Conference Scene

3rd Keystone Symposium on T regulatory cells

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The Keystone Symposia on T regulatory cells (Tregs) started in 2004 and takes place every 2 years. This year, Yasmine Belkaid, Fiona Powrie and Shimon Sakaguchi organized the 3rd Symposium, which was attended by approximately 300 participants from all over the world. The meeting was organized into plenary sessions, afternoon workshops and evening poster sessions, with a casual atmosphere that encouraged discussion and collaboration. In this article, we will highlight some of the unifying themes that provided new insight into the biology of Tregs, with a specific focus on the mechanisms of development and action of Tregs, evidence for their stability and plasticity, and results from ongoing clinical trials to test the efficacy of Treg-targeted therapies. Owing to space limitations, we apologize that there is not sufficient space to discuss all of the presentations.

Mechanisms of Treg development

■ Thymic development of 'naturally occurring' Tregs

Ludger Klein (University of Munich, Germany) began the discussion of what controls the thymic development of naturally occurring (n) Tregs by proposing three models: first, a quantitative model where development depends on the strength of the T-cell receptor (TCR) signal; second, a qualitative model where there is a dedicated Treg-inducing lineage of antigen-presenting cells (APCs); and third, a developmental model where the stage in development when self-antigen is encountered is key in defining the Treg lineage. Using a transgenic mouse expressing an influenza hemagglutinin (HA)–green fluorescent protein (GFP) fusion protein under control of the AIRE promoter, he elegantly showed that both medullary thymic epithelial cells and dendritic cells (DCs) can present self-antigen, arguing against a qualitative model. Hence, it is likely that thymic Treg development is influenced by both the strength of TCR activation and the developmental stage at which they encounter antigen.

Chan Wang Lio (Washington University, DC, USA) used Foxp3–GFP reporter mice to show that expression of CD25 precedes that of Foxp3. He proposed a two-step model where the development of a CD25^{hi}Foxp3⁺ nTreg precursor depends on the strength of TCR activation, but

that ultimately, the expression of Foxp3 is IL-2 dependent and TCR independent. Michael Farrar (University of Minnesota, MN, USA) also spoke regarding the role of IL-2 and STAT5 in regulation of Foxp3 expression and thymic Treg development. He showed that nTregs from transgenic mice expressing a constitutively active form of STAT5 have a broader TCR repertoire, arguing that IL-2 plays a role not only in the survival/expansion of nTregs, but also in their differentiation. He went on to demonstrate that IL-2 produced by thymic DCs is sufficient to drive nTreg development.

WanJun Chen (NIH, USA) spoke regarding the role of TGF- β in the thymic development of nTregs. In mice whose T cells were unable to respond to TGF- β owing to conditional deletion of *TGF- β RI*, he found that early thymic nTreg generation was largely, but not completely, impaired. However, by day 14, when the mice were suffering from acute inflammation, normal numbers of Foxp3⁺ cells were restored. He hypothesized that the restoration of normal nTreg numbers may be due to increased IL-2-stimulated proliferation of the few nTregs that developed in the absence of TGF- β signals. Indeed, if the TGF- β RI-deficient mice were crossed to IL-2-deficient mice, then the frequency of nTregs was impaired throughout life. Overall, it can be concluded that TGF- β is indeed required for thymic nTreg development and that exogenous IL-2 is required for their survival and expansion.

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■ Peripheral development of 'induced' Tregs

