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14. ABSTRACT
This represents work performed under year 2 of our Breast Cancer IMPACT award. The goals of the award are clinical trials with designer T cells (dTc) in breast cancer, but also preclinical development work to bring new configurations to the clinic. The preceding Phase Ia was completed during this period, and the DOD-supported Phase Ib initiated. In response to manufacturing challenges encountered for achieving the highest doses, however, new methods had to be developed to improve dTc production. In parallel, new vector and new methods were applied for improved modification fractions. These efforts led to delays in initiating the trial, but these development efforts were very important investments going forward to ensure the long-term success of the program. With these improvements in place, outreach has now been ramped up to generate rapid recruitments. Laboratory work was performed to continue assessing additional domains from T cell signaling molecules to improve the dTc for patient use, and new staff members hired to complete the molecular analyses. Perspectives were published on the implementation and safety of the dTc approach.

15. SUBJECT TERMS
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Annual Report

INTRODUCTION

The following are the study Aims. We report progress on three Aims: 1, 2 and 3 (marked by *).

I. Clinical (existing agent)
*Aim 1. Test 2nd gen designer T cells for OBD and efficacy in metastatic breast cancer
*Aim 2. Test ancillary procedures for improved persistence and activity of infused cells

II. Pre-clinical: Advanced Research & Development
*Aim 3. Devise and preclinically test 3rd generation CIRs
*3.1. To create and test other CIR designs with alternative co-stimulatory domains
*3.2. To create and test CIR to avoid need for IL2 supplement in vivo to sustain T cell survival

III. Clinical (new agent)
Aim 4. Test safety and efficacy of 3rd gen designer T cells

BODY

Aim 1. To test 2nd gen designer T cells in metastatic breast cancer

A. Background

This Aim applies a randomization of 12 subjects to –IL2 or +IL2 arms at the maximum tolerated dose (MTD) or maximum practical dose (MPD) of designer T cells under a Phase Ib design. This will test the optimal biologic dose (OBD) in terms of the benefit of IL2 to T cell persistence and activity in vivo.

There were three dose levels in the original Phase Ia: 10^9, 10^10 and 10^11 T cells. During this report period, we treated our final patient on the 10^10 dose (level 2), completing the first two cohorts. In the first two cohorts, all patients had good tolerance of the designer T cells. One patient had a minor response to treatment, with shrinkage of brain and lung mets, but with resurgence of disease in subsequent months. This is a situation in which we postulated that addition of IL2 will allow a more prolonged and deeper response. A second patient had stable disease without other effective treatment for 12+ months.

B. Dose preparation

There are two elements of dose preparation: (1) T cell modification (creating designer T cells), and (2) T cell expansion (to meet the dose size requirements). During the recent period, we made significant advances in both areas.
(1) T cell modification

During the Phase Ia portion of the study, we confronted modest dTc modification efficiencies (10-20%) that got worse with the mid-dose level, where more cells had to be modified. This was in large part due to poor vector titer from an error at the National Gene Vector Lab that prepared the vector under contract to us. Another vector we had made against a different tumor antigen had modifications routinely in the 50-60% range. During this period, we addressed this problem by two means:

(a) Retronectin-enhanced transduction. As previously noted, we explored the benefit of retronectin to improve transduction (Td) efficiency. Results showed a 50-100% increase in transduction efficiency. Approvals to use this agent were obtained from Takara Biotech (Tokyo) to receive clinical grade retronectin, which was consented to with a reduced price for our study; by the FDA; and by the RWMC IRB. During this period, we applied this in the final patient to complete the Phase Ia, second cohort of designer T cell treatment. Improved Td efficiency of 25-30% was obtained versus levels of 5-15% previously.

(b) New vector supernatants. As mentioned above, the NGVL initially mis-prepared our vector: they manufactured the virus at the wrong temperature (37°C), which we have shown greatly reduces titer versus our standard collection temperature (32°C). During year 2, the NGVL reprepared a new batch of vector at a reduced cost $50,000 to the grant (normally $150-200,000). The titer is 50% better than the prior batch. The batch underwent safety testing and was released in the Q6 period for human use.

(c) Vector dilutions. We have shown that the vector dilution can be performed without loss of transduction efficiency, down to 1:3. This is due to inhibitors in the medium that the VPCs produce. When the inhibitor diluted out, the transductions increase, until the virus level declines too greatly. This will allow conservation of the vector supernatants, which are very expensive.

(2) T cell expansion

(a) Background

For the Phase Ib portion under DOD funding, we needed to go up one log in our expansions to achieve dose. We were generally successful with the 10^9 and 10^10 doses. But the 10^11 dose level presented significant challenges. For example, patient #1 (CS) at the 10^11 dose level had the following transduction and expansion data, starting with 10^9 cells, our maximum for Td.

<table>
<thead>
<tr>
<th>Harvest</th>
<th>TD's performed</th>
<th>Vector used</th>
<th>Total Cells</th>
<th>% Modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harvest 1</td>
<td>4</td>
<td>300 ml</td>
<td>12.7E9</td>
<td>51.4 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>vector/TD</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1200 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harvest 2</td>
<td>4</td>
<td>300 ml</td>
<td>11.1E9</td>
<td>42.3 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>vector/TD</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1200 ml</td>
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</table>
All transduction (Td) efficiencies were very good (42-51%), some of the best we have seen with this vector. To achieve this, we applied procedures we developed using retronectin (RN) as above. However, we encountered difficulties with this patient’s T cell expansions. Our target was $10^{11}$ cells or whatever we could achieve, which we term the “maximum practical dose” (MPD). Despite two expansions and pooling them for her dose (using enormous quantities of vector, 2.4L), each dose only increased about 10-fold from the activated T cells to the expansion. This was adequate for the preceding doses of $10^{10}$ cells, but falls far short of the target dose of $10^{11}$.

(b) Strategies for improving dTc expansions.

The source of the difficulty was not obvious. We previously prepared seven doses of $10^{11}$ dTc in our prior 1st generation CEA dTc study some 10 years before. A full review of prior procedures did not reveal an obvious difference.

At this point, we undertook to collect information from diverse sources on expanding T cells. We obtained the rapid expansion protocol (REP) from Mark Dudley (Steve Rosenberg group at NCI) that used high dose IL2 to expand tumor infiltrating lymphocytes (TILs). We obtained from Dr Bruce Levine at UPenn beads coated with antibody to CD28 and CD3. These provide double signals to the T cells and allow them to be boosted in their proliferation when their expansion rate slows. We first explored high doses of IL2 to overcome the lag that occurs during the T cell expansion. This proved to be productive. The tests of beads (adding another step to the protocol) have been deferred.

As mentioned above, we had difficulty reaching our highest ($1e11$) dTc cell dose in patient #1 at this IL2 level, requiring multiple T cell activations, transductions and expansions, still falling short of target. If this was the best we could do, we would simply call that our maximum practical dose (MPD). Not satisfied with this, however, we embarked on some tests using high dose IL2 and also tried increased concentrations of activating anti-CD3 antibody, OKT3.

There were four tests:

<table>
<thead>
<tr>
<th></th>
<th>OKT3 ug/ml</th>
<th>IL2 IU/ml</th>
<th>(Standard)</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>30</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>60</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>30</td>
<td>3000</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>60</td>
<td>3000</td>
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</tbody>
</table>

Peripheral blood mononuclear cells (PBMC) were seeded at $2e6/ml$ in 20 ml AIM V clinical grade growth medium (GM) under the conditions listed. Typically, there is cell loss in the days after activation, due to adherence and removal of PBMC monocytes from liquid phase and possibly apoptosis of some T cells. Following this, there is an explosive expansion of the T cell
fraction that typically has been in the range of 10-100 fold expansion over a two week period, after which the T cells plateau then decline in number.

As seen in the experiment of Figure 1, the expansion with standard dose IL2 follows this pattern, and plateaus at 14d after a 20-fold expansion and then declines. Extraordinarily, the expansion with high dose IL2 continues for 50d in log phase and declines only after day 52, with a net expansion of 1e7-fold (10 million-fold)! We examined 30 ug/ml versus 60 ug/ml OKT3 and there was no difference in the profiles. The experiment was repeated using another normal donor with similar results, with 100-fold expansion with 300 IU/ml and 1e8-fold expansion with 3000 IU/ml.

With these data, we changed the manufacturing SOP to incorporate high IL2, effective with patient #2 on the Phase Ib. This is a significant additional cost in terms of IL2, but it is completely justified by these results.

(c) Application to Prometheus to permit high-dose IL2 for expansions.

These data were shared with Prometheus (licensed by Novartis to manage Proleukin [IL2]). After an internal review, the company consented to continue its support of the trial with IL2 under this expanded requirement. The final documents are being prepared.

(d) Patient dose

This method was applied in the instance of patient #2 (VS). She had a much improved expansion, ultimately harvesting after a 50-fold growth, >5e10 cells, which was 50% of target, in a single expansion.
Although this was a substantial improvement over the dose expansion with Patient #1, the net expansion at 32 days (50-fold) was still far less than that of the control preclinical tests we did with normal random donors (1000-fold). This difference could be due to the effect of multiple prior chemotherapies to damage the patient T cells; to determine this, we will need to see how different patient donors do in parallel tests with normal donors.

On the other hand, we did make note of a curious step-like pattern on the expansion we had not seen during the preclinical tests (Graph) that appeared to impede the net growth. This step pattern seemed unlikely to be a patient issue. We speculated that this might instead be due to a toxic effect of the added fresh media that preceded each slowing. Since the basal media itself (AIM-V) was the same for both the preclinical and clinical expansions, this could only be explained by the different sera: the preclinical tests employed fetal calf serum (FCS), which was heat-inactivated (HI), whereas our human serum (HS) supplementing the clinical culture was not HI.

Since this time, we have done an expansion in parallel with both HI and non-HI human serum. These tests showed an expansion of 1000-fold with normal donor cells by 32 days, like the expansions with FCS, but with only a two-fold benefit for using HI human serum. This confirmed a better growth of the HI serum medium (not shown), but the deficiency versus HI was only 2-fold, not 20-fold as seen in the patient #2 expansion. Therefore, it remains possible that the conditions of the patient cells themselves are a variable in the expansion kinetics. Regardless, the expansion with high dose IL2 and HI sera should bring all patients into the range of the $10^{11}$ cell dose target.

Bringing together these benefits, the next patient (#3) will have her cells expanded in AIM-V + 5% HI HS.
C. Patient Enrollments

During the past year, we enrolled and treated the final patient on the Phase Ia of anti-CEA designer T cells. We then initiated the Phase Ib portion with the $10^{11}$ T cell dose (level 3) under DOD support. This includes a randomization to –IL2 or +IL2 co-administration.

Patient #1 randomized to +IL2. She had a dose prepared that was $0.2 \times 10^{11}$ T cells, under target, for reasons discussed above. She had dTc administered without complication. She had side effects attributed to IL2, with respiratory difficulty and a brief hospitalization. Within three days, she fully recovered. She was not resumed on IL2 but continued to be monitored. She had tumor biopsy (liver) on days +2 and 14. These were stored for batch analysis with other patient samples.

During the 2 weeks following dTc administration, her tumor markers underwent a modest decline, then resumption of their upward course after this period. It is possible that with full $10^{11}$ dose of dTc and IL2 as planned, she could have had a sustained and much deeper tumor suppression.

![Tumor Marker Profile on Patient CS](image)

Figure 3. Patient #1 (CS) was treated with dTc on day 0. Prior data of tumor markers were from day -40, with line drawn on graph. Values were approximately linear in growth leading up to the day 0 values. IL2 was given only day 0-day 3. The markers had their nadir at two weeks (14 days).
Patient #2 randomized to +IL2. Her dose preparation yielded $0.5 \times 10^{11}$ cells, as documented above. Unfortunately, this patient’s clinical condition deteriorated due to cancer progression and she was excluded from treatment on the day of her scheduled infusion. (Drop-out)

Patient #3 randomized to +IL2. She enrolled on June 10, 2011. She will undergo leukopheresis on June 14, 2011, with dose preparation to follow, employing our newly improved methods (above).

C. Activities for Patient Recruiting

This is new technology, in which methods are evolving and new challenges had to be addressed at each dose level in the dTc preparation. While methods were being perfected, we did not aggressively recruit patients until we had confidence all could be treated with the high doses we planned. Although further improvements may be considered, we are satisfied with the recent changes in manufacturing procedures that all patients can now reach target doses. This will allow a test of our hypothesis of adequate T cells and benefit of IL2 co-administration.

With this positive development, we are now confident in our procedures and now begin a very active outreach to recruit patients and achieve our treatment goals. In anticipation of this eventuality, the PI met with Dr Susan Love at the AACR meeting in 2010, whose Love/Avon Army of Women foundation has a tremendous email data base of patients and support groups. During 2011, our protocol was reviewed by their Scientific Advisory Board, and a recommendation was made to assist us in this important effort. The LAAW will support our recruitment efforts with emailings and postings. This will materially improve recruitments and will supplement awareness efforts of Ms Marlene McCarthy of the RI Breast Cancer Coalition, who is on the study as Patient Advocate. The first email “blast” and newsletter listing will occur during the month of June, 2011, and we look forward to many contacts from this.

During this period, the PI presented on this protocol to the Hematology-Oncology group at the Boston Medical Center. The PI gave a seminar in Providence at Women and Infants Hospital (WIH) to the breast cancer group. Two patients (#2 and #3) can to us as a result of this. A further seminar is planned for June 15 at Lahey Clinic (Burlington MA) and June 17 at Rhode Island Hospital (Providence). The PI’s abstract was selected for oral presentation at the 2011 Era of Hope meeting in August in Orlando. The PI has contacted Drs Winer and Englehart of the Dana Farber Breast Cancer section to offer a presentation to their group.

Despite early delays for technology improvements, the patient recruitments are now underway from which we expect to catch up quickly with our patient recruitment and treatment goals.
D. Pharmacokinetics by PCR

Assays are already in place to follow DTC by flow cytometry, but this method is limited to detection when 1% or more of circulating cells. We have developed a PCR based approach that can detect at a 0.01% level. Because of homologies with endogenous elements in the human genome, we have had to adjust the primers to more selective domains. We learned there is interference of the assay from heparin in the cell collection tubes, and have now switched to sodium citrate as anti-clotting agent. During this period, we developed a new set of primers that now avoid interference from endogenous elements, and citrated samples have proven effective to work with. This improvement will be applied to the breast cancer patient samples.

E. Administrative Note

During the first year, we were engaged with the HSRRB for harmonization of the protocol between the hospital and the Army. We also treated patients on the Phase Ia under separate funding that preceded the Phase Ib of this study. During this second year, the Phase Ia had its final patient treated, the Phase Ib was initiated, and optimization studies performed based on patient dose experience. These are now optimized. During this period, we have been careful to conserve resources for the clinical trial so that the treatment goals, though delayed, will not be hampered in their accomplishment. This carry-over in direct costs are $248,000 and $214,000 for years 1 and 2 that will be applied to the patient treatments.

*Aim 2. Test ancillary procedures for improved persistence and activity of infused cells*

In the application, we proposed that IL2 + IL15 would lead to a major improvement in the expansion of the dTc for patient use. Our preliminary data are shown here.

This is reminiscent of the high dose IL2 expansion seen above. Because both IL2R and IL15R share the same beta-gamma chain, differing only in the alpha chains, it is possible that complete engagement of beta-gamma by either cytokine is sufficient for the expansion. To test this hypothesis, we have initiated a collaboration with Dr Thomas Waldmann of the NCI/NIH. He is the world’s expert in IL15, and he has supplied us with IL15 to conduct experiments to test this hypothesis. These will be outlined in the next quarterly report.

Figure 4. Activated T cell expansion in cytokines. See text. (A Lo & RPJ, unpubl. results)
**Aim 3. Devise and preclinically test next (3rd) generation product**

*Aim 3.1. Create and test additional co-stimulatory configurations for CIR*

Costimulation plays an essential role in the survival and expansion of T cells after antigen encounter. Our second generation product of the Phase Ia study is complemented with CD28 costimulation.

We proposed a plan to perform high through-put testing of second co-stimulatory molecules in the format of Ig-CD28-X-zeta, in which Ig represents the MN14 antibody recognition domain, and X is the second co-stimulatory molecule. (CD28 is the first.) Constructs have been prepared for all of the following co-stimulatory molecules. These include X = HVEM, 4-1BB, ICOS, OX40, and CD27. This work was performed by Dr Bais.

- MN14-X
- MN14-z(tm)-X
- MN14-28-X-z
- MN14-X-z

We previously reported early data on the HVEM constructs. Controls are MN14-zeta (1st generation) and MN14-CD28-zeta (2nd generation). These both express well in PG13 cells, our vector producer cell line. With HVEM, we characterized the following.

