixed with 80 µl of 10 mM Tris and kept on rotation for O/N at 4°C. The radioactivity was measured in 2 µl aliquot and the probe was diluted to 20,000 cpm / µl. If not used immediately, the radioactive probe was stored at -20°C for maximum of 3 weeks.

5.2.5.2. Electrophoretic Mobility Shift Assay (EMSA)

A 6 % polyacrylamide gel was poured and allowed to polymerize for 1 hour at RT. The polymerized gel was pre-run in 0.4x TBE at 20A (constant 200 V) till the power dropped down to 10A (usually for 2 hours) to let the salt run out off the gel. For EMSA, nuclear proteins were used. The master mix for each sample was prepared as following:

3x binding buffer	3.3 µl
poly dI/dC (1 µg/µl)	0.7 µl
dd H_2O minus nuclear extract and probe	up to 10.0 µl

The master mix for all similar samples (i.e. same probe) was mixed with radioactive probe (~20,000 cpm for each sample), aliquoted into different tubes for different nuclear extracts, mixed with respective nuclear extracts (2.0 µg) and incubated for 20-30 min on ice. For adjusting the different concentrations of nuclear extracts to the final volume of 10 µl, the buffer C was used. For “Supershift assays”, 1 µl (1 µg/µl) of antibody solution against the transcription factor to be studied was added. A 100-fold excess of cold oligos were added for competition assays. For separation of complexes, 8 µl of each sample was directly loaded on the 6% polyacrylamide gel which was run at 220 V until

the marker reaches a marked line, usually 13 cm from the wells. The glass plates were removed carefully and the gel was immersed in 10% acetic acid for 20 min to fix small fragments. A Whatman paper was placed on the gel and carefully separated from the glass plate. The gel was covered with plastic foil and dried for 60-120 min in a vacuum dryer. An autoradiogram was established by exposing the gel for 24 - 48 hours to X-ray film at -70°C with an intensifying screen.

5.2.6. Reporter Gene Analysis

5.2.6.1. Transfection of EL-4 cells

DEAE-Dextran method was used for transient transfection of EL-4 cells. One day before transfection, EL4 cells were inoculated at a density of 50 x 10^5 cells/50 ml. Before transfection, transfection cocktail per sample was made in a cryotube as following, vortexed, spinned down and left at RT:

Reporter construct	1 µg
Vector DNA	6 µg
1x TBS	up to 540 µl
DEAE	180 µl

50 ml of overnight cultured cells were centrifuged at 1200 rpm for 3-5 minutes, pooled in TBS (e.g. for 27 dishes, all cells were pooled in 27 ml TBS), aliquoted equally in the different tubes, and again centrifuged at 1200 rpm for 3 minutes to pellet down cells. The transfection cocktail was mixed with the cell pellet and incubated on ice for 20 minutes with shaking. In the meanwhile, 100 mm plate with 19 ml complete RPMI medium was incubated at 37°C. Cells were shocked by adding 400 µl of 25% DMSO (in 1x TBS) for 90 seconds, then 4.0 ml of 1x TBS was added and centrifuged at 1200 rpm for 3 minutes. The cell pellets were resuspended in RPMI medium and pooled all cells transfected with same reporter. Again pooled cells were centrifuged, resuspended in 1 ml / dish of complete RPMI medium and dropped on each plate containing 19 ml complete RPMI medium and incubated in 37°C. Transfected cells were stimulated overnight according to the protocol.

5.2.6.2. Transfection of 293T Cells

For reporter gene assay, 293 HEK cells were transiently transfected using PolyFect™ reagents. 6 x 10^5 cells were seeded in 6 well cell culture dish one day before transfection to get a confluency up to 50-60 %. Before transfection, DNA and PolyFect cocktail per well was made as following in a 1.5 ml sterile tube:

Gene of interest	4 µg
RPMI 1640 (without FCS)	up to 100 µl
SuperFect or PolyFect	10 µl

This cocktail was incubated at RT for 10-15 minutes for complex formation. After incubation, 1.9 ml of complete DMEM was added and transferred to each well for transfection. Cells were incubated for overnight in transfection cocktail, which was replaced with 2 ml of fresh complete DMEM.

