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Rapamycin-resistant effector T-cell therapy

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Summary:

Pharmacologic inhibition of the mechanistic target of rapamycin (mTOR) represents a stress test for tumor cells and T cells. Mechanisms exist that allow cells to survive this stress, including sub-optimal target block, alternative signaling pathways, and autophagy. Rapamycin-resistant effector T (T-Rapa) cells have an altered phenotype that associates with increased function. *Ex vivo* rapamycin, when used in combination with polarizing cytokines and antigen-presenting-cell free costimulation, is a flexible therapeutic approach as polarization to T-helper 1 (Th1)- or Th2-type effectors is possible. Murine T-Rapa cells skewed toward a Th2-type prevented graft rejection and graft-versus-host disease (GVHD) more potently than control Th2 cells and effectively balanced GVHD and graft-versus-tumor (GVT) effects. A phase II clinical trial using low-intensity allogeneic hematopoietic cell transplantation demonstrated that interleukin-4 polarized human T-Rapa cells had a mixed Th2/Th1 phenotype; T-Rapa cell recipients had a balanced Th2/Th1 cytokine profile, conversion of mixed chimerism toward full donor chimerism, and a potentially favorable balance between GVHD and GVT effects. In addition, a phase I clinical trial evaluating autologous T-Rapa cells skewed toward a Th1- and Tc1-type is underway. Use of *ex vivo* rapamycin to modulate effector T-cell function represents a promising new approach to transplantation therapy.

Keywords

Th1/Th2/Th17; graft versus host disease; apoptosis/autophagy; transplantation; cytokines

Rapamycin resistance and transplantation therapy, an introduction

Allogeneic hematopoietic cell transplantation (HCT) is an important arena for novel adoptive T-cell therapy efforts, because donor T cells mediate curative graft-versus-tumor (GVT) effects and prevent the host-versus-graft (HVG) immune response that restricts alloengraftment. In addition, donor T cells mediate the main toxicity of allogeneic HCT, graft-versus-host disease (GVHD). The clinical need to promote alloengraftment and mediate GVT effects with limited GVHD has prompted current investigations into the use of T cells of defined antigen specificity, cytokine phenotype, or differentiation status. A common denominator for such investigations is the requirement for *ex vivo* manufacturing, which offers an opportunity to evaluate the effect of various biologics or pharmaceutical agents on T-cell function. In our research, we have evaluated the *ex vivo* effect of rapamycin (sirolimus) on T cells in light of the long history of using this drug *in vivo* to modulate transplantation responses. Through these efforts, we have determined that primary murine

and human CD4⁺ and CD8⁺ T cells can rapidly acquire resistance to rapamycin, and in the process, undergo a diversity of functional alterations that associate with increased *in vivo* effects upon adoptive transfer. This article focuses on the biology of rapamycin resistance and summarizes progress relating to transplantation therapy using rapamycin-resistant T cells.

Rapamycin and the mechanistic target of rapamycin (mTOR)

It is fortuitous for transplant and cancer patients, physicians, and now biologists of nearly every discipline that the natural product rapamycin was discovered on Easter Island, with the first report of its anti-fungal properties published in 1975 (1). Rapamycin was approved by the Food and Drug Administration in 1999 for use as an immunosuppressant. More recently, two drugs that share the same mechanism of action as rapamycin ('rapalogs') have been approved for use in the treatment of metastatic renal cell carcinoma (2, 3). In parallel with this clinical drug development has been extensive basic research into the mechanistic target of rapamycin (mTOR), which has been summarized recently (4).

mTOR is a serine/threonine protein kinase of the phosphoinositide 3-kinase (PI3K)-related family. mTOR, which is the key catalytic domain that dictates downstream cellular programs, interacts with either six or seven proteins to form the large mTOR complexes known as mTORC1 (uniquely contains raptor) and mTORC2 (uniquely contains rictor) respectively. Rapamycin, once it binds with the intracellular 12-kDa FK506-binding protein (FKBP12) (5), can directly inhibit mTOR as it exists within the mTORC1 complex but not the mTORC2 complex. The rapamycin-FKBP12 complex stabilizes the raptor-mTOR association and compromises the structural integrity of mTORC1, thereby reducing mTOR kinase activity (6, 7). As detailed below, although rapamycin can directly influence only mTORC1, subsequent indirect modulation of mTORC2 can occur. As such, it is essential to consider both mTORC1 and mTORC2 pathways when one considers the biologic effects of rapamycin.

Summary of upstream mTORC1 events

As recently summarized (4), the mTORC1 pathway has been extensively characterized and found to integrate cellular response to growth factors and levels of energy, stress, oxygen, and amino acids. These fundamental processes are under the control of dozens of molecules that lie upstream or downstream to mTORC1, including numerous tumor-related and tumor-suppressor genes. The GTP-bound form of Rheb lies immediately upstream of mTORC1 to stimulate mTOR kinase activity; however, just upstream to Rheb is the tumor suppressor complex tuberous sclerosis 1/2 (TSC1/2) that negatively regulates mTORC1 by converting Rheb to the inactive GDP-bound form (8).

This TSC1/2 complex can be inhibited through phosphorylation by multiple pathways en route to mTORC1 activation: protein kinase B (Akt) (8), ras via extracellular-signal-regulating kinase 1/2 (ERK1/2) (9), ribosomal S6 kinase (RSK1) (10), pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) (11), and Wnt signaling (12). Converse to these processes that activate mTORC1 through TSC1/2 inhibition, increased TSC1/2 activity reduces mTORC1 signaling in various states of stress: hypoxia or low-energy, via

adenosine monophosphate-activated protein kinase (AMPK) (13); and DNA damage, through a p53-dependent mechanism (14, 15). In sum, these observations demonstrate that the TSC1/2 inhibitory complex is central to control of the majority of processes that mTORC1 regulates. However, mTORC1 function is regulated independent of TSC1/2 in the case of amino acid sensing (16, 17) and sensing of phosphatidic acid, which is a lipid critical for cell growth and signal transduction (18, 19).

