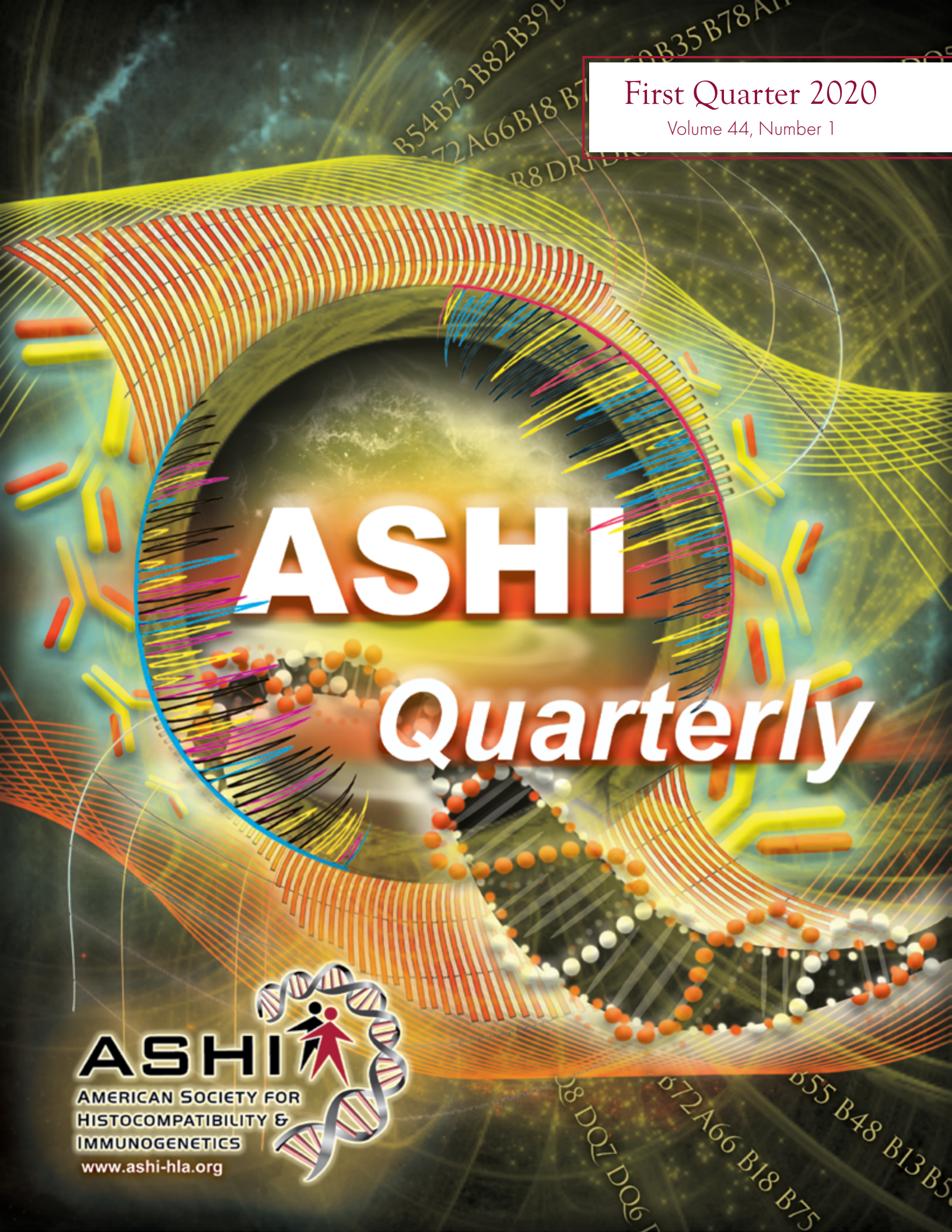
First Quarter 2020
Volume 44, Number 1



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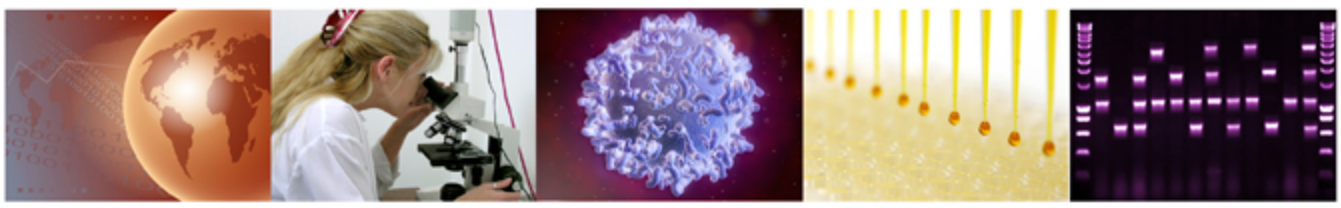

ASHI
American Society for Histocompatibility & Immunogenetics

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AMERICAN SOCIETY FOR HISTOCOMPATIBILITY AND IMMUNOGENETICS

# ASHI 46TH ANNUAL MEETING

## OCTOBER 19-23, 2020

ANAHEIM MARRIOTT | ANAHEIM, CA


#ASHI2020

# Table of Contents

ASHI Quarterly

American Society for Histocompatibility and Immunogenetics

Volume 44, Number 1



## Features



## Departments



## Editor's Note

Individuals interested in submitting articles for *ASHI Quarterly* should observe the following requirements:

- All articles must be submitted via email in Microsoft Word format
- All articles must be double-spaced

Article submissions should be forwarded to:
Editor-In-Chief
Manish J. Gandhi
Email: Gandhi.Manish@mayo.edu

Copy deadlines for future issues:
Second Quarter 2020: May 1, 2020
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EDITORIAL

# From the Editor-In-Chief

Manish J.Gandhi, *MD*

Welcome to the first *Quarterly* of 2020! In this edition we continue the trend of reprinting the papers that were awarded the best papers published in *Human Immunology* (HI). The HI editorial board recently voted for the best three papers that were published in 2018. We are publishing the second and third best papers in this issue. I would like to thank Dr. Amy Hahn, the editor-in-chief of HI for allowing us to share this with you; and to Kathy Giovetsis and Sarah Black from ASHI headquarters for facilitating this.

In this issue of *ASHI Quarterly*, in the editorial section we have the usual complement of items, including the President's Message, My way to HLA, and Committee Reports. We have an excellent article from the Technologist Affairs Committee on validating the Halifaster Flow Cytometry Crossmatch Protocol. In the Scientific Articles section we have two articles. Both are review articles focusing on the role of Regulatory T cells for tolerance and CRISPR/Cas9 and CAR-T cell technologies. Personally, I found them well written, very easy to read as well as providing a succinct overview of these innovative technologies.

As a parting note, *Quarterly* is only made possible by contributions from the community. We are in dire need for articles and I appeal to all readers to share their articles and experiences for publication. If you do not have one, look for someone who may have something that can be shared. Kindly send this to me or any member of the editorial board.

## New ABHI Certification Wallet Cards – *now digital!*

In the past, you would receive a paper card mailed to you once every 3 years, after a successful recertification. ABHI is moving paperless and has launched a new feature that allows you to download and/or print the wallet card yourself, at *any* time, not just recertification.

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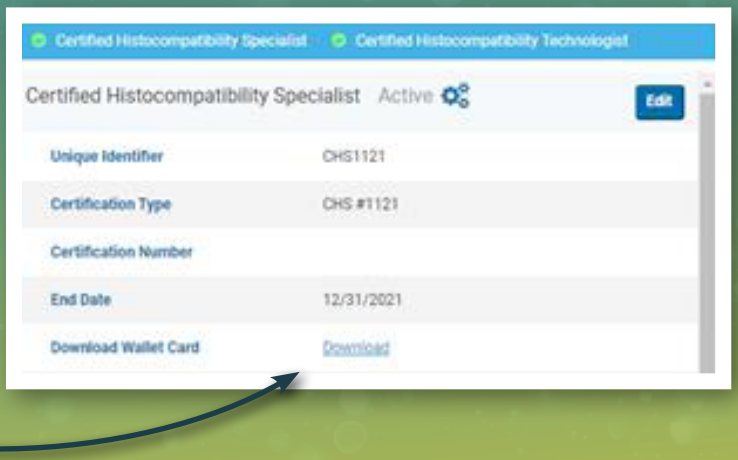

EDITORIAL

# President's Message

Medhat Askar, *MD, PhD*

Dear friends and colleagues,

I just communicated with you few days ago regarding the impact COVID-19 is having on ASHI. As we all watch new developments closely, I thought I'd try to take your mind off that even briefly by sharing some of the exciting things happening in our great society, ASHI:

**Website refresh:** The IT Committee is working with a web design team on a website refresh for ashi-hla.org. The design will feature an updated home page, a new menu bar for easier navigation and rebranding artwork consistent with the ASHI University website. We expect to unveil this new look by year's end.

**Local HLA Meetings:** The March 1 deadline for completing an application for Local HLA Meeting funding has ended and I'm thrilled to announce that the board has approved funding for 6 labs! This is double the number of labs approved in 2019. If you'd like to request funding from ASHI to host a local HLA meeting, just complete this application by the next deadline, August 1.

**Award Nominations:** The deadline for award nominations is May 15th. Please consider nominating a colleague in recognition of their exceptional contributions to our society.

**Board Nominations:** The deadline for nomination is April 10. Please consider nominating yourself or a colleague to bring unique and diverse perspectives and experiences to propel the progress of our great society even further than it has even been.

**ASHI's Support of the FOCIS Meeting:** ASHI prides itself on its collaborations with other organizations and we are dedicated to advancing the science, education and application of immunogenetics and transplant immunology. For over 10 years, ASHI has jointly sponsored two symposia at the FOCIS meeting. Organized by ASHI leader, Dr. Elaine Reed, these symposia are scheduled on Tuesday, June 23, 2020. The morning session sponsored by ASHI, The American Society of Transplantation (AST) and The Transplantation Society (TTS), is entitled "Going Viral: Modulation of Alloresponses by Viral Infection." The afternoon session, sponsored by ASHI, The Association of Medical Laboratory Immunologists (AMLI) and The Immune Transplant Network (ITN), is entitled "Engineering Immunity: Shedding Light on Potential Pathogenic Mechanisms in Autoimmunity, Cancer and Transplantation."

**2018 Best Articles in Human Immunology Awarded:** Dr. Amy Hahn, editor-in-chief of *Human Immunology*, together with the *Human Immunology* Editorial Board, have announced the best articles of 2018:

- The paper receiving the most votes was Predicting an HLA-DPB1 expression marker based on standard DPB1 genotyping: Linkage analysis of over 32,000 samples by Schöne, B., et al.
- In second place was CRISPR/Cas9 and CAR-T cell, collaboration of two revolutionary technologies in cancer immunotherapy, an instruction for successful cancer treatment by Mollanoori, H., et al.
- In third place was Regulatory T cells for tolerance by Kawai, K., et al.

Congratulations to all these esteemed colleagues, their research teams and institutions.

Wishing you all health and happiness,

Medhat Askar

## Publish your article in *ASHI Quarterly*!

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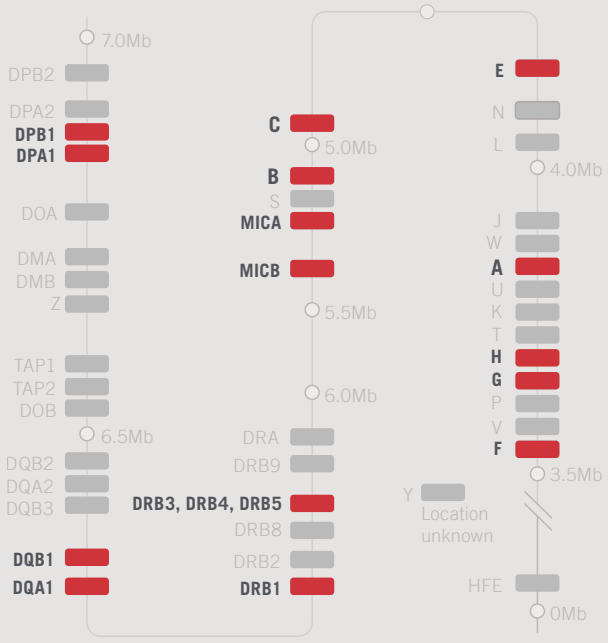


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# ASHI On the Hill

Tim Casey

While the year is still young, 2020 is shaping up to be yet another active year for federal policy developments of interest and importance to ASHI. We wanted to provide an overview of some key updates and initiatives that are shaping our policy focus this year.

## VALID Act Introduction Imminent

Following December's roundtable with Congressional staff, Food and Drug Administration (FDA) officials and key stakeholders, authors of the Verifying Accurate, Leadingedge IVCT Development (VALID) Act have been busy preparing to introduce legislation that would reform how laboratory-developed tests are regulated. Building on the compelling testimony delivered by ASHI President, Medhat Askar MD, PhD, during last year's roundtable on Capitol Hill, ASHI continues to engage in meaningful dialogue with Congressional authors ahead of the bill's unveiling.

Lawmakers are slated to introduce the newest iteration of the legislation this month with bipartisan, bicameral support. ASHI continues to fight to ensure HLA labs' ability to provide timely determinations of donor-specific histocompatibility is preserved. Be sure to check back for updates on developments surrounding this important legislation.

## President's Budget Released

On February 10, the Trump administration released its FY 2021 budget proposal, A Budget for America's Future. While the budget proposal will never carry the weight of law, it serves as a framework for the administration's priorities and provides insight on policy initiatives they may pursue. ASHI identified a number of key items of relevance for members, including the following:

### Health Resources and Services Administration (HRSA)

- **Organ Transplantation:** The president's framework includes a boost of $3 million for organ transplantation efforts. The proposed budget provides funding for the implementation of new policies and initiatives designed to expand support for living organ donation. The organ transplantation program projects that it will facilitate the transplantation of more than 32,600 deceased donor organs in FY 2021. The request also includes $18.5 million for the OPTN, SRTR and public and professional education efforts to increase public awareness about the need for organ donation and approximately $2.4 million for activities related to the Advisory Committee, interagency agreements and other internal support and program-related activities.
- **Cord Blood Inventory Program:** The Cord Blood Inventory Program would see a reduced investment of $9 million in 2021 under the proposal. It still seeks to keep pace with the statutory goal of building a genetically diverse inventory of at least 150,000 new units of high-quality cord blood for transplantation and supports collecting and banking approximately 2,400 CBUs toward the statutory goal.
- **C.W. Bill Young Cell Transplantation Program:** This budget request maintains the performance target of 4.08 million adult volunteer donors from underrepresented racial and ethnic populations listed on the program's registry and prioritizes recruiting and tissue-typing new donors.

### Department of Health and Human Services

- Advancing American Kidney Health: The budget includes $39 million in support of the president's executive order. The proposal would invest across multiple HHS agencies and requests new legislative authority in support of the Advancing American Kidney Health Initiative's three goals:
  1. Reduce the number of Americans developing end-stage renal disease (ESRD) by 25% by 2030
  2. 80% of new ESRD patients in 2025 receive dialysis at home or a transplant
  3. Double the number of kidneys available for transplant by 2030

To achieve these goals, HHS is scaling programs nationwide to optimize screening for kidney disease, educate patients on care options, support innovation and research for therapies and pioneering new payment models.

- **Extend Immunosuppressive Drug Coverage for Kidney Transplant Patients:** Kidney transplant recipients not otherwise eligible for Medicare lose coverage of immunosuppressive drugs 3 years after transplantation. The proposal creates a new federal program that provides lifetime coverage of immunosuppressive drugs for certain kidney transplant recipients until they are otherwise eligible for Medicare coverage. Coverage applies to kidney transplant recipients whose transplants were covered by Medicare and who are no longer ESRD patients or do not otherwise meet Medicare eligibility criteria.

- **Allow the Secretary to Determine Appropriate Number of Organ Procurement Organizations:** The budget proposal would allow the secretary to increase the number of Medicare-certified OPOs. The administration poses that the flexibility would provide the department with additional tools to increase the utilization of available organs from deceased donors by increasing organ recovery and reducing the organ discard rate.
- **Allow the Secretary to Determine the Appropriate Recertification Period for Organ Procurement Organizations:** The proposal would allow the Secretary to set the recertification intervals to be at least every two years but not more than every four years.
- The administration also proposed cuts to FY2021 budgets for the National Institutes of Health and the Centers for Disease Control and Prevention, with nearly 7% and 16% cuts proposed to each agency, respectively.

Now that the president's budget proposal has been released, the FY2021 appropriations process is kicking into high gear. ASHI will continue to provide updates on this process as we work to ensure that funding for priority programs remains robust.

## ASHI Advocates for Better Data Collection, Reimbursement

ASHI has long been a critic of the flawed data collection methodology brought forth by the implementation of Protecting Access to Medicare Act (PAMA) and the subsequent reimbursement cuts that followed. Last year, ASHI worked to support the passage of the Laboratory Access for Beneficiaries (LAB) Act of 2019 in order to delay the next round of PAMA reporting requirements by one year. Importantly, the new law also requires the Medicare Payment Advisory Commission (MedPAC) to conduct a thorough study of CMS' data collection methodology and report to Congress with recommendations.

Passage of the LAB Act represents a critical victory in our advocacy efforts, but much work remains. We encourage laboratories to prepare for data reporting next year in order to ensure CMS' data collection is as robust as possible. In the meantime, please take time to read ASHI's position statement, and check back for updates as ASHI continues working to reverse the trend of harmful payment cuts.

## Comprehensive Immunosuppressive Drug Coverage for Kidney Transplant Patients Act

Significant progress has been made in recent months on legislation that would remove the harmful 36-month post-transplant Medicare coverage limit on immunosuppressive drugs and ensure those receiving kidney transplants get the lifesaving medication they need.

The Comprehensive Immunosuppressive Drug Coverage for Kidney Transplant Patients Act has now been introduced in both the House and Senate with bipartisan support. ASHI is actively working to support its passage and submitted testimony to the House Energy and Commerce Committee earlier this year when the committee considered the proposal. Recent committee attention, in combination with the above-referenced support for this policy included in the president's budget request, represents meaningful momentum for this important policy change. Recognizing the significant number of patients awaiting transplants and the limited supply of transplantable kidneys, ASHI will continue working to support measures, like these, that prioritize the long-term success of kidney transplants. ASHI will keep you updated on progress of this important issue.

## The Lost Opportunities to Supply Transplantable Organs (LOST Organs) Act

Senators Todd Young (R-IN), Chris Van Hollen (D-MD) and Ben Cardin (D-MD) introduced the Lost Opportunities to Supply Transplantable Organs (LOST Organs) Act, which would require UNOS to track and publicly report on the status of all organs in transport. In the event that an organ is lost, damaged or delayed, an open and transparent process would be established to determine the cause, corrective action and recommendations to prevent it from happening again.

**If you have any questions or concerns regarding the policy initiatives ASHI is pursuing and developments we are tracking, please do not hesitate to contact ASHI Headquarters at info@ashi-hla.org.**

# My Way to HLA

Runying Tian, *CHS(ABHI)*

## New Job – Dr. Hildebrand – HLA

When I first joined Dr. William Hildebrand's lab at Oklahoma University Health Science Center in 2003, I had no clue what I was getting into career wise. I knew his lab was under contract with NMDP, switching HLA typing methodologies to capillary sequencing, and was in the process of achieving ASHI accreditation. For the first few months, I kept busy reading "HLA Beyond Tears," the ASHI manual, ASHI standards and the accreditation inspection checklist. Dr. Deborah Crowe was invited to help us, and I learned a great deal from her as well. At the end of 2003, the lab was accredited by ASHI.

For the next 8 years, I continued to learn and explore the "HLA Disneyland."

## ASHI Annual Meeting – One Lambda Party – Dancing

I attended my first ASHI Annual Meeting in 2003. I remember vividly how lost I felt internally on the first day. This field was new to me and I did not know anyone outside of my lab. I made a lot of effort during the meeting to meet people, starting by adding my Chinese name to my name tag and introducing myself to those who could speak Chinese. I made some progress and met Jennifer Zhang from UCLA, Dongfeng Chen from Duke, Ming Lin (now with Oklahoma Medical Center) and Jianping Li from Canada. My ultimate bonding with the HLA field occurred at the One Lambda party. When I entered the party, the energy I felt in the air secured my love for this field. I said to myself "*ASHI people know how to relax through music and dancing. This is our connection.*"

## Mentors – Inspiration – Advancing in HLA

My first mentor was Dr. Hildebrand. His passion for science and motivating energy were incredible. He was very assertive, and consistently challenging himself and those who were around him. It was intimidating at the beginning. But, I soon recognized that he was challenging us for the benefit of our career. I remember the first abstract I wrote, he came to me with a copy of my abstract in his hand shaking it and said, "where are the data." When Dr. Hildebrand noticed that I had not checked the oral presentation box as I submitted my abstract, he confronted me and urged me to do it. I checked it, but secretly wished I would not be chosen. When I found out that I was accepted for an oral presentation, I was so nervous and asked my church friends to pray for me. Subsequently, I joined a Toastmaster Club (training for public speaking). As I am writing this article, I realize how far I have come. I am grateful for his mentorship.

*As I am writing this article, I realize how far I have come. I am grateful for his mentorship.*

I was inspired to become an ASHI inspector by Dr. Marilyn Pollack. She came to inspect our lab in 2005. Steve Cate and I assisted her during the inspection. Something triggered my curiosity that day. I remember thinking that I want to be an inspector someday. That "someday" did not happen until more than a decade later.

As I mentioned before, Dr. Deborah Crowe helped us with our ASHI accreditation. I had an opportunity to tell her how much I appreciated her when I inspected her lab at Vanderbilt VA Hospital in 2018.

I first came to know Dr. Rene Duquesnoy during ASHI's 31st Annual Meeting in Washington, DC, in 2005. I had a very close friend in Pittsburgh. When I planned my travel for the meeting, visiting her was part of my plan. During the meeting, I looked for someone from Pittsburgh. I was only in the HLA field less than

*Continued on next page*

EDITORIAL

# Technologist Affairs

## *The View from a Travel Fund Award Recipient*

Runying Tian, MS, *CHS(ABHI)*

As a recipient of a Travel Fund Award to attend the 2019 ASHI Annual Meeting, I am privileged to share my experience with ASHI members. I heard about this award many times over the years but had never considered applying. My manager at the in the Clinical Transplantation and Immunology Lab of Duke University Medical Center, Wendy Hanshew, encouraged me to apply on July 22, 2019. I decided to give it a try, and am so glad that I did! It was not a complicated process. The questions focused on my involvement with ASHI (volunteering, committees, etc.) and my general supervisor/director's name and email address. A short time after submitting my application, my director, Dongfeng Chen, and manager were contacted to confirm my experience. The next thing I knew, I had won!