Many speakers reported studies on pathways that can lead to the peripheral development of different types of induced (i) Tregs, with a major focus on those that are Foxp3⁺. Harald von Boehmer (Harvard Medical School, MA, USA) showed that Foxp3⁺ iTregs can be generated *in vivo* by infusion of low amounts of peptides and that retinoic acid (RA) can enhance this process. He then discussed the ability of other compounds to enhance low-dose, peptide-driven iTreg conversion *in vivo* and found that in addition to RA, 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD) (an aryl hydrocarbon-receptor ligand), everolimus (a rapamycin derivative) and decitabine (a DNA methyltransferase inhibitor) could all promote Treg conversion. The overall conclusion was that compounds that block cell division generate the most potent and stable Foxp3⁺ iTregs *in vivo*. Peripheral induction of iTregs was also discussed by Rachel Gottschalk (Memorial Sloan-Kettering Cancer Center, IN, USA) who focused on the role of TCR signal strength in peripheral conversion. Using 5C.C7 TCR transgenic mice, for which three peptides with different agonistic strengths exist, she found that all three peptides induce Foxp3 expression. *In vivo* experiments, however, showed that a high dose of a weak agonist was not equal to a low dose of a strong agonist in terms of induction of Foxp3, and she speculated that this may be related to the impact of antigen density at the T cell–APC interface on the duration of TCR stimulation.

Craig Maynard (University of Alabama, AL, USA) discussed the effect of RA on induction of Foxp3⁺ iTregs versus IL-10⁺CD4⁺ Tr1 cells. He found that TGF- β in conjunction with Toll-like receptor (TLR)9 activation stimulated IL-10 production, but that RA actually inhibited this process. Therefore, the CD103⁺ RA-producing mucosal DCs, which are known to induce Foxp3⁺ Tregs, do not simultaneously induce IL-10-producing Tr1 cells. By contrast, CD103⁻ DCs that do not produce RA suppressed Foxp3 and induced IL-10 expression. Since TLR9 inhibits RA production, this might be a new approach to promote the development of IL-10-producing Tr1 cells.

Dan Littman (New York University School of Medicine, NY, USA) proposed that a common precursor gives rise to peripheral iTregs or the newly identified Th17 cells depending on environmental cues. He argued that TGF- β can induce expression of both Foxp3 and ROR- γ t, but that in the absence of inflammatory cytokines, such as IL-6, IL-21 and IL-23, Foxp3 prevails, inhibiting the function of ROR- γ t, and Tregs develop. He then spoke about fate-mapping experiments to trace cells that expressed Foxp3 at some point in their life. Surprisingly, up to 23% of IL-17⁺ cells were found to have previously expressed Foxp3, highlighting the potential plasticity of the iTreg lineage.

Treg stability & homeostasis

A topic of much discussion and debate was the stability of Foxp3⁺ Tregs and differences between nTregs and iTregs in this respect. Talal Chatila (University of California, CA, USA) developed a model to test the relative contribution and stability of nTregs versus iTregs in control of autoimmunity. By tracking the ability of nTregs versus iTregs to rescue *Foxp3*^{-/-} mice he showed that optimal regulation required both nTregs and iTregs and that, in the steady state, 85 and 15% of Foxp3⁺ cells were derived from nTregs and iTregs, respectively. In terms of stability, 8–11% of transferred nTregs lost Foxp3 expression. Notably, autoimmunity could not be rescued with iTregs alone, even though these cells were stable *in vivo* for at least 2 months and 70% of them remained Foxp3⁺.

Noriko Komatsu (RIKEN Research Center for Allergy and Immunology, Japan) provided evidence that the stability of Foxp3 expression correlates with CD25 expression. Foxp3⁺CD25^{hi} cells maintained Foxp3 expression whereas Foxp3⁺CD25⁻ cells (~10% of the total population of Foxp3⁺ cells) lost Foxp3 expression when transferred to a wild-type host. Furthermore, Xuyu Zhou (University of California, CA, USA) described fate-mapping experiments to track cells that lost Foxp3 expression and became 'exTregs'. ExTregs were found in normal mice, had a memory phenotype, produced IFN- γ and IL-17, and became pathogenic in the absence of Tregs. Although these results agree with those described above from

Littman, they contrast with data from Alexander Rudensky (Memorial Sloan Kettering Cancer Center, NY, USA) who used fate mapping to show that Foxp3⁺ cells are a stable lineage and that even in 8-month old mice there was no evidence that they acquired the capacity to produce cytokines.