- MN14-HVEM
- MN14-z(tm)-HVEM
- MN14-28-HVEM-z
- MN14-HVEM-z

Of these, the MN14-HVEM expressed the best, followed by MN14-z(tm)-HVEM (using the zeta TM domain), then MN14-28-HVEM-z, and non-expressing MN14-HVEM-z. The desired configuration with 3 signals in the single construct was thus shown to be problematic for expression. The origin of this problem is not obvious, but it suggests that this construct cannot be effectively tested in our T cells.

As an alternative, we are co-expressing MN14-28-z and MN14-HVEM in the same vector, separated by a 2A sequence for two-gene expression. This applies a novel procedure devised in this laboratory to suppress homologous recombination between the repeated MN14 sequences that leave one gene rather than two. Dr Bais undertook a mutagenesis procedure that wobbles the bases for every amino acid possible in one of the repeated elements.

We have now prepared a large number of constructs. These are shown in the following table, with more to follow. In particular, we are interested in further attempts with the CD2 construct which had problems with expression in the Aim 3.2 studies reported previously. These details will be discussed in the next quarterly report.
1. IgTCR-z
2. IgTCR-28z
3. IgTCR-hvem
4. IgTCR-hvemz
5. IgTCR-28hvemz
6. IgTCR-z(tm)hvem
7. IgTCR-41BB
8. IgTCR-OX40
9. IgTCR-ICOS
10. IgTCR-CD27

Functional tests were stalled by mycoplasma contamination of one of the parental cell lines for making VPCs. During this reporting period, new VPCs were prepared. There were still difficulties with transduction. Since the last report, we obtained again new parental helper lines to re-examine this matter, and VPCs re-prepared. The titers are improved.

Finally, an important feature of this section is the successful recruitment to the lab of Dr Agnes Lo, a researcher who formerly trained with the PI, who filled a vacancy from a departing staff member. Dr Lo is outstanding with the biologic tests of these agents, which are not trivial to accomplish. She will work with Dr Bais to complete these critical biologic studies for the benefits of costimulation. She began with the group in May, 2011, and I expect a marked increase in the results from this section. Her resume is attached in the appendix.

**Aim 3.2. Create and test products to avoid need for IL2 supplementation**

There are three parts to this Aim:
3.2.1) to examine Signal 3 (LFA1 and CD2) for their capability to yield sustained IL2 on re-stimulation with antigen,
3.2.2) to express IL2 constitutively from a promoter within the transferred transgene, and
3.2.3) to express anti-apoptotic genes the make cells resistant to IL2 withdrawal.

We have made progress on subaim #4.1, and are finalizing a manuscript for publication. We previously had shown that IL2 secretion with Signal 1+2 was abundant, but that IL2 release was lost on successive antigen restimulations [1]. We recently published that IL2 was essential for in vivo responses with 2nd generation designer T cells in animal models, despite the high IL2 secretion on antigen contact [2]. Our hope had been that Signal 3 would overcome the block to IL2 secretion on restimulations to help the T cells replicate in vivo without exogenous IL2 stimulation. [NB: the co-stimulatory molecules of Aim 3 are all considered Signal 2, although in tandem with zeta and CD28 will provide three signals. We reference CD2 and LFA1 as Signal 3 being qualitatively different, based on data of Sprent and Schlom. See grant proposal for references.]
In our experiments, we stimulated resting and activated T cells with Signal 1 or Signal 1+2 and assessed the impact of adding Signal 3 on IL2 and gamma interferon secretion. Whether with antibody stimulation or 3rd generation CIR signaling, we found improved IL2 production with 3 signals, but it was found still not to be self-sustaining. Thus, the hypothesis of sufficiency for IL2 production with three signals was falsified. This shifts the hope for avoiding systemic IL2 costs and toxicities to the subsequent subaims.

We had good expression and function of the LFA1 constructs, but, as stated above, we had problems to express a CD2 based construct. We will look at this in the context of the costimulation experiments above. Our tests showed that the IL2 secretion signal was primarily from the LFA1 beta chain, and not the alpha chain. These data were previously unknown, and speak to the outside-in signaling of the LFA1 that is primarily thought of as an adhesion molecule.

We have done nothing further with the subaims 3.2.2 and 3.2.3 that address constitutive secretion of IL2 and anti-apoptosis genes. These will follow. Crucially, however, we have added a further subaim to get at the question of IL2 secretion.

Aim 3.2.4. The signaling features of IL2 secretion shutdown.  
The failure of 3 signals that were thought the best chance for IL2 sustained secretion now begs the question to understand the mechanisms of this shutdown. IL2 is first abundant, then rapidly decline with restimulation of the T cells. This suggests an active repression mechanism, either via phosphorylation or dephosphorylation of intermediates in the signaling cascade, or chromatin remodeling or transcription factor alterations. Although the pathways to activating IL2 production are well studied, the shutdown of IL2 production has not been examined. Once this is understood, there may be ways to regulate it so that IL2 continues for as long as the T cells encounter antigen. Both signal transduction and transcription analysis will be performed.

To pursue this goal, we have recruited yet another member to the team, Dr Patrycja Dubielecki, an outstanding signal transduction expert with experience in T cells and leukemias. She will be starting in July, 2011. Her resume is attached in the Appendix. We have also arranged to collaborate with Dr Chozha Rathinam, a new junior faculty recruit at this institution in the Department of Medicine whose expertise in transcription analysis will be invaluable. His resume is attached in the Appendix.

**KEY RESEARCH ACCOMPLISHMENTS**

1. Development of procedures for improved gene modification  
   a. Use of retronectin to improve virus transduction  
   b. New vector supernatants with higher viral titer
2. Development of procedures for improved expansions (>10^6 fold)  
   a. High dose IL2  
   b. Switch to heat-inactivated serum
3. Established collaboration with Dr Waldman and obtained IL15 for Aim 2 studies
4. Coordination with Dr Susan Love of the Army of Women foundation for patient recruitments.
   a. Registration, submission of protocol and grant materials
   b. Review by Love/Avon Army of Women Scientific Advisory Committee
   c. Approval notification 6/9/2011
   d. E-blast to membership will follow
   e. This will complement efforts of Ms Marlene McCarthy of the RI Breast Cancer Coalition.
5. Clinical
   a. Phase Ia, completed with three subjects at the 10^10 dose level
   b. Phase Ib: three enrolled
   c. One treated (inevaluable for IL2), one dropped out, one awaiting dose production and treatment
   d. Recruitment mechanisms installed to ramp up patient enrollments, including successful listing with Love/Avon Army of Women.
6. Retroviral vector constructs prepared for multiple costimulatory molecules in 3-signal single gene format.
7. Recruitment of Dr Lo to assist in completing evaluation of these constructs
8. Falsification of hypothesis of Signal 3 allowing sustained IL2 secretion on restimulation.
9. Recruitment of Dr Dubielecki to investigate mechanisms of shutdown of IL2 production, and establishment of collaboration with Dr Rathinam.

REPORTABLE OUTCOMES

Publications:


CONCLUSION

Progress is being made on the Aims of the proposal. The Phase Ib study initiated during the recent year and is set to accelerate recruitments. Resources have been conserved to conduct these patient tests. The laboratory research continues to develop new agents with a plan to understand IL2 needs of dTc.

REFERENCES


APPENDICES

I. Prometheus (Novartis) Designation of new quantities of Proleukin (IL2) to support high IL2 expansions. (Verbal agreement made. Written agreement to be signed shortly; final draft attached.)

II. Resumes
   A. New Staff
      Agnes Lo, PhD
      Patrycja Dubielecki, PhD
   B. Collaborator
      Chozha Rathinam, PhD

III. Publications


June ___, 2011

Richard P. Junghans, Ph.D., M.D.
Chief, Division of Surgical Research
Associate Professor of Surgery and Medicine
Director, Biotherapeutics Development Lab
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825 Chalkstone Avenue
Providence, Rhode Island  02908

Dear Dr. Junghans,

Roger Williams Medical Center and Prometheus Laboratories Inc. (“Prometheus”) entered into a grant agreement on March 2, 2011 (the “Original Agreement”) and now desire to amend and restate the Original Agreement to address certain changes to terms of the Original Agreement.

The Investigator Initiated Trial Review and Approval Committee (“ITTRAC”) of Prometheus reviewed the Study synopsis of each of your proposed Study “Phase Ia/Ib Trial of 2nd Generation Anti-CEA Designer T Cells in Metastatic Breast Cancer- Prometheus Protocol #IIT10PLK16” on Exhibit A hereto (“the Study”), and determined that they each have scientific and medical merit. The ITTRAC of Prometheus is pleased to offer you (the “Recipient” or “you”) a research grant in-kind (the Grant”) in the form of a supply of Proleukin (aldesleukin) for use in the Study in the quantity and form as more fully described on Exhibit B hereto (the “Study Materials”).

Our agreement to provide Study Materials is conditioned on the following:

1. All necessary regulatory approvals and Institutional Review Board (“IRB”) approvals for the Study shall be obtained by Recipient, and Recipient will conduct the Study in accordance with all applicable laws, rules and regulations (including any applicable FDA and ICH guidelines), the Protocol and any applicable IRB requirements. Recipient will have obtained proper informed consent from each participant in the Study. Recipient will notify Prometheus promptly of any action by the Recipient’s IRB altering its review or approval of the Study.

2. Recipient will notify Prometheus of, and provide Prometheus with a reasonable opportunity to review and comment upon, any proposed changes to the Protocol. Recipient shall also notify the IRB and all applicable regulatory bodies of any such change. However, Recipient shall have the full and final discretion over changes to the Protocol and Recipient will notify Prometheus when any changes have been finalized.

3. Recipient will provide Prometheus with copies of all reports submitted to FDA, and other government agencies, or an IRB that are related to the Study, as well as any correspondence with such entities related to the Study.
4. Recipient will provide Prometheus with copies of unpublished manuscripts, including abstracts, as well as a summary of any presentation relating to the Study at least 30 days in advance of submission to a journal or publication (for a manuscript or abstract) and 7 days in advance of any scientific meeting (for a presentation). At the request of Prometheus, Recipient will remove any Prometheus Confidential Information. No editorial rights or control over such publication or presentation by Prometheus is implied herein.

5. Recipient shall keep Prometheus informed of the status of the Study including, without limitation, Recipient shall a) meet with Prometheus, at times mutually agreed to by the parties to advise as to the status of the Study, and b) provide Prometheus, on a confidential basis, periodic reports on the status of the Study until the Study is completed, and with a final report within 90 days after Study completion.

6. Recipient shall permit Prometheus to conduct audits from time to time to verify compliance with the terms of this grant agreement. Any such audit shall be conducted by or on behalf of Prometheus at reasonable times as agreed to by the parties and with reasonable prior written notice. Recipient shall assist Prometheus in good faith and shall provide Prometheus with all such information necessary for Prometheus to confirm compliance with the terms of this research grant agreement.

7. Records relating to the Study will be retained for at least two years after the completion or earlier termination of the Study. Source documents, such as patient charts, will be retained for not less than five years.

8. The Recipient will use the Study Materials solely for the conduct of the Study.

9. The Recipient will not distribute the Study Materials to any third party.

10. The Recipient shall return any unused supplies of Study Materials to Prometheus at the completion or earlier termination of the Study.

11. PROMETHEUS MAKES NO REPRESENTATIONS AND EXTENDS NO WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED WITH RESPECT TO THE STUDY MATERIAL INCLUDING WITHOUT LIMITATION, WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, OR THAT THE USE OF THE MATERIAL WILL NOT INFRINGE ANY PATENT, COPYRIGHT, TRADEMARK, OR OTHER PROPRIETARY RIGHTS.

12. The Recipient shall acquire no intellectual or other proprietary rights of any kind with respect to the Study Materials or other products or materials provided by Prometheus. Recipient will notify Prometheus immediately upon filing a patent application on any invention made while using the Study Materials furnished to Recipient under this Agreement. Nothing herein shall be construed as granting any assignment or license under any patent, trademark, trade secret or copyright of Prometheus.

13. You will maintain in confidence, and only use for the Study, any confidential or proprietary information we provide or disclose to you in connection with the Study.

14. Unless prohibited by law, Recipient agrees to hold harmless and indemnify Prometheus against any claims or lawsuits for damages that arise out of the Study. Prometheus agrees to notify you promptly of any such claim and to assist you and your representatives in the investigation and defense of any lawsuit and/or claim for which indemnification is provided hereunder. You shall not settle any claim for Prometheus without Prometheus’ prior written consent, which shall not be unreasonably withheld.
15. The Grant will only be used for the Study and any funds that are not used for the Study will be refunded to Prometheus upon completion or earlier termination of the Study. If the Study is not conducted in accordance with the Protocol, you will return the Grant to Prometheus promptly upon Prometheus request.

16. Prior to receiving any payment under this grant agreement, the Recipient shall provide Prometheus with a sufficiently detailed invoice for each requested payment.

17. The Grant imposes no obligation, express or implied, for you to purchase, prescribe, provide favorable formulary status for, or otherwise support Prometheus products.

The foregoing represents the entire grant agreement between us regarding the Study, and there are no further commitments, obligations or understandings of any nature regarding the Study. This grant agreement will be effective on the date of the last signature below, and will expire, unless earlier terminated in accordance with terms hereof, upon the completion of the Study and our payment of all amounts required hereunder. Prometheus shall have the right to terminate this grant agreement at anytime during the grant agreement with written notice to Recipient. The provisions of Sections 7, 8, 9, 10 and 13 shall survive the expiration or earlier termination of this grant agreement. This grant agreement may be amended with the mutual written consent of the parties.

If the above terms are acceptable to you, please sign both copies of this letter agreement in the space provided below and return one original to my attention.

Sincerely,

PROMETHEUS LABORATORIES INC.

By: ______________________________
Name: ______________________________
Title: ______________________________

Accepted and agreed to: Acknowledged and agreed to by:

By: Karen Geremia, Director Research Department ________________________________

Name Richard P. Junghans, Ph.D., M.D.

Title: ________________________________

Date: ________________________________
Exhibit A

See Attached Protocols:

“Phase Ia/Ib Trial of 2nd Generation Anti-CEA Designer T Cells in Metastatic Breast Cancer-Prometheus Protocol #IIT10PLK16”
Exhibit B

Number of patients to treat for the entire study: 12

Number of patients already enrolled treated: 2 (Non evaluable)

Number of patients yet to treat under March 15, 2010 protocol: 12

**Phase Ib - IL-2**
Per pt: 7 vials for manufacturing x 6 pts = 42 vials

**Phase Ib + IL-2**
Per pt: 7 vials for manufacturing x 6 pts = 42 vials, and 12 vials for each pt receiving IL2 x 6 pts= 72 vials

156 vials for Phase Ib
40 vials overage if any patients do not complete treatment and have to be replaced.
196 vials to complete the study
Agnes Shuk Yee Lo

Education and Fellowship

**King’s College, London, United Kingdom of Great Britain and Northern Ireland**

**Ph.D. in Immunology** Dec 2002

Dissertation: Harnessing the Immunosuppressive Tumour-Derived Cytokine, Macrophage Colony-Stimulating Factor (M-CSF), to Costimulate T Lymphocyte Growth and Activation” (co-Advisors: Professor Mike Kemeny and Dr. John Maher)


**The Chinese University of Hong Kong, Hong Kong**

**M.Phil. in Biochemistry (in Molecular Biology) ** Jun 1995

**The Chinese University of Hong Kong, Hong Kong**

**B.Sc. in Biochemistry** Aug 1995

Research Experience

**Beth Israel Deaconess Hospital, Human Monoclonal Antibody Lab, USA** Oct 2008 – Sep 2009

Research fellow, Department of Hematology-Oncology

Supervisor: Drs. Marshall Posner and Lisa Cavacini

Project: Development of anti-Dsg3 IgG (1 and 4 subclasses) and scFv for pemphigus of oral cavity and its application for the head and neck immunotherapy.

* Amplified the variable domains of antibodies from hybridomas by RT-PCT to construct full human IgG1 and IgG4 subclass antibodies. The DNA clones of these antibodies were expressed in CHO-K cells. The best expression clone was isolated by limited dilution. Antibody supernatant was purified using sepharose protein G column.
  
* Constructed scFv antibodies for expression in CHO-K cells.

* Studied pharmacokinetic of antibodies in the nude mice, immunoreactivity by live cell ELISA and toxicity using MTT assays

* Established xenograft model using matrigel to enhance the tumor growth in nude mice

* Linking scFv antibody with anti-HPV siRNA for targeting head and neck cancer (Ongoing)

**Dana-Farber Cancer Institute, Harvard Medical School, USA** Oct 2004 – Sep 2008

Research fellow, Department of Cancer Immunology and AIDS,

Supervisor: Dr. Wayne Marasco

Targeting immunotherapy for renal cell carcinoma (RCC) tumor antigen G250 using anti-G250 scFv-Fc fusion protein and lentivirus transduced T cells.