Summary of events downstream to mTORC1

mTORC1 controls many key cellular processes, including protein and lipid synthesis, metabolism, and autophagy. Protein synthesis is promoted by mTORC1 via activation of S6 kinase 1 (S6K1) and inactivation of the inhibitory molecule 4E-BP1 (reviewed in 20). Fatty acid and cholesterol synthesis is accelerated by mTORC1 through upregulation of transcription factors [sterol regulatory element-binding protein 1/2 (SREBP1/2)] (21). mTORC1 activation, through its promotion of hypoxia-inducible factor (HIF-1- α) transcription and translation, yields an increase in glycolysis; the pleiotropic effects of mTORC1 on immune cell metabolism has recently been thoroughly detailed (22). Finally, mTORC1 phosphorylates and inhibits an Atg13-containing kinase complex that initiates autophagy (23), which is a membrane cannibalization process invoked in states of cellular starvation for the purpose of restricting cell growth and generating metabolic substrates for the avoidance of cell death (reviewed in 24).

mTORC2, the lesser known complex

Relative to mTORC1, less information exists pertaining to the role of the mTORC2 complex. It is important to acknowledge that significant crosstalk occurs between mTORC1 and mTORC2, with mTORC1 activation and downstream S6K1 phosphorylation suppressing mTORC2 (26); this biology appears to represent yet another brake on immune T-cell activation. As discussed, mTORC1 senses the presence or absence of nutrients for control of T-cell expansion or contraction respectively. By comparison, mTORC2 must associate with ribosomes to receive cell surface growth factor receptor signaling for resultant downstream activation of several kinases, including Akt (27); because ribosomes are required for cellular growth, mTORC2 helps ensure that only well-nourished cells will execute growth factor signaling (28). Another interesting point arises from this information, which is relevant later when discussing rapamycin-resistance: that is, that Akt lies both upstream to mTORC1 and downstream to mTORC2.

Rapamycin directly inhibits mTORC1, indirectly inhibits mTORC2

Although the rapamycin-FKBP12 complex can physically bind to only the mTORC1 complex, the significant crosstalk between mTORC1 and mTORC2 results in substantial rapamycin-mediated effects on mTORC2. In the short-term (perhaps measured in hours), rapamycin effects can be specifically directed to mTORC1, thereby supporting the conclusion that mTORC2 is rapamycin-insensitive (29); however, long-term treatment with rapamycin (perhaps measured in days to weeks) can reduce both mTORC1 and mTORC2 signaling because prolonged mTORC1 inhibition can suppress mTORC2 assembly (30). Prolonged rapamycin therapy can inhibit Akt-driven angiogenesis, thereby indicated the physiologic significance of rapamycin-mediated mTORC2 inhibition (31). A rapalog

blocked Akt-mediated acute myelogenous leukemia growth by disrupting mTORC2 formation (32). Furthermore, rapamycin caused insulin resistance not through acute mTORC1 effects but through mTORC2 alterations (33). *In vivo*, rapamycin therapy reduced both mTORC1 activity (clinically evident by murine host longevity) and mTORC2 activity (clinically evident by impaired glucose tolerance) (34). In sum, these data indicate that in many *in vitro* and *in vivo* situations, rapamycin inhibits both mTORC1 and mTORC2.

Rapamycin-resistance mechanisms (tumor cell focus)

As early as 1994, it was understood that various cancer cell lines intrinsically possessed differential sensitivity to inhibition by rapamycin (35). Initial rapamycin-resistance research focused on the potential role of mutations in key pathway players, including FKBP12 (36) and mTOR (37). Yet, in a prescient review in 2001, at a time when a great deal of mTOR biology was still unresolved, it was fully realized that multiple mechanisms of acquired resistance to rapamycin existed (38). The topic of cancer cell rapamycin resistance, which is a deeper literature relative to T-cell rapamycin resistance, has been recently updated (39). Because similar resistance pathways likely exist in cancer cells and immune T cells, the cancer cell literature is briefly summarized.

Resistance mechanism #1: insufficient mTORC1 block

Although sophisticated mechanisms of rapamycin resistance can be entertained, perhaps the most prominent mechanism relates to sub-optimal targeting of mTORC1 (Fig. 1). Recently, the ability of rapamycin to block mTORC1 has been compared with agents that are direct mTOR kinase inhibitors, which directly inhibit both mTORC1 and mTORC2 in an FKBP12-independent manner; a recent review article (40) focused on the clinical development of various mTOR kinase inhibitors. Increasing evidence indicates that rapamycin differentially modulates mTORC1 effector function, with greater inhibition of the S6K pathway compared with the 4E-BP1 pathway (41, 42); by comparison, direct mTOR kinase inhibitors more potently and equally inhibit mTORC1-directed S6K and 4E-BP1 pathways, thereby resulting in a more profound block in protein synthesis (43).

The enhanced ability of direct mTOR kinase inhibitors to more fully inhibit mTORC1 effector function has translated into improved anti-tumor efficacy in multiple models (41, 42, 44, 45). Interestingly, the increased efficacy of these agents relative to rapamycin has been primarily attributable to more effective mTORC1 inhibition rather than the direct mTORC2 inhibition offered by these agents (41, 46). It is important to note that rapamycin and direct mTOR kinase inhibitors also differentially modulate dendritic cell (DC) function, with the latter compounds generating a more suppressive DC phenotype having increased B7-H1 expression and an increased ability to induce regulatory T cells (Tregs) (47). The dramatic difference in the capacity of rapamycin and direct mTOR kinase inhibitors to modulate mTOR effector function was quantified in a recent study using NIH-3T3 cells: relative to rapamycin, direct mTOR kinase inhibitors yielded a 20-fold increase in differentially regulated genes, including those in the 4E-BP1 pathway (48).

Resistance mechanism #2: mTORC2-mediated Akt positive feedback loop

Because of the crosstalk between the mTORC1 and mTORC2 pathways, rapamycin inhibition of mTORC1 can result in a shift toward increased mTORC2 activity, which subsequently leads to increased downstream kinase activity, including Akt (27). Prevention of this mode of resistance can be overcome by use of the following: prolonged rapamycin therapy, with resultant disruption of the mTORC2 complex (30); histone deacetylase inhibitors to block compensatory mTORC2 activation (49); direct mTOR active site inhibitors to achieve simultaneous mTORC1 and mTORC2 inhibition (44); or agents with combined targeting of mTOR and Akt (40).

Resistance mechanism #3: PIM kinases

Another form of tumor cell resistance to rapamycin is mediated by the proviral insertion site in Moloney murine leukemia virus (PIM) proteins (reviewed in 50). The PIM proteins are a family of serine/threonine kinases that are primarily regulated by gene transcription; and, these kinases are constitutively active once they are produced. The expression of PIM kinases is directly increased (independent of mTOR) by cell surface cytokine receptor signaling [such as through the IL-3 receptor (51)]. A recent review (52) further summarizes the circuitry of this pathway, namely the sequential: activation of cell surface growth factor and cytokine receptors; activation of Janus kinase (JAK) 1/2/3 molecules and signal transducer and activator of transcription (STAT) molecules (reviewed in 53); and finally, expression of the PIM kinases. This sequence of events leads to the block of cell cycle inhibitors such as p21Cip1/WAF1 (54) and p27Kip1 (55), activation of Myc with subsequent cell cycle progression (56), and inhibition of bcl-2 and other pro-apoptotic molecules (57). Indeed, the PIM genes operate as a weak oncogene and are upregulated in leukemia, lymphoma, and solid tumors, such as prostate cancer. Because of this segregation of the PIM and mTOR signaling pathways, inhibitors of the PIM kinases are being evaluated in combination with mTOR inhibitors.