Ethan Shevach (NIH, USA) proposed that iTregs are unstable and lose Foxp3 expression over time only if they are not in an inflammatory environment: when iTregs generated *in vitro* were transferred into a wild-type mouse, only 50% remained Foxp3⁺ in the lymph node and 30% in the ear after 24 days. By contrast, when iTregs were transferred into scurfy mice that suffer from acute inflammation, 90% remained Foxp3⁺ in both the lymph node and ear after 24 days. It can be concluded that Foxp3⁺ nTregs and iTregs are not necessarily fixed lineages, and more research will be required to better understand what signals and pathways can switch off their suppressive program.

Foxp3: molecular biology & epigenetics

The major advance in our understanding of the molecular biology of Foxp3 came from the first description of the crystal structure of the NFAT1:Foxp3:DNA complex, reported by Lin Chen (University of Southern California, CA, USA). He found that Foxp3 crystallized as a homodimer and formed a 'domain swap', an interaction that commonly forms in dimeric transcription factors. The domain swap involves exchange of DNA-binding domains, and the resulting homodimer forms a molecular bridge that can link two pieces of DNA. Hozefa Bandukwala (Harvard Medical School, MA, USA) investigated the relevance of domain swapping to the function of Foxp3 by mutating residues predicted to be important for domain swapping. The domain-swap mutants of Foxp3 retained their ability to bind to DNA and transactivate CD25 and cytotoxic T-lymphocyte antigen (CTLA)4, but could not suppress expression of IL-2 and, importantly, their suppressive capacity was hindered. Fortunately, these results correlated with data from Megan Levings (University of British Columbia, Canada), who described the molecular and cellular characteristics of T cells transduced with mutated forms

of Foxp3 found in immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome patients. Two of the mutants she investigated turned out to be in the predicted domain-swap region, providing a possible explanation for the finding that the IPEX mutations in question neither severely hindered the expression of Treg-associated proteins nor completely abrogated suppressive function.

Other molecular data came from Steven Ziegler (Benaroya Research Institute, WA, USA) who described a new Foxp3-interacting protein known as G4, which is expressed in humans but not mice. G4 is a splice variant of zinc finger protein (ZFP)90 that only contains a KRAB region, which is a transcriptional repressor domain that recruits the KAP1 corepressor and is known to serve as a binding platform for histone deacetylases. The interaction with G4 involves amino acids 120–198 of Foxp3; hence the alternative Foxp3b isoform is not able to associate.

Jochen Huehn (Helmholtz Centre for Infection Research, Germany) discussed epigenetic regulation of the *Foxp3* locus and how knowledge of what regulates Foxp3 expression can provide insight into the molecular differences between stable Foxp3⁺ Tregs and activated T cells that, in humans, can also transiently express Foxp3. His group identified a regulatory element upstream of exon-1 in the *Foxp3* locus that has CpG motifs that are demethylated (characteristic of an actively transcribed region) in nTregs. By contrast, the region, named as the Treg-specific demethylated region, remained methylated in iTregs and other T-cell subsets. Girdhari Lal (Mount Sinai School of Medicine, MD, USA) also discussed epigenetic regulation of *Foxp3*, and identified a region upstream of the *Foxp3* enhancer (-5786 to -5558) that is also specifically demethylated in nTregs. Thus, new methods to track the methylation status of the regulatory regions of the *Foxp3* locus could be useful diagnostic tools that would allow more specific enumeration of *bona fide* Foxp3⁺ Tregs in disease states.

Christophe Benoist (Joslin Diabetes Center, Boston, MA, USA) called into question the status of Foxp3 as a 'master regulator' of Tregs and proposed that it may instead be an amplifier of patterns set by other factors. For example, even though TGF- β -induced

Treg cells are 100% Foxp3⁺, their gene signature is significantly different from *ex vivo* nTregs. In addition, when Foxp3 was ectopically expressed, only 30–35% of the Treg signature genes were induced. He is currently using optimized algorithms to predict which other transcription factors are essential for Treg development, as discussed in more detail later.