Being able to attend the 2019 ASHI/BANFF meeting was a great blessing. The HLA field is advancing at a high speed. When I had to miss a meeting one year, I felt 10 years behind the next. The meeting allows me to learn the latest technologies and to see how the field of HLA is constantly expanding. Networking within the ASHI community is such a warm and encouraging experience. I have developed personal friendships and made professional contacts from attending ASHI meetings. The ASHI Inspectors Training Workshop refreshed my knowledge and gave me more confidence for my October 2019 inspection. The most touching and rewarding experience was hearing Dr. Robert Montgomery's story. As a transplant surgeon and a heart transplant patient, his presentation gave me a new perspective about what I am doing. Our lab was then able to send one more person to attend the ASHI meeting. I appreciate the opportunity very much.

## *My Way to HLA • Continued from previous page*

3 years at that time. Someone told me about Dr. Duquesnoy. I approached him and asked him for a ride to Pittsburgh. He did not show any hint of hesitation and accepted my request. It was one of my favorite memories in my career. I also got to know Dr. Adriana Zeevi's mother, as she rode in the car as well. I did not know much about Dr. Duquesnoy at that time. If I had known he was the 1986-1987 ASHI President, I probably would have been too intimidated to ask and would not have this story to tell. Another unforgettable thing about Dr. Duquesnoy was his view about heart transplant. He said it was a very ancient idea. He quoted Ezekiel 36:26, "I will give you a new heart and put a new spirit in you: I will remove from you your heart of stone and give you a heart of flesh." That was a refreshing perspective about heart transplant.

When I reflect on my HLA journey, not only am I impressed by the professional expertise of my colleagues, but am also inspired by their unselfish personalities. My colleagues have instilled into me the willingness to share knowledge, experience, and kindness, and to be authentic.

### Duke – Solid Organ – Expanding of my HLA career

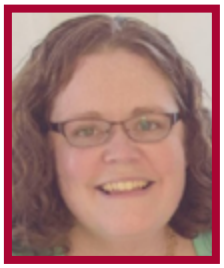
The more I learned about HLA, the more I realized that I needed to explore solid organ transplantation. A position opened at Dr. Dongfeng Chen's lab at Duke University Medical Center and I was offered a position to join their team in 2011. The last 9 years have been very rewarding. Dr. Chen and Wendy Hanshew (lab manager) have helped me to grow professionally. I have been involved in all aspects of solid organ transplantation testing, such as Flow Crossmatch, Flow PRA, Luminex Single Antigen and SSO and NGS typing. Becoming an ASHI-certified inspector and volunteering to serve on the Technologist Committee are my ways of saying "thank you" to ASHI and the HLA community.

Without my family's sacrifices and support, I would not be able to tell you all these stories. My husband, Ziyun, and two precious children, Nathanial and Nina, have been my solid mental and emotional supporters. Thank you for the opportunity to tell my HLA story!

# Technologists Affairs

## *Halifaster Flow Cytometry Crossmatch Protocol: The Art of Performing a Meaningful Validation*

Rachel Wahlert, *MS, CHS(ABHI)*
Valia Bravo-Egana, *PhD, D(ABHI), MBA*



Rachel Wahlert, MS, CHS(ABHI)

Valia Bravo-Egana, PhD, D(ABHI), MBA

Crossmatch (XM) remains very relevant to assess immunological compatibility between transplant candidates and donors. Like most of the tests performed in histocompatibility laboratories, XM is a non-waived, high-complexity test which requires a thorough validation before it can be used in a clinical setting. The main purpose of a validation is to confirm, through the establishment of objective evidence, that all requirements for the specific intended application have been fulfilled,[1] demonstrating the test is ready to be implemented for routine use. However, collecting data to prove the validity of such complex techniques can be challenging, especially when considering XM testing is performed using methods and protocols that are not standardized among different laboratories.

Modified protocols for flow cytometry XM testing have been developed. Halifaster is one such protocol, optimized for speed and efficiency by changing the assay platform from test tubes to a $^{96}$-well tray and adjusting parameters such as serum-to-cell ratio and incubation times.[2] Using this protocol, it is possible to reduce the time required to perform the test while maintaining or even improving upon the quality and sensitivity of the standard flow cytometry XM.

Despite the advantages of implementing such improved methods, various factors may play a role limiting their adoption in histocompatibility laboratories. Some labs lack the time, discretionary funding, or dedicated personnel to perform the extensive and complex validations that XM testing requires. Even labs able to embark upon this kind of project may find their staff require time-consuming direction to carry out the validation in an effective manner.

To complete a successful validation, many requirements must be met. For this purpose, ASHI and CAP standards are important tools for evaluating the results, but detailed, step-by-step guidance is outside their scope. Rather, standards are intended to establish the regulatory framework in which the testing should be used. Therefore, we perceive the need for a document outlining the validation procedure itself. With this article, we aim to provide comprehensive guidance that helps lab staff design and accomplish a successful XM validation without wasting time and efforts. While we recognize that every lab may perform validations with slight differences and adapt protocols to better fit their own workflows, here we present the Halifaster validation undertaken by our lab in 2018 as a framework for other laboratories.

The first phase of our validation focused on defining reagent volumes and dilutions; specifically, anti-human IgG-conjugated FITC, CD3 and CD19 monoclonal antibodies to detect T and B lymphocytes, and positive controls. Titrations of crossmatch reagents were done for one reagent at a time. We started with FITC, then moved to the monoclonal antibodies and eventually to class I and class II positive controls. The humanized monoclonal positive controls used are commercially available (3). Titrations were done using concentrations above and below those listed in the Halifaster protocol to ensure that selected concentrations were optimized for our lab. In the next step, serum to cell ratios were defined (Table 1).

TABLE 1
### Validation Steps

| STEPS | NUMBER OF CROSSMATCHES |
|---|---|
| 1. Titrate FITC | 5 |
| 2. Titrate monoclonal antibodies (CD3, CD19) | 5 |
| 3. Titrate positive control | 5 |
| 4. Titrate serum volume | 5 |
| 5. Generate negative and positive cutoffs (crossmatching 20 cells versus 20 sera) using the reagent titers and volumes previously determined | 400 |
| 6. Validate the established cutoffs by comparing results of Halifaster with standard XM protocol | 46 |

TABLE 2

## Selected Parameters Optimized in Our Laboratory Compared to The Standard XM Protocol and the Original Halifaster Protocol

| Parameter | Standard tube Protocol | Original Halifatser Protocol (Liwski et al.) | Halifaster Protocol optimized in our laboratory |
|---|---|---|---|
| Assay Platform | 5ml 12x75 mm tubes | 96 -well tray | 96-well tray |
| Serum volume | 20ul | 30ul | 20ul |
| Cell isolation | EasySep Direct | EasySep Direct | EasySep Direct |
| Cell number | 2.5x10^5 | 1.5x10^5 | 1.5x10^5 |
| First Incubation | 30 minutes | 20 minutes | 20 minutes |
| Washes 1st set | 4x1 minutes at 3000 RPM | 3x1 minutes at 500 g | 3x1 minutes at 500 g |
| Wash buffer volume | 1000ul | 200ul | 200ul |
| volume CD3/CD19/FITC | Added individualy 10ul/20ul/50ul (1:800 ) | 50ul cocktail (46.88ul PBS) 2ul/1ul/0.125ul | 50ul cocktail (26ul PBS) 6ul/12ul/6ul (1:100) |
| Second incubation time | 30 minutes | 5 minutes | 5 minutes |
| Washes 2nd set | 2x1 minutes at 3000 RPM | 2x1 minutes at 500 g | 2x1 minutes at 500 g |
| Final suspension volume | 500ul | 400ul | 200ul |

Once the specific conditions were defined (Table 2), flow crossmatches were performed using 20 cell preparations (10 isolated from peripheral blood and 10 from lymph nodes or spleen preparations) against 10 sera from non-transfused males and 10 sera from historically non-sensitized patients to define the cutoff for positivity.

We included sera from patients with a well-documented history of no sensitization to account for minor non-HLA related reactivity that could be detected in their serum due to interference from health conditions and/or treatments. Including samples exhibiting reactivity of this nature might better reflect the individualities observed in some patient's sera, which may differ from sera obtained from healthy individuals.

The negative cutoff was defined as the mean plus 2 standard deviations (SD) of the channel shifts obtained from the XM performed with the 10 sera from non-transfused males and the 10 sera from historically non-sensitized patients. The positive cutoff was defined as the mean channel shift plus 3SD (Table 3a). The negative range was defined as any channel shift equal to or below the negative cutoff. The positive range was defined as any channel shift equal to or above the positive cutoff. The range between the channel shift mean plus 2SD and the channel shift mean plus 3SD was defined as Questionable Negative (Table 3b). The minimum positive control shifts for the crossmatch to be accepted as valid was defined as the mean channel shift of the positive control (1:10,000 dilution) plus 2 SD. From values exhibited in table 3a, it would be 208.6+81.1=290 channel shifts for T cells and 274.5+61.9=336 channel shifts for B cells.

CS=channel shift, SD=Standard Deviation. Mean CS refers to the channel shifts obtained from the XM performed with the 10 sera from non-transfused males and the 10 sera from historically non-sensitized patients.

To validate the defined cutoffs, we determined the correlation between the interpreted results obtained with the standard method and the Halifaster protocol optimized in our lab. We also correlated each crossmatch protocol with antibody testing. We ran 46 XMs using 20 donor cells and 38 sera in parallel using both protocols; 39 XM were allogeneic and 7 were autologous; 15 of the cell preparations used were from patients and healthy donors, 3 were from deceased donors, and 2 were from proficiency surveys.

A variety of combinations of serum samples exhibited none, only one, few or multiple donor specific antibodies (DSA) with different reactivity levels expressed as Mean Fluorescence Intensity (MFI) with particular cells were selected for these XMs. Similarly, an assortment of serum samples from patients with different levels of sensitization for one or both of the HLA antigen classes were included to account for cross-reactivity, elevated background reactivity due to high level of sensitization, and etc.

To summarize the comparison of the results obtained using the Halifaster and the standard XM protocols, 39 out of the 46 XMs performed reached consensus between both protocols for

TABLE 3

## a) Defining Negative and Positive Cutoffs.

| Serum | T cells | | | B cells | | |
|---|---|---|---|---|---|---|
| | Mean CS | 2SD | 3SD | Mean CS | 2SD | 3SD |
| Negative Control | 19.1 | 50.5 | 75.8 | 11.5 | 76.7 | 115.1 |
| Positive Control 1:4000 dilution | 304.6 | 80.5 | 120.7 | 366.9 | 58.4 | 87.6 |
| Positive Control 1:10000 dilution | 208.6 | 81.1 | 121.7 | 274.5 | 61.9 | 92.9 |

## b) Result Interpretation Ranges

| Ranges | T cells | B cells |
|---|---|---|
| Negative (≤ Mean CS + 2SD) | ≤ 71 | ≤ 90 |
| Questionable Negative (Mean CS + 2SD - Mean CS + 3SD) | 72-95 | 91-128 |
| Positive (≥ Mean CS + 3SD) | ≥96 | ≥129 |

TABLE 4

### Correlations Between the 2 XM Protocols and With Antibody Testing

| CORRELATIONS | T CELLS | B CELLS |
|---|---|---|
| Standard XM protocol and Halifaster XM protocol (%) | 85 | 93 |
| Standard XM protocol with SA results (%) | 83 | 91 |
| Halifaster protocol with SA results (%) | 96 | 93 |

TABLE 5

### Sensitivity and Specificity of the Protocol Being Validated Versus the Standard Protocol and Antibody Testing

| | RESPECT TO CURRENT XM METHOD | | RESPECT TO SA TEST | |
|---|---|---|---|---|
| | T CELLS | B CELLS | T CELLS | B CELLS |
| Halifaster Sensitivity | 71% | 85% | 93% | 89% |
| Halifaster Specificity | 100% | 100% | 90% | 96% |

T cell and 43 for B cells. Therefore, the correlation between the standard protocol and the Halifaster protocol was 85% and 93% for the T and B cell XMs, respectively (Table 4).

For the cases where there was no consensus between the two protocols, we determined that the 7 T cell XM with disparate results were negative with the Halifaster protocol, while 6 were questionable positive and 1 positive with the standard protocol. Remarkably, in 6 of the 7 XMs, no positive DSA were detected in the sera used, suggesting the results obtained with Halifaster correlated better with antibody testing than those obtained with the standard protocol. In the remaining discrepant XM, DSA were detected with low to moderate MFI values. In circumstances where antibodies with low titers are detected, interpretation may be particularly difficult. Defining the nature of low reactivity might be challenging. Various factors can contribute to this complexity, such as different sensitivity of the XM and the test used to detect antibodies, different expression of HLA antigens on particular cells, false positive reactivity detected in the antibody assay, and other variables.

For all 3 B cell XMs where no consensus between protocols was found, they were negative with Halifaster protocol while questionable positive with the standard protocol. However, no positive DSA were detected in any of these three samples, which also suggests the results obtained with Halifaster correlates better with the antibody testing than those obtained with the standard protocol.

Based on this validation, we calculated the sensitivity and specificity of Halifaster with respect to the standard protocol, which was 71% and 100% respectively for T cells, and 85% and 100% for B cells. The sensitivity and specificity of Halifaster with respect to the presence of DSA detected by SA antibody testing was 93% and 90% for T cells, and 89% and 96% for B cells (Table 5).

In summary, we provided step by step instructions for validating a XM protocol. In our particular case, the protocol validated was Halifaster but the guidance presented has the potential to be used for validating other protocols. We described the test design, including the key reagents that should be titrated, the controls necessary for initial and ongoing verification of test performance, as well as ideas for sample selection.

The variety of samples and controls selected are crucial for generating data which allow determination of meaningful cutoffs and establishment of acceptable criteria for test performance. The array of surrogate donor cells selected helped to account for differences in antigen expression and other complexities related to the quality of donor cell preparations. Finally, we discussed the analysis performed to validate the cutoffs, assess sensitivity and specificity compared to an established XM protocol, and comparison with other testing such as HLA antibody identification.

Although we did not expand the scope of this article to the implementation phase, some of the elements reviewed may help in the preparation of the documents required for the accreditation process and to incorporate the test in the lab workflow.

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# CRISPR/Cas9 and CAR-T cell, collaboration of two revolutionary technologies in cancer immunotherapy, an instruction for successful cancer treatment

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ABSTRACT

Clustered regularly interspaced short palindromic repeats/CRISPR associated nuclease9 (CRISPR/Cas9) technology, an acquired immune system in bacteria and archaea, has provided a new tool for accurately genome editing. Using only a single nuclease protein in complex with 2 short RNA as a site-specific endonuclease made it a simple and flexible genome editing tool to target nearly any genomic locus. Due to recent developments in therapeutic engineered T cell and effective responses of CD19-directed chimeric antigen receptor T cells (CART19) in patients with B-cell leukemia and lymphoma, adoptive T cell immunotherapy, particularly CAR-T cell therapy became a rapidly growing field in cancer therapy and recently Kymriah and Yescarta (CD19-directed CAR-T cells) were approved by FDA. Therefore, the combination of CRISPR/Cas9 technology as a genome engineering tool and CAR-T cell therapy (engineered T cells that express chimeric antigen receptors) may lead to further improvement in efficiency and safety of CAR-T cells. This article reviews mechanism and therapeutic application of CRISPR/Cas9 technology, accuracy of this technology, cancer immunotherapy by CAR T cells, the application of CRISPR technology for the production of universal CAR T cells, improving their antitumor efficacy, and biotech companies that invested in CRISPR technology for CAR-T cell therapy.

## 1. Introduction

The increasing burden of cancer in the human population is the major concern in today's societies, therefore finding safe and effective alternative therapies had become one of the main goals of researchers throughout the world. One of these alternative approaches is harnessing the power of the body's own immune system to battle against cancers which is termed immunotherapy. Considering the use of the body's own immune system, immunotherapy promises a more effective and durable treatment than conventional treatments, and chimeric antigen receptor-T cell therapy (CAR-T), a type of immunotherapy, can transform the future of cancer treatment. CAR-T cells are genetically engineered T cells that express artificial proteins known as chimeric antigen receptors, which navigate this CAR-expressing immune cells to surface tumor antigens. Recently significant advances were reported in therapeutic engineered T cells and effective responses were shown by the use of CD19-directed chimeric antigen receptor T cells (CART19) in patients with B-cell leukemia and lymphoma. Kymriah and Yescarta are two CD19-directed genetically modified autologous T cells were recently approved by FDA as CAR-T cells that used in patients who had acute lymphoblastic leukemia (ALL) and certain types of non-Hodgkin lymphoma (NHL), respectively [1,2]. Considering the overlap between genome editing approaches and CAR-T cell therapy in the context of genetic engineering enables us to use both of them together in combination. Making targeted manipulation in the genome for therapeutic goals requires highly efficient systems to modify existing DNA patterns with great accuracy. Programmable nucleases such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR-Cas9 provide powerful tools for editing and engineering the genome that can be used to study the function of genes and therapeutic goals. Among aforementioned nucleases, the use of CRISPR/Cas9 has surpassed others for ease, pliability and the possibility of multiplex gene editing [3,4]. CRISPR-Cas9 is taken from type II acquired immune system in bacteria and archaea that used to shield

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**Table 1**
Comparison of the different types of programmable nucleases in genome editing.

| Features | CRISPR/Cas9 | TALEN | ZFN |
|---|---|---|---|
| DNA binding component | RNA | Protein | Protein |
| Cost | Low | Moderate | High |
| Nuclease | Cas9 | FokI | FokI |
| Toxicity | Low | variable – high | Low |
| System design | Simple | Complex | Complex |
| Efficiency | High | High | High |
| Multiplex editing capability | High | Low | Low |
| Disadvantages | Off targets | expensive and time consuming<br>Pairs of ZFNs are required to target<br>any specific locus<br>Complexity of protein domains<br>Off targets | time consuming<br>Pairs of TALENs are required to target<br>any specific locus<br>Complexity of protein domains<br>Large size of TALE molecules difficult to deliver to the cells |
| Limitations | Narrowed target sequences due to necessity of presence of PAM sequences | Binding efficiency depends on the presence of thymidine nucleotide before the 5′ end of a sequence | recognition 3–6 nucleotide sequences |

themselves from bacteriophage and plasmid of cellular invaders [5,6]. Among the types of CRISPR/Cas systems identified so far type II (CRISPR/Cas9) system has been studied the most and has been modified and adjusted for use in genome editing in eukaryotic systems [7,8]. Therefore, CRISPR/Cas9 technology, as a strong genome engineering platform is able to significantly expand the kind of cancers and patients that can be treated with CAR-T cells. Also, further improves the efficiency and safety of these therapies. This article explains how CRISPR/Cas9 technology works in the targeted genome editing and its therapeutic application in man, issues and problems related to the accuracy of this technology and the approaches used to solve these problems. Then, CAR-T Cells-based therapeutic technology and the use of CRISPR/Cas9 technology to produce universal CAR-T cells and increasing the potency of these therapeutic approaches are discussed. At the end, we introduce biotech companies that invested in CRISPR technology for CAR-T cell therapy (see Table 1).

## 2. Mechanism and application of CRISPR-Cas9 technology in the treatment of human diseases

This immune system in bacteria and archaea contains a short sequence of invaded bacteriophage or plasmid DNA that cleaved from them and stored in the CRISPR locus of these organisms as a novel spacer sequence. This DNA transcribed and processed into RNA for recognition of subsequent invade of the same virus or plasmid to eliminate them [9]. The Protospacer Adjacent Motif (PAM) sequence, a sequence contains a few nucleotides, is the recognition site in the invaders DNA and recognized by Cas nuclease but not integrated into the host genome with Protospacer sequence. Subsequent invades result in transcription of an RNA from the spacers in CRISPR locus which is called pre-crispr RNA (pre-crRNA). In type II of CRISPR system (CRISPR/Cas9) a second RNA, termed trans-activating CRISPR RNA (tracrRNA), is transcribed and results in the maturation of pre-crRNA by binding to the repeated sequences of pre-crRNA and forming a RNA duplex structure that is cleaved by RNase III. This structure also works as a scaffold among crRNA and Cas9 [10,11]. Finally crRNA/tracrRNA duplex recruits Cas9 nuclease to form a ribonucleoprotein complex that fulfills Cas9 function and navigates it to align with target DNA by base pairing with complementary crRNA spacer [5,6]. To further simplify this genome editing system for laboratory application, CRISPR/Cas9 diminished into 2 parts: Cas9 protein alongside a sole sgRNA, an artificial RNA containing crRNA and tracrRNA. Cas9 nuclease activity requires the presence of PAM sequence immediately after the 3′ end of the sgRNA complementary DNA sequence (5′-NGG-3′ for SpCas9), so in the presence of PAM sequence in target DNA Cas9 nuclease induces a double-strand break (DSB) at three nucleotides upstream of the PAM [7,12,13]. Generally, DSBs that initiates the process of two intrinsic mechanisms for DSB repair can be categorized into two different repair approaches: homologous directed repair (HDR) approach that is used to knock-in desire DNA and either leading to precise repair of the mutation or creating disease-specific mutations, and Non-homologous end joining (NHEJ) approach for knock outing the desire DNA or deletion of the target site (by inducing two cuts at two borders of desired locus) (Fig. 1) [14]. Therefore, by generating DSBs at the desired sites in the genome and using two aforementioned repair approaches, the genome can be edited for different purposes, recently the use of this system has become popular for therapeutic purposes (see Fig. 2).

CRISPR-Cas9 system as an adaptive immune defense system has shown therapeutic potential in manipulating and disabling viral genome to obstruct virus infection, such as HIV, human papillomavirus, Hepatitis B virus, Herpes viruses, JC Virus and hepatitis C virus [15]. Gene therapy applications have also been examined for monogenic disorders, including disruption of the PCSK9 gene for treatment of cardiovascular disease, deletion of an intronic region in the CEP290 gene that comprises a mutation which disrupts the gene coding sequence by generating an aberrant splice site for Leber Congenital Amaurosis type 10 (LCA10), correction of the sickle cell mutation and induction of fetal hemoglobin (HbF) expression for sickle cell disease (SCD), correction of point mutation in fumarylacetoacetate hydrolase (FAH) for Hereditary Tyrosinemia type I and other diseases such as cystic fibrosis, Duchenne muscular dystrophy, Epidermolysis bullosa, X-linked chronic granulomatous disease [16].