* Isolated 16 unique human anti-G250 human scFv antibodies from phage display antibody library by paramagnetic proteoliposome or immuno-tube panning. Constructed anti-G250 scFv-Fc fusion protein in mammalian pcDNA vector. Production and purification of antibodies from mammalian 293FT and CHO cell lines.

* Characterized binding affinity and binding kinetics of anti-G250-Fc fusion proteins by FACS and Biacore, respectively. Epitope mapped the functional domain by ELISA. Measured tumor killing activity by ADCC.
* Generated gene construct of anti-G250 T cells in lentivirus vector. Optimized lentivirus transduction of human T cells using DEAE-dextran and high MOI virus titer.
* Established luciferase transfected RCC tumor model in nude mice by passaging cell line.

Development of novel dendritic cell vaccine that harnesses the G250-Fc’s capability to elicit both CTL and Th responses by taking advantage of effector Fc portion of antibody.
* Constructed G250-Fc with 2A self processing peptide to facilitate equal expression of the two genes separated by 2A peptide in vitro and exponential expression of two genes in vivo.
* Co-expressed GM-CSF or IL-2 with G250-Fc with the aid of lentivirus vector.
* Measured GM-CSF and IL-2’s biofunctional activity by ELISA and effects on cell proliferation using FT-1 and CTLL-2 cell lines separately.

Participating the HIV project: Cloned the anti-CCR5 antibodies into the human full IgG vector for their expression and performed neutralization assay using pseudovirus system

**Beth Israel Deaconess Hospital, Harvard Institute of Medicine, USA**  Mar 2003 – Sep 2004
Postdoctoral fellow, Department of Hematology-Oncology
Supervisor: Dr. Richard P. Junghans
Project: Investigation of T cell adoptive immunotherapy using scFv-TCRz, scFv-TCRz/CD28 or scFv-TCRz/Bcl-XL for CEA-expressing human colorectal carcinoma and for ganglioside GD3-expressing human melanoma in nude mouse model system.
* Constructed anti-CEA and anti-GD3 designer T cells, which possess specific antibody affinity and T cell cytotoxicity potency for targeting therapy.
* Made retroviral constructs for various cancer models.
* Transduced human primary T cells using retrovirus supernatant and protamine/spinoculation.
* Studied mechanism of T cells killing of tumor in vitro by 51 chromium release assay, clonal expansion, antigen activated induced IL-2 secretion and visualization of the physical contact between T cells and tumor cells using confocal microscopy.
* Studied the effectiveness of designed T cells for tumor therapy in rodent model by caliper.

**King's College, London, United Kingdom**  Oct 1998 – Dec 2002
Ph.D. student, Department of Immunology
Project I: Assessment of the immunosuppressive effects of M-CSF on human differentiated dendritic cells.
* Isolated human monocytes for DC differentiation by depleting T cells, B cells and NK cells.
* Analyzed immunophenotype of M-CSF differentiated DC by FACS analysis, mixed lymphocyte reaction, 3H-thymine incorporation, and endocytosis assay.
* Characterized M-CSF derived DC cell death by apoptosis assays, caspase activation method, and mitochondrial potential detection. Investigated signaling pathway of non-Fas mediated cell death.

Project II: Testing of strategies to confer M-CSF responsiveness upon T lymphocytes.
* Constructed chimeric CD28/M-CSF receptors using retrovirus vector and their expression on T cell line or primary T cells via retroviral transduction.
* Established in-vivo EG7 tumor model that concomitantly express M-CSF (in secreted form) and OVA (providing signal 1). Examined the strength of signal 2 conferred by M-CSFR transduced T cells.

**The Prince of Wales Hospital, Hong Kong**  Oct 1995 – Apr 1998
Research Technician, Department of Surgery
Supervisor: Dr. Sin Ming Ip
* Compared changes in metabolic parameters following traditional (open) surgery and endoscopic surgery by monitoring hormone, cytokines, CRP, and endotoxin levels. Concentration of endotoxin was determined by Limulus lysate assay.
* Identified p53 oncogene mutations in esophageal and gastric cancer by SSCP and direct sequencing.
* Evaluated chemotherapy in cancer patients by monitoring expression of cancer drug resistance genes, such as MDR1 and MRP, by quantitative RT-PCR.
* Compared gene expression in esophageal and gastric cancer by differential display method.
* Mapped mutant genes of esophageal and gastric cancer by cytogenetic and FISH methods.
* Quantified release of mitomycin-C during chemotherapy for gastric cancer using HPLC.
* Isolated and characterized Kupffer cells obtained via bile duct surgery.

**The Chinese University of Hong Kong**, Hong Kong  
M.Phil. Student, Department of Biochemistry  
Supervisor: Dr. Mary Waye  
Project: Human heart cDNA library sequencing for the Human Genome Project

* Sequenced, verified, and submitted 500 human heart cDNA clones to the GenBank for the human genome project.
* Isolated, sequenced, (Southern and Northern) blotted, and radiation hybrid mapped the hcMDH gene.
* Raised rabbit polyclonal serum to hcMDH to measure expression level of hcMDH in various organs and in cardiomyopathy patients by Western blot and *in situ* hybridization

**PUBLICATIONS**


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**Manuscripts in preparation**


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**PATENT PENDING**

1. The Brigham and Woman’s Hospital, Inc. Corporate Sponsored Research and Licensing (“CSRL”) “Antibody Engineering of Mouse Anti-Human CCR4 Antibody 1567” Investigator Names: Jianhua Sui, Wayne Marasco, Asli Muvaffak, **Agnes Lo**, Thomas Kupper, Mei Bai


---

**CO-REVIEWER OF SCIENTIFIC PAPERS**

Co-reviewed the following original research papers with Dr. Wayne Marasco, Dana-Farber Cancer Institute.

**References:**
Letters of recommendation are available upon request.
CURRICULUM VITAE

PATRYCJA DUBIELECKA-SZCZERBA, PhD
Assistant Member
New York Blood Center
Lindsley F. Kimball Research Institute
Cell Signaling Laboratory
310E 67th Street, New York, NY, 10065
Phone: 212-570-3240, Fax: 212-570-3355
patrycja.dubielecka@gmail.com

EDUCATION
Postdoctoral Fellowship, 2005-2009, Lindsley F. Kimball Research Institute, New York Blood Center, New York, USA

Ph.D. 2005 (Graduated with honors) University of Wroclaw, Faculty of Biotechnology and Division of Hematology, Medical University of Wroclaw, Wroclaw, Poland (jointly).

M.Sc. 2001 (GPA 3.8) University of Wroclaw, Faculty of Biotechnology, Poland

B.S. 1999 University of Wroclaw, Faculty of Biotechnology, Poland

POSITIONS AND EMPLOYMENT
2009 – present, Assistant Member, Lindsley F. Kimball Research Institute, New York Blood Center, New York, USA


ADDITIONAL TRAINING
Jan-Feb 2010, Department of Microbiology and Immunology and Center for Live-Cell Imaging, University of Michigan Medical School, Ann Arbor, USA

Aug-Oct 2008, Helmholtz Center for Infectious Research, Signaling and Motility Group and Cytoskeleton Dynamics Group, Braunschweig, Germany

May-Jun 2005, Socrates/Erasmus Scholarship of EU, Charles University in Prague, Faculty of Medicine in Hradec Kralove, Department of Medical Biology and Genetics, Hradec Kralove, Czech Republic

Dec 2000 – May 2001, Socrates/Erasmus Scholarship of EU, Helsinki Biophysics & Biomembrane Group, Department of Medical Chemistry, Institute of Biomedicine, University of Helsinki, Helsinki, Finland

OTHER EXPERIENCE AND PROFESSIONAL MEMBERSHIPS
Dec 2009 – American Society for Cell Biology

May 2008 - American Association for Cancer Research

Apr 2008 - Society for Experimental Biology and Medicine
Jan 2010 – reviewer for the Journal of Pediatric Biochemistry

March 2011 - reviewer for the Bioorganic & Medicinal Chemistry Letters

HONORS AND FELLOWSHIPS
2007 Rector’s Team Scientific Achievement Award for period of 2002-2006– University of Wroclaw, Poland

The Socrates/Erasmus Program of the European Union, two scholarships in 2001 (University of Helsinki, Finland) and 2005 (Charles University, Czech Republic)

Polish Ministry of Education grant (3P04C09725) supporting PhD. project “Spectrin in early apoptosis in lymphoid and lymphoblastic cells” (2003-2005)

PATENTS
U.S. Provisional Patent Application No. 61/357,475, Regulation of macropinocytosis by p85-Abi1

P.382191 ‘Fast Chemotherapy Efficiency Test with Spectrin Involvement’ processing by the Polish National Patent Office

P.382192 ‘Fast Chemotherapy Efficiency Test with Protein Kinase C Involvement’ processing by the Polish National Patent Office

PENDING GRANT APPLICATIONS
1. PA08-165] - STEM CELLS AND CANCER (R21) - ‘Role of Abi1 in survival and quiescence of Bcr-Abl positive leukemic stem cells’ 1R21CA164735-01, PI Patrycja Dubielecka-Szczerba

PUBLISHED ARTICLES


INVITED TALKS
Max-Planck Institute of Molecular Cell Biology and Genetics Symposium, 30 May – 1 June 201, Königstein (Saxon Switzerland), Germany, ‘Abi1 in Bcr-Abl signaling’.

Helmholtz Center for Infectious Research, Braunschweig, Germany, 2008, ‘Differential regulation of macropinocytosis by Abi1/Hssh3bp1 isoforms’.
Charles University in Prague, Faculty of Medicine in Hradec Kralove, Department of Medical Biology and Genetics, Hradec Kralove, Czech Republic, 2005, ‘Changes in spectrin organization in leukemic and lymphoid cells upon chemotherapy’.


**COURSES TAUGHT**
Lectures and courses for undergraduate students:

Cell Biology, specific courses: *Apoptosis*. Series of lectures during academic years 2001-2005 – University of Wroclaw, Faculty of Biotechnology, Wroclaw, Poland

Cell Biology, specific courses: *Practical course in immunochemistry*. Course taught during academic years 2001-2005 – University of Wroclaw, Faculty of Biotechnology, Wroclaw, Poland

**MENTORSHIP**
**Mentorship for undergraduate students during Summer Internship Program at the New York Blood Center; years 2007-2011,** (*asterisk indicate mentored undergraduate student):
1. Abayomi Are, high school student associated with Harlem Children Society, New York, NY; project title: ‘Characteristics of focal adhesions in MEF cells lacking Abelson interactor protein 1 gene’. Project was awarded presentation at 2010 Sigma Xi Scientific Research Society Meeting in Raleigh, NC.


3. Dan Yi Zhao, high school student, associated with Harlem Children Society, New York, NY; project title: ‘Cell motility of MEF cells lacking Abelson interactor protein 1 gene.’


**Mentorship for undergraduate students during academic years 2001-2005,** (*asterisk indicate mentored undergraduate student):


**Hosted meetings:**
Series of Meetings for postdoctoral trainees at the New York Blood Center: *Happy Fridays*, monthly meetings; starting date Aug 2009 – to present.
PLACE OF BIRTH
Jelenia Gora, Poland (Citizenship: PL, Immigration status: US Permanent Resident)
Curriculum Vitae

Chozha Vendan Rathinam

Date of Birth: 
Nationality: Indian
Gender: Male

Education

Since May 2006-Postdoctoral Associate (Stem Cell Biology)
Yale Stem Cell Centre,
Yale School of Medicine, New Haven, U.S.A.

December 2004-April 2006, Scientist (Hematopoiesis)
Hannover Medical School, Germany

Jan-2001-November 2004 Ph.D., (Hematopoiesis)
Hannover medical school, Germany

1995-2000, Master of Science (Life Sciences)
Bharathidasan University, Trichy, India
**Distinctions & Awards**

Has always scored distinction grades in all the exams (During primary, secondary and university education)

‘Best Ph.D thesis work’ award (2007)  
Awarded by University of Tubingen, Germany

ASH Travel Award (2005)  
Awarded by American Society of Hematology, USA

**Best paper presentation Award (2000)**  
Awarded by Electronics And Instrumentation Engineering Association, Trichy, India

**Merit Scholarships**

Fellowship from the Bill and Melinda Gates foundation, USA (2007-till date)

Fellowship from the American Diabetes Association, USA (2006-2007)

Scholarship from the Varta Foundation, Germany (2003-2004)

Fellowship from the Land of Lowersaxony, Germany (2003)

Scholarship of the pro-rector of Hannover Medicine school, Germany (2002-2003)

Scholarship of the pro-rector of Hannover Medicine school, Germany (2001-2002)

University of Cambridge scholarship, The United Kingdom (2001)

International postgraduate research scholarship from the government of Australia (2001)

University of Adelaide scholarship, Australia (2001)

**Teaching Experience**

April - December 2000: Lecturer: Taught molecular biology and Immunology courses for students (class of 40 students) enrolled in the Master of Science programme during academic year 2000-2001 at Bharathidasan University, Trichy, India.
Mentorship Experience

2004-2006 - Daniel Kotlarz – MD thesis work, Hannover medical school, Germany
2005-2006 - Giri Appaswamy – PhD thesis work, Hannover medical school, Germany
2007-2008 - Nico Lachmann – M.Sc thesis work, Yale school of medicine, USA
2009-2010 - Christoph Schunemann – MD thesis work, Yale school of medicine, USA

Oral Presentations

1. Rathinam C. A new role for an old cytokine: M-CSF regulates functions of human hemetopoietic stem cells, YCEMH mini symposium, New Haven, CT, July 23rd, 2010

2. Rathinam C. c-Cbl-Cancers friend and foe? Yale Stem Cell Centre, New haven, USA, June 10th, 2009

3. Rathinam C. Gfi1- A master regulator of hematopoiesis. Institute of molecular pathology, Vienna, Austria, August 10th, 2006

4. Rathinam C. “Transcriptional repressor Gfi1 integrates cytokine-receptor signals controlling B-cell differentiation” 47th annual meeting of the American Society of Hematology, Atlanta, Georgia, USA, December 10-13, 2005

5. Rathinam C. “Transcriptional profiling reveals Gfi1 as a critical factor for STAT3-dependent dendritic cell development and function” Wilsede, Germany, 9th June, 2005

6. Rathinam C. “Transcriptional repressor Gfi1 controls STAT3 dependent dendritic cell development and function” Hannover Medical School, Germany, April 21st, 2005

7. Rathinam C. “Gfi1- A master regulator of dendropoiesis” Hannover Medical School, Germany, March 10th, 2005

8. Rathinam C. “Towards the elucidation of transcription factor controlled development of dendritic cells” Wilsede, Germany, June 25th, 2003

9. Rathinam C. “Development of dendritic cells from hematopoietic progenitor cells” Hannover Medical School, Germany, December 5th, 2001


Poster Presentations

1. 45th Annual meeting, American society of hematology, 6-9.12.2003, San Diego, USA
2. 9th Congress of the European Hematology Association, Amsterdam, The Netherlands
3. 8th International symposium on dendritic cells, 17-21.10.2004, Brugge, Belgium
4. 46th Annual meeting, American society of haematology, 4-7.12.2005, San Diego, USA

Abstracts submitted


Patents

1. Rathinam C and Flavell RA. A novel method to expand hematopoietic stem cells by inhibiting the expression and function of E3 ubiquitin ligases. (Status-Filed)

2. Rathinam C and Flavell RA. Use of M-CSF to treat human hematopoietic stem cell disorders (Status-to be filed)
Research publications

1. **Rathinam C**, Majetic L and Flavell R. The HECT domain E3 ligase Itch negatively controls haematopoietic stem cell maintenance and functions (Revision in *Nature Immunology*)


3. **Rathinam C** and Flavell R. c-Cbl deficiency leads to diminished lymphocyte development and functions. *Proc Natl Acad Sci USA* 107, 8316-21 (2010)


<table>
<thead>
<tr>
<th>Manuscripts Submitted/in Preparation</th>
</tr>
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<tbody>
<tr>
<td>2. <strong>Rathinam C</strong> and Flavell R. Deficiency of the E3 ubiquitin ligase c-Cbl results in malignant hematopoiesis originating from HSCs (Submitted)</td>
</tr>
<tr>
<td>5. <strong>Rathinam C</strong>, Eynon EE, Manz MG and Flavell RA. Identification of a novel role for</td>
</tr>
</tbody>
</table>
active collaborations and collaborators

1. Dr. Wallace Langdon, Associate Professor, Division of Pathology, The University of Western Australia (c-Cbl Leukemia Project)

