THE ROLE OF PARALLEL SIGNALING PATHWAYS IN MAINTAINING LYMPHOID CELL SURVIVAL

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INTRODUCTION

The objective of this study is to investigate the contribution of IL2-induced alternate signaling pathways to control lymphoid cell growth and survival. IL2 is a major regulator of immune homeostasis in T lymphocytes, mediating cell proliferation, survival, apoptosis and differentiation. However, uncontrolled T cell function is the underlying abnormality in a number of immune mediated disorders like allergy, leukemia, lymphoma, allograft rejection and autoimmunity. Understanding the role of key pathways critical to T cell growth and survival will contribute to the therapy of immune mediated diseases. IL2 activates a number of signaling pathways including Jak1/3, Stat5a/b and 3, Syk and Lck, Mitogen Activated Protein Kinase (Mapk), PI3K and mTor. This work attempts to dissect the role of these pathways in mediating lymphoid cell growth and survival. The ***rationale*** behind the proposed research centers on compelling new data demonstrating that mice deficient in γc, Jak3 and Stat5 transcription factors are immune suppressed. To accomplish the objective of this proposal, we will test whether targeted inhibition of cell signaling components related to the Jak/Stat signaling pathway blocks T cell activity.

The **hypothesis** to be tested is that Stat molecules are critical in maintaining T cell viability via the following specific aims:

(1) Determine whether selective disruption of γc protein expression or Jak3 activity inhibits lymphoid/T cell function.

(2) Determine whether disruption of PI3K, Mapk and Raf pathways blocks Stat5 serine kinase and lymphoid cell growth.

(3) Determine whether constitutively active Stat3 provides a survival pathway in a lymphoid tumor cell line.

IL2 receptor gamma chain (γc) is indispensable for Jak3 activation and signaling, and is a shared subunit of receptor complexes utilized by several T cell growth factors (TCGFs), including IL2, 4, 7, 9, 13, 15, 21. Jak3, which is primarily expressed in lymphocytes, binds to the γ_c and is necessary for T cell development and function. The importance of Jak3/γ_c association and signaling pathway has been demonstrated by gene targeting in mice and by the identification of mutations in the γc and Jak3 genes of humans with severe combined immunodeficiency (SCID). Since the abnormalities of such deficiencies are limited to the

immune system, we focused our attention on the inhibition of IL2 signaling at the level of γc and Jak3 as potential immunosuppressive therapy of T cell mediated disorders. These defects could be developmentally regulated. However, for a therapeutic viewpoint, the effect of blocking γc/Jak3 in mature lymphocytes is more relevant.

To do this, first we utilized selective phosphorothioate antisense oligonucleotides directed to γc and present its effects on the proliferation and survival of a human lymphoid cell line.

Next, we provide evidence that PNU156804, which is an analogue of undecylprodigiosin, and was found to block T cell proliferation, selectively blocks downstream signaling pathways of Jak3 such as the Mapk cascade and Stat5.

Stat5 is critical for immune regulation and T cell function. The binding of IL2 to its receptor results in rapid tyrosine phosphorylation of Stat5 by IL2R-associated Jak kinases. In addition to tyrosine phosphorylation, earlier work demonstrated that IL2 induces the rapid serine phosphorylation of both Stat5a and Stat5b in primary human and rat T cell lines and that this phosphoacceptor site is likely confined to the transactivation domain, but less is known about the regulation of this event. The IL2-induced serine/threonine kinases directly or indirectly may play a role in the serine phosphorylation of Stat5a and Stat5b and the mitogenic response.

We sought to identify the possible convergence of these signaling pathways on the level of serine phosphorylation of Stat5a/b, as well as their contribution to the IL2 mediated growth of lymphoid cells.

To further support current models that suggest cytokines activate Stats to regulate T cell survival, we sought to investigate whether constitutive activation of Stat3 provides a cell survival signal in a lymphoid tumor cell line (YT). YT is a human NK-like cell line originally derived from a patient with Acute Lymphoblastic Lymphoma.

To block persistent Stat3 activation, we chose to use phosphorothioate antisense oligonucleotides with 2'-methoxyethyl wings at both the 3' and 5' ends, delivered by electroporation. As a control, a 5-nucleotide mismatched oligo with the same modification was utilized.