CRISPR-Cas9 technology for treating cancer has so far been used in three areas: cancer immunotherapy, manipulation of cancer genome and epigenome, and elimination or inactivation of carcinogenic viral infections.

## 3. Obstacles and limitations of using CRISPR/Cas9 for therapeutic applications

Due to the simplicity, high efficiency, high pliability, low cost, ability to multiplex genome editing, and wide applicability (in a wide range of cell cultures and animal models) for targeted genome editing the attention of many researchers have been attracted to the CRISPR/Cas9 technology. This rapidly expanding technology has begun a new vista in the treatment of a variety of human diseases, although some technical challenges and barriers related to the accuracy, efficiency, and delivery of this technology remain ahead and delay its therapeutic potential. Accuracy of the CRISPR–Cas9 technology is defined by the ability of editing desired target locus in the genome, but we understand the Cas9-sgRNA binding to the target DNA can tolerate sequence mismatches and besides target DNA sequences, also identical, or highly

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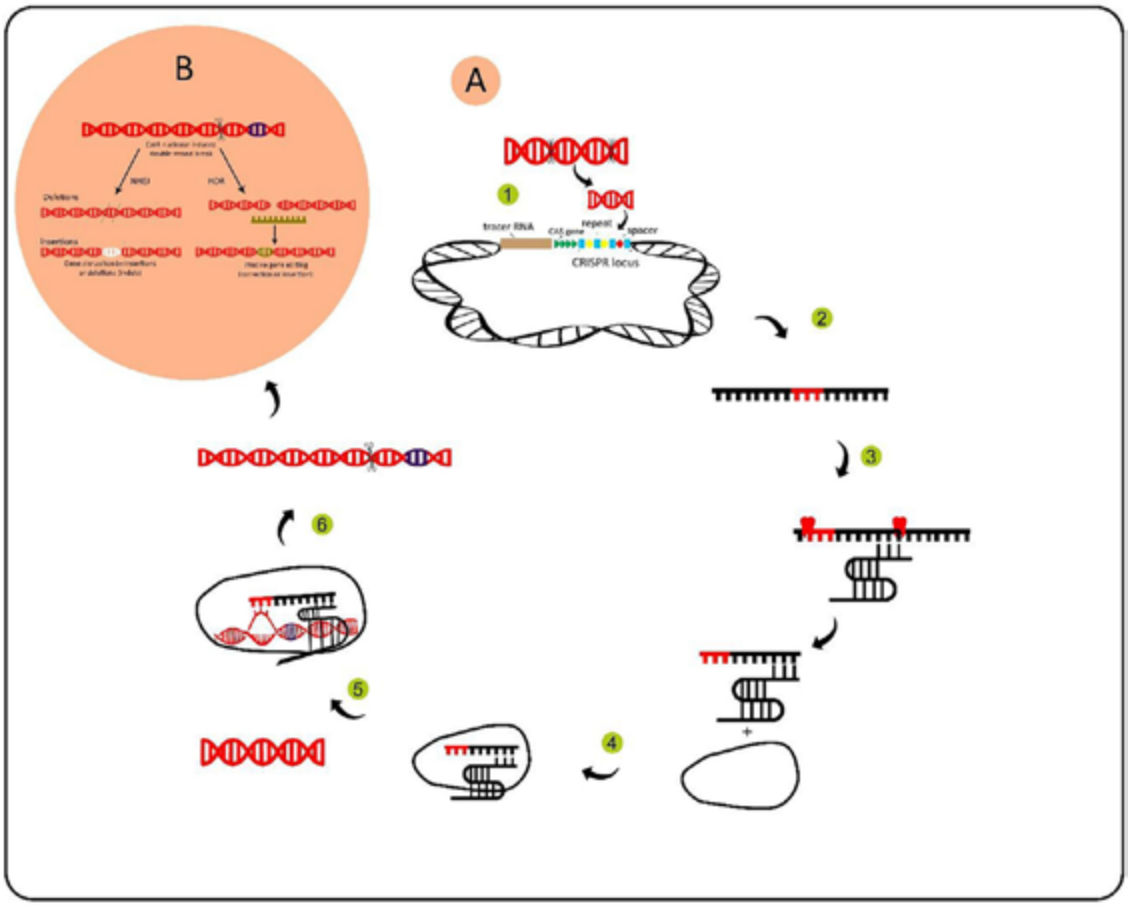


**Fig. 1.** Natural CRISPR/Cas9 pathway and its usage in gene editing approaches. A: 1. A short sequence of invaded bacteriophage or plasmid DNA incorporated into the CRISPR locus as a novel spacer sequence. 2. This sequence transcribed and processed into pre-crRNA after subsequent invade. 3. A second RNA, tracrRNA, is transcribed and results in maturation of pre-crRNA by binding to the repeated sequences of pre-crRNA and forming a RNA duplex structure that is cleaved by RNase III and produces the guide RNA 4. Inactive Cas9 nuclease recruited by guide RNA and becomes activated. 5. The activated guide RNA/cas9 complex binds to the target DNA. 6. The Cas9 nuclease cleaves the invaded DNA and inactivates it. B: In genome editing approaches DSBs initiates two intrinsic mechanisms for DSB repair: homologous directed repair (HDR) approach that used to knock-in desire DNA and either leads to precise repair of mutation or creates disease-specific mutations, and Non-homologous end joining (NHEJ) approach for knock outing desire DNA or deletion of the target site.

homologous DNA sequences can be cleaved. This will lead to mutations in undesired genomic loci, called off-target effects. Off target effects are the major barrier to the potential use of CRISPR/Cas9 technology in therapeutics approaches that lead to mutations or chromosomal re-arrangements because of re-ligation between cuts on different chromosomes. These off target effects are just as deleterious as genomic defects, so in order to have a safe therapeutic application of CRISPR/Cas9 system, reduce off-target events derived from the endonuclease remain a priority and should be diminished, as much as possible.

Various approaches have been explored to minimize Cas9-mediated off-target events that are briefly mentioned below. The precise selection of sgRNAs is the first and most effective approach for reducing potential off-target effects. Several online tools based on predictive algorithms are available that calculate the interaction between synthetic editing systems and DNA for designing sgRNAs with maximum specificity for a desired genomic locus [17]. Use of a truncated sgRNAs, 17 bp, showed similar efficiency as full-length gRNA while showed a reduced off-target effect and more sensitivity to sgRNA/DNA mismatches. The 5′-end nucleotides in sgRNAs are not essential for full sgRNA activity and may lead to mismatches at other positions along the sgRNA-target DNA interface, so the use of shorter sgRNA improves the specificity of CRISPR/Cas9 by increasing the sensitivity to mismatches. The reduction of extra binding energy of the Cas9/sgRNA complex to target DNA may lower nonspecific mismatches of the sgRNA/DNA target-locus jointing, thereby leads to ameliorated specificity of targeting [18]. Modified Cas9 proteins will produce novel Cas9 variants with altered PAM preference that have improved the range of available PAMs and present more sgRNA options for providing a better chance to recognize those with less off-target activity. CRISPR/Cas9 recognition of the target site by sgRNA will navigate Cas9 to a complementary sequence in the target site, but Cas9 requires recognition of a short neighboring PAM for its nuclease activity. This restriction leads to a challenge in the implementation of genome editing applications that require precision in a small area of the genome, such as homology-directed repair (HDR), the introduction of indel mutations into genetic elements with small size such as microRNAs, splice sites, etc., and allele-specific editing. Engineered Cas9 variants with novel PAM specificities can circumvent targeting range limitations, also by providing novel PAM sequence we can improve the precise selection of more specific sgRNAs [19]. High-fidelity Cas9 endonucleases arise from the structure-guided design of CRISPR/Cas9 system based on the detailed crystal structure of Cas9–gRNA complex bound to its target DNA. Kleinstiver et al. 2016 created multiple substitution mutations in the amino acids that bind to the phosphate backbone of the target DNA strand and found one variant

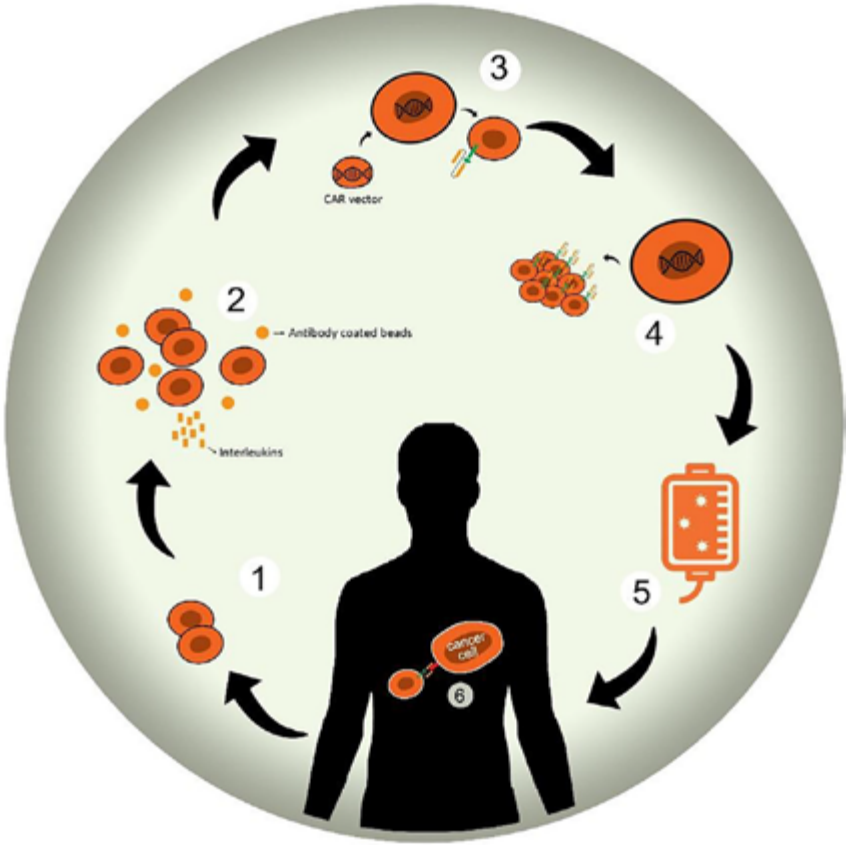


**Fig. 2.** CAR T cell therapy: 1- The first step in CAR T therapy is collection of T cells from patients by leukapheresis. 2- Collected T cells are activated and expanded by antibody coated beads and interleukins. 3- T cells transduced with CAR gene by using lentiviral vectors, retroviral vectors, or non-viral methods like electroporation and sleeping beauty system to express CAR receptor on their surface are called "CAR T cells". 4- CAR T cells are expanding. 5- After quality control CAR T cells infused into the patient. 6- In the patient's body CAR T cell track down and kill tumor cells.

with four substitutions functioned as a high fidelity Cas9 (Cas9HF) with more on-target activity and minimal off-target activity. Cas9 nuclease has some non-specific interactions with its target DNA site, such as four direct hydrogen bonds to the phosphate backbone of the target DNA strand that made by N497, R661, Q695, Q926 residues. The disruption of these non-specific contacts reduces the extra energy of the Cas9/sgRNA complex, enables it not to bind to mismatched off-target sites in the presence of a desired target DNA site [20]. With a similar approach, positively charged amino acids involved in the interaction with the complementary DNA strand mutated and demonstrated enhanced accuracy.

Nuclease function is activated through strand separation by helicase activity of Cas9. The crystal structure of Cas9/sgRNA/target DNA complex reveals a positively-charged groove, positioned between the HNH, RuvC, and PAM-interacting domains in Cas9 that is likely to be involved in stabilizing the non-target strand of the target DNA, so neutralization of these positive charges can significantly reduce off-target cutting whilst maintaining on-target activity, by that means more stringent Watson-Crick base pairing between the guide RNA and the target DNA strand produced [21]. One of the molecular modifications of Cas9 to reduce off-target effects involves inducing mutations in one of the two nuclease domains, HNH or RuvC, to produce a modified Cas9 that nicks one strand of the target DNA (termed a Cas9 nickase). In this strategy Cas9nickase has been combined with a pair of sgRNAs complementary to opposite strands of the target locus and in a way similar to dimeric ZFNs and TALENs cleaves the target locus by the synergistic interaction of two independent sgRNA, whilst nicking of both DNA strands by a pair of Cas9 nickases leads to site-specific DSBs, individual nicks are predominantly repaired by the high-fidelity base excision repair pathway (BER), thereby minimizes off-target effects by each individual Cas9n-sgRNA complex [22]. Fusion of a catalytically inactive Cas9 protein (dCas9) to the dimerization-dependent nonspecific FokI cleavage domain generates an RNA-guided nuclease that its nuclease activity depends on the presence of a pair of FokI domains to form a dimer. This approach improves CRISPR accuracy due to the fact that FokI is just active as a dimer, thereby navigated via a pair of sgRNAs to cleave a larger DNA in their target site (about 40 bps). This larger sequence fortunately almost always is unique [23]. The exposure time of genome to Cas9 or high levels of Cas9 has been associated with the off target effect, so control of Cas9 activity results in reducing the off-target effects and improving the specificity. Different strategies used for regulating Cas9 nuclease activity, for example conditional expression of Cas9 by an inducible promoter, fusion of Cas9 with an estrogen receptor domain (ERT2) that sequester Cas9 to the cytoplasm in the absence of 4-HT, control of the Cas9 enzymatic activity via self-splicing properties of inteins, control of Cas9 activity by stimulating with blue light through fusion with light-responsive elements and use of self-restricted CRISPR/Cas9 system [17].

Another barrier to the use of CRISPR/Cas9 technology in therapeutic approach is the lower HDR efficiency than NHEJ, however, so far several approaches for increasing HDR rate have been explored [16]. By solving current challenges and improving safety and delivery methods, the CRISPR-Cas9 system can be applied for clinical applications in patients. Recently this system has been approved by advisory committee at the US National Institute of Health as the first clinical trial to attack cancer cells [24].

## 4. Why CAR-T cell therapy?

Conventional therapies have several restrictions to become reliable and to produce lasting outcome in patients with advanced, relapsed and refractory malignancies, therefore the development of new therapeutic

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approaches with suitable outcomes are critical to successful cancer treatment. Immune system, particularly T lymphocytes has a critical role in identification of tumor-specific antigens, therefore harnessing the power of the patient's immune system to eradicate cancerous cells has been regarded in the scope of immunotherapy. Adoptive T cell transfer (ACT), a type of immunotherapy, involving the ex vivo expansion of fragments of resected tumors or genetically engineered patients' derived T cells and sending them back to the patients for eliminating tumor cells, divided into three scopes: tumor-infiltrating lymphocytes (TILs), T cell receptor (TCR) -engineered T cells, and chimeric antigen receptor (CAR) -modified T cells [25]. There are 2 major T cell sources that can be used in the ACT, including tumor-infiltrating lymphocytes (TILs) and peripheral blood T lymphocytes that engineered to express alpha/beta T-cell receptors (TCRs) or chimeric antigen receptors (CARs). In TIL therapy approach antigen-specific tumor-infiltrating lymphocytes (TILs) are derived from tumor tissue and cultured with lymphokines such as interleukin-2 and then these TILs infused into the patient to induce lysis of tumor cells and tumor regression. Adoptive T-cell therapy (ACT) using TILs is a powerful immunotherapeutic approach against metastatic melanoma, but several important limitations exist in TIL therapy: first the requirement to perform an invasive method to gain tumor tissue to grow TILs, second the difficulty in identifying antigen-specific T cells in other cancer types, and third failure to grow TILs in ~10% to 15% of patients with melanoma [26,27]. The ability to modify lymphocyte genes to induce expression of the desired T-cell receptor (TCR) or chimeric antigen receptor can circumvent these limitations. Chimeric antigen receptor/T cell receptor (CAR/TCR) engineered T cells are autologous T-cells that harbor a transgene encoding antigen receptors (CARs) or genetically engineered T cell to express modified T-cell receptors (TCRs) chain that will elevate antigen affinity [28]. Both CAR- and TCR-engineered T cells have unique advantages and disadvantages to each other. Engineered TCRs recognize peptides derived from either cell surface or intracellular proteins in an HLA-dependent manner that provide the option to target intracellular tumor antigen. However, restriction to HLA also has been a limitation. CAR T cell contrary to TCRs operates in an MHC-independent manner. Engineered TCRs recognize MHC restricted peptides taken from cell surface as well as intracellular proteins and transcription factors, so provides access to a greater number of targets than CARs, however CAR-T cells also can be develop against carbohydrate and glycolipid antigens to target malignant cells expressing these antigens via the activation of cytoplasmic co-stimulation [29–31]. The CAR is a single chimeric protein that artificially made by fusing an extracellular antigen-recognition moiety, single-chain variable fragment (scFv) antibody is frequently used for the antigen-recognition domain also human Fab fragments and natural ligands that engage their cognate receptor can be used, to one or several intracellular T-cell signaling and costimulatory domains [32,33]. CAR T-cells divided into four generations, the first generation has used a single chain antibody such as CD3ζ or FcεRIγ that links the immunoreceptor tyrosine-based activation motifs (ITAM) at transmembrane region, second and third generations include costimulatory molecule such as CD28, CD137(4-1BB), CD134(OX40), CD27, DAP10 and CD244 that can ameliorate proliferation, cytotoxicity, sustain response, and prolong the life of CAR-T cells in vivo (the second generation has one costimulatory molecule and the third generation has two costimulatory molecules). The fourth-generation CARs were generated by adding a second factor, including cytokines or ligands such as IL-12, IL-18, CD40L and 4-1BBL etc. to the foundation of the second-generation constructs to ameliorate efficacy and durability [34–36]. CAR-T cell therapy as one of the most novel and successful agents in cancer immunotherapy entered into the clinic and recent significant responses in clinical trial in B-cell malignancies have generated great enthusiasm to the ultimate smart cancer therapeutics which turned on the lights of hope in cancer patients. After showing complete responses in refractory ALL and CLL patients using a CD19-directed CAR T cell product, US Food and Drug Administration (FDA) recognized CAR-T cell therapy as a "breakthrough therapy" and approved it to treat leukemia and lymphoma which opened the path to commercializing CAR-T cell by a number of pharmaceutical and biotechnology companies [37,38].

## 5. Combined use of CRISPR-Cas9 and CAR-T cell in cancer immunotherapy

Conventional CARs in the majority of CAR T-cell clinical trials derived from patient's own autologous T cells, however, the generation of these autologous CAR T-cells have some limitation, including, time-consuming and expensive process, difficulty in collecting T cells with good quality and quantity in patients with crucial diseases that limit the use of these modified T cells to a low number of patients. These limitations have led to the idea of making universal or off-the-shelf T cells, genetically modified allogeneic T cells derived from healthy donors, that have the potential to overcome these limitations, simplify the manufacturing of engineered CAR-T cells and available for many number of patients. Universal CAR-T cell achievement requires passing from two main obstacles, graft-versus-host disease (GVHD), rejection of the infused allogeneic T cells in the recipient, and improved biosafety profile for more powerful disease-targeted activity. The endogenous αβ T-cell receptors (TCRs) on allogeneic T cells that responsible for antigen recognition may recognize recipient alloantigens and result in GVHD. The presence of intrinsic human leukocyte antigen (HLA) molecules on the allogeneic CAR-T cells that recognized as foreign HLA molecules, usually triggers rapid immunologic rejection in recipients and prevents their application in allogeneic setting as the off-the-shelf CAR-T cells. To solve these obstacles, researchers investigated ways to silence or disrupt both TCRs and HLA molecules in allogeneic universal T cells and as mentioned above, CRISPR/Cas9 technology because of the possibility of highly effective multiplex gene editing at a time, simplicity to use and high pliability can be used for this aim. In several studies CRISPR/Cas9 mediated multiplex knock out of TCR beta chain and beta-2-microglobulin (B2M), an essential subunit of the HLA-I molecule, have been used in the production of universal CAR-T cells. Results show these universal cells retain function both in vitro and in vivo without causing GVHD [39–41]. Knocking out B2M and the production of HLA-I negative CAR T-cells, leading to a problem in this CAR T-cells. HLA-I negative CAR T-cells are potential target for Nk cells that would lead to the rejection of theses universal T cells. There are some approaches to circumvent this problem, including applying an engineered T cell that express HLA-E or anti-NK cell depletion antibody [42].

T cell inhibitory receptors or signaling molecules, such as CTLA-4, PD-1, LAG-3, and TIM-3 are naturally occurring "off signals" to ensure proper control of T cell response. The expression of these inhibitory receptors on CAR-T cells leads to T cell exhaustion. Recent studies showed tumor cells use this characteristic for immune evasion, for example, tumor cells upregulated PD-1 ligand that causes reduced immune responses [43]. Therapeutic approaches specifically designed to target and inhibit these inhibitory receptors by immune checkpoint related antibodies, such as anti-PD-1, PD-L1 and CTLA-4, displayed great success in the treatment of solid tumors in addition to hematologic malignancies [44–46]. The reported successes of inhibiting these inhibitory signals by related antibodies have led to the use of CRISPR/Cas9 technology to destroy them. Studies indicate an improvement in the antitumor efficacy and clinical outcome of these modified CAR-T cells [39,41,47–49]. T cell response, can be mediated by Fas receptor that is a member of the tumor necrosis factor α family of death receptors. This receptor and its ligand (FasL) involved in T cell apoptosis that can attenuate CAR T cell activity because of cell Fas/FasL-dependent activation induced cell death (AICD). Therefore, ablating Fas induced cell death by knocking out the Fas receptor by CRISPR/Cas9 can result in the generation of CAR T cells that are resistant to apoptosis and enhances CAR T cell function [41].

In a new manner, recently, CRISPR technology was used to target

TRAC locus. In this approach CRISPR/Cas technology used as a delivery system to knock in a CD19-specific CAR into TRAC locus that placed the CAR expression under control of TCR promoter and caused enhanced potency, uniformed CAR expression, more memory characteristics and less exhausted phenotype in this CAR [50].

In summary, studies that used CRISPR/Cas9 technology to modify CAR T cells indicate that this technology is a highly efficient approach to generate genetically modified CAR T cells with a targeting efficiency about 90% for single gene disruption (the efficiency decreased as the number of targeted genes increased, maybe due to the competition of the gRNAs for Cas9) without impairing effector function a pure population of CAR T cells could be achieved by enriching the genetically modified T cells.

