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Interleukin-2 and STAT5 in regulatory T cell development and function

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Abbreviations: Tregs, regulatory T cells; nTregs, natural Tregs; iTregs, induced Tregs; SP, single positive; IL2, interleukin-2; IL2R, interleukin-2 receptor; STAT5, signal transducer and activator of transcription 5; FOXP3, Forkhead box P3; IL15, interleukin-15; TCR, T cell receptor; CNS, conserved non-coding DNA sequence; TGF-β, transforming growth factor-β; IL17, interleukin-17; T_H1, T helper type 1 cells; T_H17, T helper type 17 cells

Interleukin-2 and its downstream target STAT5 have effects on many aspects of immune function. This has been perhaps best documented in regulatory T cells. In this review we summarize the initial findings supporting a role for IL2 and STAT5 in regulatory T cell development and outline more recent studies describing how this critical signaling pathway entrains regulatory T cell differentiation and affects regulatory T cell function.

Introduction

It has now been 20 years since the first reports appeared demonstrating that interleukin-2 is required to prevent the development of systemic autoimmune disease.[1] Subsequent studies by Sakaguchi and colleagues identified CD25, the interleukin-2 receptor α chain (IL2Rα), as one of the first useful markers for the identification of regulatory T cells (Tregs).[2] This led to the initial hypothesis that IL2 is required for the development or function of Tregs and more recently the implementation in the clinic of agonist IL2:anti-IL2 complexes for the treatment of autoimmune and inflammatory conditions.[3,4]

Supporting the initial hypothesis that IL2 is involved in Treg development, work by Malek and colleagues demonstrated that the autoimmune disease that developed in *Il2rb*$^{-/-}$ mice could be prevented by the transfer of CD25$^+$ Tregs from WT mice into *Il2rb*$^{-/-}$ host mice. These studies demonstrated that *Il2rb*$^{-/-}$ mice lacked a functional population of Tregs.[5] Additional work by this same group demonstrated that expression of an *Il2rb* transgene that was expressed solely in the thymus was sufficient to rescue the defect in Treg development suggesting that the defect in *Il2rb*$^{-/-}$ mice is due to a failure of Treg development in the absence of IL2.[6] In contrast, Lafaille and colleagues found that transfer of CD4$^+$ T cells from *Il2*$^{-/-}$ mice into a mouse model of experimental autoimmune encephalomyelitis prevented disease, while CD4$^+$ T cells from *Il2ra*$^{-/-}$ mice did not. These results suggested that CD4$^+$ T cells in *Il2*$^{-/-}$ mice are capable of developing into and functioning as Tregs.[7] Supporting this observation, two other groups used either *Foxp3-GFP* reporter mice, or the ability to stain for intracellular FOXP3, to demonstrate that young *Il2*$^{-/-}$ mice have FOXP3$^+$ Tregs and that the defect in these mice had to do with reduced function or "fitness" of these cells.[8,9] Finally, work from our group and Steve Ziegler's was able to reconcile these findings by demonstrating that while young *Il2*$^{-/-}$ mice do not lack FOXP3$^+$ Tregs, comparable *Il2rb*$^{-/-}$ mice have a substantial defect in Treg development.[10,11] This latter result reflects redundancy between IL2 and IL15 as *Il2*$^{-/-}$ *x Il15*$^{-/-}$ mice mimic the defect in Treg development observed in *Il2rb*$^{-/-}$ mice.[10] It is important to point out that under physiological circumstances IL15 does not play a role in Treg development or function as IL2 signaling in Tregs leads to downregulation of the IL15Rα chain, thereby rendering these cells much less responsive to IL15.[12] Thus, subsequent studies have demonstrated that the original experiments by Malek and Lafaille and colleagues were both correct as IL2 plays an important role in both Treg development and function.