Resistance mechanism #4: autophagy

Autophagy can be viewed as a double-edged sword in cancer therapy, as this complex process of membrane organelle downsizing likely controls initial tumor development but may promote the growth of established tumors (58). Activation of mTORC1 limits autophagy, thereby permitting the accumulation of protein aggregates, damaged mitochondria, and reactive oxygen species that predispose to cancer development; such observations provide some support for efforts to use rapamycin as an mTORC1 inhibitor for cancer prevention. In contrast, autophagy in late-stage cancer can act as a survival mechanism, with tumor cells using the autophagic membranes as a source of substrates to fuel their high metabolic requirements (reviewed in 59, 60). Consistent with this line of logic, tumor cells that are not only inhibited at mTOR but also restricted in autophagy by exposure to molecules such as 3-methyladenine (61) or chloroquine (62) are stripped of a survival mechanism and undergo cell death. Of note, the direct mTOR kinase inhibitors are more effective than rapamycin for induction of autophagy even in cells lacking raptor, which is necessary for mTORC2 function (46); these observations represent further evidence that rapamycin only partially inhibits mTORC1. It is important to note that marked autophagy

and progressive cellular starvation can result in cell death rather than a survival advantage: as such, the more potent direct mTOR kinase inhibitors are associated with autophagy followed by cell death rather than cell survival (63).

Resistance mechanism #5: p53 deficiency and other mTOR pathway amplifiers

Dysregulation of multiple genes in the mTOR pathway, including TSC1/2, is associated with familial cancer disorders (reviewed in 64), thus making rapamycin a rational treatment approach in this setting. Beyond this, however, are the majority of human cancers that also have increased activation of the mTOR pathway, either through PI3K/Akt activation or p53 tumor suppressor gene deficiency. Indeed, p53 status helps determine the response to rapamycin (65): that is, loss of p53 promotes mTORC1 activation whereas wild-type p53 controls mTORC1 activation through upregulation of AMPK and TSC1/2 (15). Although these observations might suggest that rapalogs would be broadly effective against cancer, the existence of multiple genetic defects in tumor cells can reduce the efficacy of rapalog therapy; for example, cancer cells with loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) or PI3K gain that also harbor KRAS mutations are predisposed to developing rapamycin resistance (66). There is emerging evidence that mTORC2 also plays a role in cancer signaling, as increased rictor expression, which drives Akt signaling, is elevated in 70% of glioma cases (67), and loss of the tumor suppressor PTEN requires an intact mTORC2 pathway for tumor development in a prostate cancer model (67). Clearly, numerous molecules in the mTORC1 and mTORC2 pathways interact to modulate cancer cell sensitivity to rapamycin.

Resistance mechanism #6: growth factor receptor positive feedback loop

Previously, it has been documented that mTORC1 activation and resultant phosphorylation of S6K exerts a negative regulatory signal at the level of insulin receptor signaling (68). Conversely, mTORC1/mTORC2 inhibition can result in multiple myeloma tumor cell resistance via elevated signaling along the receptor tyrosine kinase-PI3K pathway (69). Furthermore, in a recent study, mTOR blockade promoted a cytokine-driven and insulin receptor substrate (IRS)-driven activation of the JAK/STAT signaling pathway that conferred tumor cell resistance in a model of metastatic breast cancer (70). The authors concluded that the IRS-driven events could promote growth factor signaling both through the PI3K/Akt/mTOR pathway and through the JAK/STAT pathway; the JAK/STAT pathway would then presumably operate in part by upregulating PIM kinases. In sum, these data indicate that mTORC1 puts a brake on growth factor and cytokine receptor signaling, the release of which results in a compensatory increase in signaling and resultant cellular resistance to mTOR inhibition.

Tumor cell rapamycin resistance: implications for adoptive T-cell therapy

The increasing depth and breadth of understanding regarding the intrinsic and acquired mechanisms of tumor cell resistance to mTOR inhibition has several implications for adoptive T-cell therapy efforts. First, new understandings derived from cancer cell studies may motivate new experimental avenues for better characterization of T-cell mechanisms of rapamycin resistance. Second, new approaches to avoid rapamycin resistance *in vivo* during cancer therapy may facilitate new advances in transplantation medicine (for example, an

improved ability to treat refractory GVHD). And third, new understandings in mTOR biology and the development of new agents that target various components of the mTOR pathway may help identify novel *ex vivo* approaches to the modulation of T-cell function. Conceivably, these aspects can be combined in sequence, with more effective mTOR inhibition *in vivo* for improved tumor cell sensitivity followed by adoptive cell therapy using T cells manufactured *ex vivo* to attain various forms and levels of resistance.

T-cell rapamycin resistance

mTORC1, mTORC2 influences on T-cell differentiation

In some *in vitro* conditions, inclusion of rapamycin during costimulation can result in murine T-cell anergy (71); consistent with this initial report, recent studies have demonstrated that rapamycin causes anergy in murine T cells that are activated due to TSC1 deficiency (72). More recently, the role of mTOR in murine T-cell differentiation has been clarified using cells genetically deficient in mTORC1 or mTORC2 components (reviewed in 73, 74). mTOR-deficient murine CD4⁺ T cells failed to differentiate into effector lineages even under optimal conditions, instead defaulting to Treg differentiation; this effect required not only mTORC1 but also mTORC2 ablation (75). mTORC1-deficient T cells could be polarized to a Th2-type but were not able to attain Th1 or Th17 differentiation; by comparison, mTORC2 deficient T cells could be polarized to the Th1 or Th17 lineages but were refractory to Th2 differentiation (76). In other studies, mTORC2-deficient T cells were impaired in Th1 and Th2 differentiation capacity but had relatively intact Treg and Th17 lineage commitment (77).