As above mentioned, CRISPR/Cas9 can be easily used for silencing or disrupting any desired genomic locus, so, the combination of this technology with CAR-T cell therapy can be an instruction for successful cancer treatment and as mentioned in the next section several companies invest in the improvement of CAR-T cell therapy by CRISPR/Cas9 technology.

## 6. Big biotech companies in commercializing therapeutic approach

Conventional autologous CAR T-cell therapy has some limitations and high cost, but the idea of universal allogeneic CAR T-cells that sourced from a healthy donor and undergo gene editing changed the condition and several big companies have invested in this field. Indeed, CRISPR technology by realizing the dream of manipulating the genome opened a new vista in the treatment of human disease. Unlike traditional genome-editing technologies that have had limited use due to design complexity, transfection inefficiencies and limitations in ability to multiplex gene editing, the emergence of CRISPR technology results in a significant enhance in genome editing investment in the last years because of its extraordinary potential in genome editing, simplicity and low cost [51]. Editas Medicine (Editas medicine. 2018), Intellia Therapeutics (Intellia Therapeutics. 2018) and CRISPR Therapeutics (CRISPR Therapeutics. 2018) are three big biotech companies that invested in CRISPR-based gene-editing and play a major role in this field. Intellia established in 2014 by Caribou Biosciences to expand the Caribou CRISPR-Cas9 platform and in January 2015, Intellia initiated collaboration with Novartis to explore options for using CRISPR to engineer chimeric antigen receptor (CAR) T cells and hematopoietic stem cells (HSCs). Intellia Therapeutics uses in vivo approaches for targeting liver diseases, including transthyretin amyloidosis, alpha-1 antitrypsin deficiency, hepatitis B virus, and inborn errors of metabolism. Its ex vivo approaches include chimeric antigen receptor T cells and hematopoietic stem cells. Editas Medicine targets diverse range of diseases including LCA10, β-thalassemia, SCD, DMD, AATD, CF and cancer (chimeric antigen receptor/T cell receptor (CAR/TCR) engineered T cells). In May 2015, Editas Medicine entered into collaboration with Juno Therapeutics (a leader in the emerging field of immuno-oncology) to ameliorate the efficacy and safety of Juno's chimeric antigen receptor/T cell receptor (CAR/TCR) engineered T cells by its own CRISPR platform. CRISPR Therapeutics develops CTX101, a donor-derived allogeneic anti-CD19 CAR-T cell for targeting CD19-positive malignancies and also anti-CD70 CAR-T cell for both hematologic malignancies and solid tumors. Anti-B-cell maturation antigen (BCMA) for multiple myeloma. Due to the potential of CRISPR/Cas9 technology to target any desire locus in the genome and abundance of genetic based diseases, including monogenic disease, and common diseases such as cancer, CRISPR market worth's billion dollars globally.

## 7. Conclusion and outlooks

The rapidly growing field of immunotherapy has created many hopes in the treatment of cancer and CAR-T19 has recently achieved FDA approval. So, development of this therapeutic approach has attracted much attention nowadays. Furthermore, CRISPR-Cas9 technology has opened a new vista to genome editing and its utilization in the engineering of T cells provides an extraordinary potential to build CAR-T cell and streamlined the emerging field of immunotherapy. But several technical challenges still need to be solved regarding accuracy, safety and efficiency issues of this technology. After solving these current challenges and improvement of the delivery methods, the CRISPR-Cas9 system can apply for clinical applications in patients.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2018.09.007.

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Review

# Regulatory T cells for tolerance

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ABSTRACT

Regulatory T cells (Tregs) are critical mediators of immune homeostasis and hold significant promise in the quest for transplantation tolerance. Progress has now reached a critical threshold as techniques for production of clinical therapies are optimised and Phase I/II clinical trials are in full swing. Initial safety and efficacy data are being reported, with trials assessing a number of different strategies for the introduction of Treg therapy. It is now more crucial than ever to elucidate further the function and behaviour of Tregs *in vivo* and ensure safe delivery. This review will discuss the current state of the art and future directions in Treg therapy.

## 1. Introduction

The past few decades have marked an era of promising advances in the quest for clinical tolerance, with much of the progress attributed to the growing knowledge of regulatory T cells (Tregs). Findings within that demonstrate the role of Tregs as crucial mediators of immune homeostasis have led many to speculate these cells to be key to tolerance induction. Indeed, from the identification of specific Treg markers to the implementation of Treg cellular therapies in pioneering preliminary clinical studies, the addition of Tregs to the clinical armamentarium is on the horizon. There is still much to learn in the study of Tregs, ranging from optimizing expansion mechanisms to implementing appropriate therapies in clinical trials. This review will focus on the knowledge gained in Treg research thus far, ongoing clinical trials for Treg-based cell therapies, and prospective areas of research.

### 1.1. Regulatory T cells (Tregs): A brief background

#### 1.1.1. Origin

Although the concept of T cells that act as suppressors had been proposed as far back as the 1970's and 1980's with contributions from groups Gershon/Kondo [1], Okumura/Tada [2], and Hall/Jelbart/Dorsch [3], research in Tregs only really started to gain traction in the 1990's after the discovery of specific markers that distinguished this suppressor population from other T cells [4]. It was in this period that the $CD4^+CD25^+$ T cell population was identified as having potent regulatory properties in self-tolerance and autoimmune suppression [5]. While a number of other non-CD4 expressing Treg subtypes exist, this review will focus on the extensively studied $CD4^+$ Treg subset. CD25 not only serves as a useful marker for Treg identification, but due to its high affinity for interleukin-2 (IL-2), it is also important for the expansion and maintenance of Tregs [4].

The identification of the FOXP3 gene helped to spearhead investigation into the characterization and function of Tregs due to its role as a 'master regulator' [6,7]. Foxp3-mutant scurfy mice and Foxp3-null mice demonstrate $CD4^+CD25^+$ regulatory T cell deficiency that quickly leads to aggressive lymphoproliferative autoimmune syndromes that is reversible with restoration of the Treg compartment. In immunodeficient mice, Tregs can also be generated with the addition of a Foxp3 transgene [8]. In a similar manner, a FOXP3 mutation in humans leads to an X-linked autoimmune lymphoproliferative disorder called immune dysregulation, polyendocrinopathy, enteropathy, X-linked

*Abbreviations:* aGvHD, acute graft-versus-host disease; aGvHD, acute graft-vs-host disease; AL, acute leukemia; AL, acute leukemia; APC, antigen presenting cells; arTreg, alloantigen reactive T regulatory cell; arTreg, alloantigen-reactive T regulatory cell; ATG, anti-thymocyte globulin; aTreg, activated T regulatory cell; aTreg, activated Treg; BMT, bone marrow transplantation; CAR, chimeric antigen receptor; cGvHD, chronic graft-versus-host disease; CNI, calcineurin inhibitors; CTLA-4, cytotoxic T-lymphocyte antigen 4; DC, dendritic cell; FACS, fluorescent-activated cell sorting; GMP, good manufacturing practice; GvHD, graft-versus-host disease; HLA, human lymphocyte antigen; HSC, hematopoietic stem cells; IDO, indoleamine 2,3-dioxygenase; IL, interleukin; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; iTreg, induced regulatory cell; LDLT, living donor liver transplantation; mAb complex, monoclonal antibody complex; MMF, mycophenolate mofetil; NHP, nonhuman primate; nTreg, natural T regulatory cell; PBL, peripheral blood lymphocyte; PBSCT, peripheral blood stem cell transplant; pTreg, peripheral T regulatory cell; rTreg, resting Treg; SOT, solid organ transplantation; Tconv, T conventional cell; ThRIL, TReg Immunotherapy in the setting of Liver transplantation; Tr1, type 1 regulatory cell; TSDR, T regulatory cell-specific demethylated region; tTreg, thymus-derived T regulatory cell; UCB, umbilical cord blood

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(IPEX) [9]. Variability in FoxP3 gene expression can lead to differential Treg suppressive function. In particular, the methylation status of the Treg-specific demethylated region (TSDR) within the FoxP3 gene locus in both mice and humans correlates with suppressive function. The demethylation of this evolutionarily conserved region not only serves to regulate FoxP3 gene transcription and the conversion of non-Treg cells to Tregs, but also plays a factor in maintaining suppressive function. However, the mechanisms by which it serves this role is not yet completely understood [10–12].

Tregs may be classified into two main subpopulations, the more conventional naturally-occurring thymus-derived Tregs (nTregs), and induced Tregs (iTregs), which represent a population of suppressive Tregs that are differentiated from $CD4^+$ T cells in the periphery. nTregs and iTregs are also commonly referred to as thymus-derived Tregs (tTregs) and peripherally-induced Tregs (pTregs), respectively [13]. A number of other regulatory $CD4^+$ T cells exist, including Type 1 regulatory T cells (Tr1) and Th3 cells, which exhibit suppressive functions but do not express FOXP3. There have been interesting findings that suggest that transient FOXP3 expression may be necessary for the suppressive function of these cells, but further discussion of non-FOXP3 expressing Tregs is beyond the scope of this review. [14,15]

*1.1.2. Suppressive mechanisms*

Tregs display significant versatility in their suppressive mechanisms, which can depend on the immune environment, the Treg activation state, and target cell type [16]. In transplantation, for example, the suppressive mechanisms can depend on donor-specificity [17]. There are four main processes by which Tregs suppress immune responses: (1) modulation of dendritic cell (DC) function or maturation, (2) inhibitory cytokine release, (3) cytolysis, and (4) metabolic disruption, [18].

(1) Investigation of specific pathways involving cytotoxic T-lymphocyte antigen 4 (CTLA-4) has demonstrated that Tregs can hinder the activation of effector T cells by modulating DCs. Multiple studies have supported the finding that Tregs may downregulate B7 costimulatory molecules CD80 and CD86 on DCs in a CTLA-4 dependent manner to disrupt the activation of effector T cells by antigen presenting cells (APC). CTLA-4 interacts with CD80/CD86 ligands with high affinity to deprive naive T cells of CD28-mediated costimulation. [19]. Studies indicate that although CD80/86 is mainly expressed by APCs, naive T cells can also express these molecules, providing a further target for CTLA-4 [20]. Through CTLA-4 ligation, Tregs may also induce DCs to express the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO), which converts tryptophan to kynurerine, a molecule toxic to T cells [16]. Another relevant molecule expressed on Tregs is LAG-3, which binds to MHC-II molecules to suppress DC maturation [18].
(2) TGF-β, IL-10, and IL-35 are considered to be the main regulatory cytokines released by Tregs. These cytokines are involved in tasks such as the direct suppression of effector T cell signaling, regulation of IFN-γ, the induction of Tregs, and the maintenance of FoxP3 expression [16,21,22].
(3) Tregs may exert suppression through expression of granzymes that induce programmed cell death. By this mechanism Tregs are able to kill and suppress B cells and possibly hinder effector T cell function through apoptosis [23].
(4) Metabolic disruption is another potent mechanism of suppression. A number of studies have demonstrated that Tregs can induce apoptosis of effector T cells through their high expression of CD25 that allows them to deprive effector T cells of IL-2 [24].

*1.1.3. Treg therapy in transplantation*

The overarching goal in Treg therapy in transplantation has been to promote a tolerogenic state, while reducing the dependence on toxic immunosuppressive medication [25,26]

Treg therapies could bring advantages in terms of practicality, such as cost, availability, specificity, and applicability across different organs, due to their potential to be standardized and manufactured industrially [27]. As a select few Treg therapy studies enter Phase II clinical trials, the present stage marks a significant milestone [28]. It is therefore critical that the operational machinery of Tregs is elucidated for safe and effective use.

An important aspect of clinical translation is development of Good Manufacturing Practice (GMP) methods for Treg therapy production [29]. This is complicated by controversies over which subsets of Tregs are best suited for therapy in terms of function and stability.

When isolating Tregs for clinical cell therapy, it is important to be careful of contamination with non-Tregs in the final product. The presence of populations such as effector cells, for example, may be proinflammatory [30,31]. Thus, the isolation of suppressive Treg cell populations is much more complicated than simply the identification of previously discussed markers such as CD4/CD25/FOXP3. Although FOXP3 is constitutively expressed in suppressive Tregs, it is an intracellular marker that cannot be used in the context of isolation. Moreover, CD25, although expressed highly in Tregs, is not exclusive to Tregs and includes other cell types, such as activated effector T cells [6,15,32]. Necessarily, other markers must be used to further purify the desired population. CD127, the α-chain of the IL-17 receptor, inversely correlates with FoxP3 expression, and its low expression can be used in conjunction with CD4 and CD25 to isolate a highly suppressive Treg population [33–35].

Isolation of Tregs is also complicated by the need to consider the different subsets within the heterogeneous Treg population that would best function in transplantation. Depending on various environmental and pathological conditions, different Treg populations can express a number of transcription factors, microRNA, chemokine receptors, suppressor molecules, cytokines, and other immune-related proteins [36]. However, the identification of many new markers in recent years has made it possible to dissect this heterogeneity and to hone Treg therapy to identify and select for specific populations that will function optimally in organ transplantation. CD45RA, for example, distinguishes three Treg subpopulations with functionally different properties: $CD45RA^+FoxP3^{lo}$ resting Tregs (rTregs), $CD45RA^-FoxP3^{hi}$ activated Tregs (aTregs), and cytokine-secreting $CD45RA^-FoxP3^{lo}$ non-suppressive Tregs [12,37]. However, there are challenges in Treg isolation through CD45RA because naive Treg numbers in the peripheral blood diminish with age [38,39].

Treg stability is critical in clinical therapy. As a whole, previous studies have demonstrated the durable stability of Tregs, but there remain findings that suggest that at least some subsets of Tregs display a loss of suppressive function or FOXP3 expression. There is evidence that a minor population of Tregs, which have lower expression of CD25, will not only lose their suppressive function but also differentiate into effector T cells in certain conditions, particularly when Tregs are adoptively transferred into lymphopenic recipients [40]. Loss of FoxP3 expression after infusion may be attributed to reduced IL-2 production within the host due to the Tregs not being able to produce IL-2 themselves [41]. Some studies have also indicated that Tregs lose their FoxP3 expression through stress signals elicited by proinflammatory cytokines and liposaccharides, where FoxP3 is ubiquinated by the E3 ligase Stub1 [42]. Some of these “ex-Tregs” may be able to regain their suppressive function, but the exact mechanisms and conditions in which this occurs is not yet clearly defined [40]. A large obstacle in measuring the stability of Tregs post-infusion is the lack of a safe and effective GMP-approved strategy to track the Tregs’ survival in humans. While there have been some studies that label Tregs to provide insight into their behaviour post-infusion [43,44], an effective method that can be used in the clinical setting would be crucial in guiding treatment post-transplantation.

One marker that has been associated with stability of suppressive function in Tregs is the transcription factor *Helios*. Some have also suggested *Helios* as a marker for distinguishing nTregs from iTregs [45];

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however, other recent studies have challenged this notion that the lack of *Helios* expression does not necessarily indicate an iTreg population [46]. The TSDR, which is perhaps the most commonly identified marker for stability of FoxP3 expression, has been demonstrated to be fully demethylated in nTregs, while markedly more methylated in iTregs [47]. In general, nTregs are thought to be able to maintain FOXP3 expression better than iTregs and have been the focus of many isolation protocols. However, iTreg generation *in vivo* using induction agents may be a useful and perhaps more economically viable strategy in comparison to adoptive cellular therapy [48].

### 1.2. Animal models

#### 1.2.1. Mice

In terms of elucidating potential mechanisms and new targets of research, Treg cell therapy in mouse models has offered valuable insights. The pretreatment of mice with infusion of donor alloantigen and anti-CD4 mAb can generate alloantigen-specific CD4 + CD25+ T cells that potently regulate rejection of skin allografts in a CTLA-4 and IL-10 dependent manner [49]. In bone-marrow allograft transplantation murine models, transfer of either freshly isolated or *ex vivo* expanded Treg cells promotes tolerance of the allograft while hindering GvHD [50,51]. In another approach, pretreatment with IL-2/IL-2 monoclonal antibody (mAb complexes) expands Tregs and induces tolerance to pancreatic islets [52]. The adoptive transfer of Tregs in mouse models has also been used to track Treg migration patterns that suggest that Tregs have suppressive functions in both the transplanted graft and secondary lymphoid organs [53].

#### 1.2.2. Humanized mice

The majority of studies that have facilitated the translation towards clinical studies and the necessary regulatory body approvals have been performed in humanized mouse models and non-human primates. The differences between mouse and human Tregs necessitate this. For example, human Tregs typically express a memory phenotype, while mouse Tregs are generally antigen-inexperienced due to their housing in controlled environments [54]. Moreover, unlike mouse Tregs that can be clearly recognized with distinct markers such as FoxP3, there is yet to be a more definitive and defining marker for human Tregs. For example, FOXP3 in humans can be expressed by non-Tregs that may also upregulate CD25 while not being suppressive, bringing clear challenges in terms of identification [55].

Humanized mice are created through the engraftment of immunodeficient mice with human mononuclear cells, leukocytes, or hematopoietic stem cells (HSC). Our group has used humanized mice to demonstrate that treatment with *ex-vivo* expanded human Tregs could prevent human transplant arteriosclerosis, skin rejection, and islet rejection [35,56,57]. Using this model we have also demonstrated that different Treg populations have distinct tissue-specific homing markers that dictate their migration patterns and regulatory efficacy *in vivo* [58]. Treg populations that were initially assessed in these models have now been taken through to clinical trials in renal transplantation.

#### 1.2.3. Nonhuman primates (NHP)

Nonhuman primate Tregs have provided useful insights into human Tregs and accelerated clinical translation [59]. Bashuda et al. first demonstrated that the adoptive transfer of immunosuppressive anergic T cells that were generated by co-culture with donor alloantigen in the presence of anti-CD80/CD86 mAbs to suppress renal allograft rejection [60]. These cells have now progressed to clinical trials in kidney transplant recipients. In 2011, Ma et al. pretreated NHP cells with antithymocyte globulin (ATG) and then performed an adoptive transfer of donor alloantigen-specific Tregs combined with low-dose sirolimus, delaying acute rejection of renal allografts in *Cynomogulous* monkeys [61].

A number of NHP studies have focused on tracking Tregs after infusion, highlighting the challenges in sustaining the suppressive function of Tregs for use in therapy due to their transient nature [62]. Ezzelarab et al. infused multiple doses of *ex vivo*-expanded Tregs into lymphodepleted, MHC-mismatched *cynomogulous* monkey heart graft recipients early post-transplant and treated with tacrolimus, anti-IL-6 mAb, and rapamycin. Here, unlike the previous two NHP trials, the adoptive transfer of Tregs led to a relative decline in graft function along with induction of proinflammatory cytokine expression, anti-donor alloantibody production, and an increase in effector memory cells [63]. IFN-γ, IL-6, and IL-15 levels were elevated, which likely led to enhanced Tmem and alloAb responses and destabilization of Tregs. In the study, the authors reason that it is possible that a re-expansion of effector T cells was associated with the immunosuppressive tapering strategy [64]. The different results between these studies may be attributed to factors such as timing, frequency, and dosage of the injections, as well as the absence of splenectomy and the specific immunosuppressive regimen used.

### 1.3. Clinical trials of Treg cellular therapy

From the first report of human Treg expansion in 2001 and the subsequent first large-scale expansion of human Tregs in 2004 [65,66], progress has accelerated swiftly towards clinical trials. Research centres worldwide have now begun to translate their Treg cell therapies to the bedside with varying yet promising results [Table 1].

In 2009, Trzonkowski et al. published the first clinical report using *ex vivo* expanded Tregs in two patients with graft-versus-host disease (GvHD) [28]. Peripheral blood was isolated from family donors of patients and cells were flow sorted for $CD4^+CD25^{hi}CD127^-$ Tregs, and expanded *ex vivo* for adoptive transfer. After the transplantation, the patients were treated with increasing immunosuppression comprised of solumedrol, tacrolimus, mycophenolate (MMF), and ATG, and then were infused with Tregs ($6 \times 10^7$ Tregs per infusion) three times (days +75, +82, and +93 post-transplant). In a patient who had developed chronic GvHD (cGvHD) following bone marrow transplantation (BMT) to treat myelodysplastic syndrome, the infusion of *ex vivo* expanded Tregs led to a marked decrease in dependency on suppressive drug treatment as well as alleviation of GvHD complications. The other patient, who had developed acute GvHD (aGvHD) following allogeneic peripheral blood stem cell transplant (PBSCT) for chronic myelocytic leukemia, was treated with three doses of donor expanded Tregs that moderately improved the patient's condition. However, the patient did not survive long after available donor Tregs for infusion were depleted, indicating that more doses may be necessary to sustain suppression. This first clinical trial revealed vital information of the feasibility of adoptive Treg cell therapy in the context of transplantation and also highlighted some areas of required improvement.