STAT5 Activation Drives Thymic Treg Lineage Commitment

CD4$^+$CD25$^+$FOXP3$^+$ Tregs that develop in the thymus (also known as "natural Tregs") constitute 2–4% of CD4 single positive (CD4SP) thymocytes, yet this relatively small population plays a critical role in maintaining peripheral tolerance and preventing autoimmunity. The T cell receptor (TCR) repertoire of these natural Tregs overlaps with that of non-regulatory T cell populations but is skewed to favor TCRs that interact with higher affinity to self-antigens in the thymus.[13-18] The molecular mechanisms that drive Treg development have been tied to three primary signaling modules. First, TCR signaling plays a key role as TCRs with higher affinity for self-antigen are preferentially selected into the Treg lineage.[15,19] Second, the costimulatory receptor CD28 also plays an important role as *Cd28*$^{-/-}$ and *B7-1/B7-2*$^{-/-}$ mice both show clear defects in Treg development.[20-23] Third, signals emanating from

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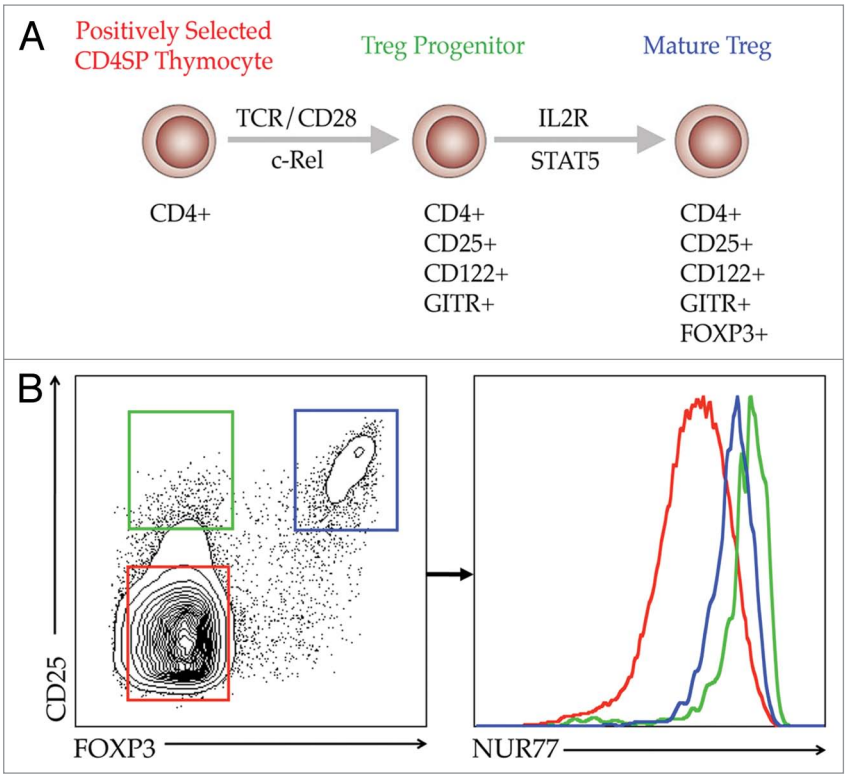

Figure 1. Two-step model of thymic Treg development. (**A**) CD4SP thymocytes perceiving high affinity/avidity signals emanating from TCR/CD28 are first programmed via the NFκB pathway to express IL2Rα and IL2Rβ, rendering them highly responsive to IL2. A second step, which is TCR-independent, but cytokine-dependent, is completed when Treg progenitors receive IL2 signals transmitted via STAT5 to subsequently drive expression of *Foxp3*. This second step yields mature, fully functional FOXP3$^+$ Tregs. (**B**) CD4SP thymocytes plotted on the basis of CD25 and FOXP3 expression can be categorized into (1) conventional or non-Treg cells which are CD4$^+$CD25$^-$FOXP3$^-$ (gated in red), (2) CD4$^+$CD25$^+$FOXP3$^-$ Treg progenitors, which are also CD122^{hi} and GITR^{hi} (gated in green) and (3) CD4$^+$CD25$^+$FOXP3$^+$ mature Tregs (gated in blue). The representative TCR signal strength of each of these populations, reported via NUR77-GFP expression, is shown in the histogram on the right.

the interleukin-2 receptor are also required for Treg differentiation in the thymus.[10,11] These observations culminated in the development of a two-step model of thymic Treg development, in which a TCR- and CD28-dependent, but cytokine-independent first step generates an IL2-responsive intermediate "Treg progenitor" that lacks FOXP3 expression. Subsequently, a TCR-independent, IL2/STAT5-dependent second step results in the rapid conversion of Treg progenitors into mature FOXP3$^+$ Tregs[24,25] (**Fig. 1**). We examine this model in further detail below.