2. Dr. Pietro De Camilli, Professor, Department of Cell Biology, Yale School of Medicine (Endopilin Project)

3. Dr. Agnes Vignery, Associate Professor, Department of Orthopaedics and Rehabilitation, Yale School of Medicine (HSC Niche Project)

4. Dr. Eswarakumar V.P., Assistant Professor, Department of Orthopaedics and Rehabilitation, Yale School of Medicine (MSC Project)

5. Dr. Sherman Weissman, Professor, Department of Genetics, Yale School of Medicine (Fat10 Project)

6. Dr. Stephanie Halene, Assistant Professor, Yale Stem Cell Center, Yale School of Medicine (Human Leukemia Project)
### Academic Referees

1. **Prof. Richard Flavell Ph.D., FRS (Postdoc mentor)**  
   Chair, Department of Immunobiology  
   Investigator, Howard Hughes Medical Institute  
   Yale School of Medicine  
   New Haven, CT- 06520, USA  
   Phone: 203-737-2216  
   Fax: 203-785-2958  
   Email: richard.flavell@yale.edu

2. **Prof. Christoph Klein MD, Ph.D. (Ph.D mentor)**  
   Chair & Director, Department Of Pediatric Hematology and Oncology  
   Hannover Medical School, Carl Neuberg Strasse 1  
   D – 30625 Hannover, Germany  
   Phone: 49-511-532-6718  
   Fax: 49-511-532-9120  
   Email: klein.christoph@mh-hannover.de

3. **Prof. Agnes Vignery Ph.D. (Collaborator)**  
   Yale Orthopaedics and Rehabilitation, and Molecular and Cellular Biology  
   Yale School of Medicine  
   New Haven, CT- 06520 USA  
   Phone: 203-785-5968  
   Fax: 203-737-2701  
   Email: agnes.vignery@yale.edu
Autoimmunity associated with immunotherapy of cancer

Sally M. Amos, Connie P.M. Duong, Jennifer A. Westwood, David S. Ritchie, Richard P. Junghans, Phillip K. Darcy and Michael H. Kershaw

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include the digital object identifier (DOIs) and date of initial publication.
Autoimmunity associated with immunotherapy of cancer

Sally M. Amos¹,*, Connie P.M. Duong¹,*, Jennifer A. Westwood¹, David S. Ritchie², Richard P. Junghans³, Phillip K. Darcy¹,4,5,6 and Michael H. Kershaw¹,4,5,6

¹Cancer Immunology Research Program, Peter MacCallum Cancer Centre, Melbourne, Australia, ²Department of Hematology and Medical Oncology, Peter MacCallum Cancer Centre, ³Roger Williams Medical Center, Providence, RI, USA ⁴Department of Pathology, University of Melbourne, Australia. ⁵Department of Immunology, Monash University, Clayton, Australia. ⁶Contributed equally as senior authors. *Contributed equally as first authors.

Corresponding author: Michael H. Kershaw, Cancer Immunology Research Program, Peter MacCallum Cancer Centre, St. Andrews Place, Melbourne, Victoria, Australia 3002. Telephone: (613) 96561177, Fax: (613) 96561411, E-mail: michael.kershaw@petermac.org.

Short title: Autoimmunity from immunotherapy
Abstract

In this age of promise of new therapies for cancer, immunotherapy is emerging as an exciting treatment option for patients. Vaccines and cytokines are being tested extensively in clinical trials and strategies using monoclonal antibodies and cell transfer are mediating dramatic regression of tumors in patients with certain malignancies. However, although initially advocated as being more specific for cancer and having fewer side effects than conventional therapies, it is becoming increasingly clear that many immunotherapies can lead to immune reactions against normal tissues. Immunotoxicities resulting from treatment can range from relatively minor conditions, such as skin depigmentation, to severe toxicities against crucial organ systems, such as liver, bowel and lung. Treatment-related toxicity has correlated with better responses in some cases, and it is likely that serious adverse events from immune-mediated reactions will increase in frequency and severity as immunotherapeutic approaches become more effective. This review introduces immunotherapeutic approaches to cancer treatment, provides details of toxicities arising from therapy and discusses future potential ways to avoid or circumvent these side effects.
Introduction

Immunotherapy holds much promise for the treatment of cancer. The immune system is capable of dramatic and decisive responses against infectious disease, which is accomplished with exquisite specificity against antigen. Components of immunity are seen as potentially more specific weapons to direct against tumors than chemotherapy or radiation. With our expanding knowledge of tumor-associated antigens (TAA), there are many different approaches being developed to direct immunity against transformed cells.

Immunotherapies may involve the active generation of immunity to TAA, via vaccination with peptides or peptide-pulsed dendritic cells.\(^1\) In addition, administration of immune modulators such as cytokines can boost existing anti-tumor immunity and target immune effector cells to sites of tumor growth.\(^2\) Monoclonal antibodies harness both innate and adaptive immune mechanisms and direct them against tumor cells.\(^3\) Additionally, the effector functions of cytotoxic T lymphocytes (CTL) have proven them to be particularly useful in targeting TAA in adoptive immunotherapeutic protocols.\(^4\)

Some TAA are tumor specific, whose expression is entirely limited to tumors, examples of which include viral antigens expressed on cells in which viral oncogenes have contributed to cellular transformation. In these cases, immunotherapy can be employed with fine specificity and very little toxicity against normal tissues.\(^5\) However, most TAA are expressed by some cells of normal tissues and the potential exists for on-target toxicity against these tissues. These on-target toxicities can be assigned to two broad categories. Firstly, they can comprise “true” autoimmunity, involving a fundamental induction of endogenous immunity against self antigens, and we will refer to this type as “autoimmunity”. Secondly, they can be more “drug-like” in nature, where damage is mediated directly by the immunomodulatory agent, and these toxicities
will be referred to as “immune-mediated”. Toxicities have been described in a proportion of patients using a range of immunotherapeutic approaches. As immune-based cancer therapies become more potent, it is likely that autoimmune and immune-mediated toxicities will become more severe. Indeed, these toxicities can be associated with better anti-tumor responses resulting from immunotherapy.

In this review, we will introduce various immunotherapeutic approaches and provide details of toxicity to normal tissues resulting from these approaches, as well as describe potential ways to overcome these toxicities.

**Autoimmunity associated with adoptive immunotherapy**

Adoptive immunotherapy is a very promising approach to treating cancer that involves the isolation of leukocytes and their activation and expansion *in vitro* followed by infusion into patients. Advantages of this type of approach include the opportunity to manipulate and activate lymphocytes away from the *in vivo* immunosuppressive environment, and their expansion to vast numbers, thereby circumventing many regulatory checkpoints and delivering “instant” immunity. Adoptive immunotherapy has been demonstrated to induce regression of established tumors, often complete, in both mouse models of disease and in patients. However, apart from EBV-associated malignancies, this form of therapy targets antigens expressed on some normal tissues besides tumor cells, and immune-mediated toxicity has been observed in the treatment of both mice and humans (Table 1, Figure 1).
**Tumor-infiltrating lymphocytes and melanoma**

Tumor inhibition has been described using adoptive immunotherapy in various animal models over the past 50 years, but antigen specificity and its expression on normal tissue was largely undefined in earlier models. More recently, mouse tumor models with known tumor antigens also expressed on self tissues have become available and effective anti-tumor responses following adoptive immunotherapy have been observed. However, immune-mediated toxicity has been observed in these mouse models targeting normal tissues including skin, eye, colon and the B cell compartment as summarized in Table 1. In the following discussion, we will focus on these toxicities following adoptive immunotherapy in humans.

One of the most promising applications of adoptive immunotherapy in the clinic involves the use of tumor-infiltrating lymphocytes (TIL) to treat melanoma. Lymphocytes derived from tumors can be expanded to yield many billions of cells that are reactive with a range of melanoma antigens including gp100, MART-1 and tyrosinase.

Following demonstrations that prior lymphodepletion could lead to enhanced persistence and activity of transferred T cells, adoptive immunotherapy protocols were modified to include preconditioning regimens to deplete cells of the hematopoietic system. Depletion regimens ranged from non-myeloablative, using cyclophosphamide and fludarabine, to fully ablative regimens using chemotherapy and whole body irradiation, together with stem cell support. Objective response rates of adoptive immunotherapy reached 70% with these modifications, but immune-mediated toxicities were also observed, with reports of vitiligo and occasional reports of ocular toxicity, involving responses against melanin-containing cells important in retina function.
In cases where endogenous TIL are difficult to obtain, tumor-specific T cells can be generated from peripheral blood lymphocytes by genetic modification with genes encoding specific TCR α and β chains. In a 2009 trial, melanoma patients treated with gene-modified T cells reactive with melanoma/melanocyte antigens, experienced destruction of normal melanocytes in the skin (27 of 36 patients), as well as responses against normal cells of the eye and ear, which occurred in approximately 50% of 20 patients receiving T cells modified with a highly reactive TCR.10 These patients were administered steroids to inhibit T cell activity, which led to resolution of both uveitis and hearing loss.

Interestingly, in addition to the above on-target immune toxicities, the potential for immune-mediated off-target toxicity using adoptive transfer of TCR gene-modified T cells has been demonstrated in mice. In a study using mouse T cells genetically engineered to express TCR alpha and beta chains specific for ovalbumin, severe toxicity was observed including cachexia, anemia, pancreatitis and colitis.11 Reactivity against these normal tissues was thought to be due to mispairing of introduced and endogenous TCR chains leading to T cells with neo-specificities. These findings were also extended to include T cells modified to express other TCRs including those specific for gp100, SV40 large T antigen, TRP-2 and influenza virus nucleoprotein, although the incidence of lethal toxicity varied depending on the TCR. The propensity of TCR mispairing to induce immune-mediated toxicity in humans has yet to be determined fully but it has not been observed to date in clinical trial.12

**Genetically redirected T cells in adoptive immunotherapy**

By far the greatest application of adoptive immunotherapy has been in the melanoma setting as described above. This is largely due to the availability of specific T cells, and
extension to other common cancers is restricted by a lack of availability of endogenous T cells of appropriate specificity. However, T cells reactive with a range of common cancers can be generated by genetic modification of peripheral blood lymphocytes with chimeric antigen receptors (CARs), whose specificity is derived from monoclonal antibodies specific for cell surface TAA. CAR-modified T cells have been used in clinical trials for a range of cancers including ovarian cancer, neuroblastoma, colon cancer and lymphoma. The use of CAR-modified T cells is in its infancy and only limited anti-tumor effects have been described to date. Nevertheless, in a phase I study for renal cell carcinoma (RCC) targeting the TAA carbonic anhydrase IX, adoptive transfer of gene-modified T cells led to grade 3-4 liver toxicity in three of seven patients treated. Toxicity was thought to be due to the T cells targeting the CAIX antigen also present on bile ducts. Toxicity was resolved in this study after cessation of adoptive T cell transfer or administration of steroids.

CAR-modified T cell activity against normal cells was also observed in a clinical study targeting CD19 for the treatment of follicular lymphoma. The CAR was composed of a single-chain anti-CD19 antibody linked to CD28 and the zeta chain of the CD3-TCR complex. In this study, dramatic regression of malignant cells was observed, but a prolonged depletion of normal B cells was also observed leading to greatly decreased levels of serum immunoglobulin. While low levels of serum antibody is concerning, administration of exogenous immunoglobulin can correct for this deficiency thereby providing protection against infection. In another study targeting CD19 with CAR-modified T cells, this time for chronic lymphocytic leukemia, treatment was well tolerated in 3 patients receiving T cell transfer in the absence of prior lymphodepletion, with only transient fevers experienced. However, a patient receiving T cells following lymphodepletion developed hypotension, dyspnea and renal failure and died four days
after treatment.\textsuperscript{16} Death in this case was not thought to be due to treatment and was attributed to sepsis due to infection.

More recently, adoptive transfer of CAR-modified T cells, specific for the TAA Her-2, led to death of a patient receiving treatment for colon cancer.\textsuperscript{17} Toxicity involved respiratory distress and may have been due to CAR-expressing T cell activity against normal Her-2-expressing cells of the lung. Increased levels of serum cytokines including IFN-\(\gamma\), GM-CSF, IL-6 and TNF-\(\alpha\) were observed, together with hypotension, brachycardia and gastrointestinal bleeding, which led to cardiac arrest. In another study targeting Her-2 no toxicity was observed,\textsuperscript{18} although comparisons between this study using autologous CTL clones and the CAR-expressing study are difficult due to significant differences in the studies, including the use of lymphodepleting conditioning and greater numbers of T cells in the CAR study. Thus, many different organ systems can become targets of immune-mediated damage following adoptive immunotherapy, a recent example of which involved severe, yet transient, colitis in 3 of 3 patients receiving T cells specific for the colon cancer-associated antigen, CEA.\textsuperscript{19}

In the above examples, the target tumor antigens were also expressed by a range of normal tissues. However, antigens with greater tumor specificity are emerging as more desirable targets. The cancer/testes antigen, NY-ESO-1, represents such a target that is expressed on a range of tumors, but whose normal tissue expression is limited to testes.\textsuperscript{20} In a phase I study, over 50\% of patients with either melanoma or synovial cell sarcoma experienced objective clinical responses in the absence of any immune-mediated toxicity.\textsuperscript{21} Similarly, in a phase I study targeting the carbohydrate antigen, GD2, with genetically redirected T cells, no T cell-related adverse events were observed, despite apparent anti-tumor activity demonstrated in 4 of 8...
evaluable patients. GD2 is an attractive target since it is expressed on neuroblastoma and many melanomas but normal tissue expression is largely limited to brain and peripheral nerves.

**Autoimmunity associated with antibody therapy**

There is much promise and excitement in the use of monoclonal antibodies for immunotherapy, with 10 approved by the FDA for the treatment of cancer (reviewed by Weiner *et al* 23). These antibodies are specific for a variety of molecular targets expressed on a range of cancers including lymphomas, leukemias, breast cancer and colorectal cancer. A variety of effector mechanisms are employed by antibodies against tumor cells, which include antagonizing growth factors and their receptors, or inducing their degradation. Alternatively, antibodies may activate antibody-dependent cell-mediated cytotoxicity (ADCC) or the complement pathway. Finally, antibodies may also be used to antagonize receptors such as cytotoxic T lymphocyte antigen-4 (CTLA-4), which normally downregulate immune responses.

Clinical trials are currently under way to test the potential of antibodies to mediate tumor regression. However, just as spontaneously arising tumor-specific antibodies have been shown to induce autoimmune pathologies in paraneoplastic neurologic disorders,24 toxicities against normal tissues have also been observed in a proportion of patients receiving exogenous antibody (Table 2).

Toxicities arising from antibody administration can occur in various ways. Firstly, toxicity can follow the induction of potent endogenous autoimmunity against both tumor antigens and other self antigens, resulting in both on- and off-target toxicities. Secondly, toxicities can involve on-target depletion of normal cell subsets, compromising normal tissue
function. In this case, cell depletion and resulting toxicity is generally transient, persisting only during antibody administration after which normal cells are replaced from precursors. Nevertheless, even transient depletion can have severe consequences.