Role of Helios in Treg development & function

Beyond Foxp3, there is now evidence that several other transcription factors are involved in regulating the development and function of Tregs, and many speakers specifically discussed the role of the Helios, an Ikaros-family protein well known for its role in lymphoid development and differentiation. Tregs express high levels of Helios, and Christophe Benoist revealed that over-expression of Helios alone only induced 15–20% of the Treg gene signature, but that coexpression with *Foxp3* significantly amplified the effects. He argued that these data support the concept that Foxp3 amplifies patterns set by other transcription factors rather than acting as master regulator of Treg development. Ethan Shevach also discussed Helios and, using a newly generated monoclonal antibody (mAb), demonstrated that in the periphery, 60–70% of Foxp3⁺ cells express Helios in mice and humans. By contrast, iTregs generated *in vitro* did not express Helios, and in MHC class II-deficient mice, all Foxp3⁺ cells were Helios⁺, suggesting that Helios may be specifically expressed by nTregs. Derese Getnet (John Hopkins School of Medicine, MD, USA) reported that siRNA-mediated knock-down of Helios resulted in a decrease in Foxp3 expression and reversed suppression *in vitro*. He also mapped a Helios-binding site in the *Foxp3* promoter between bases -692 and -355. The fact that Helios can directly regulate Foxp3 expression further supports the notion that other transcription factors act in concert with Foxp3 to define the Treg lineage.

Mechanisms of suppression

It is increasingly clear that Foxp3⁺ Tregs do not use a single mechanism to suppress immune responses; rather, depending on the context, they have an arsenal

of many different suppressive molecules and pathways that can be activated. This concept was elegantly reviewed by Shimon Sakaguchi (Kyoto University, Japan) who proposed that Tregs have core mechanisms that are supplemented by auxiliary mechanisms depending on the stimulus intensity, type of tissue and microenvironment.

■ Interactions with APCs

A general conclusion was that effects of Tregs on APCs have been underappreciated, and several speakers presented convincing data demonstrating that Tregs directly impact APC function. Juan Lafaille (New York University School of Medicine, NY, USA) described a novel mechanism whereby Tregs induced the apoptosis of antigen-loaded DCs. He used two-photon microscopy to demonstrate that if Tregs were present there was a shorter contact time between T cells and DCs. In addition, mice harboring Tregs had a higher rate of DC turnover and lower recovery of injected DC. Because Tregs express granzymes, he proposed that they may kill DC-presenting cognate antigens and suggested a licensing model for Tregs where, upon antigen recognition, Tregs acquire the capacity to induce DC apoptosis and indirectly inhibit the activation of conventional T (Tconv) cells.

Sakaguchi discussed evidence that interactions between Tregs and DCs are LFA-1 dependent but CTLA4 independent. He also showed that Tregs downregulate expression of CD80 and CD86 on DCs via an unknown mechanism that is dependent on antigen, LFA-1 and CTLA4. In a system where he attempted to recapitulate the suppressive capacity of Tregs in the absence of Foxp3 expression, he showed that T cells from *CTLA4*-transgenic mice crossed to IL-2-deficient mice were suppressive and could also downregulate expression of CD80 and CD86 on DCs. He argued that these data suggested that the core mechanism of Treg suppression is inhibition of IL-2 production and downregulation of DCs. Ashley Martin (University of San Francisco, CA, USA) found that Tregs prevented DC maturation in inflamed islets, lending further support for the role of Treg–APC interactions in suppression.