Upon interacting with medullary antigen presenting cells (APC) presenting self-peptide:MHC II complexes, strong TCR signals in a fraction of CD4SP thymocytes cause them to differentiate into Treg progenitors, marked by elevated expression of the high-affinity IL2Rα chain (CD25), the IL2Rβ chain (CD122), and the costimulatory TNF receptor superfamily member, glucocorticoid-induced TNF-related protein (GITR).[24,25] The emergence of this CD4$^+$CD25$^+$CD122^{hi}GITR^{hi}FOXP3 Treg progenitor population requires canonical activation of the NFκB pathway downstream of TCR and CD28 ligation. Paired activation of LCK from these receptors signals through the canonical NFκB pathway to ultimately promote nuclear translocation of c-REL and REL-A.[22,26-29] The requirement for NFκB activation in Treg differentiation is demonstrated by the absence of thymic Tregs—and importantly, Treg progenitors—in animals deficient in *Cd28*, *Prkcq*, *Carma1*, *Bcl10* and *Rel*.[21,22,30-32] Further studies revealed that c-REL binds the conserved non-coding sequence 3 (CNS3) located in the *Foxp3* gene to promote epigenetic modification of *Foxp3* rendering it permissive for subsequent transcription initiation.[33]

The conversion of FOXP3$^-$ Treg progenitors into mature FOXP3$^+$ Tregs in the thymus occurs via a TCR-independent but IL2/STAT5-dependent process.[24,25] Ligand binding by the high affinity IL2R complex leads to phosphorylation of three key tyrosine residues located in the cytoplasmic domain of IL2Rβ by the kinases JAK1 and JAK3. Phosphorylation of Tyr-338 recruits the SH2-containing adaptor molecule, SHC, facilitating activation of the RAS/MAPK/ERK and PI3K/AKT pathways via GRB2 and GAB2, respectively. Phosphorylation of IL2Rβ at Tyr-510 (and to a lesser degree Tyr-392) is critical for recruiting and activating STAT5.[34] The importance of IL2R signaling in thymic Treg differentiation is clearly demonstrated by the fact that the lethal autoimmunity in mice lacking *Il2rb* is due to a failure to generate thymic Tregs, and this phenotype is completely restored by adoptive transfer of small numbers of wild type Tregs.[5] Moreover, retroviral transduction of *Il2rb*$^{-/-}$ bone marrow with wild type *Il2rb*, or a mutant construct capable of activating only STAT5 via Tyr-510, restored thymic Treg generation in bone marrow chimeric mice. In contrast, restoration of Treg development did not occur when mutant constructs capable of activating RAS/PI3K, but not STAT5, were transduced into *Il2rb*$^{-/-}$ bone marrow cells and engrafted into recipient mice.[10] Likewise, crossing *Il2rb*$^{-/-}$ mice to transgenic mice expressing a constitutively active form of STAT5b (*Stat5b-CA* mice) restored Treg development in the thymus.[10] Additional support for the role of STAT5 in Treg development came from two studies that demonstrated that conditional deletion of STAT5 in DP thymocytes (i.e., *Cd4-Cre* × *Stat5a/b*$^{FL/FL}$ mice) had minimal effects on CD4SP thymocytes with the exception of CD4$^+$FOXP3$^+$ thymic Tregs.[10,35] Together, these findings indicate that STAT5 activation downstream of IL2R is required for thymic Treg development.

Two groups have demonstrated that CD4$^+$CD25$^+$FOXP3$^-$ thymocytes are direct precursors of FOXP3$^+$ Tregs, which require only an additional IL2R/STAT5-dependent signal to express FOXP3 (**Fig. 1**). First, Hsieh and colleagues showed that adoptive transfer of CD4$^+$CD25$^+$FOXP3$^-$ thymocytes, but not CD4$^+$CD25$^-$FOXP3$^-$ thymocytes, into the thymii of wild-type hosts resulted in the development of CD4$^+$FOXP3$^+$