Because rapamycin has only partial inhibitory effects on mTORC1 and can indirectly inhibit mTORC2, there exist significant differences in methodology when one compares genetic deletion versus pharmacologic approaches to the study of mTOR as it relates to T-cell biology. Perhaps the direct mTOR kinase inhibitors, which more profoundly inhibit both mTORC1 and mTORC2, mimic such genetic-deletion studies to a greater extent than rapamycin. It should also be noted that to this date, there are no reports of pharmacologic agents that specifically target either mTORC1 or mTORC2. Nevertheless, it was reported that the combination of rapamycin with sub-optimal dosing of a direct mTOR kinase inhibitor resulted in preferential and marked mTORC1 inhibition, with resultant potent downstream effects on autophagy induction and restriction of protein synthesis (78).

Previous studies describing rapamycin-resistant effector T cells

It has long been known that the culture of human T-cell lines with rapamycin for several months can yield rapamycin-resistant cell expansion (79). Initial observations determined that human T cell IL-2 receptor signaling could be resistant to the inhibitory effects of rapamycin (80) and that rapamycin resistance in murine T cells was mediated in part by downregulation of the cell cycle inhibitor, p27kip1 (81). In other studies, human CD8⁺ T cells rendered rapamycin-resistant relied upon PI3K signaling for survival and were characterized by downregulation of p27kip1 and increased expression of anti-apoptotic bcl-xL (82); in addition, rapamycin-resistant human CD8⁺ T cells maintained antigen-driven proliferation in spite of effective S6K blockade (83). In contrast with these prior studies,

where rapamycin-resistance was achieved by *ex vivo* drug exposure, investigators have recently introduced resistance into human T cells by transfer of a transgene encoding a rapamycin-resistant mutant of mTOR; such T cells, which were also modified with a chimeric antigen receptor against CD19, avoided the suppressive effects of rapamycin and mediated increased *in vitro* killing against leukemia targets (84).

Rapamycin and regulatory T-cell differentiation *ex vivo*

As previously discussed, genetic-deficiency in mTORC1 and mTORC2 constrains murine effector CD4⁺ T-cell differentiation *in vitro*, resulting in a shift toward immunosuppressive Treg cells (75). Rapamycin, when combined with antigen-presenting cell (APC)-free T-cell activation in the absence of polarizing cytokines, can selectively expand Treg cells (85). Human CD4⁺ T cells, when cultured in the presence of rapamycin and the absence of polarizing cytokines, can yield rapamycin-resistant Treg cells through a mechanism mediated in part by Foxp3 transcription factor upregulation of PIM2 kinase (86). An absence of polarizing cytokines appears to be an important key to an ability to promote Tregs *in vitro*: polarization with IL-4 leads to GATA3 transcription factor expression, which inhibits Treg development (87), and IL-12 leads to T-bet transcription factor expression, which can also limit Treg differentiation (88, 89). In sum, these data indicate that an absence of polarizing cytokines, when combined with effector differentiation inhibition through rapamycin-induced mTOR blockade, promotes Treg differentiation.

Rapamycin modulation of effector T-cell differentiation and metabolism

In contrast with this information regarding the role of rapamycin in promoting immunosuppressive Treg cells, an emerging literature indicates that *in vivo* rapamycin can improve CD8⁺ T-cell immunity through its effects on differentiation and metabolism (reviewed in 22, 90). *In vivo* rapamycin treatment, acting through the mTORC1 complex in murine CD8⁺ T cells, increased the quantity and quality of anti-viral memory cells (91). Rapamycin blocked IL-12 (92)- or IL-7 (93)-induced effector CD8⁺ T-cell maturation, with a resultant shift in transcription factor expression from T-bet to eomesodermin and an increase of *in vivo* memory function and anti-tumor efficacy.

More recently, the rapamycin-mediated promotion of a CD8⁺ T-cell memory response was attributed to increased expression of the Foxo1 transcription factor (94). Rapamycin promotes the expression of yet another transcription factor, KLF2, which helps execute the memory T-cell program (91), including increased transcription of the lymph node homing molecule CD62L (95). *Ex vivo* rapamycin and subsequent adoptive transfer of LCMV-specific murine CD8⁺ T cells also conferred an increase in memory T cells *in vivo* (96). Such rapamycin-resistant CD8⁺ T cells had metabolic changes associated with increased T-cell function, including both increased glycolysis and oxidative phosphorylation; of note, increased oxidative phosphorylation is a metabolic attribute that also associates with increased T-cell-mediated experimental GVHD (97). In sum, these data indicate that rapamycin can improve T-cell function through its effects on differentiation and metabolism.

Rapamycin and experimental allogeneic BMT

We have implemented our studies of cytokine-polarized, rapamycin-resistant T cells in the context of experimental allogeneic bone marrow transplantation (BMT) (Fig. 2). In particular, host elements are comprised of tumor cells (which are the focus of the GVT response), dendritic cells (which initiate GVHD) (98), and T cells remaining after host conditioning (which mediate graft rejection) (99). Relevant components from the donor standpoint include hematopoietic progenitor cells and unmanipulated CD4⁺ and CD8⁺ T cells, which initiate GVHD (100).

Experimental allogeneic BMT: *in vivo* rapamycin drug therapy

There has been a long history of studying rapamycin in the context of experimental allogeneic BMT, with initial studies identifying the ability of post-transplant rapamycin to prevent graft rejection (101) and prevent both GVHD and a graft-versus-leukemia response (102). In this latter study, rapamycin therapy shifted the T-cell cytokine phenotype from a Th1- to a Th2-type; this result is consistent with multiple lines of investigation that support a shared biology of GVHD and GVT effects (reviewed in 103). More recent studies have identified multiple additional mechanisms whereby *in vivo* rapamycin therapy can reduce experimental GVHD, including a promotion of CD4⁺ Treg cells, particularly when combined with IL-2 therapy (104). An ability of rapamycin to preferentially support Treg cell expansion post-transplant has been attributed in part to Treg cell preferential expression of PTEN, which is an upstream inhibitor of mTORC1 activity (105). Interestingly, rapamycin in combination with an IL-2 pathway activator also promoted the expansion of a CD8⁺ Treg cell population that reduced experimental murine GVHD (106). In marked contrast with these results, rapamycin actually inhibited Treg cell expansion in the non-transplant setting involving lymphoreplete hosts (107); as such, an ability of rapamycin therapy to promote Treg cells clearly depends on the clinical context. Finally, yet another mechanism whereby rapamycin therapy can reduce experimental GVHD includes modulation of APC populations, as adoptive transfer of rapamycin-exposed dendritic cells reduced GVHD (108). Taken together, these studies highlight distinct but potentially inter-related mechanisms whereby rapamycin therapy might prevent GVHD, including preferential inhibition of effector T cells relative to Treg cells and modulation of APC.