■ Antigen specificity of Treg suppression

Several speakers discussed the relative ability of polyclonal versus antigen-specific Tregs to suppress immune responses. Somewhat surprisingly, Shevach presented data demonstrating that although polyclonal and antigen-specific Tregs can both suppress autoimmunity, they do so by different mechanisms of action. Specifically, polyclonal nTregs trapped Tconv cells in the lymph node and prevented them from moving to the target organ whereas antigen-specific Tregs inhibited Tconv cell priming and differentiation, probably via an APC-dependent mechanism. Antigen-specificity was also discussed by Soyoung Oh (University of Pennsylvania, PA, USA) who reported that although polyclonal Tregs prevented arthritis in a model where mice coexpress influenza virus HA and a transgenic TCR with high or low reactivity for HA, counterintuitively, HA-specific Tregs did not prevent disease even though they could suppress B-cell anti-influenza antibody response. She speculated that high antigen levels may diminish Foxp3 expression and impair suppression. Also addressing the topic of antigen specificity, Maria Grazia Roncarolo (San Raffaele Telethon Institute for Gene Therapy, Italy) discussed that although polyclonal Tr1 cells could prevent graft rejection in a less stringent model, they were unable to prevent rejection in a stringent model. By contrast, alloantigen-specific Tr1 cells were able to prevent rejection in both models. She cautioned, however, that these data do not rule out the possibility that polyclonal nTregs could be more protective in the setting of autoimmunity.

■ Suppression mediated by secreted proteins

Two novel mechanisms of action of note included release of soluble (s) CTLA4 and the newly identified cytokine IL-35. Robert Barker (University of Aberdeen, UK) developed a new mAb to revisit the biology of sCTLA4, a splice variant lacking the transmembrane region, and challenged the notion that this is an unimportant molecule. Classically, it was thought that sCTLA4 was only secreted by resting T cells, but Barker found that IL-10-secreting Tr1 cells are also a major

source of sCTLA4. Notably, sCTLA4 had potent inhibitory effects on T cells, at least partly via its ability to stimulate expression of IL-10 and indoleamine 2,3 deoxygenase. With this new mAb, it will be of considerable interest to define whether some effects previously attributed to membrane-bound CTLA4 may in fact be due to sCTLA4.

Dario Vignali (St Jude Children's Research Hospital, TN, USA) presented an update on his investigation of the role of IL-35, a heterodimeric cytokine composed of Ebi3 and p35, in Tregs. Ebi3- or p35-deficient Foxp3⁺ Tregs were not suppressive *in vitro* or *in vivo*, and recombinant IL-35 inhibited the proliferation of Th1 and Th2 but not Th17 cells *in vitro*. He argued that since there is a role for IL-35 in suppression mediated by Foxp3⁺ Tregs, it is possible that the induction of suppression is contact-dependent whereas suppression itself could be mediated by soluble factors. Interestingly, suppressed T cells also expressed Ebi3 and p35, but not Foxp3 or IL-10, and acquired cytokine-mediated suppressive capacity. This novel subset of Tregs, denoted iTr35 cells, was able to reduce autoimmunity *in vivo* and, moreover, was found among tumor-infiltrating cells in a model of melanoma.

Class-specific suppression

In his keynote address, Rudensky proposed the idea of class-specific suppression where specific transcription factors are required not only to differentiate Th cell subsets, but also to confer Tregs with the ability to suppress the corresponding Th cells. For example, he showed that interferon regulatory factor (IRF)4, which is required for Th2 cell differentiation, is also required for suppression of the Th2 cell response. Another example of class-specific suppression was illustrated by studying mice with a Treg-specific deletion of STAT-3, a transcription factor required for Th17 cell development. He showed that if Tregs lacked STAT3, mice developed fatal colitis and had increased numbers of Th17 cells in the gut-associated lymphoid tissue. Evidence that colitis could be rescued by neutralizing IL-17 further demonstrated the specific loss of control over fatal Th17 cell-driven responses.

Daniel Campbell (Benaroya Research Institute, WA, USA) provided further evidence for the concept of class-specific suppression and discussed the role of T-bet, a Th1-associated transcription factor, in Tregs. Campbell proposed that, during a Th1 response, Tregs respond to IFN- γ by upregulating expression of T-bet and CXCR3, ultimately allowing Tregs to accumulate at sites of infection. The biological relevance of this model was illustrated in a model of *Mycobacterium tuberculosis* where he showed that *T-bet*^{-/-} Tregs were outcompeted by wild-type Tregs, and moreover, they could not rescue autoimmunity in scurfy mice. He proposed that Tregs must express T-bet in order to survive and proliferate in a Th1-dominated environment. Yasmine Belkaid (NIH, USA) also found that Foxp3⁺ T cells expressed T-bet and produced IFN- γ during acute infection with *Toxoplasma gondii* but ultimately died due to a decline in IL-2 production by T effectors.