5.2.6.3. Luciferase Reporter Gene Assay

Bioluminescence is characterized by light emission catalyzed by an enzyme. Firefly (*Photinus pyralis*) luciferase catalyzes the release of light upon addition of luciferin and ATP. The luciferase activity was assayed and quantified by measurement of light production. The photon production by catalytic oxidation of luciferin occurs from an enzyme intermediate, luciferyl-AMP. Luciferin was first activated by the addition of ATP and the activated luciferin, in turn, reacted with oxygen to form dioxetane. Dioxetane decomposes and excites the molecule, which transfers to its ground state by emission of fluorescence light.

$$\text{Luciferin} + \text{ATP} \rightarrow \text{luciferyl-AMP} + \text{PP}$$

$$\text{Luciferyl-AMP} + O_2 \rightarrow \text{oxyluciferin} + \text{light}$$

Cells were collected in 50 ml tube, centrifuged at 1200 rpm for 3 minutes, resuspended in 1 ml of PBS, and transferred to 1.5 ml tube. Cells were again centrifuged at 6,000 rpm for 2 minutes, added 100 µl of harvesting buffer to the pellet, mixed thoroughly by vortexing and centrifuged at 14,000 rpm for 4 minutes. Now 50 µl of this supernatant was mixed with 50 µl of assay buffer in the luciferase plate, which was placed in a luminometer, injected with 100 µl of luciferin solution and luciferase activity was measured.

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7. Appendix

7.1. Abbreviation Index

A	Adenosine
Aa	Amino acid
Ab	Antibody
AEBSF	4-(2-Aminoethyl)-benzensulfonylfluoride hydrochloride
Ag	Antigen
AHR	Airway hyperresponsiveness
AP	Alkaline Phosphatase
APC	Antigen-presenting cell
AP-1	Activator protein 1
APS	Ammoniumperoxidisulfate
Aqua dest.	distilled water
ATP	Adenosine triphosphate
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cDNA	complementary DNA
CR	Complement receptor
C	Cytosine
°C	Degree Celsius
CMV	Cytomegalovirus
CNS	Central nervous system
ConA	Concanavaline A
cpm	Counts per minute
CREB	cAMP response element binding protein
CTL	Cytotoxic T lymphocytes
CTLA	Cytotoxic T lymphocyte-associated antigen
Da	Dalton
dd	double distilled
DEPC	Diethylpyrocarbonate

DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DNP	Dinitrophenyl
dNTP	2´-deoxynucleoside 5´-triphosphate
ds	double-stranded
DTT	Dithiothreitol
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescence
EDTA	Ethylendiamintetraaceticacid (Disodiumsalt)
e.g	for example (*L. exampli gratia*)
EGTA	Ethyleneglycol-bis(ß-aminoethylester)N,N,N´,N´-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic moility shift assay
ER	Endoplasmic reticulum
et al.	and others
F	Forskolin
FACS	Fluorescence-activated cell sorter
FcR	Fc Receptor
FBS	Fetal bovine serum
FCS	Fetal calf serum
FITC	Fluorescine isothiocyanate
FL	Fluorescence
FSC	Forward Scatter Light
G	Guanosine
g	Gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GST	Glutathione S-transferase
H-89	N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinoline-sulfonamide hydrochloride
HBSS	Hanks´ balanced Salt solution
H_2O	Distilled Water
HEK	Human Embryonic Kidney Cell line

HEPES	N-2-hydroxyethylpiperazine-N´-2-ethansulfonic Acid
HRP	Horse radish peroxidase
IFN	Interferon
IgG	Immunglobulin G
IκB	Inhibitory NF-κB
IL	Interleukin
i.p.	intraperitoneal
IPTG	Isopropyl-β-D-thiogalactoside
I	Ionomycin
i.v.	intravenous
Kb	Kilobase
Kbp	Kilobase pair
Kd	Dissociation constant
KDa	Kilodalton
LB	Luria-Bertani
LFA-1	Leukocyte function associated antigen-1
LPS	Lipopolysaccharide
Luciferin	4, 5-dihydro-2-[6-hydroxy-2-benzothiazolyl]-4-thiazol-carboxylacid; C11H8N2O3S2
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MEM	Minimal essential medium
2-ME	2-mercaptoethanol
MES	Morpholinoethansulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
MHC	Major histocompatibility complex
ml	Milliliter
μg	microgram
mg	milligram
μl	microliter
μM	micromolar
mM	millimolar
M_r	Relative molecular mass