Cytokine-polarized, rapamycin-resistant T cells: differentiation status

In initial experiments, we found that primary murine CD4⁺ and CD8⁺ T cells could be rendered rapamycin-resistant using APC-free costimulation in media containing either IL-4 or IL-12 to generate polarized Th2/Tc2 or Th1/Tc1 populations respectively (109). Such rapamycin-resistant and cytokine polarized T cells secreted minimal levels of cytokines at the end of culture and were characterized by an increased frequency and increased intensity of expression of CD62L that is characteristic of T central memory (T_{CM}) cells. This CD62L induction occurred by day 2 of culture and did not require cell division; these observations are consistent with the now known role of rapamycin in altering T-cell memory status at a transcriptional level (95).

In contrast with previous studies, where rapamycin caused anergy in spite of costimulation (71), we found that rapamycin-resistant (RR) and cytokine-polarized T cells actually had increased effector function. For example, relative to control Th1/Tc1 cells not generated in rapamycin, the RR-Th1/Tc1 cells had increased *ex vivo* expansion once they were removed from rapamycin, increased expansion *in vivo* after allogeneic BMT that resulted in an increased ability to out-compete control Th2/Tc2 cells, and an increased capacity to mediate lethal GVHD. Similarly, the RR-Th2/Tc2 cells had increased expansion *ex vivo* and *in vivo*, which correlated with an increased ability to out-compete control Th1/Tc1 cells. Of note, *ex vivo* acquired rapamycin resistance did not confer *in vivo* resistance to rapamycin, as each polarized population, particularly the Th1/Tc1 subset, was susceptible to *in vivo* suppression by rapamycin. This lack of *in vivo* resistance in spite of *ex vivo* resistance likely relates to rapamycin suppression of allogeneic APC post-transplant, with a resultant decrease in alloantigen-driven T-cell expansion.

These initial findings harmonize to a great deal with current results in adoptive T-cell therapy research, particularly as it applies to the role of T-cell differentiation status. Specifically, in the setting of a murine melanoma model, anti-tumor CD8⁺ T cells with increased CD62L expression and minimal effector function just prior to adoptive transfer mediated enhanced anti-tumor effects (110). In more recent studies, a pharmacologic inhibitor to glycogen synthase kinase 3 β (GSK3- β) mimicked Wnt pathway signaling to endow anti-tumor CD8⁺ T cells with increased CD62L expression and a stem cell phenotype that conferred increased anti-tumor effects upon adoptive transfer (111). GSK3- β promotes TSC2 activity to reduce mTORC1 (12); as such, rapamycin (which reduces mTORC1) and GSK3- β inhibition [which increases mTORC1 (112)] offer very different routes to the pharmacologic modulation of T-cell differentiation. Indeed, combination of GSK3- β inhibition plus rapamycin optimized an expansion of the hematopoietic stem cell pool *in vivo* (112). With respect to adoptive T-cell therapy, it will be interesting then to evaluate mTOR inhibitors in combination with other differentiation pathway modulators (reviewed in 113).

The topic of memory status is a burgeoning area of research in adoptive T-cell therapy. In primates, adoptively transferred central memory CD8⁺ T cells (Tcm) (CD62L-expressing) had increased persistence *in vivo* relative to effector memory cells (Tem) (CD62L-negative) (114). In the setting of experimental allogeneic transplantation, allogeneic murine Tem cells that were CD62L-negative conferred protective immunity without GVHD (115); similarly, both allogeneic CD4⁺ (116) and CD8⁺ T cells (117) of Tcm phenotype mediated increased GVHD relative to their Tem counterparts. In contrast, adoptive transfer of regulatory T cells of Tcm status were enriched for their capacity to prevent GVHD (118); this enhanced *in vivo* efficacy can be attributed to the broad functional phenotype associated with the central memory Treg cells rather than to the CD62L molecule itself (119). In sum, these data fit with a model whereby T cells with a Tcm phenotype in general are more robust *in vivo* after adoptive transfer, with different clinical results being determined by other functional attributes (increased GVHD from non-polarized or Th1-skewed Tcm cells; reduced GVHD from Th2-type or Treg Tcm cells).

Rapamycin-resistant CD4⁺ Th2 cells: prevention and treatment of GVHD

In an initial study, we found that combination of high-dose rapamycin, costimulation, and IL-4 polarization yielded murine CD4⁺ T cells that were enriched in their capacity to prevent experimental acute GVHD. Rapamycin-resistant Th2 cells had minimal effector function at the time of adoptive transfer, as evidenced by minimal cytokine secretion; however, *in vivo*, recipients of the RR-Th2 cells had approximately a 10-fold increase in type II cytokine secretion, a more marked drop in IFN- γ secretion, and enhanced protection against GVHD (120). This anti-GVHD effect was abrogated in the setting of RR-Th2 cell IL-4 deficiency, thereby indicating that the RR-Th2 cells operating by a Th2-type effector mechanism rather than a conventional Treg mechanism. Of note, abrogation of GVHD was also associated with inhibition of a GVT effect; however, delayed infusion of Th2 cells allowed for an anti-tumor effect to first be realized with subsequent modulation of established GVHD (sequential Th1 $\rightarrow$ Th2 strategy). Further studies (121) performed to evaluate additional mechanisms whereby RR-Th2 cells treated established GVHD identified that the anti-GVHD effect required both Th2 cell IL-4 and IL-10 production and was reversed by adoptive transfer of immune-competent host APC; this latter result suggests that donor RR-Th2 cells worked in part, perhaps via IL-10 secretion, to modulate host APC for reduced GVHD. In addition, exogenous administration of IL-2 rescued alloreactive T cells from the RR-Th2 cell inhibition, thereby exacerbating GVHD; these observations suggest that RR-Th2 cells can outcompete other T-cell populations for limiting growth factors. The schema in Fig. 2 summarizes mechanisms whereby RR-Th2 cells can modulate GVHD.

RR-Th2 cells: prevention of graft rejection

Host T cells are also a relevant consideration in adoptive T-cell therapy in the allogeneic transplantation setting because of their capacity to mediate graft rejection. One direction in allogeneic transplantation has been to reduce the conditioning intensity in an attempt to limit morbidity and mortality, particularly in patients of advanced age or organ dysfunction. However, minimization of host conditioning results in higher levels of host immunity and a commensurate increase in T-cell-mediated HVG reactivity that primarily accounts for graft rejection. In previous experimental models, the mechanism whereby allogeneic T cells prevented graft rejection was primarily attributed to cytotoxic mechanisms involving host T-cell deletion (122). Given the enhanced potency of RR-Th2 cells in terms of GVHD modulation through an IL-4 dependent mechanism, we evaluated whether this donor T-cell population might be able to prevent fully MHC-disparate graft rejection through a similar mechanism.