Benoist described the presence of numerous Tregs interspersed with the adipocytes of abdominal fat. Of particular interest in the context of class-specific suppression was the finding that Tregs isolated from fat have a unique genomic profile and share expression of many genes normally expressed by adipocytes and associated with lipid metabolism. The concept that Tregs are plastic and change in response to their environment in order to become functionally specialized was one of the most innovative new concepts discussed at the meeting.

Immune regulation in infectious disease

In line with the notion of plasticity and specialization, several speakers presented data describing conditions under which Th1 cells may acquire regulatory functions. Dragana Jankovic (NIH, USA) focused on IFN- γ IL-10⁺ Th1 as regulatory cells in the context of *T. gondii* infection. Protective immunity during this infection relies on T-cell-derived IFN- γ , but host survival depends on coexpression of IL-10 from Tbet⁺IFN- γ Foxp3⁺ T cells. IL-10 expression in these cells was transient, suggesting that these cells do not represent a unique subtype but rather a unique state of 'immunoregulatory' Th1

cells. Eleanor Riley (London School of Hygiene and Tropical Medicine, UK) found evidence for similar IL-10⁺IFN- γ ⁺ Th1 cells during acute malaria infection, as did Anne O'Garra (National Institute for Medical Research, UK) who found that they do not express FOXP3 or GATA3. Alexander Scheffold (Miltenyi Biotec, Germany) suggested that signaling stimulated by Notch ligand delta-like 4 could be responsible for the induction of IL-10 in Th1 cells. There was some discussion regarding whether these IL-10-producing Th1 cells may be related to Tr1 cells, but Roncarolo pointed out that although human Tr1 cells do indeed make IFN- γ , the amounts are significantly less than in Th1 cells. Thus, the ability of Th1 cells to produce IL-10 most likely serves as a negative-feedback pathway to limit inflammation in acute infections.

Richard Maizels (University of Edinburgh, UK) described three possible outcomes upon helminth exposure in humans: uninfected despite exposure, nonpathogenic infection, or pathogenic infection. Notably, T cells from individuals with nonpathogenic infections become hyporesponsive even to nonparasite antigens. For example, children infected with schistosomes have a decreased incidence of allergy, and multiple sclerosis patients who are infected with gut helminths are protected from progression of disease. This concept of 'spillover' suppression was further illustrated in a presentation from Linda Wammes (Leiden University Medical Center, The Netherlands). She found that lymphocytes from children infected with helminths proliferated less in response to stimulation with bacillus Calmette–Guerrin or *Plasmodium falciparum* antigens compared with cells from uninfected children; moreover, this proliferative defect was restored by depletion of Tregs. Therefore, particularly in environments where parasitic infections are endemic, this bystander suppressive effect of Tregs should be considered when developing vaccination strategies for other infectious agents.

Maizels went on to discuss mechanistic studies designed to determine how infection with *Helicobacter polygyne*, a chronic gut nematode, could suppress allergy in mice. He investigated whether

the nematodes might secrete immuno-suppressive molecules and indeed found that *H. polygyne* secreted a TGF- β analog that can signal to mammalian cells via TGF- β RII. Interestingly, conditioned medium from the worms also promoted Th17 development in the presence of IL-6, and parasite infection induced a short-lived IL-17 response *in vivo*. He hypothesized that an early Th17 response could serve to limit a Th2 response, allowing the parasite to avoid entrapment in granulomas before ultimately promoting Tregs and chronic infection.