In 2011, Di Ianni et al. reported the first clinical trial of adoptive transfer of Tregs to prevent GvHD in the absence of any post-transplantation immunosuppression in 28 patients with acute leukemia (AL) [67]. Their method centred around the early infusion of freshly isolated donor Tregs, followed by conventional T cells (Tconv) at the time of full-haplotype-mismatched HSC, and ultimately demonstrated that this prevented GvHD while also favouring Tconv-mediated post-transplantation immune reconstitution. The same group have continued with a Phase II trial in which they investigated whether the Treg-Tconv adoptive immunotherapy prevents post-transplant leukemia relapse [68]. In this trial with 43 adults with high-risk AL, patients were conditioned with a total-body irradiation-based regimen followed by grafts of $CD34^+$ cells, Tregs ($2 \times 10^6$/kg), and Tconvs ($1 \times 10^6$/kg) isolated from donors. The findings were promising in that with this treatment, almost 90% of patients were protected against GvHD, further supporting the safety and efficacy of using freshly isolated Tregs. Notably, in a 46-month follow up of AL patients, the use of Treg-Tcov adoptive therapy favoured post-transplant immunological reconstitution and led to an extremely low cumulative incidence of post-transplant leukemia

**Table 1**
Clinical trials of Treg cellular therapy.

| Study ID | Trial title [location] | Phase | Intervention | Study Start and estimated complete date | Condition [estimated enrolment] | Specificity | Doses |
|---|---|---|---|---|---|---|---|
| NCT01624077 | Safety Study of Using Regulatory T Cells Induce Liver Transplantation Tolerance (Treg) [China, Jiangsu] | I | iTregs | Dec 2014 to Dec 2015 | Liver Tx [n = 1] | Alloantigen specificity | $1 \times 10^6$/kg |
| NCT01660607 | Phase I/II MAHCT w/T Cell Depleted Graft w/Simultaneous Infusion Conventional and Regulatory T Cell [United States, California] | I/II | Fresh nTregs | Dec 2011 to March 2018 | Acute GVHD prevention [n = 54] | Non-antigen specificity | $1.0 \times 10^6$/kg (Initial dose) |
| NCT01795573 | Ex-vivo Expanded Donor Regulatory T Cells for Prevention of Acute Graft-versus-Host Disease (GVHD) [United States, Florida] | I | Donor alloantigen-reactive Treg (arTreg) | June 2014 to Jun 2019 | Acute GVHD prevention [n = 48] | Alloantigen specificity | – |
| NCT01903473 | Donor Regulatory T Cells Infusion in Patients With Chronic Graft-versus-host Disease [Belgium, Leuven and Liège] | II | Donor Treg | Jul 2013 to Dec 2016 | Steroid -refractory chronic GVHD [n = 35] | Non-antigen specificity | $0.5 \times 10^6$/kg |
| NCT01911039 | Phase 1 Infused Donor T Regulatory Cells in Steroid Dependent/ Refractory Chronic GVHD [United States, California] | I | Fresh nTregs (donor Treg) | Jul 2013 to Jul 2016 | Steroid-dependent/refractory chronic GVHD [n = 20] | Non-antigen specificity | 1, 5, and $15 \times 10^5$/kg |
| NCT01937468 | Trial of Regulatory T-cells Plus Low-Dose Interleukin-2 for Steroid-Refractory Chronic GVHD [United States, Massachusetts] | I | Fresh nTregs and IL-2 | Nov 2013 to Nov 2020 | Steroid -refractory chronic GVHD [n = 25] | Non-antigen specificity | – |
| NCT02088931 | Treg Adoptive Therapy for Subclinical Inflammation in Kidney Transplantation (TASK) [United States, California] | I | Polyclonal nTregs | Mar 2014 to Dec 2016 | Living donor kidney Tx [n = 3] | Non-antigen specificity | $320 \times 10^6$/total |
| NCT02091232 | Infusion of T-Regulatory Cells in Kidney Transplant Recipients (The ONE Study) [United States, Massachusetts] | I | Belatacept-induced nTregs | May 2014 to May 2018 | Living donor kidney Tx [n = 8] | Non-antigen specificity | – |
| NCT02129881 | ONE Study UK Treg Trial (ONETreg1) [United Kingdom, London and Oxford] | I/II | Polyclonal nTregs | Apr 2014to March 2017 | Living donor kidney Tx [n = 12] | Non-antigen specificity | $1–10 \times 10^6$/kg |
| NCT02145325 | Trial of Adoptive Immunotherapy With TRACT to Prevent Rejection in Living Donor Kidney Transplant Recipients (TRACT) [United States, Illinois] | I | Polyclonal nTregs | Apr 2014 to Dec 2021 | Living donor kidney Tx [n = 10] | Non-antigen specificity | – |
| NCT02166177 | Safety and Efficacy Study of Regulatory T Cell Therapy in Liver Transplant Patients (ThRIL) [United Kingdom, London] | I/II | Polyclonal nTregs | Jun 2014 to Jun 2019 | Liver Tx [n = 26] | Non-antigen specificity | 1 and $4.5 \times 10^6$/total |
| NCT02188719 | Donor-Alloantigen-Reactive Regulatory T Cell (darTregs) in Liver Transplantation (deLTa) [United States, California and Minnesota] | I | Donor arTreg | Dec 2014 to Jan 2022 | Liver Tx [n = 24] | Alloantigen specificity | 50, 200, and $800 \times 10^6$/total |
| NCT02244801 | Donor-Alloantigen-Reactive Regulatory T Cell (darTreg) Therapy in Renal Transplantation (The ONE Study) (DART) [United States, California] | I | Donor arTreg | Nov 2014 to Jun 2018 | Living donor kidney Tx [n = 16] | Alloantigen specificity | 300 and $900 \times 10^6$/ total |
| NCT02371434 | The ONE Study nTreg Trial (ONEnTreg13) [Germany, Berlin] | I/II | Polyclonal nTregs | Jan 2015 to Dec 2017 | Living donor kidney Tx [n = 9] | Non-antigen specificity | 0.5, 1.0 and $3.0 \times 10^6$/ kg |
| NCT02385019 | A Phase 1/2 Trial of Donor Regulatory T-cells for Steroid-Refractory Chronic GVHD (TREGeneration) [Portugal, Lisboa and Porto] | I/II | Fresh nTregs (donor Treg) | Mar 2015 to Dec 2019 | Steroid -refractory chronic GVHD [n = 22] | Non-antigen specificity | 0.5, 1, 2, and $3 \times 10^6$/ kg |
| NCT 02423915 | Fucosylated T Cells for Graft versus Host Disease (GVHD) Prevention [United States, Texas] | I/II | Fucosylated Tregs | Jul 2015 to Jul 2019 | GvHD prevention [n = 47] | Non-antigen specificity | 1 and $10 \times 10^6$/kg |
| NCT02474199 | Donor Alloantigen Reactive Tregs (darTregs) for Calcineurin Inhibitor (CNI) Reduction (ARTEMIS) [United States, California, Illinois, and Minnesota] | I/II | Donor arTreg | Sep 2015 to Dec 2018 | Liver Tx [n = 18] (Immunosuppression reduction) | Alloantigen specificity | $400 \times 10^6$/total |
| NCT02519816 | Continuous Alloreactive T Cell Depletion and Regulatory T Cell Expansion for the Treatment of Steroid-refractory or Dependent Chronic GVHD (CARE) [Canada, Quebec] | II | Polyclonal nTregs | Mar 2016 to Aug 2017 | Steroid-dependent/refractory chronic GVHD [n = 25] | Non-antigen specificity | – |
| NCT02711826 | Treg Therapy in Subclinical Inflammation in Kidney Transplantation (TASK) [United States, California] | I/II | I: Polyclonal nTregs II: Donor arTreg | May 2016 to Oct 2021 | Subclinical inflammation in kidney Tx [n = 3] | Alloantigen specificity | $320 \times 10^6$/total |
| NCT02749084 | Multiple Donor Treg DLI for Severe Refractory Chronic GVHD (TREG2015001) [Italy, Bologna] | I/II | Fresh nTregs (donor Treg) | Aug 2016 to Mar 2022 | Refractory chronic GVHD [n = 20] | Non-antigen specificity | 0.5, 1.0, and $2.0 \times 10^6$/kg |
| NCT02991898 | Treg Cell With IL-2 to Suppress aGVHD After Umbilical Cord Blood Transplantation [United States, Minnesota] | II | Fresh nTregs and IL-2 | Feb 2017 to Jan 2025 | Acute GVHD prevention [n = 10] | Non-antigen specificity | – |
| NCT 03162237 | Safety and Efficacy Study of Islets Xenotransplantation [China, Changsha] | I | Fresh nTregs | Jul 2013 to Dec 2020 | Type I Diabetes | Non-antigen specificity | $2 \times 10^6$/kg |

(*continued on next page*)

**Table 1** (*continued*)

| Study ID | Trial title [location] | Phase | Intervention | Study Start and estimated complete date | Condition [estimated enrolment] | Specificity | Doses |
|---|---|---|---|---|---|---|---|
| NCT03284242 | A Pilot Study Using Autologous Regulatory T Cell Infusion Zortress (Everolimus) in Renal Transplant Recipients [United States, Kentucky] | I | Fresh nTregs | Dec 2017 to Dec 2019 | Kidney Tx [n = 12] | Non-antigen specificity | – |
| UMIN000015789 | Tolerance induction by a regulatory T cell-based cell therapy in living donor liver transplantation [Japan, Hokkaido] | I/II | Donor arTreg (iTreg) | Nov 2012 to - | Living donor liver Tx [n = 40] | Alloantigen specificity | 1.3–5.4 × $10^6$/kg |

relapse.

Brunstein et al. have also reported the successful use of *ex-vivo* expanded, partially human leukocyte antigen (HLA)-matched nTregs derived from umbilical cord blood (UCB) in a phase I dose-escalation trial in 23 patients with aGvHD [69]. Interestingly, UCB has become a popular source for Tregs due to its reduced proportion of activated effector T cells and ease of Treg isolation. In this preliminary trial, nTregs were obtained through CD25 isolation, expanded with anti-CD3/anti-CD28-beads and IL-2, and infused at doses of 0.1–30 × $10^5$ Tregs/kg. Compared with 108 historical controls, treatment with UCB-derived Tregs reduced the incidence of grade II-IV GvHD with no indication of toxicity. Moreover, in a two-year follow up of the patients, there was no increased incidence of opportunistic infections or relapse in the long-term [70]. Recently, utilizing a novel technique of nTreg expansion, patients were also infused with UCB-derived nTregs expanded in the presence of KT64/86 cells (K562 cells modified to express CD64, a high-affinity Fc receptor, and the CD28 ligand CD86). KT64/86 cells displayed a higher effectiveness at expanding Tregs, while maintaining FOXP3 expression and suppressive function [71].

Recently, Johnston et al. completed a Phase I trial to treat cGvHD with adoptive transfer of donor Tregs in 10 match-related donor recipients [72]. This 3 + 3 dose escalation trial (1, 5, 15 × $10^5$ Treg cells/kg) was the first clinical study with the use of highly purified donor-derived Tregs isolated through high-speed cell sorting, in which cells were enriched for CD25, purified for $CD4^+CD127^{dim}$, and infused without expansion. They reported no toxicity from the infusions with 6 of the patients having stable to improved cGvHD, while the other 4 had unresponsive or progressive disease requiring a new immunosuppressive regimen.

In terms of **solid organ transplantation** (SOT), although met with a separate set of challenges compared to HSCT, implementation of Treg cell therapies have currently demonstrated encouraging results in phase I/II clinical trials and potential for the reduction in the dependency of immunosuppressive drugs. Many trials for Treg therapy in SOT are currently in the initial phases or ongoing, with published data not yet available.

In Japan, 10 end-stage liver failure patients undergoing living donor liver transplantation (LDLT) were treated with a Treg-like cellular therapy [73]. Patients received an adoptive transfer of this *ex-vivo*-generated Treg-like cell product at doses of 0.23–6.37 × $10^6$ Tregs/kg, generated through co-culture of recipient lymphocytes with irradiated donor cells in the presence of anti-CD80/86 monoclonal antibodies. The immunosuppressive regimen, which consisted of steroids, MMF, and tacrolimus, was tapered 6 months post-cellular infusion and stopped at 18 months. All patients also underwent splenectomy. Under this treatment, 7 out of the 10 patients achieved complete immunosuppression withdrawal, ranging from 16 to 33 months post-transplantation, and have displayed normal graft function and histology. However, 3 patients presented with signs of acute cellular rejection and had immunosuppression re-started. Interestingly, these 3 patients had an autoimmune cause for their liver disease (primary biliary cirrhosis or primary sclerosing cholangitis). Unfortunately, due to this, the trial has been suspended and the authors currently cannot extend to the 40 patients as originally planned [73].

In another clinical trial, Bashuda's group in Japan has attempted the adoptive transfer of self-anergic cells in kidney transplant recipients from HLA-mismatched living donors. In the generation of the anergic T cells, both recipients and donors underwent lymphocytapheresis treatment before the transplant and PBMCs from both were co-cultured in the presence of anti-CD80/CD86 mAb, which modulates Treg function, and infused 12 days after the transplant as immunosuppression was also gradually tapered. All 12 recipients who received a splenectomy or rituximab and cyclophosphamide displayed graft survival, but further immunosuppression reduction was withheld due to a high incidence of biopsy-proven acute rejection. Data from the 40 recipients who received rituximab and rATG instead of cyclophosphamide, have yet to be

reported. This new approach may be promising for SOT if the optimal balance with immunosuppression can be further clarified [74].

Two groups from the U.S. have recently shared results from their trials of adoptive cellular therapy with Tregs in kidney transplant recipients. The TRACT trial assessed the safety of the infusion of autologous, polyclonally expanded Tregs (0.5, 1, and 5 × $10^9$ cells) in living donor kidney transplant recipients [75]. Here, patients received alemtuzumab induction for lymphodepletion before being infused with Tregs, which were isolated by $CD25^+$ enrichment following $CD8^+$ and $CD19^+$ depletion by CliniMACS and then expanded with anti-CD3/anti-CD28-coated beads, IL-2, and sirolimus. Thereafter, patients received tacrolimus and mycophenolate-based immunosuppression followed by conversion to sirolimus 30 days post-transplantation, in an effort to aid Treg survival. All 9 patients received this therapy and displayed a 9–20-fold increase in circulating $CD4^+CD25^{hi}CD127^-FOXP3^+$ Tregs in the peripheral blood with no signs of rejection or serious adverse effects.

Similarly, the TASK pilot trial sought to assess the safety and feasibility of injecting $CD4^+CD25^+127^-$ peripheral blood Tregs (320 x $10^6$ cells) that were FACS-sorted and expanded polyclonally *ex vivo* with a deuterium label into kidney transplant recipients with subclinical inflammation on a 6-month protocol biopsy [76]. Two of the patients displayed reduced graft inflammation and improved rejection scores with no incidence of acute rejection, while one patient displayed signs of subclinical acute cellular rejection at 6-month post-Treg infusion and was treated with steroids and modified maintenance therapy. The authors mentioned that the third patient possibly had worsened outcomes because he started to develop *de novo* donor-specific antibodies prior to the infusion. The group have also recently initiated a trial to compare the efficacy of infused polyclonal Tregs versus donor alloantigen-reactive Tregs in kidney transplant recipients with subclinical inflammation [NCT02088931].

Our group is currently a partner in the EU-funded ONE Study (Phase I) and MRC-funded TWO Study (Phase II) trials, which aim to investigate the safety and therapeutic efficacy of Tregs in living donor renal transplantation. The central focus of the ONE study, a consortium coordinated in Regensburg and including centres located in the UK, Germany, France, Italy, and the US, is to test and compare a variety of immunoregulatory cell products in the context of a similar patient population, with a reference group of patients [77]. Both the cellular therapy and reference groups received low dose tacrolimus, MMF, and steroids, with the experimental group receiving regulatory cell therapy and the reference group receiving anti-CD25 antibody induction. These immunosuppressive regimens were held consistent throughout all centres. The manufactured cellular products included polyclonal and alloantigen-specific nTregs, Tr1 cells, tolerogenic dendritic cells, and regulatory macrophages. Additionally, Prof Lombardi's group, a ONE Study collaborator, has also initiated an independent clinical trial called ThRIL (TReg Immunotherapy in the setting of Liver transplantation), which similarly tests the safety of Treg therapy in liver transplant recipients [78].

The TWO Study aims to assess the efficacy of Tregs in renal transplant recipients. In this randomized controlled trial, patients in the therapy arm will receive expanded nTregs and immunosuppression reduced to a monotherapy. The primary outcome measure is biopsy-proven acute rejection within 18 months of transplantation and patients will also be intensively monitored for detection of changes in immune phenotype.

#### *1.3.1. Current methods of Treg isolation for cell therapy*

Due to the low frequency of nTregs in the peripheral blood, cells must be purified and then expanded *ex vivo* or pooled [Fig. 1]. A common GMP-approved method for isolation is CliniMACS (Miltenyi Biotech), in which cells are isolated with the use of magnetic beads. Studies have used this for the depletion of $CD8^+$ effector T cells and the positive selection for $CD25^+$ cells to purify a Treg population. More recently, some have found success in depleting Treg populations from $CD19^+$ and $CD127^+$ cells before Treg enrichment to isolate a more suppressive population with increased purity [34,79]. However, methods such as CliniMACS that are based on magnetic bead-based isolation are limited. Although purification levels are being improved, there are restrictions in the number of parameters that may be used for isolation and there is little 'fine tuning' in the isolation of cells with intermediate expression levels.

Conversely, fluorescent-activated cell sorting (FACS)-based purification has the potential to yield cell products with higher purity because of the ability to isolate on a greater number of markers than CliniMACS. Not only would this generate populations of higher purity, but the use of different parameters for isolation allow for greater specificity and potency. Indeed, there is specific focus on the development of reliable, high performance clinically-approved FACS-based methods, such as the Cytonome GigaSort system and MACS Quant Tyto. Nonetheless, there remains a gulf to bridge developing the technology to meet GMP requirements, particularly in Europe. Thus, recent research in Treg therapy has focused on optimizing these two main methods.

#### *1.3.2. Clonality (Polyclonal vs. Alloantigen Reactive Tregs)*

Recent studies have debated the use of polyclonal versus alloantigen-reactive Tregs (arTregs) [80]. Polyclonal Tregs can be expanded commonly with the use of anti-CD28/anti-CD3 beads/antibodies and IL-2, with a few groups demonstrating methods for expanding cells in substantial numbers [81]. However, the use of polyclonal Tregs has its disadvantages in that large populations are necessary for adequate suppression because only a certain number of clones are likely to be donor-reactive. This non-specific reactivity may have detrimental effects through the suppression of other immune responses, leading to vulnerability to opportunistic pathogens and tumours, although in practice experimental studies have not been able to demonstrate this vulnerability [82].

Conversely, arTregs are selected due to their enhanced responsiveness to donor antigens and theoretical reduction in the risk of suppression of third-party immune responses. Thus, studies have found them to be as much as 10 times more suppressive than polyclonal Tregs in preclinical and clinical models [29,34,83], with fewer cells necessary to prevent rejection in mice [29,57]. However, because they are generally expanded using allogeneic APCs, this strategy is difficult to apply to deceased donor SOTs [25]. Additionally, arTregs are generally more expensive and difficult to isolate with great frequency, especially because increased HLA-matching leads to decreased stimulation and expansion of arTregs [29].

### *1.4. Production/Manufacturing*

#### *1.4.1. Cost*

Currently, the manufacturing costs of patient-tailored Treg therapies can vary widely depending on isolation procedures, GMP facility costs, incubation period, dosage, and characteristics of Tregs isolated. However, there is potential for such a therapy to be economical in the long-term due to reduced reliance on immunosuppression, complications and hospitalization [25,84]. In general, the manufacturing costs mean that autologous Treg infusions are more expensive than allogeneic infusions. As the manufacturing of Tregs become more standardized and commercialized, it is anticipated that these treatments will become cheaper. An outstanding question remains whether such therapies will be produced centrally or within each hospital's GMP facility. We believe that the central production of a Treg product has benefits in terms of standardization of production, quality control, enhanced throughput and concentration of expertise.

#### *1.4.2. Dosage*

The dosage necessary for Treg therapy is also an area that requires further investigation, for it is dependent on the characteristics of the

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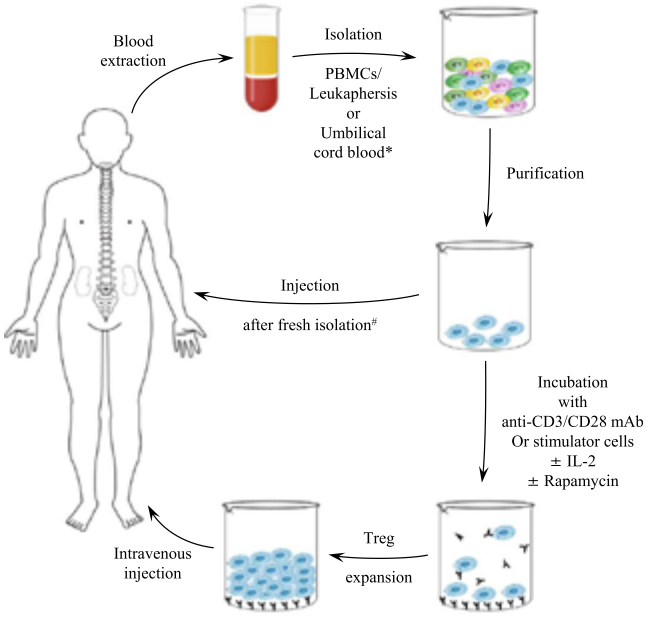
A




B

【Representative methods of Treg expansion or induction】

| Trials | Method of expansion |
|---|---|
| ·The ONE study & TWO study [77] | Incubation with IL-2, anti-CD3/CD28 coated beads & rapamycin |
| ·Trzonkowski et al. [28] | Incubation with IL-2 and anti-CD3/CD28 coated beads |
| ·Di Ianni et al. [67] | Isolated freshly# (no incubation) |
| ·Safinia et al. [78]<br>·ThRIL | Incubation with IL-2, anti-CD3/CD28 coated beads, and Rapamycin |
| ·Safety Study of Using Regulatory T Cells Induce Liver Transplantation Tolerance (Treg)** | Incubation with IL-2, anti-CD3/CD28 coated beads, and TGF-β |
| ·Brunstein et al. [69]* | Incubation with IL-2 and anti-CD3/CD28 coated beads |
| ·Todo et al. [73] | Incubation with blocking anti-CD80/86 mAb |
| ·Koyama et al. [74] | Incubation with blocking anti-CD80/86 mAb |

**Study ID: NCT01624077

**Fig. 1.** Treg isolation/expansion methods. (A) Treg isolation/expansion pathways for various methods in Treg cell therapy. (B) Representative methods of Treg expansion or induction used in clinical trials.

infused Tregs (stability, purity, and type), as well as the patient's condition and timing of injection. On the one hand, because polyclonal cells are less specifically suppressive than arTregs, they require a higher dosage. Previous mouse studies have demonstrated that a polyclonal Treg to Tconv ratio of 1:1–1:2 is sufficient to prevent allograft rejection [83,85,86]. Thus, assuming this proportion is consistent for humans, at least 49 to 79 $\times$ $10^9$ Tregs will be needed to fulfil this requirement in humans [83]. If lymphodepletion to reduce the Tconv population is performed prior to Treg infusion in the patient, 10% of the total number of Tregs will be necessary to balance the scales. With recently reported strategies that can expand polyclonal Tregs to around 1 $\times$ $10^8$ cells/100 mL of peripheral blood, the production of high numbers of polyclonal Tregs is technically feasible [29,81,87]. arTregs require a fraction of the polyclonal Treg numbers necessary in order to induce the same suppressive potency, but are more expensive and difficult to expand with high purity under GMP conditions.