RESULTS AND DISCUSSION

The primary goal and objective of this study was to determine the role of possible parallel cell signaling pathways in maintaining lymphoid cell survival, primarily focusing on the Jak/Stat signaling pathway. The challenge of undertaking this project was the development of novel tools and reagents to inactivate these molecules. Our approach was to develop and then utilize pharmacological inhibitors or selective antisense oligonucleotides to determine the role of these specific molecules.

Effects of selective inhibition of γc receptor and Jak3 on lymphoid cell survival

To initiate these studies, we first determined the effects of disrupting the expression of γc in a human NK-like tumor cell line YT, by using phosphorothioate antisense oligonucleotides; comparing all effects to the scrambled oligo treated or electroporated control samples.

Our findings were:

(1) Antisense to γc (15μM) delivered via electroporation successfully inhibited protein expression (40%) at 72 hrs post-transfection.

(2) Bcl-2 protein level was unchanged.

(3) This treatment decreased the cell viability (40%) and increased the number of apoptotic cells (30%) compared to the scrambled control treated cells at kinetically equivalent points.

(4) Disruption of the γc induced cell cycle arrest at 36 hrs following electroporation and increased the number of cells in subG_1 (15%) and G_2-M (12%) phases, and decreasing in $G_{0\text{-}1}$ (21%).

Results of Jak3 inhibition utilizing a novel pharmacological inhibitor PNU156804:

(1) PNU156804, but not the inactive control compound PNU159744, blocks the activation of Jak3 substrates (Stat5a and b), as assessed by phosphotyrosine and phosphoserine Western blots.

(2) PNU156804 disrupts the activity of p44/Erk1 and p42/Erk2 Ser/Thr kinases.

Long-term use of PNU156804 induces T cell apoptosis (~ 40%) at 48hrs (data not shown, personal communication with R. A. Kirken).

The γc receptor is central to cytokine mediated T cell proliferation. A large body of literature has accumulated over the past few years that has attempted to reveal its exact role in cytokine signaling. However, most findings are derived from murine gene deletion experiments. In order to unveil γc function in human mature cells, the application of antisense oligonucleotides was invaluable to this process. As we presented here, antisense targeted against γc successfully inhibited protein expression. There are several advantages in the use of antisense oligonucleotides such as the sequence specificity and relatively low expenses of the synthesis of the oligos. It provides a powerful technique for the short-term modulation of the expression of the gene of interest, once an optimal target sequence is found and a successful method for delivery is optimized. However, as with all other techniques, antisense approaches have some inherent risks. The disruption of the target mRNA or the blockade of the target protein expression may not be complete. The occurrence of some non-antisense effects cannot be ruled out, although the application of appropriate controls with the same modifications can minimize some of these problems. Therefore, it is important to confirm observations made with antisense oligonucleotides by other independent techniques, such as monoclonal antibodies, dominant negative constructs, overexpression of the protein-of-interest, or small molecule inhibitors. However, it should be noted that all of these techniques have their own limitations. The above results suggested that activation of hyperactive T cells found in allergy, asthma or allograft rejection could be corrected by the application of γc antisense, or at least targeted inhibition of this protein. Given the above-mentioned technical hardships of antisense therapy new methods of delivery are required to make it a relevant therapeutical treatment strategy. Nonetheless, the fundamental problem for all currently available immunosuppressants is the ubiquitous distribution of their targets, which ultimately results in toxicity; therefore we must continue to identify highly specific agents that can inactivate molecules uniquely expressed in resting T cells (e.g. Zap70) or in activated T and B cells (e.g. Jak3) and validate these potentially novel targets through in vitro and in vivo studies.

Although understanding the signaling pathways activated by Jak3 (directly or indirectly) is far from complete, Jak3 signaling by Stat5a/b is necessary to regulate genes required for cellular proliferation. As shown here, PNU156804 abolished IL2-dependent Stat5a/b tyrosine phosphorylation and activation. Interestingly, blockade of Jak3 by PNU156804 also inhibited the phosphorylation of a conserved proline-serine-proline (Pro-

Ser-Pro) motif in Stat5a and Stat5b and induced cell death within 48 h. Given the limited pattern of Jak3 expression, the γc-Jak3-Stat5 pathway is likely to represent a convergence point by which TCGFs drive T cell clonal expansion, thereby making it a preferred pathway for novel and selective immunosuppression. The limitation of validating this model is which Jak3 substrate is critical for regulating T cell activity. While it is known that Jak3 regulates Stat5, Mapk and PI3K signaling pathways, is one more crucial than the other for maintaining cell survival?