RR-Th2 cells (but not control Th2 cells not generated in rapamycin) indeed effectively abrogated T-cell-mediated graft rejection mediated by host Th1/Tc1 cells that were dependent upon STAT1 signaling (123). The mechanism of rejection abrogation involved RR-Th2 cell secretion of IL-4 and host T-cell signaling via the STAT6 pathway, which yielded host T cells that were Th2-polarized and reduced in their capacity to cause graft rejection. More recently, we have demonstrated that RR-Th2 cells may also represent a strategy for the prevention of solid organ allograft rejection: that is, adoptive transfer of host-type RR-Th2 cells prevented rejection in a rat model of cardiac allografting (124).

As such, RR-Th2 cells modulated both GVHD and graft rejection in part through their ability to secrete high levels of IL-4 *in vivo* for the modulation of donor or host T cells respectively. In other studies, we found that rapamycin-resistant donor T cells expressed a multi-faceted anti-apoptotic phenotype that facilitated multiple rounds of T-cell expansion *in vivo* and T-cell persistence (125). In addition, in this study, we determined that the rapamycin-resistant donor T-cell product had reduced expression of the activated, phosphorylated forms of molecules downstream to mTORC1 (S6K and 4E-BP1) and downstream to mTORC2 (Akt); these findings indicated that high-dose rapamycin for the 6-day T-cell culture interval effectively blocked both mTOR pathways. In contrast, RR-T cells had sustained expression of PIM2, thereby offering an alternative signaling pathway to allow T-cell growth and differentiation during potent mTOR inhibition.

Role of autophagy in rapamycin-resistant T-cell therapy

In subsequent research, we shifted our attention to human T cells, with a particular focus on determining whether autophagy, which is promoted during mTORC1 inhibition, might be playing a role in the rapamycin-resistant phenotype of cytokine polarized effector cells. Similar to our murine results, we determined that primary human CD4⁺ and CD8⁺ T cells could be polarized to a type I cytokine phenotype in high-dose rapamycin provided that APC-free costimulation was utilized in combination with high doses of the STAT1/STAT4 signaling cytokines IL-12 or IFN- α (126). Rapamycin-resistant human Th1/Tc1 cells expressed high levels of T-bet and minimal GATA3 or FOXP3; after adoptive transfer into immunodeficient murine hosts, such RR-Th1/Tc1 cells maintained their type I cytokine phenotype and cytolytic effector function. Relative to control Th1/Tc1 cells not generated in rapamycin, RR-Th1/Tc1 cells had increased expression of the T_{CM} markers CD62L and CCR7, increased persistence *in vivo*, and an increased capacity to mediate lethal xenogeneic GVHD. In sum, these data are consistent with our murine observations and indicate that APC-free costimulation in the presence of optimal polarizing cytokines can facilitate the development of rapamycin-resistant T cells of various effector lineages that have an altered differentiation status and enhanced *in vivo* function.

In these studies, we investigated several different pathways that may have contributed to the human effector T-cell acquisition of rapamycin resistance. First, using autophagy inhibition by both a pharmacologic approach (3-methyladenine) and an siRNA approach (to the Atg protein Beclin-1), we determined that rapamycin resistance required autophagy. Mitochondrial autophagy was operational, as the RR-Th1/Tc1 cells had reduced mitochondrial mass. It is interesting to note that induction of mitophagy improved the quality of T-cell mitochondria, as the RR-Th1/Tc1 cells had stabilization of mitochondrial membrane potential, a favorable balance of pro- and anti-apoptotic members of the bcl-2 family member gene family, and reduced apoptosis.

These results support the conclusion that autophagy can be harnessed in attempts to improve adoptive T-cell therapy (reviewed in 127). In addition to invoking autophagy, we found that other survival mechanisms were likely at play that permitted human T-cell polarization and cell survival in the face of high-dose rapamycin, including inefficient blockade of the mTORC1 target by rapamycin, particularly with respect to the 4E-BP1 pathway, maintained

signaling through the PI3 kinase pathway, and preserved expression of the PIM1/PIM2 kinases. This latter finding is consistent with a recent report that found the PIM kinases to be critical for human Th1 differentiation via IL-12/STAT4 signaling (128).

Clinical trials using allogeneic rapamycin-resistant Th2/Th1 cells

In an initial clinical trial that did not use *ex vivo* rapamycin, we evaluated the infusion of IL-4 polarized and costimulated donor CD4⁺ T cells in the setting of allogeneic HCT using reduced-intensity conditioning. The manufactured T-cell products were maintained for 20-days in culture with two rounds of costimulation; as a result, the T-cell products produced relatively high levels of cytokines commensurate with a Tem differentiation state. Recipients of the manufactured T cells had rates of T-cell-mediated toxicity (engraftment syndrome and acute GVHD) that were comparable to the protocol control group that did not receive the polarized T cells (129). This protocol utilized relatively stringent pretransplant host CD4⁺ T-cell depletion (to levels <50 cells/ μ l) and conditioning that consisted of fludarabine combined with cyclophosphamide at a total dose of 4800 mg/m². As a consequence, rapid full donor engraftment was uniformly achieved in both cohorts; thus, it was not possible to detect a pro-engraftment effect of the manufactured T cells. Similarly, there was no clear difference between the cohorts in terms of anti-tumor effects. This protocol established the safety of the manufactured T cells, but a clinical benefit was not realized.

In light of our murine data indicating an improved ability of RR-Th2 cells to both reduce GVHD and prevent graft rejection, we developed a new protocol (www.cancer.gov/clinicaltrials; #) that incorporated rapamycin during *ex vivo* manufacturing (Fig. 3A). In addition, the transplant platform was modified in an attempt to improve safety and improve an ability to detect any potential pro-engraftment effect of the manufactured T cells (Fig. 3C shows a schema of the protocol design). First, in terms of protocol platform modification, the requirement for host CD4⁺ T-cell depletion was made less stringent (to a CD4 count <200 cells/ μ l) and the conditioning intensity in terms of cyclophosphamide dose was reduced by 75% (to a total dose of 1200 mg/m²), thereby permitting outpatient conditioning. These protocol changes were implemented in an attempt to achieve an initial post-transplant state of mixed donor/host chimerism, which would both predictably reduce GVHD and allow for identification of engraftment promotion as a protocol endpoint.

We added a short-course of sirolimus drug therapy in the first 14 days post-transplant to complement standard cyclosporine GVHD prophylaxis. The goal of this intervention was to control GVHD initiated by the unmanipulated T cells contained in the G-CSF-mobilized peripheral blood allograft. In collaboration, we have piloted a short-course sirolimus GVHD prophylaxis approach (130). The typical use of sirolimus after transplantation consists of prolonged drug administration, which has been associated with effective prevention of acute GVHD (131) and improved anti-tumor outcome in lymphoma patients (132).