*1.4.3. Timing*

The timing of infusion can impact Tregs' effectiveness *in vivo*. It is generally thought that Tregs should be infused around the time of transplantation to optimally exert their suppressive effects. Given that the Treg response and infiltration into grafts is typically delayed compared to conventional T cells [88], it can be argued that Tregs must be infused early enough to counteract effector T cell activity before damage has commenced. The delayed migration may be due to differences in their lymphotoxin-dependent migratory patterns [89]. In cases where expansion of donor-specific Tregs is necessary prior to infusion, treatment before or at the time of transplantation is logistically difficult and therefore the therapy can only be given to recipients of living donor transplants where the donor HLA type is known [90]. Moreover, allografts that are normally subject to the trauma of surgery, can promote a proinflammatory microenvironment that destabilizes Tregs through the loss of FoxP3 expression and the induction of an effector phenotype [91,92]. The timing of infusion also needs to be coordinated with induction therapy that results in lymphodepletion. Given that a high Treg:Tconv ratio plays a vital role in tolerance induction, lymphodepletion may offer an effective therapeutic window for Tregs to function, particularly when Treg expansion processes are limited [83,86,93]. However, induction therapies used at the time of transplantation may also themselves be detrimental to Treg expansion and function [76,94]. Tregs may therefore need be infused after induction therapy is complete. However, there are insufficient studies focused on varying the timing of Treg therapy in humans, let alone any studies focused on the optimal timing for multiple infusions.

*1.4.4. Cryopreservation*

Methods for optimizing efficient and effective cryopreservation of Tregs can also open up many possibilities for Treg therapies. Reliable storage can alleviate issues of timing of infusion and depletion of the stock during the treatment protocol. Initial studies have shown that cryopreservation of human Tregs reduces suppressive potency and the CD4 + and FOXP3 + population, most likely due to the fragility of the Tregs [95]. However, using different preservation strategies, some groups, including ours, have found success in the recovery of cryopreserved Tregs, which could be used to prevent GvHD [81] and rejection of vessels [56], skin [35], or islet [96] grafts. Based on recent studies that demonstrate effective strategies for cryopreservation of human Tregs, the safety and efficacy of cryopreserved Tregs is being assessed in clinical trials such as the ONE/TWO Study and ThRIL [78].

*1.4.5. Source*

The source by which Tregs are isolated from also needs to be taken in consideration. nTregs are commonly isolated from peripheral blood, but they may also be taken from umbilical cord blood. Because cord blood is generally immunologically naive, the $CD4^+CD25^+$ population lacks the non-suppressive memory and activated T cells that usually coexist within this population in peripheral blood [97]. Thus, unlike in peripheral blood, $CD4^+CD25^+$ expression can reliably be used for

isolation of Tregs. Moreover, with less contamination within the isolated population, *ex vivo*-expanded UCB Tregs are generally more potent in suppression than peripheral blood Tregs [98–100]. Pooling of UCB Tregs from multiple sources is a possible strategy for the production of large numbers for therapy. In a comparative study of pooled human UCB versus peripheral blood Tregs, the former was found to be more suppressive *in vivo* [101]. Multiple clinical trials are now using UCB as a source of Tregs.

*1.4.6. Immunosuppression*

The optimal Treg cell therapy would completely eliminate the use of immunosuppressive drugs due to their adverse effects, but studies have indicated that Tregs cannot prevent rejection without at least some form of adjunctive immunosuppression. Recent findings indicate that some of the commonly used immunosuppressive agents have detrimental effects on human Tregs *in vivo*. For example, basiliximab and calcineurin inhibitors (CNI) such as tacrolimus block IL-2 production, which is necessary for Treg proliferation and function [94,102]. Addition of IL-2 to the therapeutic regimen may therefore be an option to counteract this effect. Low-dose IL-2 is already being used in clinical trials to enhance Treg stability and proliferation through the activation of the STAT5 pathway [103–105].

Thus, the optimal therapy should also consider which drugs can benefit Treg survival or function [106]. This section focuses on immunosuppressive drugs that have shown promise for Treg cell therapy, with more extensive investigations outlining various other drugs' effects on Tregs to be found in other reviews [106,107].

ATG, a polyclonal rabbit or horse-derived antibody, is a commonly used induction agent in transplantation that has also shown ability to expand $CD4^+CD25^+FOXP3^+$ cells. Lopez et al. found that *ex vivo* expansion of human peripheral blood lymphocytes (PBL) stimulated with rabbit-derived ATG expanded Tregs primarily through the conversion of $CD4^+CD25^-$ cells to $CD4^+CD25^+$ cells [108]. Later findings confirmed these results, suggesting that expansion may be through transcriptional regulation by increased NFAT1, a FOXP3 inducer [109–111]. Clinical studies with ATG induction indicate that both Tconvs and Tregs are depleted, but that Tregs may repopulate faster than Tconvs and be functionally suppressive [102,112,113]. Thus, Treg infusion alongside this treatment may be a suitable strategy in shifting the balance towards Tregs over Tconvs.

Rapamycin (sirolimus), has shown much promise to be used concurrently with Treg treatment and has already been implemented in a few clinical trials. Many studies have found that this mechanistic target of rapamycin (mTOR) inhibitor can preferentially promote the survival of Tregs over Tconvs [62,114–116] and is beneficial in Treg cell therapy in animal models [117]. This effect may be due to its inhibition of the PI3K/Akt/mTOR pathway, which the Tconvs are more dependent on than the Tregs for survival, making Tconvs more prone to apoptosis in the presence of rapamycin [118,119]. Therapy that is based on targeting this pathway may provide a conducive environment for Treg survival. Rapamycin is commonly used in Treg expansion protocols to reduce effector T cell contamination. There are however findings that rapamycin administration can enhance memory CD8 + T cell responses during viral or bacterial infection [120]. The specific effects of rapamycin in the context of clinical transplantation therefore need further clarification.

It is important to note that many studies indicate contradicting effects of how a certain immunosuppressive drug or regimen affects Treg differentiation and function. This is likely attributed to the differences in timing, dosage, and combination of drugs. These factors need to be carefully considered in future investigations.

*1.5. Future of Treg cell therapy*

Many questions remain regarding the specificity, stability and function of Tregs, but this has led to an avenue of exciting and novel innovation that can potentially address these concerns. For example, a significant development has been the investigation of chimeric antigen receptor (CAR) Tregs, in which nTregs are transduced with a vector containing a chimeric receptor to produce cells that are genetically modified to target donor antigens with high specificity [121]. Macdonald et al. demonstrated the generation of CAR Tregs generated with HLA-A2, a commonly mismatched antigen in transplantation, that is highly donor antigen-specific and maintains FOXP3 expression while preferentially localizing to the transplanted allograft [122]. This method is therefore able to overcome many of the limitations posed by arTregs, for it is not dependent on the presence of donor professional APCs [123]. More recently, CAR Tregs have been shown to be efficacious in a human xenograft transplant model [124].

To address the issue of stability of Tregs *in vivo*, there are some recent studies that suggest the use of Treg-derived exosomes. Exosomes are extracellular vesicles that contain proteins and RNA and are known to be secreted by most cells in order to modulate immune response. Unlike human Tregs that may be vulnerable to the loss of suppressive functions under inflammatory conditions, exosomes are unlikely to have their immune functions disrupted. Adoptive transfer of Treg-derived exosomes has been tested in a rat kidney transplant model, where the treatment inhibited T cell proliferation and suppressed acute rejection [125].

After Tregs are infused, tracking their migratory patterns *in vivo* is also crucial to understanding their survival and function within the graft, and different technological strategies have been proposed ranging from the labelling of Tregs during expansion to tomography imaging after infusion [126–128]. Further work must also focus on assessing safety due to the potential toxicities of labelling and imaging.

## 2. Conclusion

The evidence for Treg therapy in transplantation is mounting, with clinical studies now progressing through to early phases. The challenges that remain centre of the methodology for introduction of Tregs into clinical therapy and their cost. The latter may be resolved once the former is better understood. If Tregs hold significant benefits to post-transplantation outcomes and facilitate a reduction in immunosuppression, the costs for production may become relatively less significant. A scale up of the therapy may reduce costs, although it is not clear whether this is imminent given that much of the process cannot yet be automated. A greater understanding is required regarding the relative efficacy of Tregs in transplantation and whether their use will completely eliminate or only reduce the need for immunosuppression. There are scant data available to assist in determining dosages, timings and efficacy of the various Treg populations in different transplant types with the majority of data being in animal models, leading to speculative clinical studies assessing feasibility and only rarely efficacy. We are now truly on the cusp of one of the first breakthroughs in transplantation since the introduction of modern immunosuppression. For the first time the pipeline of novel therapeutics contains not molecules, but cells.

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# *ASHI Quarterly* Continuing Education Quiz

**Quiz Instructions:** The multiple-choice quiz below is provided as an alternative method of earning continuing education (CE) credit (CEC) hours. Read each article then select the ONE best answer to each question. The questions are based solely on the content of the article. Answer all of the questions and send the quiz answer sheet and fee (if applicable) to the ASHI Executive Office before the processing date listed on the answer sheet.

To be eligible for 3.0 credit/contact hours or 0.45 CECs, ALL questions must be answered, a passing score of 70% must be obtained and the answer sheet must be submitted with fee (if applicable) before the deadline. Quizzes may not be retaken, nor can ASHI staff respond to questions. Allow six weeks for paper processing following the submission deadline to receive return notification of your completion of the CE process. The CEC will be dated when it is submitted for grading. That date will determine the CE cycle year.

**Quiz may be taken online at ashi-u.com.**

## CRISPR/Cas9 and CAR-T Cell, Collaboration of Two Revolutionary Technologies in Cancer Immunotherapy, an Instruction for Successful Cancer Treatment

1. All of the following are true for CAR-T cells *except*:
   A. They are used in the treatment of HIV
   B. They are genetically engineered T-cells
   C. They express an artificial protein called chimeric antigen receptor
   D. Cells navigated to surface expressing tumor antigens
   E. Used in the treatment of lymphoma and leukemia

2. All of the following are true for CRISPR-Cas9 technology *except*:
   A. Acquired immune system in bacteria
   B. Provides platform for precise genome editing
   C. Used in treatment of HIV
   D. Used in treatment of leukemia and lymphoma

3. **True or False?** Homologous directed repair and non-homologous end joining are two repair approaches using CRISPR/Cas9 technology.
   A. True
   B. False

4. **True or False?** Off target effects are the major barrier to the potential use of CRISPR/Cas9 technology in therapeutics approaches. Off target effects are when mutations happen in undesired genomic loci using this technology.
   A. True
   B. False

5. All of the following are true about current FDA approved CAR-T cell therapy *except*:
   A. T cells are collected from healthy donors
   B. T cells are collected from the patient
   C. Collected T cells are activated and expanded
   D. T cells are transduced with CAR gene by different methods to produce CAR-T cells
   E. Expanded CAR-T cells are infused in the patient

6. **True or False?** First generation CAR-T cells used single chain antibody and costimulatory molecules like CD28.
   A. True
   B. False

7. **True or False?** Universal CAR-T cells can be produced by disrupting T cell receptors and HLA molecules using CRISPR-Cas9 technology
   A. True
   B. False

## Regulatory T Cells for Tolerance

8. Which of the following is not a marker for regulatory T cells (Tregs)?
   A. CD4
   B. CD25
   C. FOXP3
   D. CD8

9. **True or False?** Tregs are T-cells with suppressor function
   A. True
   B. False

10. **True or False?** The goal of Treg therapy in transplantation is to reduce dependence on immunosuppressive medication.
    A. True
    B. False

11. **True or False?** CD4+CD25+FOXP3+ cell isolation is successful method to produce Tregs for transplantation therapy.
    A. True
    B. False

12. **True or False?** Unlike the mouse Tregs, human Tregs express a memory phenotype on their surface
    A. True
    B. False

13. **True or False?** Challenges of Tregs therapy in transplantation include the cost of manufacturing and the majority of the data being in animal models.
    A. True
    B. False

# Answer Sheet

*Please print clearly in black ballpoint pen*

First Name | M.I. | Last Name

Institution

Address

Address

City | State/Province | Zip Code/Postal Code

Country

ASHI Membership Number (**MUST** complete if requesting fee waiver)

**Instructions: Mark your answers clearly by filling in the correct answer, like this ■, not like this ☒. 3.0 credit/contact hours or 0.45 CECs earned with passing score (70%).**

*Please use black ballpoint pen.*

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | A | B | C | D | E |
| 2 | A | B | C | D | |
| 3 | A | B | | | |
| 4 | A | B | | | |
| 5 | A | B | C | D | E |
| 6 | A | B | | | |
| 7 | A | B | | | |
| 8 | A | B | C | D | |
| 9 | A | B | | | |
| 10 | A | B | | | |
| 11 | A | B | | | |
| 12 | A | B | | | |
| 13 | A | B | | | |

**The quiz answer sheet will not be graded, no CEC will be awarded and the processing fee will be forfeited unless postmarked by:**

**July 1, 2020**

**Quiz Identification Number:**

**44-1-2020**

| **ABHI Program Number:** | **CA Provider Number:** | **FL Provider Number:** |
|---|---|---|
| ABHI 20XX-0# | 0085 | 50-2203 |

❑ Processing Fee: **ASHI Member – no fee** **Nonmember – $30**

❑ Payment is due with submission of answer sheet. Make check or money order payable to ASHI (U.S. currency only). Do not send cash. No refund under any circumstances. Please allow six weeks following the submission deadline for paper processing.

❑ Individuals paying by credit card may fax the answer sheet to 856-439-0525.

***Please check one:***

❑ Enclosed is check #_____ (payable to ASHI)

❑ Charge to the following card:

❑ MasterCard (16 digits) ❑ Visa (13 or 16 digits) ❑ Amex

Card Number_____ Exp. Date _____

Signature _____

Print Cardholder's Name _____

**For Internal Use Only**

Date Received: _____

Amount Received: _____

Notification Mailed: _____

**Answers to the 4th Quarter 2019 *ASHI Quarterly* Quiz:**

1. C; 2. E; 3. B; 4. D; 5. D; 6. A+B; 7. A+B; 8. D; 9. B; 10. B; 11. A; 12. A

*Mail to:* *ASHI Executive Office*
*ASHI CE Quiz*
*1120 Rte. 73, Suite 200*
*Mount Laurel, NJ 08054*

*Fax:* *856–439–0525*

## SAN DIEGO, CA

April 23–25, 2020
Wyndham San Diego Bayside

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# 2020 REGIONAL EDUCATION WORKSHOPS

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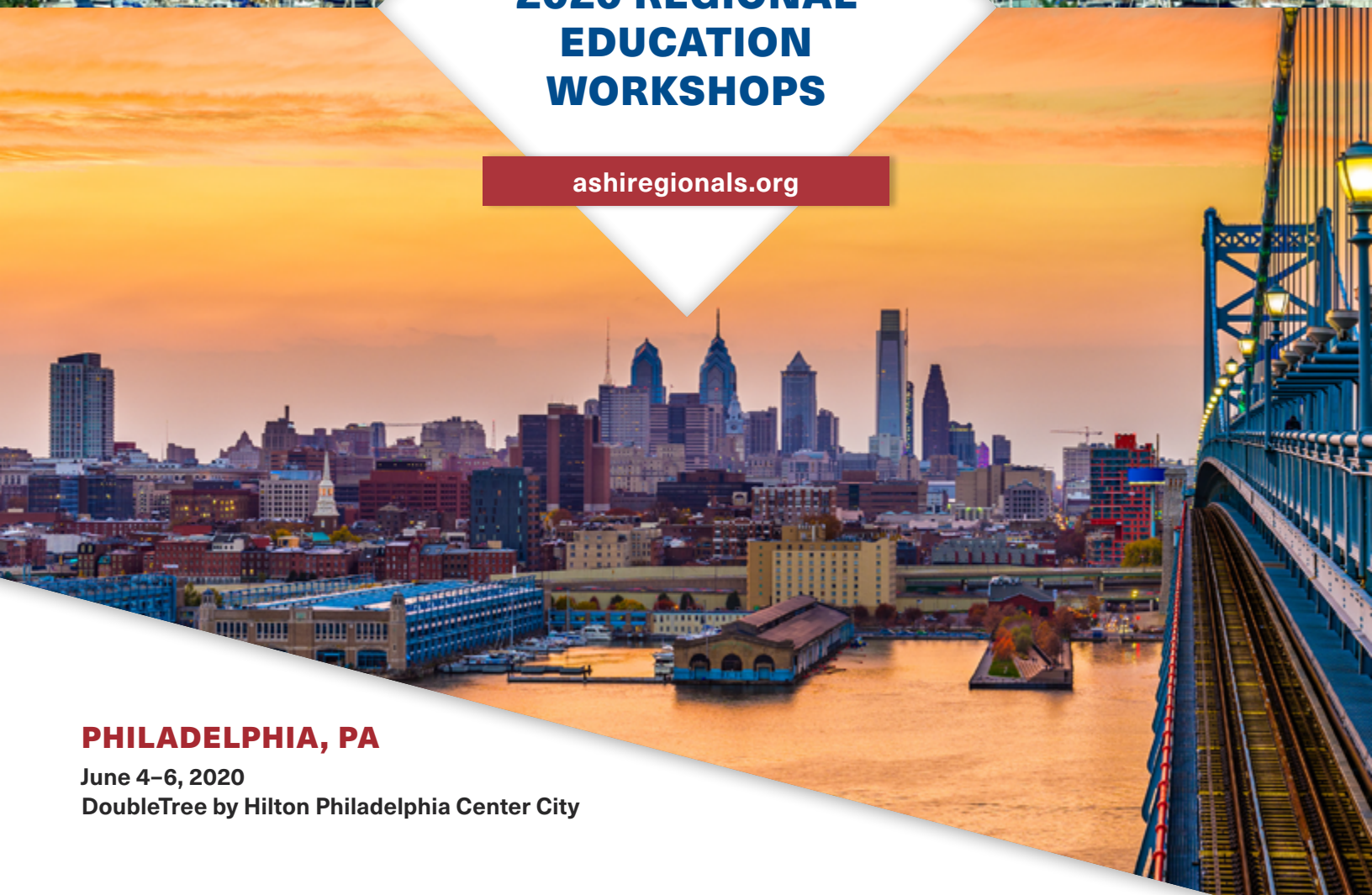


## PHILADELPHIA, PA

June 4–6, 2020
DoubleTree by Hilton Philadelphia Center City

# ABHI Update

Melissa Weeks, *ABHI Executive Director*
David Kiger, *CHS(ABHI), ABHI President*

Melissa Weeks

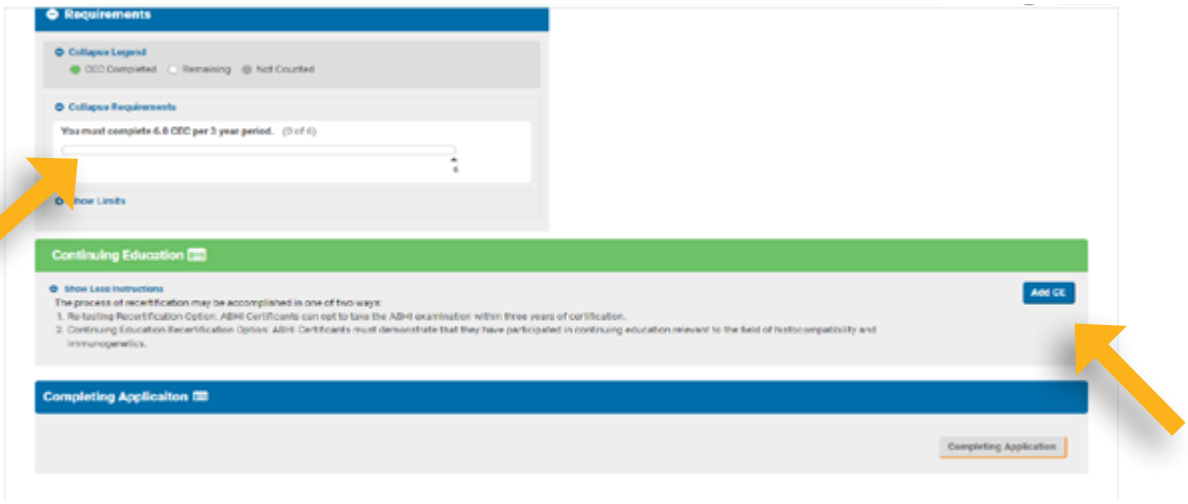
David Kiger, CHS(ABHI)


**ABHI CERTIFICATION** is separate from ASHI membership. There are fees you will pay for each, but different amounts and at different times.

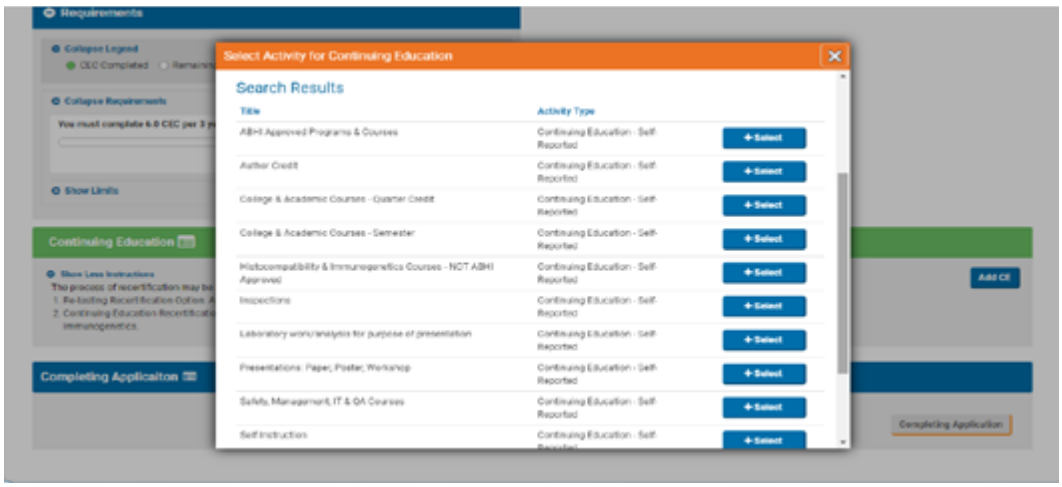
## Inside the CE Center

New this year: all courses taken in ASHI U will automatically transfer over to your ABHI account! The following guides users through adding courses to transcripts.