Uncoupling Stat5 activation from parallel signaling pathways in lymphoid cells

To address this question, we determined whether disruption of PI3K, Mapk and Raf pathways blocks Stat5 activity, a Stat5 serine kinase and possible effects on lymphoid cell growth.

The results of these studies are as follows:

(1) Serine phosphorylation of Stat5a-S726 and Stat5b-S731 occurs within a PSP-motif that is regulated by multiple TCGFs including IL2, IL4, IL7, IL9, or IL15.

(2) While Stat5a/b serine phosphorylation was not inducible following cross-linking of the T cell receptor via αCD3 ligation, it was readily activated by PMA in the absence of tyrosine phosphorylation.

(3) While several Stat dependent serine kinases including PI3K, Erk1/2 and mTor regulate Stat1, 3 and 4 phosphorylation, this positionally conserved serine residue in Stat5a/b does not appear to be one of their targets.

(4) Novel evidence was presented that IL2 is competent to activate all three Raf-isoforms (A-, B-, and C-), but that their inactivation failed to influence Stat5a/b (S725/S731 or Y699/701) phosphorylation.

(5) Based on [^{3}H]-thymidine uptake assays, inhibition of each of these serine kinases failed to significantly affect cytokine-mediated cell proliferation of human NK and murine T cell lines within the time frame tested.

Given the present findings that Stat5a/b activation remains intact upon disruption of Mapk, Raf and PI3K pathways, we conclude that these transcription factors are dependent on Jak3 but not on several IL2 mediated serine/threonine kinase pathways.

The limited region of sequence divergence existing within Stat5a/b is localized to their transactivation domains that accommodate the conserved PSP-motif. While Stat5a/b share

95% sequence identity, they display differences in biological activity since mice void of Stat5a develop mammary gland defects while Stat5b null mice manifest a runted phenotype. The current work adds to a series of Stat5a versus Stat5b dissimilarities also localized within the transactivation domain, since both displayed different serine phosphorylation kinetics. The main implication of this dissimilarity in phosphorylation for Stat5a/b is that they operate differently, explaining in part the lack of compensatory gene expression displayed in Stat5 single knockout mice. However, differences in immune-regulatory cells isolated from Stat5a or Stat5b null mice fail to display obvious cell defects. Taken together, these findings suggest each transcription factor is redundant or may act to "fine-tune" T cell gene expression. Reconstitution assays employing the prolactin receptor and serine mutants of Stat5a (S726) and Stat5b (S731) failed to identify a significant loss in Stat5a/b transactivation potential. The cellular location where Stat5a and Stat5b become serine phosphorylated is not clear, but it does not appear to be entirely dependent on an activated receptor complex since PMA alone could induce this event. These findings parallel earlier findings of phorbol-ester activation of Stat3. It has been shown that no membrane distal domains of the IL2Rβ were required to activate the kinase, the only prerequisite for this event was a functional Jak3. We are left to conclude that the Stat5a/b serine kinase is not directly associated with TCGF receptor complexes but activated distal to the receptor.

Since T cells isolated from Stat5a/b-deficient mice fail to respond to the mitogenic effects of IL2 and likely other TCGFs, possibly due to reduced T cell expression of cdk6 and cyclins A, D2, D3, and E, Stat5a/b probably regulates their transcription and therefore critical for cell cycle progression. Based on these observations, one preliminary model that could be envisioned is that Sta5a/b represents a convergence point by which TCGFs drive T cell clonal expansion or protect against pro-apoptotic pathways. Indeed, given the diversity of the carboxyl termini shared between each Stat family member it seems plausible to expect that unique sets of genes could be controlled, in part, by distinct Stat proline-directed serine kinases. Indeed inhibitors to Mek, PI3K and mTor inhibited IL2-mediated Stat3 activation but did not affect Stat5 serine phosphorylation or cell proliferation.