Some therapeutic antibodies mediate their anti-tumor activity by blocking growth factor receptors. Antibodies included in this category include bevacizumab, cetuximab and trastuzumab, which target vascular endothelial growth factor receptor (VEGF-R), epidermal growth factor receptor (EGF-R) and human epidermal growth factor receptor-2 (Her-2) respectively. Toxicities against a range of normal tissues including gastrointestinal tract, skin and lung have been observed using these antibodies for cancer therapy. However, since these antibodies mediate their effects largely by blocking the signaling ability of growth factor receptors, which is reminiscent of the action of drugs rather than immunity, we have not discussed these antibodies here, but cite references as information for the reader.25-27

**Autoimmunity involving on- and off-target toxicities.**

An immune response is a complex process that is subject to many controls and balances, including the involvement of inhibitory molecules on lymphocytes that downregulate responses (upon elimination of antigen) or prevent inappropriate activity against self antigens. CTLA-4 is one such regulatory molecule expressed by T cells that transmits an inhibitory signal to T cells upon binding to CD80 and CD86 on antigen presenting cells. The targeting of this inhibitory receptor in immunotherapy has been used to break immune tolerance of T cells to TAA, resulting in the expansion of T cells that elicit anti-tumor effects.28 However, in addition to tumor regression, anti-CTLA-4 antibodies, such as ipilimumab and tremelimumab, have been associated with autoimmunity affecting tissues including the thyroid, lung, joints, gastric mucosa
and liver (Table 2). In a clinical study using ipilimumab to treat hematopoietic malignancy after recurrence following allogeneic hematopoietic cell transplantation, complete remissions were observed in two Hodgkin’s disease patients (2 of 29) and a partial remission in a patient with mantle cell lymphoma. However, grade 2 to 4 autoimmune events thought to be treatment-related observed in 4 patients in this study, included arthritis, hyperthyroidism and recurring pneumonitis.29

Toxicities can be common and sometimes severe, as seen in a study using ipilimumab alone or together with vaccine for the treatment of melanoma or renal cell carcinoma (RCC). In this study, 21% of 198 patients developed grade 3/4 enterocolitis, with 4 patients suffering colonic perforation leading to 2 deaths and one colectomy.30 Enterocolitis resolved in most patients following administration of corticosteroids, providing further evidence of immune involvement. However, it was not clear if lymphocyte responses were directed against endogenous antigens from normal bowel or antigens derived from intestinal microflora. Gastrointestinal toxicity was also observed in another study, where 11 of 71 patients receiving ipilimumab developed grade 3-4 diarrhea, and gastrointestinal bleeding required colectomy in one patient.31

Of significant interest is that autoimmunity has been demonstrated to be associated with clinical response, suggesting that the greater the immune dysregulation mediated by anti-CTLA-4, the greater the anti-tumor effect. In some of these cases specific T cells have been identified that were associated with both anti-tumor activity and autoimmunity, where Melan-A specific cytotoxic T cells were observed infiltrating tumors and skin rashes together with a 30-fold increase in circulating Melan-A specific cytotoxic T cells following treatment with ipilimumab.32
Further randomized clinical studies continue in order to determine if the benefits of anti-CTLA-4 therapy justify the risk of autoimmune side effects. In a recent phase III study involving 676 melanoma patients, ipilimumab (with or without gp100 vaccine) was demonstrated to significantly improve survival of patients compared to patients receiving vaccine alone (10.1 months median survival compared with 6.4 months). Complete or partial tumor responses were observed in 9.4% of patients receiving treatment that included ipilimumab compared with 1.5% responses in patients not receiving the antibody. Immune-related adverse events occurred in 60% of ipilimumab treated patients, with approximately 15% of patients experiencing grade 3 or 4 immune-related adverse events. Toxicity was largely reversed with appropriate treatment, although 7 deaths occurred that were thought to be immune-related due to conditions including colitis, bowel perforation, liver failure and one patient with Guillain-Barré syndrome.

A range of measures can be taken to manage immune-mediated toxicities, including the use of corticosteroids, which can decrease the severity of toxicity. Interestingly, anti-tumor responses have been observed to be maintained in the presence of steroid treatment, suggesting that anti-tumor activity can be separated from auto-reactivity, at least in some circumstances.

Anti-CTLA-4 antibodies have induced anti-tumor responses most markedly against RCC and melanoma, which suggests that these two cancers are exceptionally immunogenic. Induction of autoimmunity by anti-CTLA-4 may occur through dysregulation of a pre-existing immune response to self antigens, which was held in check by CTLA-4, or through a de novo expansion of self-reactive T cells from naïve precursors in the absence of the CTLA-4 checkpoint.

Immune checkpoints are attractive targets for immunotherapies and much interest is being expressed in targeting immune inhibitory pathways other than CTLA-4. Programmed
death-1 (PD-1) is expressed by activated T cells and expression of its ligand PD-1-L is expressed by a range of cell types including antigen presenting cells and sometimes by tumor cells themselves. Interaction of PD-1 with its ligand inhibits T cell responses and downregulates immunity.

In a clinical study MDX-1106, a PD-1-blocking antibody, was used for the treatment of a range of malignancies including melanoma, renal cell carcinoma, non-small cell lung carcinoma and prostate cancer. Results from the first 39 patients indicate that 3 patients had objective tumor responses. Treatment was well tolerated with a single case of colitis the only grade 3/4 adverse event reported potentially related to antibody administration. A more detailed knowledge of immune checkpoints may lead to effective and safe immunotherapies.

**On-target toxicities from depletion of normal cell subsets**

In order to apply effector mechanisms of ADCC and complement against malignant cells, the identification of cell surface markers is necessary. However, most molecular targets of antibodies against hematologic cancers are lineage-specific rather than tumor-specific, and consequently immune-mediated toxicity is induced against normal blood cells, which can compromise immunity or hematopoiesis. Fortunately, target markers can sometimes be selected that are not expressed on stem cells or lineage precursors, therefore normal cells can often be reconstituted after cessation of therapy. Nevertheless, toxicities can occur due to lowered immunity following immune-mediated depletion of normal leukocytes, as seen when using antibodies for the treatment of some hematologic malignancies.

Gemtuzumab Ozogamicin (Mylotarg ®) is an antibody-calicheamicin conjugate that targets the CD33 surface antigen. Gemtuzumab treatment has been successful in the treatment of
CD33 positive AML, following internalization of the conjugate. However, the expression of CD33 on normal cells (myeloid progenitor cells and monocytes) has resulted in a large proportion of patients experiencing neutropenia (>90%) resulting in pneumonia and infections (~20%) that can be life threatening. Another serious effect of Gemtuzumab is the development of hepatotoxicity such as vascular obstructive disease (VOD), which damages the hepatic sinusoidal epithelium. This is thought to occur via receptor-mediated uptake of the antibody–calicheamicin complex through CD33 expression on liver cell populations such as Kupffer cells (up to 48% of patients in some studies). Although CD33 is still a target of interest for treating AML and some patients can benefit from treatment with gemtuzumab, Mylotarg was voluntarily withdrawn from the market in June 2010 because no significant benefit of the antibody conjugate in combination with chemotherapy was demonstrated above chemotherapy alone.

Alemtuzumab (MabCampath ®) provides another example where subsets of normal leukocytes are depleted following treatment of hematologic malignancy. Alemtuzumab is used in the treatment of chronic lymphocytic leukemia (CLL) and binds to CD52, which is present on both normal and malignant B and T lymphocytes, as well as monocytes, macrophages and eosinophils. The use of alemtuzumab has yielded promising results with objective response rates of up to 90% in CLL and encouraging results against other malignancies such as T cell prolymphocytic leukemia and peripheral T cell lymphoma. Due to expression of CD52 on normal leukocytes, alemtuzumab also depletes normal immune cell populations, which can result in severe infections that can be fatal.

On-target depletion of normal leukocytes has also been observed using Rituximab, which is specific for CD20 expressed on a variety of non-Hodgkin’s lymphomas and normal mature B
cells. Rituximab has been used in the treatment of malignancies including follicular and diffuse large B cell lymphoma. It has been shown to mediate lysis of lymphoma cells through a complement-dependent mechanism, and it has substantially increased the survival rates of patients with B cell lymphomas since its approval by the FDA in 1997. However, the use of this antibody has been associated with toxicities including tumor lysis syndrome, infections and reactivation of viruses such as Hepatitis B and Herpes Simplex virus. Although antibody-producing plasma cells are not depleted with Rituximab, several other cell subsets including mature B cells are depleted, which is thought to be responsible for increased rates of infection. Interestingly, depletion of neutrophils has also been observed following the use of rituximab. The extent of neutropenia did not generally have clinical significance in these cases, and although the mechanism of neutrophil depletion were not clear, the production of anti-neutrophil antibodies and disruption of neutrophil homeostasis resulting from B cell depletion have been proposed as potential contributing causes.

**Autoimmunity from cytokine administration**

A range of cytokines are being tested in the clinic for their ability to recruit immunity against tumor cells by inducing the activation, proliferation and survival of tumor-specific lymphocyte populations. The complexity of cytokine interactions with the immune system means that often the outcomes of their use cannot be fully predicted. It is therefore not entirely unexpected that autoimmune pathologies have been encountered in a number of cytokine trials to date (Table 3).
Interferon-α

IFN-α is a prominent and sometimes first-line therapy against many cancers, including melanoma, RCC, cutaneous T cell lymphoma, chronic myeloid leukemia, bladder and ovarian cancer. The receptor for IFN-α is almost ubiquitously expressed, and can signal to leukocytes and tumors in a variety of ways. It can have pro- or anti-proliferative effects, pro- or anti-apoptotic effects, modulate cell immunogenicity, promote immune responses and inhibit angiogenesis.

An extensive range of autoimmune complications have been reported following IFN-α therapy for cancer (Table 3). Adverse events include diabetes and vitiligo, as well as the aggravation of pre-existing autoimmune diseases. Prospective studies have indicated that autoimmunity in patients correlates with prolonged relapse-free and overall survival. This provides evidence that in humans IFN-α is working against cancer at least in part by boosting the immune response. The mechanisms by which IFN-α induces cancer regression remain the subject of ongoing investigation. It has been shown to act both on host leukocytes and directly on tumor cells.

Both B and T cell-mediated autoimmunities have been reported in response to IFN-α, which is consistent with known activities of type-I interferons. Type-I interferons can promote skewing of T cells to a cytotoxic phenotype by stimulating their expression of the IL-12R. They complement this by also inducing genes coding for MHC-I and TAAs on tumor and non-tumor populations. IFN-α may indirectly promote the proliferation of memory T cell populations and also promotes B-cell switching to the IgG2a isotype. IgG2a is capable of targeting cells for phagocytosis and triggering complement-mediated cytotoxicity.
In summary, type-I IFNs are powerful modulators of anti-tumor immunity. The information available regarding their effects on leukocytes, host stroma and tumor cells serves as a further example of the pleiotropic effects that cytokines can mediate.

**Interleukin-2**

IL-2 has been widely applied in the clinic in the treatment of melanoma, RCC and AML and is one of only a few cytokines that has progressed to FDA approval in the treatment of cancer. Used alone in the treatment of metastatic melanoma it has produced objective response rates of up to 16%.59 The anti-tumor effect of IL-2 stems at least in part from its ability to act as a growth factor for T cells and to enhance the cytolytic activity of NK cells.

Non-immune systemic toxicities associated with high-dose IL-2 therapy are well appreciated. IL-2 therapy induces systemic inflammation, the exact mechanism of which is poorly understood, and the main symptomatic outcomes are hypotension and capillary leak syndrome, accompanied by flu-like symptoms, vomiting and diarrhea. Despite its toxicity, advances in managing symptoms, and the resolution of toxicity following cessation of administration, have led to its continued use in the cancer setting.60,61

In addition to these systemic toxicities, IL-2 therapy is known to both exacerbate autoimmunity and trigger it *de novo* (Table 3). In a notable case study, both of these outcomes were detected within the same patient following high-dose IL-2 therapy for RCC, with worsening of diabetes and induction of myasthenia gravis and polymyositis.62 There is evidence that IL-2 can augment humoral responses specific for autoantigens and drive expansion of NK and T cells.63-65 Thus, both humoral and cell-mediated immune mechanisms may contribute to autoimmunity resulting from cytokine therapy.66 Interestingly, similar to observations using
antibodies or adoptive immunotherapy for cancer treatment as discussed above, anti-tumor responses in patients receiving cytokines can correlate with autoimmunity.\textsuperscript{63,64}

The best evidence that high-dose IL-2 can induce on-target (tumor antigen) autoimmunity stems from a prospective study of patients treated for metastatic melanoma and RCC.\textsuperscript{67} Here, vitiligo (autoimmune destruction of melanocytes) was observed in 11/74 of patients treated for melanoma, but 0/104 treated for RCC. Development of vitiligo in melanoma patients correlated with objective responses to therapy, indicating that immune targeting of melanoma differentiation antigens was underpinning both outcomes. Other autoimmune toxicities reported in conjunction with IL-2 therapy include diabetes mellitus and hypothyroidism (Table 3). These are presumed to represent examples of off-target autoimmunity in the absence of reported antigen sharing between tumors and the affected tissues.

**Autoimmunity associated with cancer vaccines**

Vaccines are a major focus of cancer immunotherapy, and lessons learned from successful vaccines against infectious disease are being applied to the treatment of malignant disease. Antigen formulations used in cancer vaccines include peptides and whole proteins in combination with adjuvants to improve immunogenicity or pulsed directly onto dendritic cells to facilitate antigen presentation. In addition, antigen has been encoded in recombinant viruses in attempts to generate robust immune responses to TAA in parallel to viral antigens.

The vaccination approach has shown considerable promise in preclinical murine models, although breaking of tolerance towards TAA expressed by both the tumor and healthy peripheral tissues of mice has been observed to result in autoimmune disease in some cases (summarized in Table 4). In humans, cancer vaccines have been studied intensively, particularly in the
melanoma setting. Increased frequencies of antigen-specific circulating T cells have often been observed following vaccine administration, although overall clinical responses rates of only 2.6% have been achieved.\(^68\) Perhaps in keeping with the low response rate, there have been few reports of autoimmunity arising in patients following vaccination with TAA. An evaluation of autoimmunity in melanoma patients treated with IL-2 and vaccines reported the occurrence of autoimmune thyroiditis and autoimmune insulitis, but these events were infrequent.\(^69\) Perhaps as cancer vaccines become more potent as they can be in animal models, we might see a concomitant increase in autoimmune sequelae.

**Concluding remarks and future perspective**

In summary, while immunotherapy holds great promise for the treatment of a range of malignancies, many of these therapies can be associated with autoimmunity against normal self tissues (Figure 1). Mechanisms involved in immune-mediated damage of normal tissues are varied, and a greater understanding of these mechanisms will enable enhanced and more specific forms of immunotherapy for cancer.

The most obvious means of toxicity results from expression of TAA on normal tissue (Figure 2). Thus, antibodies raised against these antigens can react against normal cells either by inducing complement-mediated lysis or facilitating ADCC by innate leukocytes. Adoptively transferred T cells specific for TAA can likewise react directly against normal tissues expressing those antigens as part of their normal proteome. However, toxicity can also result from de novo induction of immune responses against other antigens on normal tissues. For example, inhibition of CTLA-4 can dysregulate normal lymphocyte homeostasis, leading to expansion of self-reactive T cells able to respond against normal tissues that do not express TAA.\(^70\) Similarly,
immunotherapy can lead to epitope spreading in which immune responses can be raised against additional molecular targets, including those expressed on normal tissues.\textsuperscript{71}

As our knowledge of the human proteome and the cancer genome increases, opportunities are arising to refine the choice of antigens for immunotherapy. Next-generation genome sequencing allows cataloging of all the mutations within any given tumor cell.\textsuperscript{72} This could allow the rapid identification of truly tumor-unique antigens and greater personalization of vaccine and adoptive immunotherapies in the near-future. Already anti-idiotype vaccines are showing promise for the specific targeting of malignant B cell lymphomas.\textsuperscript{73,74}

Methods to reduce immune-mediated toxicity in adoptive immunotherapy may involve identifying multiple antigens that together constitute a tumor signature or “barcode”.\textsuperscript{75} Gene constructs encoding several antigen receptors can be developed where the threshold for cytotoxic activity is only reached if the complete “barcode” is recognized. Alternatively, constructs can be designed which shut down T cell activity if a barcode indicative of normal healthy tissue is encountered.

In the case of cytokine therapy, systemic toxicities and the deregulation of immune responses may also be reduced through more targeted delivery of cytokines to the tumor site, rather than systemically.\textsuperscript{76}

In antibody therapy, it is useful to consider that not all antibodies for a specific target molecule are equal in their immune side effects, as seen using TGN1412, a monoclonal antibody specific for the T cell costimulatory molecule CD28. TGN1412 was thought to hold promise as a treatment for cancer and rheumatoid arthritis, but a safety trial performed in 6 healthy volunteers demonstrated severe toxicity including hypotension and respiratory distress.\textsuperscript{77} Toxicity was thought to be due to high levels of serum cytokines in response to CD28 ligation occurring
within hours of antibody administration. TGN1412 differed from other anti-CD28 antibodies in that it had superagonist qualities, not possessed by all anti-CD28 antibodies. Similarly, individual anti-CTLA-4 antibodies can differ in their capacity to induce autoimmunity, as demonstrated in mice engineered to express human CTLA-4. In this mouse model, several antibodies varied in their relative abilities to induce autoimmunity and protection against tumor. An antibody was identified that produced the strongest anti-tumor activity and the least autoimmunity. Thus, it may be possible to separate anti-tumor activity from autoimmunity by antibody selection.

Selective downregulation of autoimmunity may also be possible using immunosuppressants. The use of corticosteroids to counteract severe immune-mediated toxicity has surprisingly indicated that steroid treatment may not always signal the end of an immunotherapy’s beneficial impact, with tumor responses being maintained after resolution of autoimmunity using steroids. A further consideration is whether it is possible to kinetically separate anti-tumor immunity from autoimmunity. Regulatory mechanisms on healthy tissues may allow them to withstand immune toxicity for a period of time.