A third protocol modification consisting of moving the infusion of manufactured T cells from day 0 of transplant to day 14 post-transplant for a preemptive donor lymphocyte infusion (DLI) strategy. This latter maneuver allowed us to separate potential side effects of the manufactured T cells from side effects attributable to the conditioning and the

unmanipulated T cells, incorporate an early confirmation assay of mixed chimerism at day 14 post-transplant prior to the DLI, and adoptively transfer T-Rapa cells without concurrent sirolimus drug administration, which might inhibit the manufactured T cells *in vivo*.

We have recently reported results from a phase II study of $n = 40$ patients that received pre-emptive DLI at day 14 post-transplant with rapamycin-resistant donor T cells (133). This protocol was conducted at the NIH Clinical Center and at Hackensack University Medical Center as a multi-center site; the manufactured T cells were produced centrally at the NIH Department of Transfusion Medicine. The manufactured T-cell product, which was generated in 12-days of culture using a single round of co-stimulation was characterized by a mix of $CD4^+$ Th2 and Th1 cells as determined by GATA3 to T-bet transcription factor ratio (Fig. 3B) and cytokine secretion profile; because the cell products contained both Th2 and Th1 effectors, we have referred to the products as 'T-Rapa' cells. Similar to our murine studies, the T-Rapa cell products were minimally differentiated, as indicated by very low (pg/ml) quantities of cytokine secretion; however, with further time in culture in the absence of rapamycin, T-Rapa cells had an increased magnitude of cytokine secretion, again in a balanced Th2 to Th1 profile. By gene expression micro-array analysis, we found that there was minimal variability in phenotype between T-Rapa cell products, with consistent gene family upregulation (for example, genes involved in cell cycle, stress response, and glucose catabolism) and gene family downregulation (for example, genes involved in apoptosis, inflammation, and cytokine production).

In this single-arm phase II study, several observations suggest that the T-Rapa cell infusions resulted in distinct modulation of transplantation responses. However, firm conclusions will require subsequent randomized studies to compare T-Rapa cell DLI with standard DLI using unmanipulated donor T cells. First, T-Rapa cell recipients had mixed donor/host chimerism at day 14 post-transplant at the time of DLI, with a relatively marked shift toward full donor elements by day 28 post-transplant; again, controlled studies will be required to determine whether such an increase in donor chimerism would also be mediated by a standard DLI. Second, the immune reconstitution pattern in T-Rapa cell recipients was characterized by preferential expansion of donor $CD4^+$ T cells with constrained expansion of donor $CD8^+$ T cells (the T-Rapa cell products were $>99\%$ $CD4^+$); in addition, T-Rapa cell recipients had a balance between Th2 and Th1 subset reconstitution post-transplant, as determined by transcription factor analysis (Fig. 3D) and cytokine secretion profile. Third, the overall transplant approach was relatively safe, as there was no engraftment syndrome, a rate and severity of GVHD that was not excessive, and no transplant-related mortality. Finally, sustained complete remissions were attained in a subset of patients with hematologic malignancy, including some patients with chemotherapy-refractory disease or high-risk diagnoses.

Current evaluation of T-Rapa cells manufactured for 6 days in culture

As discussed above, a significant literature indicates that T-cell differentiation status can help determine the efficacy of adoptive T-cell therapy, and as such, we have reasoned that truncation of the T-Rapa cell manufacturing interval might generate a unique cell product with increased *in vivo* function. This effort is analogous to a recent effort whereby tumor-

infiltrating-lymphocytes cultured for a shorter interval than previous studies were shown to be efficacious in the treatment of metastatic melanoma (134). Toward this end, we developed a new manufacturing method where the T-Rapa cells are cultured for 6 days (T-Rapa₆ cells) instead of the 12-day interval used in our initial study (T-Rapa₁₂ cells).

In a recent study (135), we found that the T-Rapa₆ and T-Rapa₁₂ cell products were very similar in terms of standard assays such as cytokine secretion profile but differed dramatically upon more detailed comparison using gene expression microarray. Compared with culture input CD4⁺ T cells, the T-Rapa₆ and T-Rapa₁₂ populations were similar in terms of major changes in gene expression. For example, each T-Rapa population had similar: increase in expression of Th1 and Th2 genes and lack of induction of Treg genes; upregulation of cell cycle, stress response, and glucose catabolism genes; and downregulation of apoptosis, inflammation, and cytokine production genes. Nevertheless, approximately 200 genes were consistently and markedly different between the T-Rapa₆ and T-Rapa₁₂ cell products; many of these genes related to relevant pathways such as apoptosis and metabolism, suggesting that the differences between the cell products might translate into differential *in vivo* effects. Clinical trials evaluating the T-Rapa₆ cell product in the exact same transplant platform that we have developed are being implemented.

Ongoing and future directions in rapamycin-resistant T-cell therapy

In addition to further evaluation of the T-Rapa₆ cell product in the allogeneic transplantation setting, we have recently initiated a phase I study of rapamycin-resistant CD4⁺ and CD8⁺ T cells of Th1/Tc1 phenotype after autologous HCT for the treatment of high-risk multiple myeloma (www.cancer.gov/clinicaltrials; #; see protocol schema and T-cell manufacturing, Fig. 4A). Multiple myeloma is a disease that nearly universally relapses after autologous HCT (136); of note, costimulated T-cell infusion in the autologous peri-transplant setting has been previously pioneered, with interesting results in terms of enhancement of immune reconstitution (137) and cytokine-activation that manifested as autologous GVHD and engraftment syndrome (138). Because of this prior information and our preclinical data demonstrating that the RR-Th1/Tc1 population had enhanced engraftment and mediated increased xenogeneic GVHD, we have elected to adoptively transfer the cells remote from host conditioning at day 42 post-transplant (Fig. 4B).

Additional future directions in T-Rapa cell therapy

Future studies will be guided by emerging clinical trial data. In the event that T-Rapa cells are found to mediate beneficial immune effects relative to standard DLI, then next generation efforts can be envisioned to further advance this novel treatment approach. Next, if it is confirmed that the T-Rapa cells are a more robust population for cell therapy (for example, increased ability to convert mixed chimerism relative to standard DLI), then next clinical efforts should begin to reduce the number of unmanipulated T cells transferred along with the mobilized allograft. It may also be important to investigate the role of alternative conditioning regimens prior to T-Rapa cell infusion, such as the pentostatin plus cyclophosphamide regimen we have developed in preclinical models of graft rejection (139) and foreign protein immunogenicity (140). Finally, it further may be important to define the

role of distinct immunosuppressive interventions prior to or during T-Rapa cell therapy, such as use of high-dose sirolimus therapy that has been pioneered for therapy of sickle cell anemia (141).