Tregs in allergy & autoimmunity

Catherine Hawrylowicz (King's College London, UK) described IL-10-secreting Tregs in asthma and the role of vitamin D3 in their development. She reported that steroid insensitive patients who did not respond to treatment with dexamethasone were also impaired in steroid-induced production of IL-10. She discussed results from a clinical study that showed that, depending on the dose, oral vitamin D3 could reverse this impaired steroid-induced IL-10 production. Moreover, at high concentrations, vitamin D3 also induced Foxp3 via a TGF- β -dependent mechanism in these subjects. In addition, vitamin D3 induced TLR9 expression, and ligation of TLR9 on IL-10-producing Tregs inhibited their regulatory capacity and production of IL-10. Hawrylowicz ended the discussion by suggesting that perhaps vitamin D3 deficiency could be a driving force in the asthma epidemic.

Fiona Powrie (Oxford University, UK) reported new data on the role of OX40 in intestinal regulation. She found that 80–90% of Foxp3⁺ Tregs expressed high levels of OX40, but previous data were conflicting as to whether this molecule was inhibitory or stimulatory on these cells. She found that OX40-deficient mice had a decreased frequency of Foxp3⁺ cells in the colon, but not in the mesenteric lymph nodes or spleen, and that *OX40*^{-/-} Tregs could not control colitis. Bone marrow chimera experiments showed that the *OX40*^{-/-} Tregs failed to compete with wild type Tregs in the colon, indicating that OX40 is required for the competitive fitness and function of Tregs.

The role of Tregs in the immune privileged eye was presented by Rachel Caspi (NIH, USA). Human autoimmune uveitis is mediated by T cells, and mouse models have shown that Tregs control this disease since their depletion increases the severity of experimental autoimmune uveitis. Notably, the aqueous humour has a high concentration of RA (1 μ M), and she investigated whether this might contribute to Treg development. Indeed, CD4⁺ T cells activated in the presence of aqueous humour expressed Foxp3 at the expense of IL-17 and IFN- γ production and were suppressive. This induction of Foxp3 required both RA and TGF- β in the aqueous humour. Interestingly, previously polarized T-effector cells were resistant to the suppressive effects of the aqueous humour, suggesting that autoimmune uveitis might be induced by cells that were activated in the periphery.

Clinical trials based on manipulating Tregs

In comparison to the previous Keystone Symposium on Tregs 2 years ago, a major advance has been the translation of methods to boost or diminish Treg function in the clinic. For example, mouse models have clearly demonstrated that Tregs are detrimental in the context of cancer, and the first clinical trials to investigate whether this is also the case in humans are currently being performed. Jason Chesney (University of Louisville, IL, USA) reported data from a Phase II trial using ONTAK (an IL-2–diphtheria toxin fusion protein) to deplete Tregs in patients with metastatic melanoma. A total of 37 patients were treated, and cellular assays revealed that treatment caused a transient depletion of both Foxp3⁺ Tregs and CD4⁺ Tconv cells followed by the *de novo* appearance of melanoma-specific CD8⁺ T cells. He showed remarkable CT and positron emission tomography imaging data that demonstrated regression of melanoma metastases in 12 patients, with four patients exhibiting a complete response.

Roncarolo described a tolerogenic DC subset called DC-10 that was found in the peripheral blood and spleen of humans and that could also be induced *in vitro* by culturing CD14⁺ monocytes with IL-4, GM-CSF and IL-10. DC-10 primed

T cells to become Tr1 cells that suppressed proliferation and cytokine production via an IL-10 and TGF- β -dependent mechanism. The DC-10-induced Tr1 cells also expressed high levels of granzymes A and B and perforin and could specifically kill monocytic cell lines. A clinical trial to test whether T cells stimulated by DC-10 can suppress graft-versus-host disease (GvHD) in patients undergoing haploidentical hematopoietic stem cell transplant is currently ongoing.