Immunotherapy is an exciting and increasingly effective treatment option for cancer. However, it is becoming increasingly clear that cancer immunotherapy is a balancing act between anti-tumor immunity and immune toxicity. The association between immune toxicity and increased anti-tumor effects following immunotherapy highlights the need for strategies that can mitigate the risk of these toxicities during immunotherapy, while preserving activity against malignancy.
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References


### Table 1: Immune-mediated toxicities associated with adoptive immunotherapy of cancer

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Species</th>
<th>Target antigen</th>
<th>Tumor</th>
<th>Toxicities</th>
<th>References</th>
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<td>CTL</td>
<td>Mouse (or rat)</td>
<td>Pnma-1</td>
<td>Paraneoplastic syndrome (rat)</td>
<td>CNS inflammation</td>
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<td>Melanoma</td>
<td>Melanocyte destruction, ocular toxicity</td>
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<td>Renal cell carcinoma</td>
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Table 2. Immune-mediated toxicities associated with antibody therapy of cancer

<table>
<thead>
<tr>
<th>Antibody regimen</th>
<th>Target antigen</th>
<th>Cancer type</th>
<th>Toxicities</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ipilimumab</td>
<td>CTLA-4</td>
<td>Hodgkin’s disease, myeloma, AML, CML, CLL, NHL</td>
<td>Arthritis, hyperthyroidism, pneumonitis</td>
<td>29</td>
</tr>
<tr>
<td>Ipilimumab</td>
<td>CTLA-4</td>
<td>Melanoma</td>
<td>Colitis, bowel perforation, vitiligo, hypophysitis</td>
<td>33</td>
</tr>
<tr>
<td>Tremelimumab or ipilimumab</td>
<td>CTLA-4</td>
<td>Melanoma</td>
<td>Colitis, dermatitis</td>
<td>30,32,95</td>
</tr>
<tr>
<td>Anti-CTLA-4 + TAA peptides</td>
<td>CTLA-4</td>
<td>Melanoma</td>
<td>Colitis, bowel perforation, vitiligo, hypophysitis, colitis, dermatitis</td>
<td>28,96-99</td>
</tr>
<tr>
<td>Anti-CTLA-4 (with vaccines and cytokines)</td>
<td>CTLA-4</td>
<td>Melanoma (mouse and human)</td>
<td>Melanocyte destruction, enteritis</td>
<td>31,100-102</td>
</tr>
<tr>
<td>Anti-CTLA-4, irradiated tumor cells, GM-CSF</td>
<td>CTLA-4</td>
<td>C2 prostate cancer (mouse)</td>
<td>Prostatitis, destruction of prostate epithelium</td>
<td>103</td>
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<tr>
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<td>PD-1</td>
<td>Melanoma, RCC, NSCLC, prostate</td>
<td>Colitis</td>
<td>34</td>
</tr>
<tr>
<td>Alemtuzumab</td>
<td>CD52</td>
<td>Chronic lymphocytic leukemia</td>
<td>Destruction of normal leukocytes, leading to susceptibility to infections</td>
<td>42,44,104</td>
</tr>
<tr>
<td>Gemtuzumab</td>
<td>CD33</td>
<td>Acute myeloid leukemia</td>
<td>Infection, sepsis, pneumonia</td>
<td>36-38</td>
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<tr>
<td>Rituxumab</td>
<td>CD20</td>
<td>Lymphoma</td>
<td>Suppression of B cells leading to deficiency in immunoglobulin and infections</td>
<td>49,105-107</td>
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Table 3. Autoimmunity associated with cytokine administration in cancer in humans

<table>
<thead>
<tr>
<th>Cytokine administered</th>
<th>Cancer Targeted</th>
<th>Autoimmune events</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>IFN-α</td>
<td>Chronic myeloid leukemia</td>
<td>Thyroiditis, sarcoidosis, systemic lupus erythmatosis associated with auto-antibodies, autoimmune thrombocytopenic purpura, acute pancreatitis, hyperthyroidism, aggravation of psoriasis</td>
<td>51,108-111</td>
</tr>
<tr>
<td>IFN-α</td>
<td>Cutaneous T cell lymphoma</td>
<td>Psoriasis</td>
<td>112</td>
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<tr>
<td>IFN-α</td>
<td>Mid-gut carcinoid tumors</td>
<td>Systemic lupus erythmatosis, pernicious anaemia, thyroid disease</td>
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<tr>
<td>IFN-α2b</td>
<td>Melanoma</td>
<td>Hypothyroidism</td>
<td>114</td>
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<tr>
<td>IFN-α2b and piroxicam</td>
<td>Melanoma</td>
<td>Vitiligo, pulmonary interstitial fibrosis</td>
<td>115</td>
</tr>
<tr>
<td>GM-CSF with gp100 and tyrosinase peptides</td>
<td>Melanoma</td>
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<tr>
<td>IL-2</td>
<td>Renal cell carcinoma</td>
<td>Hypothyroidism, myasthenia gravis, diabetes mellitus (insulin dependent) and myositis</td>
<td>62-64,117</td>
</tr>
<tr>
<td>IL-2</td>
<td>Melanoma</td>
<td>Vitiligo</td>
<td>67</td>
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<tr>
<td>IL-2</td>
<td>Colorectal cancer</td>
<td>Diabetes mellitus</td>
<td>118</td>
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<tr>
<td>IL-2 and IFN-γ</td>
<td>Renal cell carcinoma</td>
<td>Auto-antibodies against erythrocytes</td>
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<tr>
<td>IL-2 and IFN-α2b</td>
<td>Renal cell carcinoma, melanoma</td>
<td>Anti-thyroid antibodies, hypothyroidism, autoimmune thyroiditis</td>
<td>63,120,121</td>
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Table 4. Autoimmunity associated with cancer vaccines

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Malignancy (species)</th>
<th>Autoimmune events</th>
<th>References</th>
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<tbody>
<tr>
<td>TRP-1 encoded by virus</td>
<td>Melanoma (mouse)</td>
<td>Vitiligo</td>
<td>122,123</td>
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<tr>
<td>CD20 + adjuvant</td>
<td>Lymphoma (mouse)</td>
<td>Depletion of normal B cells</td>
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<tr>
<td>Her-2 encoded by plasmids</td>
<td>Breast cancer expressing Her-2 (mouse)</td>
<td>Impaired lactation, accelerated involution of mammary gland</td>
<td>125,126</td>
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<tr>
<td>DC (peptide pulsed)</td>
<td>Lymphoblastic leukaemia (mouse)</td>
<td>Systemic autoimmunity, immunity against fibroblasts, hepatomegaly, splenomegaly fur loss, cachexia,</td>
<td>127</td>
</tr>
<tr>
<td>CD40 &amp; IL-2 (fibroblasts)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC (peptide-pulsed) β-gal and LCMV model antigens</td>
<td>EL-4 thymoma (mouse)</td>
<td>Diabetes, myocarditis</td>
<td>128</td>
</tr>
<tr>
<td>Pulsed DCs or tumor cells and either GM-CSF and adjuvant or GM-CSF, adjuvant and peptides</td>
<td>Melanoma (human)</td>
<td>Vitiligo, diabetes, thyroiditis, ocular toxicity</td>
<td>69</td>
</tr>
</tbody>
</table>
Figure legends

Figure 1. Immune toxicity associated with immunotherapy of cancer. Therapeutic strategies including vaccines, adoptive immunotherapy, cytokines and antibodies can induce immunity against tumor antigens. However, these immune responses can also cause damage to a variety of organ systems as shown.

Figure 2. Mechanisms of immune toxicity associated with immunotherapy. (1) The delivery of exogenous antibody specific for TAA expressed on both tumor and normal tissue can result in damage to normal tissue mediated by complement or ADCC mediated by innate immune cells such as macrophages. (2) Similarly, transfer of TAA-specific T cells can lead to destruction of normal tissue if the TAA is also expressed on cells of normal tissue. (3) Alternate ways of inducing immune reactivity towards normal tissue include dysregulation of normal immune homeostasis using anti-CTLA-4, resulting in expansion of self-reactive T cells. (4) It is also possible that induction of immunity to tumor cells could lead to epitope spreading whereby T cells reactive with self antigens are generated.
Figure 1
Figure 2

1. Antibodies

2. Adoptive immunotherapy

3. Dysregulated immune homeostasis

4. Epitope spreading
Is It Safer CARs That We Need, or Safer Rules of the Road?

To the editor:

The recent deaths with chimeric antigen receptor–modified T cells ("T-bodies," "designer T cells") have been a wake-up call for all of us to the potential for toxicity of these therapies.1,2 This follows on a previous report of hepatotoxicity from anti-G250 designer T cells that did not result in deaths.3 In the April 2010 issue of *Molecular Therapy*, Dr. Heslop wrote an excellent Commentary on the application of these cells and potential means to improve their safety.4 Some additional thoughts may be warranted that go beyond the issues of target safety. In particular, two points could be made: (i) components useful for expanding tumor-infiltrating lymphocytes (TILs) and first-generation designer T cells (i.e., lymphodepletion and engraftment) may be unwarranted for newer, second-generation agents, and (ii) initial patient exposures for these new agents are most safely initiated at lower levels for untested antigens.

The major focus of preclinical research over the past decade has been to improve designer T-cell "quality" by supplementing with signals that co-opt components of the interaction between antigen-presenting cells and T cells.5 Like TILs, first-generation designer T cells with signal 1 provided antitumor cytotoxicity for a limited time but ultimately succumbed to activation-induced cell death (AICD) or passed to a resting, anergic state. In contrast, the advanced second-generation agents in these studies added one or more costimulatory signals to obtain signal 1+2 that conferred a new potential to respond to antigen with proliferation and sustained cytotoxicity, with escape from AICD and resistance to regulatory T-cell suppression. Consequently, even a few T cells trafficking into tumor had the potential to respond *selectively* to antigen with local intratumoral expansion until tumor elimination. The application of lymphodepletion6 before T-cell infusion vastly increases T-cell "quantity," superimposing a nonelective and systemic expansion ("engraftment") on these agents that were designed not to need it.

From the details presented, it is likely that the CD19 death was not due to T-cell toxicity but rather to a recognized complication of the conditioning regimen,1 a reminder that conditioning, integral to engraftment strategies, is not a benign intervention. In contrast, the Her2 death appears to have been the result of on-target toxicity against normal tissues (lung, bowel, heart) previously known to express antigen.2 This is reminiscent of the G250 study with toxicity from limited doses of designer T cells by infusion,3 but in the Her2 case not reversible by steroids due to the vast numbers of Her2 self-reactive T-cells in the engraftment setting and the vigor of second-generation design.

The most instructive clinical parallel is that of donor lymphocyte infusion (DLI) in allo–bone marrow transplantation settings. Patients are engrafted with T cell–depleted marrow to create a chimeric state of donor and host tolerance, whereupon small numbers (<10^9) of allo-donor T cells are subsequently infused. With a fully competent allo-immune reaction, these exposures of allo–T cells can be safely managed with a balance of graft–versus-host reaction and antitumor benefit.7,8 In this, we know that size of dose matters: too high a DLI dose kills. Translated to second-generation designer T cells, infusions under a graded dose-escalation plan should allow recognition of on-target autoimmune toxicities before grade V (death) in the same way that DLIIs are "tuned,"7 where high doses (as in engraftment) may be lethal.

In line with phase I goals, we seek means to safely increment patient exposures while advancing therapeutic aims. In contrast to the natural targets for TILs that were extensively vetted for safety via infusions before the first engraftment was ever tried, the targets of designer T cells are artificially selected and may be unsafe—especially for second-generation agents with their powerful engines for self-perpetuation under incorporated costimulation—thus warranting a cautious exposure. Accordingly, safety testing could be pursued via simple infusions, employing lower starting doses (perhaps 10^8 or 10^9 cells) in line with DLI dosing protocols.7 The G250 on-target toxicities were recognized early and safely in exactly such a dose-escalation infusion protocol; the system worked: no one died.3 Engraftment, by contrast, leads to much higher exposures that can be hard to project, with on-target toxicities that can be hard to control, as in the Her2 death.2

In terms of efficacy, there is as yet no proof that any of the second-generation designer T cells, with their incorporated costimulation signals, even *need* engraftment, a procedure devised in response to deficiencies of signal 1–only T cells (e.g., TILs). The first studies with second-generation signal 1+2 designer T cells under infusion protocols have just gotten under way, and it is too early to infer either sufficiency or deficiency of any existing second-generation reagents to eliminate tumors without systemic engraftment. Accordingly, it is plausible by this conception that a DLI-type infusion dose escalation could still be productive for Her2 targeting with this advanced agent, with a margin for antitumor benefit and safety, although the engraftment death may have the regrettable result of impeding this path.

In the end, I believe that these new agents merit new thinking, taking a step back from engrafting to permit them to reveal the potential they were designed for. T cells engaged by antigen-presenting cell costimulation eliminate infections with very few starting cognate effectors, and when we have successfully adopted those features into our engineering, I believe that we will likewise be able to eliminate cancers as efficiently, without engraftment. The proposed infusion escalations can be performed intrapatient, so that a personalized, optimally "tuned" dose can be delivered in the manner of DLI. But where infusions with these more advanced reagents are proven therapeutically inadequate at full doses (e.g., 10^11 cells with cytokine support) and safe, then engraftment, with its higher cost and hazard, is a justifiable next step in the risk escalation.9
Finally, if a designer T cell is fully escalated under simple infusions, in which suicide genes are generally not needed, then a suicide gene suggested for engraftment is also not needed if proceeding to this step, because safety of the target will have been established.

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REFERENCES


Research Article

Cell Density Plays a Critical Role in Ex Vivo Expansion of T Cells for Adoptive Immunotherapy

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The successful ex vivo expansion of a large numbers of T cells is a prerequisite for adoptive immunotherapy. In this study, we found that cell density had important effects on the process of expansion of T cells in vitro. Resting T cells were activated to expand at high cell density but failed to be activated at low cell density. Activated T cells (ATCs) expanded rapidly at high cell density but underwent apoptosis at low cell density. Our studies indicated that low-cell-density related ATC death is mediated by oxidative stress. Antioxidants N-acetylcysteine, catalase, and albumin suppressed elevated reactive oxygen species (ROS) levels in low-density cultures and protected ATCs from apoptosis. The viability of ATCs at low density was preserved by conditioned medium from high-density cultures of ATCs in which the autocrine survival factor was identified as catalase. We also found that costimulatory signal CD28 increases T cell activation at lower cell density, paralleled by an increase in catalase secretion. Our findings highlight the importance of cell density in T cell activation, proliferation, survival and apoptosis and support the importance of maintaining T cells at high density for their successful expansion in vitro.

1. Introduction

T cells are a critical component of the cellular immune response. In the past two decades, adoptive transfer of tumor reactive T cells into cancer patients has been created as an immunotherapy method to combat cancer [1]. This includes the early studies with lymphokine-activated killer (LAK) cells derived from ex vivo amplification of autologous lymphocytes with interleukin-2 (IL-2), late studies with tumor infiltrating lymphocytes (TILs) isolated from tumor specimens, and recent studies with genetically modified tumor reactive T cells [2]. The prerequisite for the success of adoptive immunotherapy relies on the successful ex vivo expansion of a large amount of T cells (up to $10^{11}$).

The ex vivo expansion of T cells for adoptive immunotherapy usually involves two phases. The first phase is T cell activation, in which resting T cells are activated with anti-CD3 antibody or plus anti-CD28 antibody supplemented with IL-2. The second phase is activated T cell (ATC) proliferation. After activation, resting T cells become ATCs and undergo vigorous cell proliferation for about two to three weeks and the ATCs lose their proliferation capacity in about four weeks. Maintaining high cell density has been considered important among investigators performing ex vivo T cell expansions for clinical therapeutic applications. This report addresses formally the basis for this observation.

Cell density has been reported to be an important factor in maintaining certain T and B cells in vitro. Resting T cells die rapidly by apoptosis when cultured under diluted conditions but survive for extended periods when cultured at high cell density [3]. This effect was found to be mediated by soluble factors and independent of integrin-mediated signals. An acute T-lymphocytic leukemia cell line, CCRF-CEM, was reported to display a cell density-dependent growth characteristic [4]. CEM cells grow well at cell density $>2 \times 10^5$ cells per mL, but at low cell densities the
cultures rapidly undergo apoptosis. The viability of low-density CEM cells could be preserved by supplementing with “conditioned” medium from high-density CEM cultures. Catalase was identified as the active component in the conditioned medium. B cell chronic lymphocytic leukemia (CLL) was reported to be dependent on cell density for surviving in cultures [5]. CLL cells survival was strongly enhanced at high cell density. Conditioned medium from high cell density CLL cells produced a marked increase in the viability of low cell density autologous cells. Again, autocrine catalase was identified as the survival factor in the high cell density cultures.