Tregs. Similar results were observed upon adoptive transfer into MHCII-deficient mice demonstrating that the conversion process did not require additional signals via the TCR.[24] In addition, stimulation of sorted Treg progenitors with IL2 in vitro led to induction of *Foxp3* mRNA within a few hours followed by the development of $CD4^+FOXP3^+$ Tregs 24 h later. These findings were subsequently confirmed by Burchill and colleagues.[25]

Interestingly, IL2R/STAT5 signaling also influences selection of the thymic Treg TCR repertoire. Several studies indicate that the Treg TCR repertoire is biased toward self-reactivity, although there is some overlap with the conventional $CD4^+FOXP3^-$ TCR repertoire.[16-18] Initial studies by Burchill et al. found that augmented STAT5 signaling clearly altered the Treg TCR repertoire. Specifically, this study evaluated the effect of forced STAT5 activation on the Treg TCR repertoire by tracking the frequency of $CD4^+FOXP3^+$ cells specific for a peptide called 2W1S bound to $I\text{-}A^b$ MHC class II molecules using peptide:MHCII tetramers (in this study the $2W1S\text{:}I\text{-}A^b$ tetramer) in littermate control and *Stat5b-CA* mice.[25] A simple comparison of the ratio of $CD4^+FOXP3^-$ to $CD4^+FOXP3^+$ cells among total and 2W1S-specific T cells revealed that this TCR is dramatically underrepresented in the Treg pool of wild type mice. The frequency of $CD4^+FOXP3^+$ T cells specific for $2W1S\text{:}I\text{-}A^b$ in WT mice (~3–6%) was much lower than that observed for the average of all other Treg TCR specificities (~15%); in contrast, in *Stat5b-CA* mice the frequency of $2W1S\text{:}I\text{-}A^b$ specific Tregs was identical to the average of all other Treg TCR specificities. This finding demonstrated that for at least one TCR specificity regulation of STAT5 signaling had a profound effect on its distribution within the Treg repertoire. Thus, ectopic STAT5 activation removed the TCR selection bias that typically results in underrepresentation of $2W1S\text{:}I\text{-}A^b$ specific T cells in the Treg TCR repertoire.[25]

To extend the observations on the role of STAT5 and the Treg TCR repertoire beyond a single TCR, a fixed TCRβ transgenic system was employed to partially restrict the repertoire, and sequencing was performed on over 1,000 productive Vα2 rearrangements from thymocytes isolated from *Stat5b-CA* mice or their WT littermate controls. Tonic STAT5 signaling in *Stat5b*-CA mice led to a dramatic expansion in the diversity of productive rearrangements among $CD4^+FOXP3^+$ Tregs including substantial numbers of TCRs that were typically not found in the Treg TCR repertoire.[25] These initial studies have been confirmed more recently by Moran and Hogquist using an independent approach.[15] This latter study used BAC transgenic mice in which a *GFP* reporter had been knocked into the *Nur77* gene locus. These mice accurately measure TCR signal strength as assessed by overall GFP expression. Importantly, this study confirmed that signal strength for TCRs expressed by Tregs is higher than that for conventional $CD4^+$ T cells (**Fig. 1**). When the *Nur77-GFP* mice were crossed to *Stat5b-CA* mice, it was observed that the Treg TCR repertoire was much broader and included substantial numbers of T cells with TCRs that signaled with significantly lower signal strength as assessed using the NUR77-GFP reporter.[15] Together, these observations indicate that limiting IL2R/STAT5 signaling helps to focus the thymic Treg TCR repertoire on TCRs with higher intrinsic signal strength.[25] This suggests that IL2R/STAT5 signaling plays an important role in ensuring the preferential development of Tregs with higher reactivity to self-antigens, which may be important in preventing autoimmunity.

Several questions remain unanswered pertaining to the role of IL2 and STAT5 in promoting thymic Treg differentiation. First, what cell subsets in the thymus actually synthesize the IL2 required for completing the second step of Treg development? IL2 was originally described as a cytokine made by activated T cells to drive proliferation and survival of T cells, thus amplifying effector responses.[36] In this regard, a reasonable assumption might be that developing thymocytes produce the IL2 needed for thymic Treg development. However, thymocytes make exceedingly low levels of IL2 (if any) relative to splenocytes upon stimulation (S.A.M. and M.A.F., unpublished observation). The observation that dendritic cells and B cells can also make IL2 suggest that these cell subsets might play an important role in producing the IL2 needed for Treg development in the thymus.[37,38] Further work in which IL2 can be conditionally deleted in T cells, dendritic cells and B cells will be needed to definitively address this question. A second question has to do with what signals actually induce or regulate IL2 production in the thymus; such signals remain undefined. Finally, a defining feature of Treg progenitors is their high expression of GITR. However, the functional significance of this high level upregulation of GITR remains unclear.