Markus Kleinewietfeld (Max Delbrueck Center for Molecular Medicine, Germany) described a new method for purifying human Tregs for clinical use based on depletion of CD49- and CD127-expressing cells. Since the vast majority of Foxp3⁺ cells do not express either protein, this method generated highly pure Tregs that were suppressive *in vitro* and *in vivo* in a xenogeneic model of GvHD. A major advantage of this approach over sorting for CD25⁺ cells is that the isolated Tregs are 'untouched' since they are never bound by antibodies during the purification procedure. A Phase I clinical trial is underway using CD49d⁻CD127⁻ Tregs in combination with rapamycin to treat patients with GvHD.

Matthias Edinger (University Hospital Regensburg, Germany) also discussed a Phase I clinical trial to test the safety and feasibility of Treg therapy in hematopoietic stem cell transplantation. The trial involved tapering cyclosporine treatment after transplant and infusion of 130–350 million donor-derived CD4⁺CD25⁺ T cells, more than 55% of which were Foxp3⁺ and not expanded *in vitro*. None of the patients developed GvHD, infections or early disease relapse, demonstrating that the approach is safe. Edinger also discussed methods to expand highly purified populations of CD25^{hi}CD45⁺ Tregs, and illustrated that sorting CD45RA⁺ cells rather than CD127^{low} cells resulted in a much more homogeneous and stable population of Foxp3⁺ Tregs. He reported exciting results from a single patient with chronic GvHD who was infused with *in vitro*-expanded CD4⁺CD45RA⁺ Tregs from the stem cell donor. Remarkably, the Tregs were detectable in circulation as soon as 1 h after infusion, and after 3 months 30% of his peripheral T cells were still Foxp3⁺. Clinically, all symptoms

of GvHD were cleared and steroid/mycophenolate mofetil treatment did not appear to have an effect on the transferred cells. Unfortunately, the patient recently succumbed to sepsis; nevertheless, these remarkable results provide great hope that Treg-based cell therapies will indeed be effective in humans.

Highlights & future perspective

A major message from the meeting was that the search for a standard and unifying mechanism of Treg-mediated suppression is probably futile. The molecules and pathways that Tregs use to regulate immune responses and homeostasis clearly depend on a number of factors including the target tissue, microenvironment and stimulus strength. Possible mechanisms discussed included suppression dependent on sCTLA4, IL-35 or other cytokines, and direct interactions with APCs and effector cells. Sakaguchi hypothesized that inhibition of IL-2 production and downregulation of CD80 and CD86 on DCs are the core suppressive mechanisms of all Tregs, but that environment-specific auxiliary mechanisms were likely also essential. The notion of class-specific suppression, highlighted by Rudensky during his keynote address, elegantly described this concept and he presented convincing data that Tregs adapt their function in response to the environment in order to specifically limit certain types of immune responses.

As the best-accepted marker of Tregs, Foxp3 was a central element in many talks, but surprisingly few data on how this protein works at the molecular level were presented. The most notable advance was the report of the crystal structure of the NFAT1:Foxp3:DNA complex, which revealed that Foxp3 acts as a homodimer and forms a domain swap. While the importance of Foxp3 to Treg function is not debated, its status as the master regulator of Tregs is in question. This is largely due to the inability of ectopic expression of *Foxp3* to fully recapitulate the Treg gene signature and the fact that a significant portion of the Treg program appears to be established before it is expressed. Hence, other transcription factors currently being investigated, such as Helios, may also play major roles in Treg development.

Despite their elusive mechanisms of action and the challenges in purifying and expanding *ex vivo* Tregs, early results from clinical trials using Tregs as a cellular therapy in the setting of hematopoietic stem cell transplantation are promising. These clinical studies will probably benefit from new approaches to distinguish between nTregs and iTregs and activated T effectors by molecular techniques, such as examining the methylation status of defined regions

of the *Foxp3* locus. We look forward to the next Keystone Symposium on T regulatory cells when the results of these ongoing trials will reveal the true potential and feasibility of Treg-targeted therapies to treat a variety of human diseases.

Website

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www.selectbiosciences.com/marketreports/StemCellsCardiovascular2009.aspx