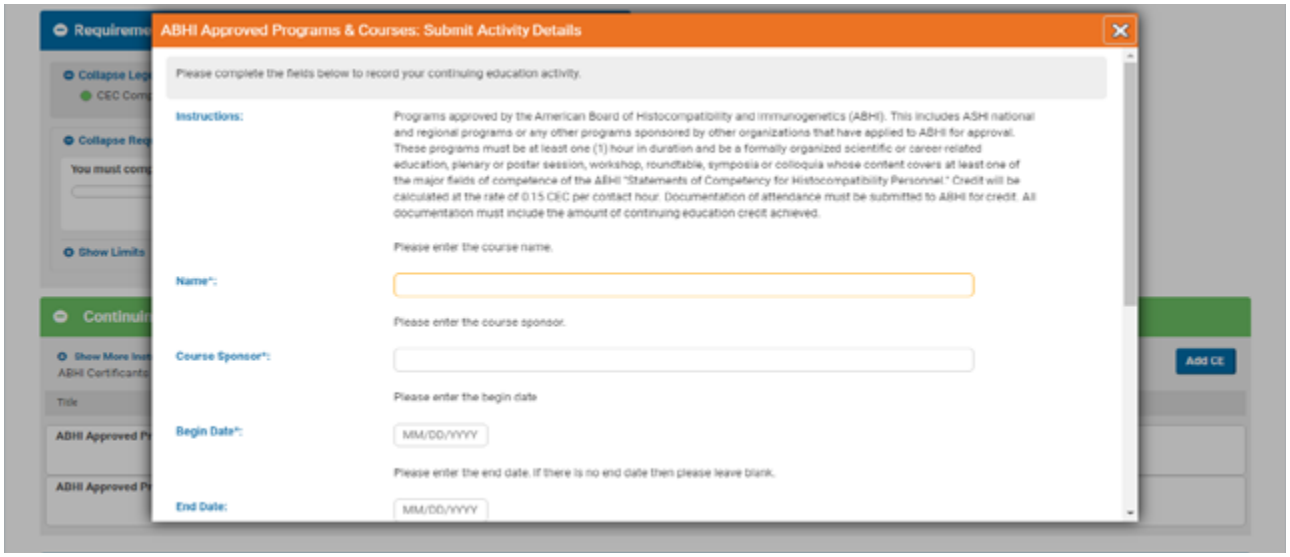
The main page showing the list of courses added and how many more credits you need to complete recertification in your 3-year period:




Next, you're able to see all the types of CE credit you can add to your transcript. The first category listed is for ABHI-approved courses – you can add an unlimited number of these types of courses. The other categories below it has limits on the amount of credit you can add:



This is where you will add more detail on each course – name, date, etc. and have a chance to upload supporting documentation like certificates of completion:




**You can keep up on your certification at the CE Center here:** **abhi.learningbuilder.com**

## Approved Providers & Courses

Labs can apply to become ABHI approved CE providers, if they offer weekly staff lectures, quarterly meetings, etc. It's easy!

Once ABHI approved, the course(s) are assigned a special number and are allowed to offer CECs to attendees.

This helps YOU, the CHA/CHT/CHS, get more credit toward your renewal, as you can add an unlimited number of ABHI approved courses to your transcript in the CE Center.

The application can be found here:
https://cdn.ymaws.com/www.ashi-hla.org/resource/resmgr/docs/abhi/3_2_2018Updates/3-2-2018_ABHI_Approved_Provi.pdf

## Coming Soon

ABHI and ASHI are excited to announce that later this year we will be launching the new American College of Histocompatibility & Immunogenetics. The new College will combine the efforts of ABHI's certification program with the standards & processes of ASHI's Director Training Review & Credentialing committee (the DTRC). Details to follow, we are excited to make everyone's lives much easier.

## Thank you to the volunteers on the ABHI Board of Directors!

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# Proficiency Testing Committee

## *ASHI 2019 Virtual Crossmatch Educational Challenge-2*

Mary Carmelle Philogene, *PhD, D(ABHI)*
Junior Co-Chair and the ASHI Proficiency Testing Executive Committee

The 2019 ASHI Virtual Crossmatch-2 (VXM-2) Educational Challenge was designed in Google Forms as a questionnaire. Participants in this challenge were asked to perform a virtual crossmatch assessment using HLA antibody data obtained from the ASHI AC-2 2019 survey. The virtual crossmatch assessment included a prediction of the outcome of physical flow cytometric (FCXM) or complement dependent cytotoxic (CDCXM) crossmatches. Although an additional question requested information regarding the use of antihuman globulin crossmatch (AHG-CDC), laboratories did not provide a response to this question. The questions also probed participants' use of one versus 2 single antigen panels for HLA antibody testing, serum treatment, and determination of donor-specific HLA antibodies (DSA). Finally, the participating laboratories were asked to use the data from the virtual crossmatch to provide a risk assessment in the case of kidney, heart, pancreas, kidney/heart and kidney/liver transplantation.

The VXM-2 consisted of 5 sera tested in the ASHI 2019 AC-2 survey (AC-515 -519) and three AC-2 donor cells (AC-135, AC-138, AC-140). The HLA typing for donor cells was performed at intermediate resolution by SSOP and the results were provided in the survey. Of 152 participants from the 2019 AC- 2 survey, forty-nine (32 %) subscribers participated in the VXM-2 survey. This represents a decrease from the previous year (84 participants in 2018; and 67 participants in 2019). As shown in Table 1, the participants were divided into 3 groups. Each group was assigned a donor cell that is different from the donor cell assigned for the ASHI 2019 AC-2 survey and, therefore, participants were blinded to the physical crossmatch results. The virtual crossmatch assessments were compared to the physical CDCXM and FCXM results obtained from the 2019 AC-2 survey. All the 2019 VXM-2 data collected were linked to Google sheets and exported to Microsoft Excel and GraphPad Prism for data analysis.

## Serum Treatment for Antibody Assessment

Participants were first asked to report laboratory standard serum treatments used for antibody testing. The majority of laboratories reported use of a single serum treatment; 46% used EDTA, 19% used DTT and 12% used heat inactivation (HI). The use of freeze/thaw centrifugation, dilution with fetal calf serum, Adsorb-out-beads or a combination of two treatments was reported by 7% of the participants. Additionally, 27% of laboratories did not use any serum treatment (untreated serum) prior to antibody testing. The distribution of serum treatment per group is detailed in Figure 1.

## Single vs Multiple Assays Used for Antibody Assessment

96% of the participants reported using only one single antigen panel, while 4% of the participants used 2 different single antigen panels to report antibody. The distribution of assay per group is shown in Figure 2.

## Donor Specific Antibody (DSA) Detected and Crossmatch Comparison

The participants were asked to identify any DSA detected for each serum/donor cell combination with MFI values deemed "Positive" based on their established MFI cutoff. DSA and MFI reported are listed for each group in figures 3A (Group A), 4A (Group B) and 5A (Group C). Box plots show mean MFI values and ranges per specificity. Based on the reported DSAs, participants performed a virtual crossmatch assessment. Only antigens for which at least one laboratory reported DSA are represented in in box plots. Laboratories also reported virtual crossmatch results based on their own MFI thresholds. The virtual crossmatches were compared to the results of the physical crossmatches in Figures 3B (Group A), 4B (Group B) and 5B (Group C). Finally, risk assessments for each solid organ group and based on virtual crossmatch results are listed per group in figures 3C (Group A), 4C (Group B) and 5C (Group C).

## Group A Data Summary

Although flow crossmatch results were concordant within all labs, most discordant risk assessment were noted with sera AC-518 and AC-519, primarily with the kidney/liver transplants. For serum AC-518 versus AC-135, reactivity against B71, and DR4 at MFI values on a single antigen bead panel ranging between 2000 MFI and 8000 MFI were not considered to be a risk factor for simultaneous kidney/liver transplants. Interestingly, 5% of participants reported the strength and specificities as intermediate risk. Positive flow crossmatches (physical and virtual) for AC-519 versus AC-135 were due to strong reactivity against HLA-DQ8 and DQ9. Surprisingly, 13% and 33% of participants reported low risk for pancreas and kidney/liver transplants respectively for this serum-cell combination. Of note, laboratories that reported "low risk" for kidney/liver transplants reported a mean MFI value of 8000 for HLA-DQ8 and DQ9.

## Group B Data Summary

Discordant results with AC-519 versus AC-138. This is consistent with discordant results observed with the DSA reported for this serum.

## Group C Data Summary

The CDC physical crossmatch was not reported for this cell-serum set as too few laboratories reported this data. There were some discordant responses in the risk assessment for kidney transplantation for AC-515 with cell AC-140 and AC519 with cell AC-140. While in both cases 100% of the laboratories reported presence of DSA, 10% of the labs for AC515 vs AC 140 (DSA against B13, mean MFI 9836) reported an intermediate risk for kidney transplantation; and 50% of the labs reported an intermediate risk for AC519 versus AC 140 (DSA against HLA-A3).

TABLE 1

### Number of VXM-2 Participants and Cell Assignments

| # OF RESPONSES PER GROUP | DONOR CELL ASSIGNMENT |
|---|---|
| Group A (N=22) | AC-135 |
| Group B (N=14) | AC-138 |
| Group C (N=13) | AC-140 |

Each group was assigned a donor cell that was not tested by this group for their cell-based crossmatch in the AC-2 survey.

FIGURE 1

### Serum Treatment Practice per Group

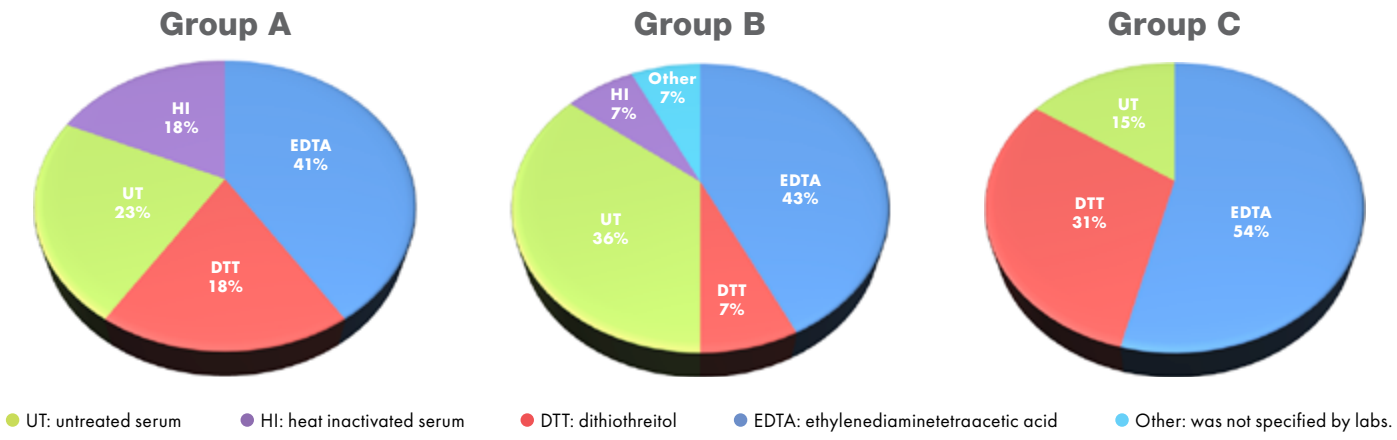



FIGURE 2

### Assay Vendor per Group

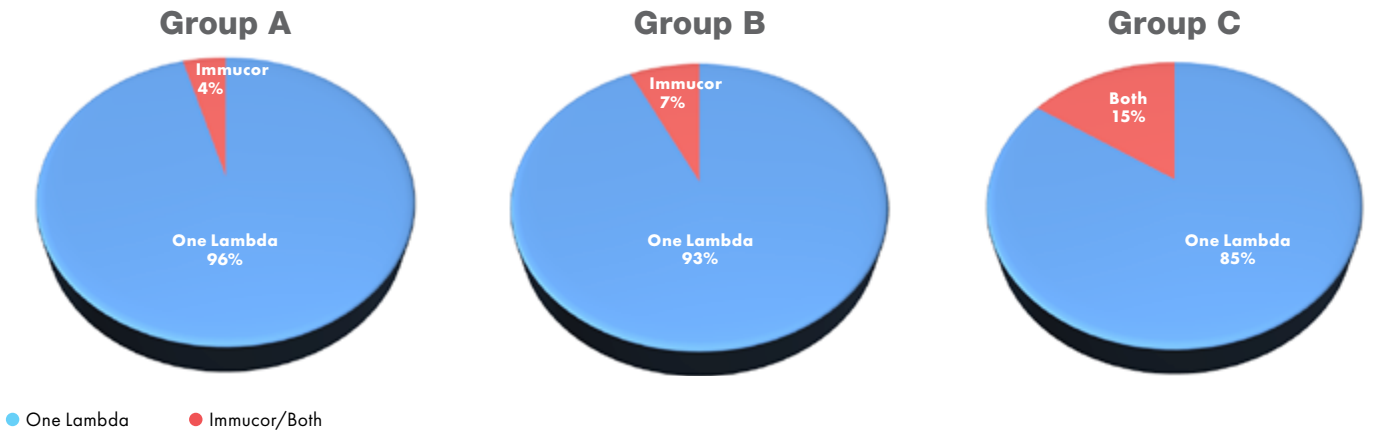


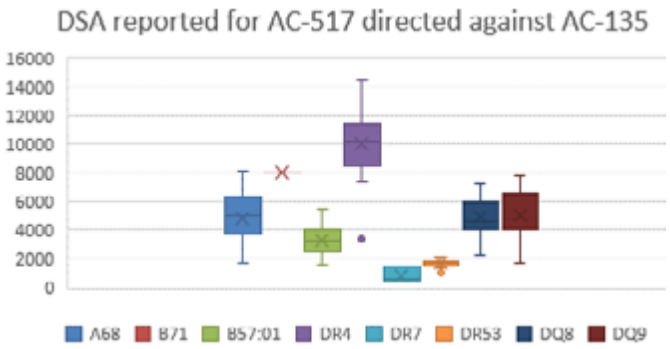
TABLE 3-1

## HLA typing for Cell AC: 135: Group A

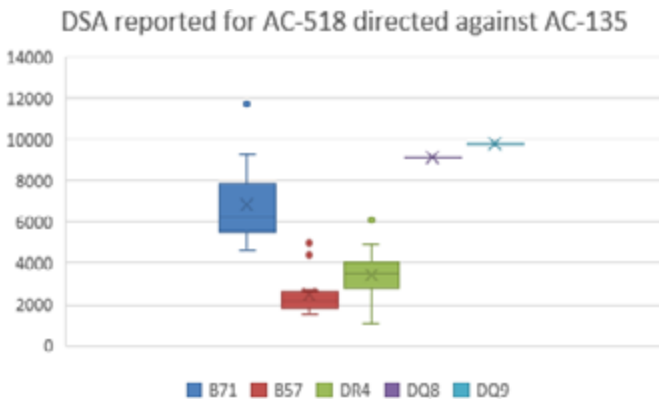
| HLA-A | HLA-B | HLA-C | HLA-DRB1 | HLA-DRB3/4/5 | HLA-DQB1 | HLA-DQA1 | HLA-DPB1 | HLA-DPA1 |
|---|---|---|---|---|---|---|---|---|
| 1 | 71 | 6 | 4 | 4(53) | 8 | 2 | 04:01 | 01:03 |
| 68 | 57 | 7 | 7 | 4(53N) | 9 | 3 | 06:01 | – |

FIGURE 3A

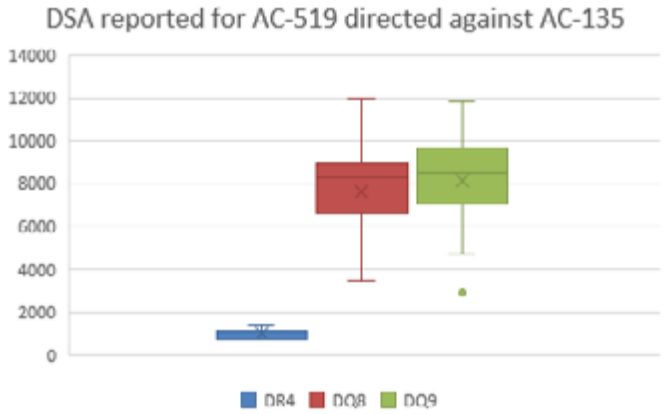
## DSA Reported Per Serum for Group A




No antibody directed against cell HLA-AC-135 antigens was detected in sera AC-515 and AC-516. Donor specific antibody against AC-135 was detected in sera AC-517, AC-518 and AC-519. Largest range in MFI values were observed for A68 (MFI 2102-8170). Only one lab reported reactivity with B71 (8000 MFI). When comparing MFI levels based on serum treatment, mean MFI for EDTA, DTT, HI, and untreated sera were respectively 5339, 3269, 6447, 3713 for antibody against HLA-**A68**; 3383, 2310, 3992, 3080 for **B57** and 10296, 9127, 10753, 10627 for **DR4**.



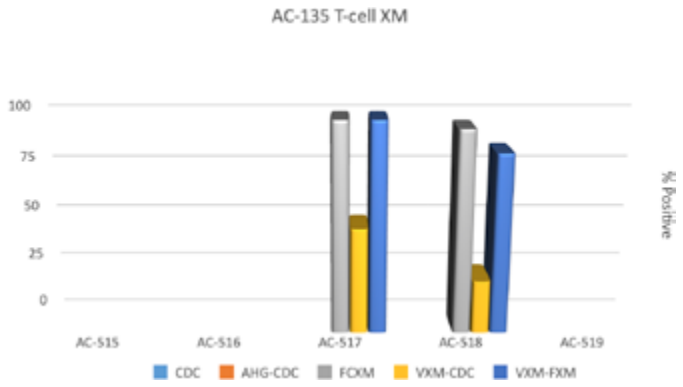

Only one laboratory reported reactivity against DQ8 and DQ9 (MFI>9000). Mean MFI values for EDTA, DTT, HI and untreated sera are as follows: 6070, 5912, 7021 and 8694 for **B71**; 2086, 1857, 2993, 3144 for **B57** and 3585, 2087, 3585, 4780 for **DR4**.

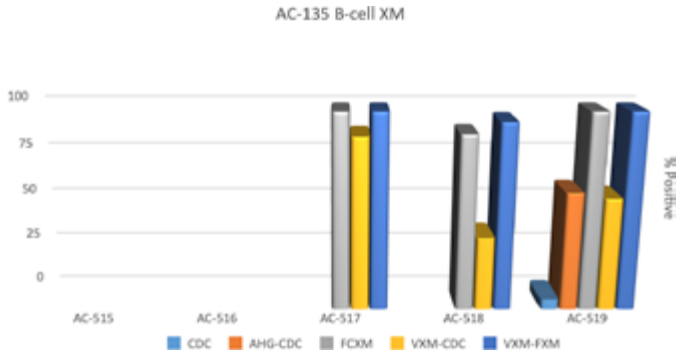



Two labs reported reactivity against DR4 below 2000 MFI. All labs reported reactivity against DR8 (range 4000-12000 MFI) and DQ9 (range 5300-11900 MFI).

FIGURE 3B

## Virtual Crossmatch Comparisons for Group A




All T cell FCXM VXM predictions were concordant with the physical crossmatches. For AC-517, the VXM results were determined based on presence of antibody against HLA-A68 (2000-8000 MFI) and B57 (2000- 5000 MFI). For AC-518 reactivity was due to B71 and B57 detected by all labs. The low consensus with the CDC-T cell crossmatch for AC135 and AC518 and AC-519 was due to low number of responses. Fifteen of the 23 labs commented that they do not perform CDC XM. Three of these labs added notes that they would predict a TAHG -CDC crossmatch positive although they reported a standard CDC as negative.

The B cell CDC and FCXM VXM predictions were concordant with the physical B cell crossmatches and were explained with reactivity against HLA class I and class II.

FIGURE 3C

## Group A Risk assessment

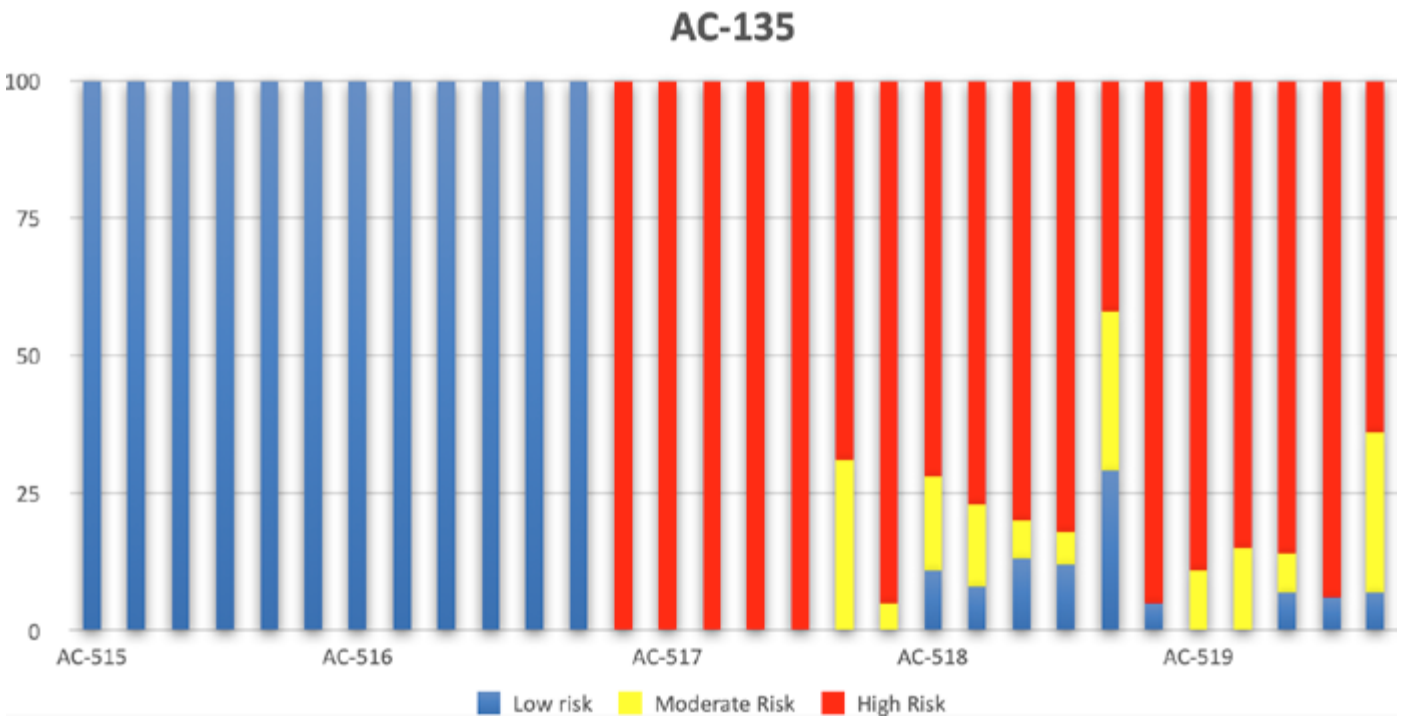


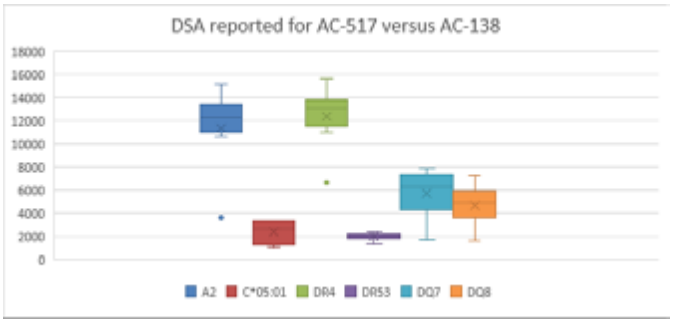
TABLE 4-1

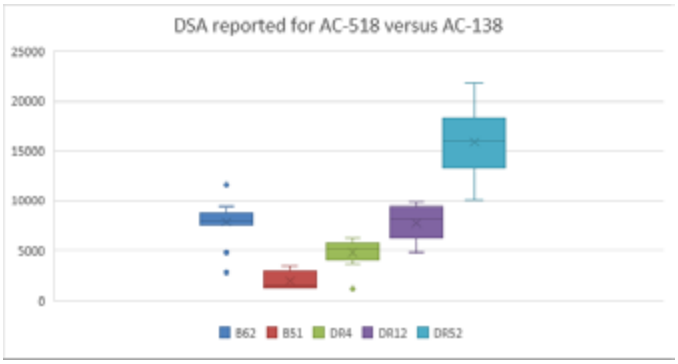
## HLA typing for Cell AC: 138: Group B

| HLA-A | HLA-B | HLA-C | HLA-DRB1 | HLA-DRB3/4/5 | HLA-DQB1 | HLA-DQA1 | HLA-DPB1 | HLA-DPA1 |
|---|---|---|---|---|---|---|---|---|
| 02:01 | 15:01(62) | 03:04 (10) | 04:01 | 4*01:01P(53) | 03:02 (8) | 03:01 | 04:01 | 01:03 |
| NA | 51:01 | 05:01 | 12:01P | 3*02:02 (52) | 03:01(7) | 05 | 05:01 | 02:02P |

FIGURE 4A

## DSA Assignments for Group B




One lab reported C1q positive for reactivity against DR52.