The molecular mechanism by which STAT5 affects *Foxp3* transcription is also unclear. STAT5 binding sites have been found in the *Foxp3* promoter region as well as within the CNS2 region of intron 1 in the *Foxp3* gene and several studies have shown STAT5 binding to those sites.[10,35] The effect of STAT5 binding to these sites is not yet clear. Deletion of the entire CNS2 region including the STAT5 binding sites did not prevent Treg development although it did have an effect on stability of FOXP3 expression.[33] However, the CNS2 region is highly methylated in non-Tregs and typically completely demethylated in natural $FOXP3^+$ Tregs. If methylation of this region normally represses *Foxp3* transcription, then deletion of the entire region would remove the need for any factors that typically reverse this methylation state. Thus, whether STAT5 binding to CNS2 plays a role in Treg development is difficult to determine based on studies deleting the entire CNS2 region.

The critical co-factors that interact with STAT5 to promote Treg development are also poorly characterized. STAT5 is known to interact with a variety of both co-activators, such as CBP and p300, and co-repressors such as NCOR2.[39,40] How these function in Treg differentiation remains untested. Intriguingly, treatment of Treg progenitors with two distinct histone deactylase (HDAC) inhibitors prevented the IL2/STAT5-dependent conversion of Treg progenitors into Tregs.[25] Although this result at first appears counterintuitive, it is consistent with several reports demonstrating that STAT-dependent gene transcription frequently requires HDAC activity.[41,42] Whether NCOR2 and associated HDACs are recruited to the *Foxp3* locus by STAT5 during thymic Treg differentiation, and if so, how this complex regulates *Foxp3* transcription, remains to be elucidated. Thus, the molecular mechanisms by which STAT5 alters transcription of genes involved in Treg differentiation remains to be established.

IL2, STAT5 and Induced Tregs

An important feature of peripheral tolerance is the conversion of naïve $CD4^+$ T cells into induced regulatory T cells (iTregs) in peripheral lymphoid organs. iTregs have important roles in protecting against chronic inflammatory conditions, and likely play a key role in regulating immune responses to commensal microorganisms.[43,44] The differentiation of iTregs, like nTregs in the thymus, requires both TCR- and IL2-dependent signals. However, unlike nTregs, iTregs require transforming growth factor-β (TGFβ) for their differentiation.[45] Moreover, while the CARMA1/NFB pathway is required for the development of nTregs it actually antagonizes iTreg differentiation.[46] Finally, the stability of iTregs is lower than that of nTregs,[47] a feature which correlates with the greater degree of DNA methylation of the CNS2 region of the *Foxp3* gene in iTregs vs. nTregs.[48,49] Thus, iTregs differ in several ways from nTregs.

A role for IL2 in iTreg development was established many years ago in studies documenting the role of both TGFβ and IL2 in iTreg differentiation.[50,51] Likewise, STAT5 also plays an important role in iTreg differentiation in vitro.[35] More recent studies have demonstrated that IL2 and STAT5 also play critical roles in maintaining stability of the iTreg lineage.[47] Specifically, these studies demonstrated that transfer of iTregs into congenic hosts resulted in loss of FOXP3 expression in the transferred iTregs. This result could be blocked by co-administration of agonist IL2: anti-IL2 complexes indicating that IL2 was required to maintain FOXP3 expression in iTregs in vivo. These studies further documented that the loss of *Foxp3* expression correlated with re-methylation of the CNS2 region (also referred to as regulatory **T** cell **s**pecific **d**emethylated **r**egion or TSDR) of the *Foxp3* gene, and that IL2 stimulation prevented this re-methylation process. The mechanism by which this occurs remains unclear. However, STAT5 binding sites are found in the CNS2 region, which may be important for maintaining *Foxp3* expression. It is also possible that STAT5 directly initiates demethylation of this region in naïve $CD4^+FOXP3^-$ T cells as they are being converted into $CD4^+FOXP3^+$ iTregs. Arguing against this possibility is evidence that STAT5 binds poorly to its cognate DNA binding site when it is methylated.[52,53] However, only one of the three potential STAT5 binding sites found in the CNS2 region contains a CpG motif that could be methylated.[10] Thus, whether IL2 and STAT5 promote demethylation of CNS2 requires additional study.