Reactive oxygen species (ROS) have been shown to contribute to the death of CEM cells and CLL cells at low cell density [4, 5]. ROS are highly reactive metabolites that are generated during normal cell metabolism. Intracellular ROS derive mainly from leakage of electrons from mitochondrial electron transport chains that reduce molecular oxygen to superoxide ions. Cells possess antioxidant systems to control their redox state, to reduce oxidative stress and to maintain cell survival [6]. Superoxide ions are converted to hydrogen peroxide (H2O2) by the action of Cu2+/Zn2+-dependent or Mn2+-dependent superoxide dismutases, and H2O2 is then detoxified by catalase or glutathione peroxidase. H2O2 can also react in vivo to generate the highly damaging hydroxyl radical by the Fe2+-dependent Fenton reaction or the Fe2+-catalyzed Haber-Weiss reaction [6, 7]. At subtoxic levels, ROS may play an essential signaling role in cell growth and differentiation [8–11]. At elevated levels, however, intracellular ROS are sufficient to trigger cell death [12–16]. Antioxidants that limit ROS-induced cell damage can suppress apoptosis in many systems. For example, N-acetylcysteine (NAC), which elevates intracellular glutathione levels, delays activation-induced cell death of a T cell hybridoma [17]. NAC or the iron chelator pyrrolidine dithiocarbamate (PDTC) or enforced expression of Mn2+-dependent superoxide dismutase inhibits apoptosis induced by TNF-α which can stimulate ROS production [18–21]. Similarly, cell death through oxidative mechanisms has been shown to be opposed by protein albumin at physiologic concentrations directly by scavenging for free oxygen radicals through the free cysteinyl sulfhydryl moiety and indirectly by maintaining the reduced state of cellular proteins [22–24].

ROS have also been shown to be the decisive contributors to the death of activated T cells (ATCs) [25–28]. First, the ATCs have increased levels of ROS [25, 26, 29–31]. Second, ATC death is prevented by manganese (III) tetrakis(4-benzoic acid) porphyrin (MnTBAP), an antioxidant that has been shown to inhibit ROS-induced death in different types of cells [25]. Evidence shows that ROS lead to ATC death by at least two pathways, one mediated by caspase activation and subsequent proteolytic cellular disintegration and the other driven by ROS themselves [25].

While cell density has been found to be important for the survival of resting T cell and certain but not all leukemic T and B lines, it remains unclear if normal T cells behave in a cell density-dependent manner during T cell expansion. In this study, we confirm a critical role of cell density in resting T cell activation and ATC expansion. We found that resting T cells need to be kept at high cell density for optimized activation and ATCs need to be kept at high cell density for optimized expansion. We show that the cell density-related ATC apoptosis need to be kept at high cell density for optimized expansion. We show that the cell density-related ATC apoptosis need to be kept at high cell density for optimized expansion. We show that the cell density-related ATC apoptosis need to be kept at high cell density for optimized expansion. Further, we confirm the antioxidative activity of added NAC or of serum albumin at high concentrations that protects ATCs from apoptosis when cultured at low cell density.

2. Materials and Methods

2.1. Reagents. N-acetylcysteine (NAC), catalase, and 3′-amino-1,2,4-triazole (ATZ) were purchased from Sigma Chemical (St Louis, MO, USA). Human serum albumin was from Bayer Corporation (Elkhart, IN, USA).

2.2. Cell Purification, Activation, and Culture. Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood from healthy adults by centrifugation over Histopaque-1083 (Sigma). For resting T cells activation study, PBMCs were cultured in 6-well plates in different cell densities and were activated in serum-free AIM V media (Gibco, Gaithersburg, MD, USA) supplemented with 100 U/ml IL-2, 100 ng/mL mouse antihuman CD3 antibody OKT3 (Ortho Biotech, Raritan, NJ, USA) only or with the addition of 100 ng/mL mouse antihuman CD28 antibody mAb9.3 (gift from Dr. Carl H. June, University of Pennsylvania, Philadelphia, PA, USA). For preparation of ATCs, PBMCs were activated at 1 × 10^6/mL cells in serum-free AIM V media supplemented with 100 U/ml IL-2, 100 ng/mL OKT3 in 75 cm² flasks for 3 days. ATCs were then washed and cultured in the AIM V medium supplemented with 100 U/mL IL-2 for an additional 7 days before use in experiments.

2.3. Monitor of T Cell Activation and Cell Division. Accompanied with T cell activation, there is a significant cell size enlargement from resting T cells to ATCs, with cytokines such as IL-2 and IFN-γ production and surface molecules such as CD69 and CD25 expression. ATCs can be distinguished from resting T cells and other types of PBMCs as the enlargement in forward-angle light scatter (FS) by flow cytometric analysis and it is identical with CD69 or CD25 staining. T cell activation was monitored by cell division was analyzed by the dilution of CFSE in the daughter cells by flow cytometer.

2.4. Detection of Apoptosis. ATC apoptosis was determined by cell shrinkage and DNA cleavage. Cell viability of ATCs was analyzed by flow cytometry [5, 33, 34]. Cell shrinkage
accompanying ATC apoptosis was detected as a reduction in forward-angle light scatter (FS) by flow cytometric analysis. Agarose gel electrophoresis was used to detect internucleosomal cleavage fragments of DNA following apoptosis [4, 35]. 2 × 10^6 cells were pelleted and resuspended in 500 μL of ice-cold lysis buffer (20 mM Tris·HCl, 10 mM EDTA, and 0.2% Triton X-100, pH 7.4). Proteinase K was added at 100 μg/mL and incubated at 50°C overnight, followed by further incubation at 37°C for 2 hours with the addition of RNase. DNA was extracted twice with phenol/chloroform at 1:1 and precipitated with isopropanol. The DNA was electrophoresed through a 1% agarose gel and stained with ethidium bromide.

2.5. *Measurement of Intracellular Oxidative Stress*. Cells were gently resuspended in 10 μM dihydrorhodamine 123 (DHR) (Molecular Probes) and incubated for 30 minutes and then analyzed by flow cytometry [5, 7]. The level of intracellular ROS was inferred from the mean fluorescence intensity (MFI) of DHR-stained cells. Dead cells and debris were excluded from analysis by electronic gating of forward and side scatter measurements.

2.6. *Generation of Conditioned Medium*. Cells were cultured at 1 × 10^6/mL (ATCs) or confluent (MIP101 cells, a human colon carcinoma cell line) in 75 cm^2 flasks. After 2 days in culture, the conditioned medium (CM) was removed from the culture flasks and centrifuged at 1500 rpm for 10 minutes. The CM was then passed through a sterile 0.45 μm filter.

2.7. Detection of Catalase. Detection of catalase was performed by western blot and dot blot. For western blot, control medium, ATCs CM, and cell lysates from ATCs were run on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and electrotransferred to nitrocellulose membranes. For dot blot, 100 μL culture medium from untreated, anti-CD3 activated and anti-CD3 plus anti-CD28 activated PBMCs were blotted on to nitrocellulose membrane by using a dot blot apparatus (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (20 mM Tris/500 mM NaCl, pH 7.5) for 1-2 hours. Membranes were washed with TTBS (0.05% Tween-20 in Tris-buffered saline), and mouse antihuman catalase mAb antibody (Sigma) was added at a dilution of 1:1000 for 1 hour, followed by incubation with HRP-conjugated goat antimouse IgG antibody at a dilution of 1:1000 for 1 hour, all in 1% non-fat dry milk in TTBS. The membrane was developed with enhanced chemiluminescence reagent (Amersham, England, UK) and exposed to X-ray film for 5–60 seconds.

2.8. Detection of Intracellular Albumin. Analysis of intracellular albumin was performed using immunofluorescence and flow cytometry. Cells were fixed and permeabilized with a Fix & Perm kit (Caltag Laboratories, Burlingame, CA, USA) and then stained with mouse antihuman albumin (Sigma), followed by staining with a secondary goat antimouse FITC-conjugated antibody (Caltag Laboratories).

### 3. Results

The objective of this study was to determine the role of cell density in ex vivo expansion of T cells. To mimic the actual situation in preparation for clinical applications, resting T cells were not further purified from the bulk PBMCs in the experiments. Typically, PBMC are composed of 50–70% T cells, and smaller numbers of B cells, NK cells, and monocytes.

#### 3.1. Cell Density Determines the Fate of T Cells Activation

Efficient activation of resting T cells requires signal 1 (TCR, CD3) and signal 2 (CD28) costimulation [36]. Activation of T cells through soluble OKT3 (anti-CD3) antibody depends on crosslinking of the antibody through Fc receptor on monocytes present in PBMCs preparations that also provide costimulation through B7 to CD28 on the T cells [37]. Typically, PBMCs cell concentrations of at least 1 × 10^6/mL are specified for this T cell activation. At lower cell densities, the opportunities for cell-cell contact are diminished and activation is incomplete or absent.

This is confirmed in our results. Following activation with OKT3 and IL-2 at 1 × 10^6 cells/mL for 6 days, 61% of cells in the culture showed a T blastic morphology by flow cytometry versus 23%, 9%, and 8% for the lower PBMCs densities of 1 × 10^5, 1 × 10^4 and 1 × 10^3/mL, respectively (Figure 1). At an intermediate T cell density of 1 × 10^6/mL, addition of an agonist anti-CD28 antibody that bypasses need for B7 on monocytes partially compensates for the reduced cell contacts, doubling the activated fraction from 23% to 47%. At the lowest cell densities, this maneuver was not effective and at the highest it was unnecessary, where the activated phenotype was maximal with OKT3 alone. At higher density, there are more chances of cell contacts and B7-CD28 interactions, and costimulation is maximal without added anti-CD28 antibody. Such maximally activated T cells (ATC) were applied throughout this study.

As analyzed by flow cytometry from one typical experiment, the PBMCs isolated from normal human blood were 57.2% CD3+, 38.8% CD4+, 21.6% CD8+, 0.37% CD4+CD8+, 39.2% CD4–CD8–, 49.8% CD28+, 53.6% CD2+, and 60.6% CD11a+. After 3 days activation with OKT3 and expansion for 7 days (total 10 days), the cell populations were 99.7% CD3+, 81.7% CD4+, 15.6% CD8+, 1.9% CD4+CD8+, 0.76% CD4–CD8–, 99.4% CD28+, 99.5% CD2+, and 100% CD11a+. Such 10-day cultures were used in the ATC studies that follow.

#### 3.2. ATCs Proliferate at High Cell Density

To examine the relation between cell density and ATC proliferation, T cells were activated under high density conditions as in Section 3.1 and then reseeded at varying cell densities and monitored over time for viable cell numbers. There was a progressive increase in the number of viable cells when ATCs were cultured at 1 × 10^5/mL, but a progressive decline in viable cells when ATCs were cultured at 1 × 10^4/mL. After 6 days, the number of viable ATCs increased 340% when cultured at 1 × 10^5/mL but decreased by 75% when cultured at 1 × 10^4/mL.
defined by the orderly sequence of ultrastructural changes from degenerative cell death or necrosis and was originally shown to occur in viable cells in our tests. At cell density of 1 to 62% and 34% when ATCs were cultured at 5 × 10^4/mL over the same time interval (Figure 3(a)). Increasing IL-2 100-fold from 100 IU/mL to 10,000 IU/mL did not rescue ATCs from death at low cell density (data not shown).

To determine by another measure whether ATCs died by apoptosis when cultured at low cell density, a DNA fragmentation assay was employed. Apoptosis is a distinctive form of cell death that occurs in a wide range of physiological and pathological situations [38]. It differs fundamentally from degenerative cell death or necrosis and was originally defined by the orderly sequence of ultrastructural changes that accompanies cell elimination during development [39]. Biochemically, it is best characterized by the presence of internucleosomal cleavage of DNA into 180–200 base-pair fragments [39, 40], which can be demonstrated by gel electrophoresis. As shown in Figure 3(b), DNA was fragmented when ATCs were cultured at low cell density (1 × 10^4/mL) but remained intact at high cell density (1 × 10^6/mL), confirming that apoptosis is the mechanism of ATC death at low cell density.

3.4. ROS Are the Mediators of ATC Apoptosis at Low Cell Density. Elevated intracellular ROS can trigger cell death [12–16]. We hypothesized that ROS were the mediators of ATC apoptosis at low cell density. To test this hypothesis, we measured the levels of intracellular ROS in ATCs cultured at different cell densities and tested if antioxidants can block ATC apoptosis at low cell density.

ROS include superoxide and hydroxyl-free radicals and H₂O₂. To measure intracellular ROS, we used the oxidation-sensitive fluorescent probe DHR [7]. DHR is nonfluorescent, uncharged, and accumulates within cells, whereas R123, the product of intracellular DHR oxidation, is fluorescent, positively charged, and trapped within cells [41]. ATCs cultured at high cell density (1 × 10^5/mL) and low cell density (1 × 10^4/mL) for 24 hours were incubated with DHR, and R123 fluorescence was measured by flow cytometry. The rate of DHR oxidation was significantly greater in ATCs cultured at low cell density than in ATCs cultured at high cell density (Figure 4(a)), confirming a correlation of intracellular ROS with apoptosis.

Antioxidants inhibit ROS-mediated apoptosis in many systems by limiting ROS-induced cell damage [16]. We therefore tested the effects of three antioxidants on ATC apoptosis at low cell density. NAC elevates intracellular glutathione [17] that is a substrate for glutathione peroxidase to catalyze the breakdown of H₂O₂; catalase detoxifies H₂O₂ produced by superoxide ions [7]; and albumin can directly scavenge reactive oxygen species through its free cysteinyl—SH [42]. ATCs were cultured at low cell density (1 × 10^5/mL) in the presence of antioxidants at different concentrations for 24 hours. Cell viability and intracellular ROS were measured by flow cytometry. All agents significantly protected cells from apoptosis at low cell density (Figure 4(b)). This further supports the hypothesis of ROS as a mediator of apoptosis in ATCs cultured at low cell density.

Albumin is the most abundant plasma protein and has been suggested to constitute an important extracellular antioxidant [43, 44]. Interestingly, the reduction in ROS with albumin appeared somewhat less than predicted versus the survival benefit observed (Figure 4(b)), suggesting that there may be additional, downstream means by which albumin may mitigate the harmful effects of ROS. A further feature of interest was the demonstration of increased albumin endocytosis in the low density ATCs versus those grown at high density (Figure 4(c)). Whether this was a generalized effect on endocytosis or selectively related to albumin was not

3.3. ATCs Undergo Apoptosis at Low Cell Density. To examine the relation between cell density and cell viability, ATCs cultured at different cell densities were evaluated after 24 hours. A condensed cell size by morphology on flow cytometry has been shown to correlate with apoptosis/cell death markers under experimental conditions close to our own [5, 33, 34] and was used to distinguish apoptotic from viable cells in our tests. At cell density of 1 × 10^5/mL, viable cells remained unchanged at 92% after 24 hours but declined to 62% and 34% when ATCs were cultured at 5 × 10^4/mL and 1 × 10^4/mL over the same time interval (Figure 3(a)). Increasing IL-2 100-fold from 100 IU/mL to 10,000 IU/mL did not rescue ATCs from death at low cell density (data not shown).

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examined. It was also not discriminated whether it was the internalized albumin or the external protein that mediated the survival benefits; its mechanism of action was not further investigated.

3.5. Soluble Factor Secreted by ATCs at High Cell Density Prevents Apoptosis of ATCs at Low Cell Density. The protective effect of high cell density indicated that either cell-to-cell contacts or soluble factor(s) produced by ATCs were inhibiting ATC apoptosis under this condition. To assess whether soluble factor(s) were involved, conditioned medium from ATCs cultured at high cell density (1 × 10^6/mL) was collected and added to ATCs cultured at low cell density (1 × 10^4/mL). With increased fractions of conditioned medium in the total medium, the intracellular ROS levels decreased in ATCs cultured at low cell density and their viability increased (Figure 5(a)). These data confirmed the secretion of one or more soluble factors at high cell density that functioned as antioxidant to protect ATCs from apoptosis.

A similar protective effect was observed with conditioned medium from a human colon carcinoma cell line, MIP101 (Figure 5(b)) and from a human T cell leukemia cell line (data not shown). These results indicate that the protective soluble factor(s) secreted by ATCs at high cell density are not ATC-specific. This result is compatible with the potential of diverse cell lines to function as feeder cells during T cell cloning procedures [40].

3.6. Autocrine Catalase Protects ATCs from Apoptosis. Based on prior studies of cytoprotective effects of catalase in cultures of lymphoid leukemia cells [5, 40], we hypothesized that the cell density effect on ATC survival was also mediated by secreted catalase. To determine whether catalase was one of the autocrine survival factors, western blot was performed (Figure 6(a)). The enzyme was clearly detected in cell lysates...
and conditioned medium, but not in control medium. In two separate assays, catalase in CM (e.g., lane 2) was estimated at 2.5–5 μg/mL (6–12 units/mL) in comparison with control purified catalase. Estimates from cell lysate (lane 3) are 2 μg per 10⁶ cells. This means that in 24 hours 10⁶ cells secrete into 1 mL of medium, a quantity (2.5–5 μg) that equals or exceeds what is present in the cells themselves. Further, this concentration of catalase in CM corresponds closely with levels that give maximum benefit to ROS control and cell viability in Figure 4(b) (e.g., ∼10 units/mL).