Our findings suggest that Mek1/2, PI3-K, mTor and Raf (A-, B-, and C-) pathways act to encourage other cell processes and gene transcription events not linked to immediate cell

survival as opposed to Stat5a/b which likely plays central role in acute cell survival and proliferative gene expression.

Lastly, studies presented herein suggest that Stat5a and Stat5b serine phosphorylation are regulated differently than other Stats. These findings have direct therapeutic consequences since inhibition of this putative proline-directed Stat5a/b serine kinase could be used to manipulate T cell expansion via G1-S cell cycle transition as found within graft versus host disease. Recent findings that Stat6 is also serine phosphorylated and void of a PSP or PXSP motif, suggest that its selective uncoupling might prove useful in combating Th2 diseases such as allergy or airway hypersensitivity.

Selective Disruption of Constitutively Active Stat3 by Antisense Oligonucleotides in a Human NK-like Tumor Cell Line

Lastly, we investigated whether constitutively active Stat3 provides a survival pathway in a lymphoid tumor cell line, suggesting that it alone could protect a cell against apoptosis, regardless of the activation state of PI3K, Mapk or Jak3.

We showed that:

(1) Stat3 is constitutively phosphorylated on the highly conserved Y701 and S727 residues. IL2 was able to further induce phosphorylation of both residues, most likely via Jak1 and Jak3, which are upstream tyrosine kinases of Stat3 in the IL2 signaling cascade.

(2) None of the Jak family members exhibit baseline enzyme activity in these cells; therefore, we conclude that an independent pathway is responsible for the hyperactivity of Stat3.

(3) Antisense to Stat3 successfully disrupted its DNA binding activity but failed to affect Stat5.

(4) Moreover, deletion of Stat3 protein promotes apoptotic cell death.

(5) Culturing Stat3-depleted cells in the presence of physiological amounts (1nM) IL2 was able to rescue the cells from apoptosis.

(6) This event correlated with the hyperphosphorylation of Stat5a and b, suggesting that the IL2-rescue mechanism is mediated by Stat5. Bcl-2 upregulation also correlated with Stat5 activity in Stat3-deleted cells suggesting a possible target gene regulated within this process.

Stat proteins are unique among signaling molecules in their ability to transmit signals from the cell surface to the nucleus and directly participate in the regulation of gene expression. Increasing evidence indicates that Stats signaling contributes to cancer formation and progression. Stat3 is known to be constitutively active in numerous malignancies, including leukemias, lymphomas, breast carcinoma, multiple myeloma, head and neck cancers, brain, lung and prostate cancers. Stat3 is thought to protect cells against apoptosis. The mechanism underlying cell survival supported by Stat3 has been linked to the transcriptional control of apoptotic regulatory genes, such as bcl-xL, bax and mcl-1; as well as cell cycle regulator $p21^{waf1}$. Another interesting feature of Stat3 that it can also participate in apoptosis promoting cellular processes. Similarly, Stat5 activation is associated with survival of some leukemic cells and likely contributes to their resistance to undergo apoptosis by regulating bcl-xL.

The paradoxical effects induced by Stat3 calls for further clarification of its role in certain types of malignant and normal cells. Possible redundant functions may contribute to the wide range of effects dictated by Stat family members, which should be the subject of future investigations.

The role of alternate signaling pathways in maintaining lymphoid cell survival

These findings led us conclude that Stats play a critical role in the growth and early survival of lymphoid cells (i.e. days).

What is the role of PI3K, Mapk and mTor? Likely they contribute to long-term cellular processes and survival. Our studies focused on relatively short kinetics 24-72 h. Studies to address this issue such as treating cells for several days to one week with Mek, mTor or PI3K inhibitors was not investigated. Likely such a treatment would have a deleterious effect on cell function and survival. Given the ubiquitous expression of these pathways, long-term therapeutic strategies blocking their activity would likely prove too toxic to the patient. Our findings suggest that inhibition of acute Stats responses and their limited expression/function profile in non-hematopoietic cells, likely represent a more practical approach to regulating lymphoid derived diseases.

If the aforementioned conclusions are true then identification of Stat regulatory mechanisms such as kinases, phosphatases, nuclear transport proteins and their target genes will provide critical new targets and means for devising novel therapeutic strategies to control allergy, asthma, graft rejection, autoimmunity, and other debilitating diseases.