mRNA	messenger RNA
mw	Molecular weight
N	Nuclear
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor κB
n.d	not determined
ng	nanogram
nM	nanomoles
NK cells	Natural killer cells
nt	Nucleotide
OCT	Octamer-binding factor
OD	Optical density
OVA	Ovalbumin
PAGE	Polyacrylamide gel electrophoresis
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrine
PEG	Polyethylene glycol
per se	intrinsically or by itself (*L.per se*)
PFA	Paraformaldehyde
PI	Propidium iodide
PIPES	Piperazine-N,N´-bis(2-ethanesulfonic acid)
Polybrene	1,5-dimethyl-1,5-diazaundecamethylene polymethobromide
PMA	Phorbol myristate acetate
pmol	picomoles
PMSF	Phenylmethylsulfonyl fluoride
p-ONPG	O-nitrophenyl-β-D-galactopyranoside
r	Recombinant
RANTES	Regulated upon activation, normal T cell expressed and secreted
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	revolutions per minute

RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SB 203580	4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole powder
s.c.	Subcutaneous
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
SSC	Sideways scatter light
STAT	Signal transducer and activator of transcription
SV40	Simian virus 40
T	Thiamidine
Tab.	Table
TBS	Tris-buffered saline
TBS-T	TBS with Tween 20
TCA	Trichloroacetic acid
TCR	T cell receptor for antigen
TEMED	N,N,N´,N´-tetramethylethylenediamine
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Th	T helper cells
T/I	TPA and ionomycin
TPA	12-O-tetradecanoyl-phorbol-13-acetate
Tris	2-Amino-2-(hydroxylmethyl)-1,3-propandiol
Triton X-100	Alkylphenylpolyethyleneglycol
tRNA	Transfer RNA
U	Unit
U/min	Revolutions per minute
U/ml	Unit per millilitre
UV	Ultraviolet
vs.	versus
WT	Wild type
w/v	Weight per Volume
YFP	Yellow fluorescence protein

7.2. Professional Profile

Personal Information

Mithilesh Kumar Jha (Mr.)

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Born : April 11, 1972

Civil Status : Married, no dependants

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Academic Qualification

M.Sc. Biotechnology, 1996
Banaras Hindu University
Varanasi, India.
Overall Average for M.Sc.: 67% marks (First Division)

B.Sc. (Honours.), Botany, 1994
Banaras Hindu University
Varanasi, India.
Overall College Average: 71.4% marks (First Division)
Major Subject Grade Average: 73% marks (First Division)

Scholastic Achievements

- Qualified the Joint All India CSIR-UGC test for Junior Research Fellowship of CSIR and eligibility test for Lectureship held on December 22, 1996 in India.
- Secured 89.09 percentile score in GATE'96 (Graduate Aptitude Test in Engineering) in India.
- Recipient of National Rural Talent Search Scholarship from 1983 to 1987 in India.
- Recipient of Senior Primary School Level Scholarship from 1981 to 1983 in India.

Work Experience

Since March 2000 :Graduate student at the Institut für Pathologie, Universität Würzburg, leading to the qualification Doktor rer.nat. Thesis title "Role of cAMP/PKA signaling in Th2 effector functions".

Feb. 1998 - Feb. 2000 :CSIR Junior Research Fellow at the Virology Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India. Project title “Molecular Characterization of Hepatitis B Virus-Induced Hepatocellular Carcinoma in a Transgenic Mouse Model”.