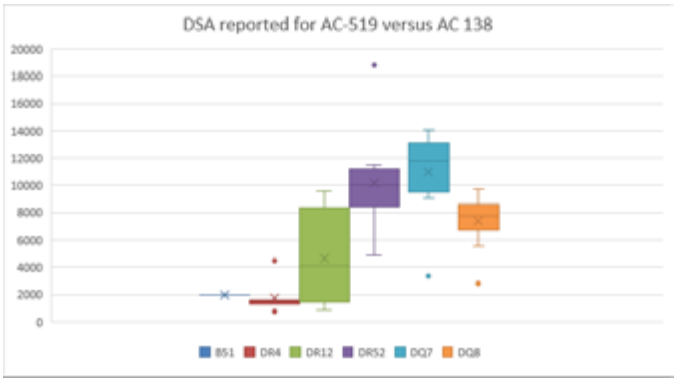


FIGURE 4B

## Group B with Cell AC-138 Comparison Between Virtual Crossmatch and Physical Crossmatch

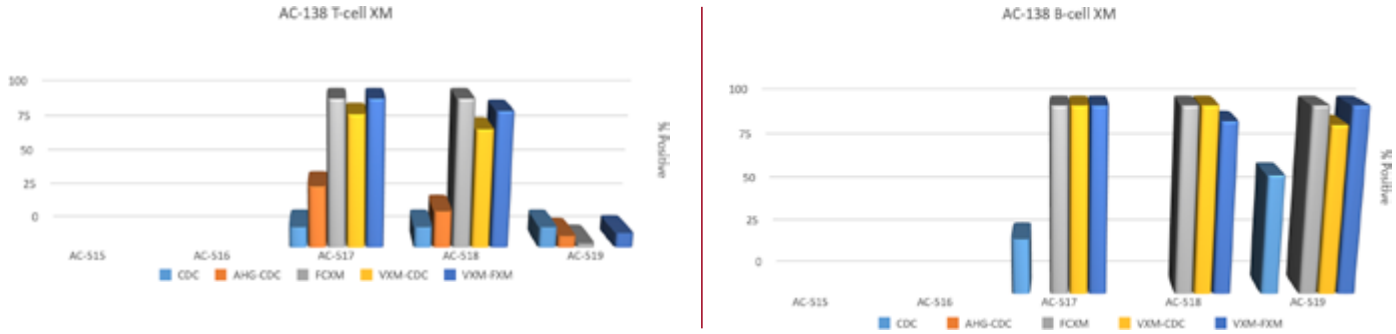



FIGURE 4C

## Group B Risk Assessments

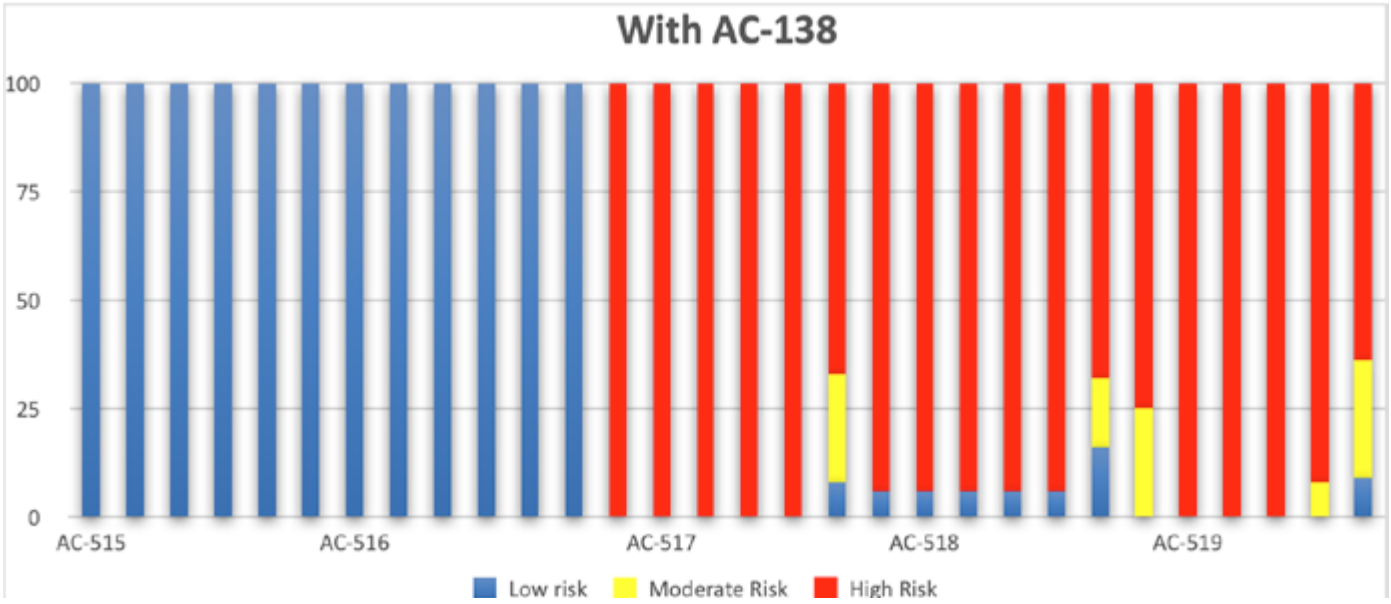


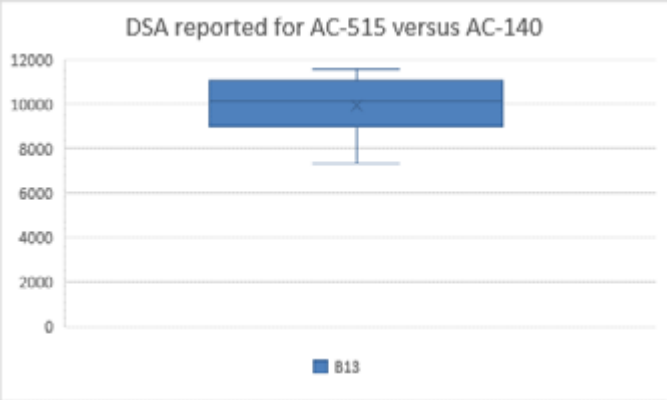
TABLE 5.1

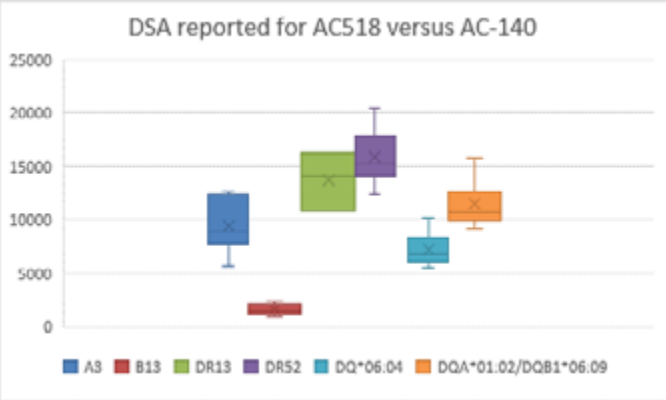
## HLA Typing for AC-140

| HLA-A | HLA-B | HLA-C | HLA-DRB1 | HLA-DRB3/4/5 | HLA-DQB1 | HLA-DQA1 | HLA-DPB1 | HLA-DPA1 |
|---|---|---|---|---|---|---|---|---|
| 03:01 | 13:.2 | 06:02 | 13:02 | 3*03:01 (52) | 06:04 | 01:02 | 03:01P | 01:03 |
| | 14:02 (65) | 08:02 | | | 06:09 | 15:01 | | 02:02P |

FIGURE 5A

## DSA Assignments for Group C




B*13:02 was only detected with one single antigen assay while the reactivity was negative among those who used a different single antigen bead assay. Participants reported the absence of a DRB1*13:02 on the single antigen bead panel and commented on presence or reactivity for DRB1*13:01 and *13:03 beads. Response based on both DR13 beads present.

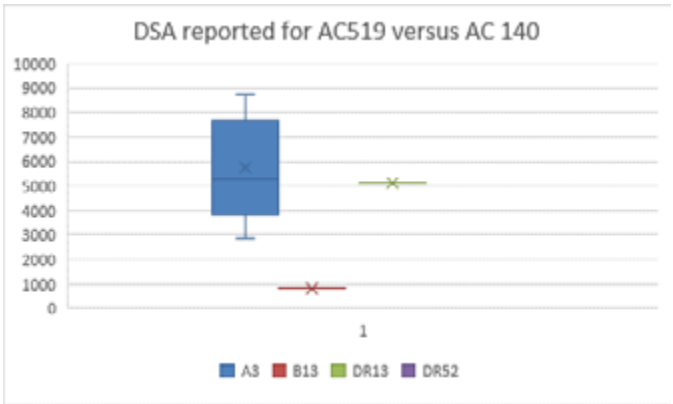


FIGURE 5B
## Group C

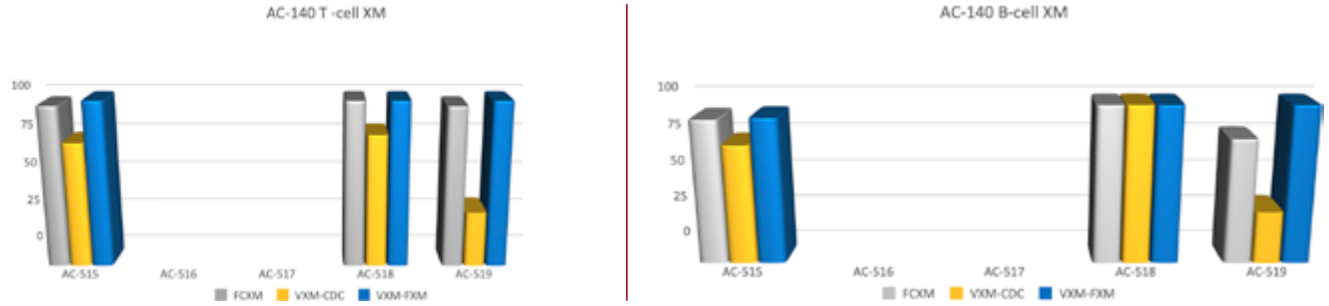
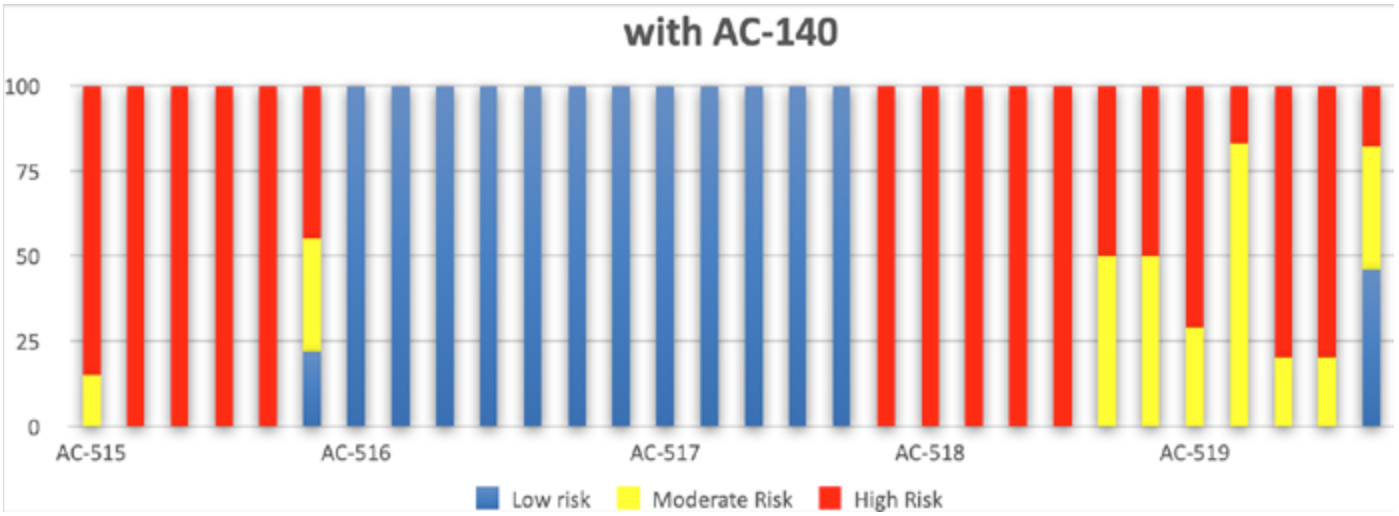



FIGURE 5C
## Group C Risk Assessments



# ARB News

Dr. John Gerlach
ARB Program Director

Welcome to the *Quarterly* Accreditation Review Board update. While I may be biased, but believe the ARB is the "best" committee ASHI has, it is probably one that is the most dynamic. What I mean is, there are always changes, reactions and responses. In this article you will note a few changes, but please, take note of the one concerning personnel files and the accreditation application in LearningBuilder. As a laboratory, there are things to be done as you enter your data this year. It may appear to be extra work, it may appear to be busy work, but it really will be work that helps all of us meet CMS/CLIA standards. Personnel records need to be reviewed for all employees during every inspection. For a small lab, easy-peasy! But for a big lab, it takes an hour or more. If the records can be reviewed electronically by your commissioner and/or inspector during the preparation for the inspection, there will be more time for the inspector to really do an inspection and more of a chance to interact with staff. You know, the real reason to be on-site. As a general comment or observation, I do want to say that having the opportunity to see all the labs during review, ASHI accredited laboratories really are a set of very well run, quality conscious, patient centric and safety minded laboratories. We do provide a level of service we should make us all proud. When we do the final review at our meetings every few months, it is rare there is a "really serious" issue, thank you for making our lives on the ARB easier.

The ARB has been busy with business as usual. Here are some updates for the ASHI membership:

- **ASHI Inspections & COVID-19:** The ARB is monitoring the situation surrounding the COVID-19 virus and its implications on travel, both domestic and international. We are receiving updates and guidance from the CDC and CMS at least weekly, if not more often, as the situation changes often. Our priority is the safety of our inspectors first.
- **Inspector Training Modules online:** In the coming months you will see updated Inspection modules in ASHI U to help you stay up to date on Inspection processes and to maintain your Inspector training. Inspectors & trainees who have attended a full day workshop in the past are considered "active" and to maintain that status must attend a half day workshop in person once every 2 years OR complete a couple of these modules in ASHI U.
- **Personnel files:** All files in the laboratory must be kept up to date, and contain proper education verification, most often in the form of transcripts. If the degree is obtained outside of the US, foreign equivalency must also be included in the file. While onsite, inspectors should be going through each personnel record, but we know this is not the best use of anyone's time, so coming in 2020, accreditation applications that are filled out annually in LearningBuilder will have a new field to upload transcripts for all testing personnel in the laboratory. This will be a one-time task in LearningBuilder for any current employee and any new employees will have to be entered as they join your laboratory – the site will store those records indefinitely.
- **State of California:** ASHI applied to the State of California for deemed status in February 2017. This is still pending.
- **Regulatory Relationships:** Melissa Weeks (accreditation manager) and I participate in the Joint Commission's Laboratory Advisory Council, with one face-to-face meeting each year and quarterly conference calls. With CMS, there are also quarterly conference calls that we participate in, plus the annual Partner's Meeting, a great and collaborative opportunity to meet with our government regulators and our peer accrediting organizations and exempt states.

## Laboratory Cycle Updates

### Cycle 1

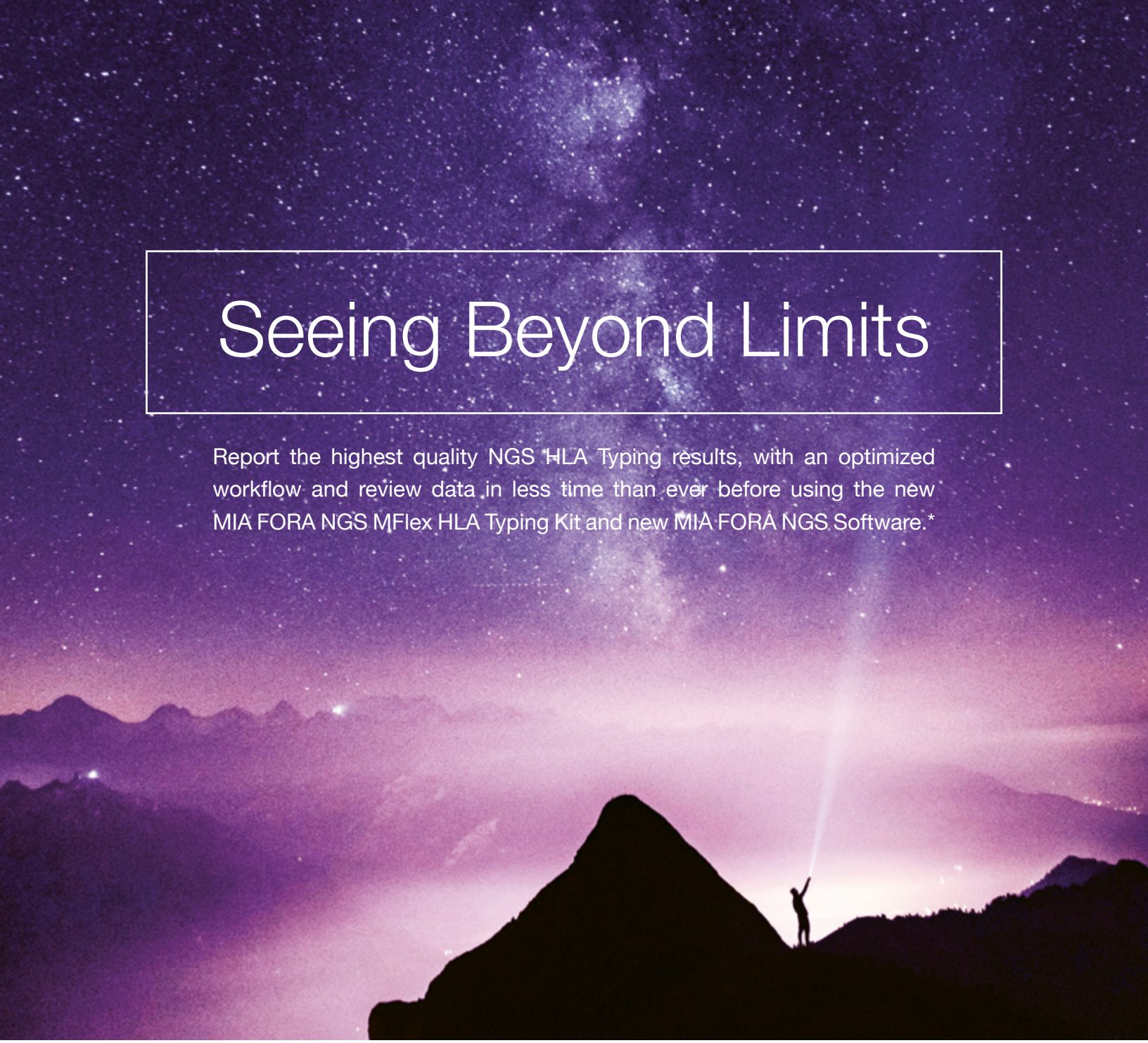
Laboratories in cycle 1 were sent links to their accreditation applications on January 1, 2020 due back on March 2, 2020. Onsite labs in this cycle will be inspected between the dates of April 15 and June 15, 2020. Laboratories in this cycle will receive their new accreditation letters and/or certificates at the end of August 2020.

### Cycle 2

Laboratories in cycle 2 received their accreditation letters and certificates in December 2019. The ARB met in San Antonio, on December 5-7 to discuss this cycle. Cycle 2 will be inactive until May of 2020 when the application & inspection process starts up again.

### Cycle 3

pplications for the cycle 3 group of laboratories were due in the accreditation office on November 1, 2019. Onsite laboratories in this cycle were inspected over the winter through February 15, 2020. The ARB will be meeting in Phoenix during the first weekend in April to review this group of laboratories – new letters and certificates were sent by the end of April 2020.

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