As we have recently shown (142), T-Rapa cells are amenable to lentiviral-mediated transfer of a human, modified thymidylate kinase (TMPK) transgene that efficiently activates the pro-drug AZT to execute a new cell fate, suicide gene axis (143). Further studies will be required to determine the merits of this approach relative to previously described suicide gene axes (144, 145). An ability to modulate the fate of T-Rapa cells *in vivo* may improve the safety of this cell therapy in transplantation settings involving increased HLA-disparity, such as haplo-identical transplantation (146). Furthermore, through a similar gene transfer approach, we have demonstrated that T-Rapa cells are amenable to enforced co-expression of TMPK for cell fate control in combination with programmed death ligand-1, which allowed the T-Rapa cells to function as a surrogate T_{REG}-like cell for the prevention of xenogeneic GVHD (147). Extrapolating from these results, it is possible to envision that T-Rapa cells might serve as a suitable cellular vehicle for chimeric antigen receptors to incorporate an antigen specificity component into T-Rapa cell therapy.

Finally, as an understanding of mTOR biology evolves and new agents to modulate this biology are developed, it will be important to determine whether new variations of T cells with heightened resistance and optimized function might be produced *ex vivo* for the enhancement of adoptive T-cell therapy.

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Fig. 1. Potential mechanisms of resistance to rapamycin.

Mechanism #1: rapamycin only partially inhibits mTORC1, with potent blockade of the S6K pathway and relative sparing of the 4E-BP1 pathway. Mechanism #2: prolonged rapamycin inhibition of mTORC1 leads to sequential inhibition of mTORC2, with inhibition of some kinases (SGK1, PKC α) but compensatory increase in the Akt kinase pathway. Mechanism #3: cell surface cytokine receptors and growth factor receptors activate specific JAK/STAT pathways, with the STAT molecules acting as transcription factors for the direct upregulation of PIM kinases, which are constitutively active once expressed. Mechanism #4: potent inhibition of mTORC1 releases the brake on autophagy, thereby downsizing cellular energy requirements while providing substrates for metabolic fuel. Mechanism #5: increased PTEN activity can dampen mTOR signaling in regulatory T cells, whereas loss of tumor suppressor activity (PTEN, TSC1/2, p53) or activation of oncogenes (KRAS) can amplify mTOR signaling. Mechanism #6: inhibition of S6K can release mTORC1-mediated steady-state inhibition of insulin receptor substrate (IRS), with the increased IRS activity pathway potentiating cell surface signaling.

Fig. 2. Rapamycin-resistant murine Th2 cells: mechanism of prevention/treatment of graft-versus-host disease (GVHD) and prevention of graft rejection.

Rapamycin-resistant Th2 cells (Th2.R) have been found to reduce both the graft-versus-host reaction (GVHR) and the host-versus-graft reaction (HVGR). With respect to GVHR, Th2.R cells secrete IL-4, which shifts effector Th1 and Tc1 cells toward a type II cytokine phenotype. Also, Th2.R cells secrete IL-10, which may operate through modulation of host antigen-presenting cells (APCs); IL-10 is known to reduce APC expression of major histocompatibility complex (MHC) molecules, costimulatory molecules (CD80), and type I polarizing cytokines (IL-12). Th2.R cell anti-GVHD effects were abrogated by exogenous administration of IL-2 *in vivo*, suggesting that the Th2.R cell population can outcompete pathogenic Th1/Tc1 effectors for limiting cytokines. With respect to HVGR, IL-4 produced from donor Th2.R cells binds to the IL-4 receptor on host precursor cytotoxic T lymphocytes (pCTLs), thereby activating STAT6 signaling in the host T cells. This sequence of events shifts host pCTLs toward Th2/Tc2 differentiation, with such Th2/Tc2 cells having reduced capacity to mediate rejection of the donor hematopoietic stem cell (HSC) pool.

Fig. 3. Phase II clinical trial of allogeneic T-Rapa cells.

(A) Prior to collection of the allogeneic peripheral blood stem cell product, donors underwent steady-state apheresis with subsequent purification of CD4⁺ T cells by positive selection. T cells were costimulated using an APC-free system (anti-CD3, anti-CD28-coated magnetic beads); culture media contained high-dose rapamycin and recombinant human IL-4 and IL-2 to promote Th2 polarization. (B) After 12 days of culture, the CD4⁺ T-cell products primarily expressed effector-type transcription factors by intracellular flow cytometry analysis (GATA3 > T-bet) with minimal expression of FOXP3. Because of this mixed Th2/Th1 composition, the cells have been termed 'T-Rapa' cells. (C) Patients with refractory hematologic malignancy proceeded to transplant once they reached a moderate degree of immune depletion (CD4 count < 200 cells/ μ l). Patients received a low-intensity chemotherapy regimen just prior to transplant, with the total cyclophosphamide dose being 1200 mg/m², which is a dose that is 75% less than our previous studies. Graft-versus-host disease prophylaxis consisted of standard cyclosporine therapy and a short-course of sirolimus until day +14 post-transplant. The transplant consisted of a T-cell replete, G-CSF mobilized peripheral blood stem cell allograft (PBSCT; HLA-matched sibling donor). (D) At day 30 post-transplant, T-Rapa cell recipients had a balanced CD4⁺ T-cell immune reconstitution pattern, with increased expression of GATA3⁺ cells relative to T-bet⁺ and FOXP3⁺ cells.

Fig. 4. Phase I clinical trial of autologous, rapamycin-resistant T cells polarized toward a Th1/Tc1 phenotype.

(A) To generate the experimental T-cell product, T cells are harvested by steady-state apheresis and costimulated using an APC-free system (anti-CD3, anti-CD28-coated magnetic beads); culture media contains high-dose rapamycin and recombinant human IFN- α and IL-2 to promote Th1 and Tc1 cell polarization. (B) Prior to autologous hematopoietic cell transplantation (HCT), patients with high-risk multiple myeloma undergo apheresis with subsequent manufacture of the rapamycin-resistant Th1 and Tc1 (RR-Th1/Tc1) cells, which are then cryopreserved. The patient then undergoes induction therapy and transplantation; the RR-Th1/Tc1 cells are then thawed and administered in a phase I dose escalation manner at a time point remote from host conditioning (day 42 post-HCT).