7.3. Publications and Other Scientific Activities

Research Publications

- Klein-Hessling, S†., **Jha, M.K†.**, Berberich-Siebelt, F., Santner-Nannan, B., Schimpl, A., and Serfling, E. Protein Kinase A (PKA) regulates GATA-3 dependent activation of IL-5 gene expression in murine Th2 lymphocytes. **Journal of Immunology, *In press* (†The first two authors contributed equally to this work)**
- Schulze-Luehrmann, J., Santner-Nanan, B., **Jha, M.K.**, Schimpl, A., and Avots, A., and Serfling, E. (2002). Hematopoietic progenitor kinase 1 supports apoptosis of T lymphocytes. **Blood** *100*, 954-960.
- Chuvpilo, S., Jankevics, E., Tyrsin, D., Akimzhanov, A., Moroz, D., **Jha, M.K**., Schulze-Luehrmann, J., Santner-Nanan, B., Feoktistova, E., König, T., Avots, A., Schmitt, E., Berberich-Siebelt, F., Schimpl, A., and Serfling, E. (2002). Autoregulation of NFATc1/A Expression Facilitates Effector T Cells to Escape from Rapid Apoptosis. **Immunity** *16*, 881-895.

Oral Presentations

- Jha, M.K., Klein-Hessling, S., Hein, L., Fischer, W., Berberich-Siebelt, F., Santner-Nannan, B., Schimpl, A., and Serfling, E. Blockade of β-adrenergic receptor inhibits IL-5 gene Expression in Primary Murine Th2 Lymphocytes. *Th1/Th2-Zell-Forschung in Deutschland; 17 - 18 June 2002, Immunologische Nachrichten 2002: in press, Marburg, Germany.*
- Jha, M.K., Klein-Hessling, S., Berberich-Siebelt, F., Santner-Nannan, B., Schimpl, A., and Serfling, E. Protein Kinase A (PKA) supports the GATA-3 mediated activation of Th2-type lymphokine genes. *32nd Annual Meeting of the German Society of Immunology; 26 - 29 September 2001, B10: 17-18, Dresden, Germany.*

Poster Presentations

- Jha, M.K., Klein-Hessling, S., Berberich-Siebelt, F., Santner-Nannan, B., Schimpl, A., and Serfling, E. Protein Kinase A (PKA) supports the GATA-3 mediated activation of Th2-type lymphokine genes. *2nd Lymphocyte Signal Transduction Workshop; 13 - 17 October 2002, Santorini, Greece).*
- Jha, M.K., Klein-Hessling, S., Berberich-Siebelt, F., Santner-Nannan, B., Schimpl, A., and Serfling, E. Protein Kinase A (PKA) supports the GATA-3 mediated activation of Th2-type lymphokine genes. *33rd Annual Meeting of the German Society of Immunology; 26 - 29 September 2002, J7: 133-134, Marburg, Germany.*
- Jha, M.K., Klein-Hessling, S., Berberich-Siebelt, F., Santner-Nannan, B., Schimpl, A., and Serfling, E. Protein Kinase A (PKA) supports the GATA-3 mediated activation of Th2-type lymphokine genes. *32nd Annual Meeting of the*

German Society of Immunology; 26 - 29 September 2001, B10: 17-18, Dresden, Germany.

- Klein-Hessling, S., Jha, M.K., Berberich-Siebelt, F., Santner-Nannan, B., and Serfling, E. Protein Kinase A (PKA) supports the GATA-3 mediated activation of Th2-type lymphokine genes. *5th Joint Meeting of the Signal Transduction Society (STS); 8 - 10 November 2001, G4: 5, Weimer, Germany.*
- Schulze-Luehrmann, J., Santner-Nanan, B., Jha, M.K., Schimpl, A., Avots, A. and Serfling, E. Hematopoietic progenitor kinase 1 (HPK1) supports Activation Induced Cell Death (AICD) of T Lymphocytes. *32nd Annual Meeting of the German Society of Immunology; 26 - 29 September 2001, P21: 270, Dresden, Germany.*
- Klein-Hessling, S., Jha, M.K., Berberich-Siebelt, F., Santner-Nannan, B., and Serfling, E. Protein Kinase A (PKA) enhances the inducible expression of Th2-specific genes. *Millennium Meeting Signal Transduction – Receptors, Mediators and Genes; 2 - 4 November 2000, P57: 152, Berlin-Müggelsee, Germany.*