STAT5 also governs Treg function. For example, Blazar and colleagues demonstrated that Tregs expressing a constitutively active form of STAT5b (*Stat5b-CA* mice) are superior to WT Tregs in protecting mice from graft-versus-host disease.[54] This was due to a number of factors including (1) improved homeostasis of transferred Tregs, (2) augmented Treg suppressor function and (3) reduced ability of *Stat5b-CA* effector T cells to differentiate into T_H1 and T_H17 cells. Supporting these observations, Malek and colleagues demonstrated that IL2 and STAT5 signaling are required for development of a population of $KLRG1^+$ Treg cells that appear to express elevated levels of many factors required for Treg function, such as IL10.[55] Interestingly, this report documented that quite modest levels of IL2/STAT5 signals are required for thymic Treg development and peripheral survival. In contrast, the development of $KLRG1^+$ Tregs, which represent a terminally differentiated form of Treg with augmented suppressor function, require much stronger IL2/STAT5 dependent signaling. In many ways this latter population resembles $KLRG1^+$ effector $CD8^+$ T cells suggesting that these two populations may develop via similar mechanisms.

STAT Family Influences on Treg Differentiation

As mentioned above, STAT5 has been shown to inhibit the production of IL17 both in vivo and in vitro. The mechanism underlying this has been attributed to the ability of STAT5 to directly compete with IL6-dependent STAT3 binding to enhancers within the *Il17* gene locus.[56] STAT5 binding within the *Il17* gene locus correlated with recruitment of the co-repressor NCOR2, which is known to interact with STAT5. Furthermore, IL2 downregulates IL6 receptor expression on iTregs which further acts to prevent iTregs from differentiating into T_H17 cells.[57] Thus the balance between STAT5 and STAT3 signaling plays an important role in directing iTreg development.

Other cytokines including IL4 and IL12 have also been shown to antagonize Treg development.[58,59] These cytokines activate STAT6 and STAT4 respectively, which are required for inhibiting Treg differentiation.[58] While the mechanism by which STAT6 and STAT4 inhibit Treg development remains to be precisely defined, both of these factors have been shown to reduce STAT5 binding to the promoter and/or CNS2 region of the *Foxp3* gene.[58] Thus, cytokine crosstalk plays an important role in directing the differentiation of iTregs.

As mentioned above with regard to thymic Treg development, STAT5 interacts with a number of potential binding partners. The role of these binding partners in iTreg development remains to be defined. Interestingly, recent work from the O'Shea lab has shown that micro RNAs activated by TGFβ and retinoic acid receptor α (RARα) suppress the expression of one of these binding partners, the co-repressor NCOR2.[60] Specifically, this study demonstrated that conversion of iTregs into T follicular helper cells (T_{FH}) is limited when *mir10a* is expressed at high levels, such as is the case following TGFβ and RARα signaling in the periphery. It appears that *mir10a* may have a role in fixing the iTreg cell lineage by suppressing conversion of iTregs into either T_H17 or T_{FH} cells. Additional studies are needed to more precisely define the role of NCOR2 and other STAT5 interacting partners on the development and maintenance of regulatory T cells.

Conclusions

Work over the past 20 years has clearly documented an important role for IL2 and STAT5 in shaping the development of both natural and induced Tregs. More recent studies point to a critical role for IL2/STAT5 signals in modifying the functional activity of regulatory T cells. However, the molecular mechanisms by which STAT5 and its many potential binding partners influence Treg biology are just beginning to be explored. A better understanding

of how the IL2/STAT5 pathway governs Treg biology may allow for more targeted approaches for augmenting or inhibiting Treg function. Such information could be particularly useful in developing strategies to inhibit Treg responses to tumors or to augment Treg responses in autoimmunity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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