We next sought to establish whether catalase contributed to the survival-enhancing effect of the conditioned medium. To address this question, the effect of the selective catalase inhibitor, 3′-amino-1,2,4-triazole (ATZ) [5, 45–48] was examined. ATZ significantly blocked the survival-enhancing activity of the conditioned medium (Figure 6(b)), indicating that catalase plays the major role in this cytoprotective effect.

3.7. Elevated Autocrine Catalase Accumulation in CD28 Costimulated T Cell Activation. Having established the role of autocrine catalase as a cell survival factor in ATC proliferation, an interesting question arises: whether autocrine catalase also plays a role in CD28 costimulated T cell activation. An intermediate cell concentration was shown in Figure 1 to benefit from CD28 costimulation, PBMCs at 1 × 10⁶/mL were activated with anti-CD3 antibody without or with anti-CD28 antibody in the presence of IL-2. Compared with anti-CD3 antibody activation alone, there was a significant more amount of autocrine catalase accumulation in the anti-CD28 antibody costimulated cell cultures (Figure 7). These results indicate that the improved T cell activation at lower cell density with CD28 is paralleled by an enhanced autocrine catalase secretion.

4. Discussion

Cell density has been reported to be important for cell survival in cultures of resting T cells [3] and certain leukemic T and B cell lines [4] but not reported in other leukemic T cell lines such as Jurkat and H9 T cells [5]. Although
FIGURE 4: Reactive oxygen species (ROS) are the mediators of ATC apoptosis at low cell density. (a) Intracellular ROS levels in ATCs cultured at different cell densities. ATCs were cultured at high cell density ($1 \times 10^5$/mL) or low cell density ($1 \times 10^4$/mL) in 75 cm$^2$ flasks for 24 hours. After staining with DHR for 30 minutes, intracellular ROS levels of ATCs were analyzed by flow cytometry. Nonstaining ATCs were used as negative control. (b) Antioxidants protect activated T cells from apoptosis at low cell density. ATCs were cultured at $1 \times 10^4$/mL in 75 cm$^2$ flasks in the presence of catalase, NAC, and human serum albumin (HSA) in different concentrations. HSA in unsupplemented serum-free medium was measured at 3 mg/mL by Bradford assay. Both cell viability and intracellular ROS levels were analyzed by flow cytometry as in (a). ROS levels were indicated as mean fluorescence intensity (MFI) of DHR-stained cells. Similar results were obtained in 2 independent experiments. (c) Elevated intracellular albumin in ATCs cultured at low cell density. ATCs were cultured at high cell density ($1 \times 10^5$/mL) or low cell density ($1 \times 10^4$/mL) in 75 cm$^2$ flasks for 24 hours. Cells were fixed and permeabilized and then stained with mouse antihuman albumin antibody, followed by staining with a secondary goat antimouse FITC-conjugated antibody. Cells stained with only secondary antibodies were used as negative controls.
there are frequent communications between investigators performing ex vivo T cell expansions for clinical therapeutic applications that maintaining high cell density is an important consideration factor in T cell expansion, the relation between cell density and T cell expansion remains unclear. In this study, we systematically examined the relation between cell density and normal human T cell expansion in vitro, providing evidence for optimizing T cell expansion protocols for clinical applications. From our results, PBMCs have to be seeded at high cell density (≥1 × 10^6/mL) for optimal T cell activation. The addition of CD28 costimulation helps resting T cells to be activated at lower cell density and coordinately yields elevated catalase secretion and accumulation in the cultures. Maintaining high cell density is also important for ATC proliferation. ATCs undergo apoptosis when cultured at cell density of 1 × 10^6/mL or less. Our mechanistic studies support the role of ROS and oxidative stress apoptosis in ATC death at low density. At high cell density, the extracellular accumulation of secreted catalase reduces intracellular ROS species and alleviates their toxic effects.

4.1. Cell Density Plays a Critical Role in Ex Vivo Expansion of T Cells. Current protocols for ex vivo expansion of T cells for clinical adoptive immunotherapy usually involve the activation of PBMCs with OKT3 alone or plus anti-CD28 antibody in the presence of IL-2. When expanding T cells in vitro, our results indicate that it is critical to maintain the cells at high cell density during both T cell activation and ATC expansion phases. It is reported that OKT3 has to be immobilized on plastic or crosslinked via accessory cells in PBMCs through Fc receptor binding for the activation of T cells [37]. At low cell density, where the cell-cell contact is poor, the OKT3 may not be efficiently crosslinked to activate resting T cells. We also found that the addition of costimulation through anti-CD28 antibody improves the activation of resting T cells at lower cell density. Unlike the OKT3, the soluble form of mAb9.3 can induce costimulation directly without the need for crosslinking [37]. Interestingly, there is a correlation between CD28 costimulation and elevated catalase accumulation in the cultures. At present, there is no basis on which to infer whether catalase is in the CD28 pathway or merely one of the many downstream components of T cell activation. Once being activated, the ATCs have to be kept at high cell density for survival and efficient proliferation.

When T cells are activated, T cells secrete abundant cytokines such as IL-2 and other growth factors for cell proliferation and survival. Maintaining high cell density in culture may enable the accumulation of such autocrine products to reach a relatively high concentration to support the proliferation and survival of ATCs. It is interesting to notice that under physiological conditions, T cell immune responses can only be exclusively induced in organized lymphoid tissues but not elsewhere in the body [49–53]. Naïve T cells traffic constitutively through secondary lymphoid organs where they encounter antigen-loaded dendritic cells and are activated to proliferate and differentiate into activated effector T cells. Following this, effector T cells then migrate to peripheral tissues to perform their function [54]. Is it true that at locations such as the T cell areas of secondary lymphoid organs, high cell density enables the initiation of T cell responses with supported T cell survival and proliferation, whereas at sites of peripheral tissues, low cell density prevents T cell proliferation and minimizes immunopathology? The question remains to be answered.

4.2. ROS Are the Mediators of ATC Apoptosis at Low Cell Density. To understand why T cells have to be maintained at high cell density for optimal expansion, it is important to know why the T cells die at low cell density. ROS such as superoxide and hydroxyl radicals and H_2O_2 are continuously produced by cells, and their levels are regulated by a number of enzymes and physiological antioxidants. Excessive generation of ROS or failure to suppress elevated
intracellular ROS by the cellular regulatory systems has been associated with cell death [12–15].

Differential effects of ROS on cell death are observed depending on the level of ROS within the cell [26, 55]. High levels of ROS lead to lipid peroxidation, damage to cellular membranes, inactivation of caspase enzymes, and necrotic cell death. Low levels of ROS can activate protein kinases and phosphatases, mobilize Ca^{2+} stores, activate or inactivate transcription factors, and lead to apoptotic cell death. ATCs have been shown to have increased levels of ROS [25, 26, 29–31] and ROS have been shown to be one of the decisive contributors to the death of ATCs [25–28].

ROS are intermediates in the induction of FasL after TCR engagement during activation induced cell death [25]. ROS-driven Bcl-2 downregulation is a necessary signal for activated T cell autonomous death [25]. Besides, ROS may affect many other molecules, such as membrane lipids, transcriptional factors, and signal transduction proteins that are involved in T cell apoptosis [25].

Our data demonstrate that ATCs cultured at low cell density have higher levels of ROS than ATCs cultured at high cell density and that reversal of high ROS in culture improves T cell proliferative response and survival, implying that ATC apoptosis at low cell density is triggered by ROS.

4.3. Antioxidants Promote ATC Survival at Low Cell Density. Glutathione (GSH) is the major intracellular redox buffer and plays an essential role in protecting cells against oxidative damage [56]. In addition, changes in the intracellular GSH levels modulate the expression of several genes involved in the control of cell growth and differentiation [57, 58]. In T lymphocytes, intracellular GSH is critical for the proliferative response to mitogens or antigens [59–62]. Our experiments demonstrate that by supplementing the GSH precursor, NAC, ATCs can be protected from apoptosis at low cell density, suggesting that the GSH peroxidase antioxidant system may play an important role in ATC survival.

Other interventions that reduce intracellular ROS were also effective in reversing the effect of low cell density to inhibit T cell proliferation and survival. These included the supplementation of cultures with purified catalase, which detoxifies H_{2}O_{2}, and addition of high concentrations of serum albumin, which contributes reducing cysteines that can scavenge oxygen radicals.

One of the striking features of human serum albumin is the presence of 34 cysteine residues forming 17 disulfide bonds, and one free thiol at the Cys-34 position [63]. One-third of the albumin molecules form mixed disulfides with either GSH or half-cystine. The remaining sulfhydryl group of the Cys-34 residue of albumin constitutes the major extracellular source of reactive free thiol [64]. In this context, it has been suggested that albumin constitutes an important extracellular antioxidant in plasma [43]. The role of albumin as an ROS scavenger has been confirmed in cell-free systems with a wide variety of oxidative species, including HOCl, H_{2}O_{2}, *OH, carbon radicals, and peroxynitrite [22, 65, 66], as well as in intact cell systems such as macrophages and renal tubular epithelium [42]. However, the mechanism by which albumin exerts its antioxidant effects is most likely multifactorial [42]. It is possible that the free sulfhydryl group of albumin enables it to act not only as an antioxidant but also as a reducing agent via modulation of cellular GSH levels [44]. GSH in turn affects a wide variety of cell proteins, the function of which is dependent on redox state, such as the N-methyl-D-aspartic acid receptor, the DNA binding protein activator protein-1, and NF-κB [67, 68].

Albumin has been an essential component of non-serum culture mediums for expansion of T cells for clinical applications. We explored the antioxidant role of albumin in cell density-related death of ATCs. Concentration-dependent reduction of intracellular ROS in ATCs cultured at low cell density suggests that albumin reacted as an antioxidant to...
sodium pyruvate, not investigated further. Whether increased internalization of albumin is finally released mainly in a degraded form from the cells [70]. Whether increased albumin binding proteins and the binding of serum albumin increased considerably upon blastic transformation [70]. Albumin is endocytosed and the internalized albumin is detected in peroxidase-conjugated form in lysosome-like bodies by ultrastructural cytochemistry. Pulse-chase experiments show that internalized albumin is finally released mainly in a degraded form from the cells [70]. Whether increased endocytosis of albumin under low-density conditions is an adaptive response to elevated intracellular ROS to import further reducing species into the cell is uncertain and was not investigated further. Interestingly, elevated intracellular albumin was detected in ATCs cultured at low cell density, implicating increased endocytosis under this condition, possibly in response to elevated intracellular ROS. Albumin is bound on the surface of lymphoid cells of all mammalian species tested [69], but its function is relatively unknown. ATCs express albumin binding proteins and the binding of serum albumin increased considerably upon blastic transformation [70]. Albumin is endocytosed and the internalized albumin is detected in peroxidase-conjugated form in lysosome-like bodies by ultrastructural cytochemistry. Pulse-chase experiments show that internalized albumin is finally released mainly in a degraded form from the cells [70]. Whether increased endocytosis of albumin under low-density conditions is an adaptive response to elevated intracellular ROS to import further reducing species into the cell is uncertain and was not investigated further.

It is likely that those nontoxic antioxidants such as penicillamine and thiolactate that exhibit protective effects against ROS may have the same effect to protect ATCs from apoptosis at low cell density. Whether these agents are useful in the maintenance and growth of ATCs remains to be determined.

4.4. Autocrine Catalase Protects ATCs from Apoptosis. Among the naturally occurring variables examined, only one correlated with the reversal of high levels of ROS under conditions of high ATC cell density: the accumulation of secreted catalase that raised extracellular levels of this potent antioxidant enzyme. This extends studies of cultured leukemic T and B cells [4, 5] suggesting that autocrine catalase functions as a cytoprotective antioxidant in protecting cells at high cell density from apoptosis. Furthermore, our results and others’ indicate that this factor is not cell-type restricted and is compatible with the observation that so-called “feeder” cells of various origins can function to support T cells under single-cell cloning conditions. We may infer that secretion of catalase to suppress intracellular oxidative stress is a key component of the supportive role of feeder cells.

How catalase is released from the cells is unclear. Catalase lacks a leader sequence and cannot therefore be secreted by the classic endoplasmic reticulum-Golgi secretory pathway [73]. It is suggested that, like some cytokines, catalase may be secreted via a leaderless secretory pathway [5]. It is unlikely that the appearance of catalase in the medium derives from dying cells. First, at high cell density, ATCs only begin to die at late stages of expansion (after 3-4 weeks). The CM we collected are from ATCs cultured at early stage of expansion (2 weeks) with good viability. Second, we have shown that CM from other cell lines that have very limited cell death can also prevent ATC apoptosis. It was noted that the endogenous cellular catalase was similar in quantity to that secreted by the cells in 24 hours. Whether the intracellular enzyme is in a compartment that is functional or inactive is not addressed by these studies. However, it is clear that cellular catalase is not a substitute for the secreted component for maintaining cellular health; otherwise, the cell concentration effects would not be observed and CM would not rescue lower ATC densities from apoptosis.

Finally, the quantities of catalase in the CM (6–12 units/mL) (Figure 6(a)) corresponded closely to levels of purified catalase (∼10 units/mL) that provide maximum control of ROS and high ATC viability (Figure 4(b)).

Regarding how extracellular catalase activity might regulate intracellular oxidative stress, it is suggested that the extracellular decomposition of H₂O₂ may create a concentration gradient favoring the diffusion of H₂O₂ out of the cells [5].

A final speculation is warranted on the relevance of these findings to the in vivo setting. The autocrine of catalase by T cells may be an important factor for their proliferation. At the time of activation, ATCs are at the highest level for ROS generation and most vulnerable to oxidative cell death [29]. At sites of high cellular density and low fluid efflux, such as lymph node paracortex, catalase could be expected to accumulate to high levels and support ATCs viability and proliferation. On the other hand, ATCs trafficking through the peripheral circulation will be dependent upon blood/tissue levels of ROS and tissue secretion of catalase, as well as serum albumin.

Another question remains to be answered is that the elevated ROS in ATCs and an increased rate of their death at low cell density may be a result of or partly related to excessive oxygen concentration in the growth medium cultivating in traditional incubators in comparison with oxygen concentration in the blood. Nevertheless, our studies clearly indicate that it is important to keep an antioxidant environment for optimized expansion of T cells in vitro.

In conclusion, the present study examined the role of cell density in T cell expansion in vitro. Several conclusions are drawn from this work. First, we confirm that cell density plays a critical role in T cell activation and ATC proliferation. Resting T cells were activated to expand at high cell density but failed to be activated at low cell density. ATCs grew
rapidly at high cell density but underwent apoptosis at low cell density in culture. Second, apoptosis of ATCs cultured at low cell density correlated with elevated intracellular ROS levels and was reversed by antioxidants NAC, catalase, and albumin, indicating that the apoptosis of ATCs at low cell density was mediated by ROS. Third, the increased survival of ATCs at high cell density was due to non-IL-2 factor(s) secreted by ATCs and non-ATCs alike. Fourth, autocrine catalase was demonstrated to be the key survival factor regulating ATC survival at high density by suppressing intracellular ROS. Fifth, CD28 costimulation that improves T cell activation at lower cell density is accompanied by enhanced autocrine catalase secretion. Our findings highlight the importance of cell density in T cell activation, proliferation, survival, and apoptosis and suggest that it is critical to maintain T cells at high cell density for the successful expansion of T cells ex vivo for adoptive immunotherapy.

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References


Strategy Escalation: An emerging paradigm for safe clinical development of T cell gene therapies

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Abstract
Gene therapy techniques are being applied to modify T cells with chimeric antigen receptors (CARs) for therapeutic ends. The versatility of this platform has spawned multiple options for their application with new permutations in strategies continually being invented, a testimony to the creative energies of many investigators. The field is rapidly expanding with immense potential for impact against diverse cancers. But this rapid expansion, like the Big Bang, comes with a somewhat chaotic evolution of its therapeutic universe that can also be dangerous, as seen by recently publicized deaths. Time-honored methods for new drug testing embodied in Dose Escalation that were suitable for traditional inert agents are now inadequate for these novel "living drugs". In the following, I propose an approach to escalating risk for patient exposures with these new immuno-gene therapy agents, termed Strategy Escalation, that accounts for the molecular and biological features of the modified cells and the methods of their administration. This proposal is offered not as a prescriptive but as a discussion framework that investigators may wish to consider in configuring their intended clinical applications.

Introduction
Gene therapy techniques are being applied to modify T cells with chimeric antigen receptors (CARs) for therapeutic ends (designer T cells, T-bodies). At their simplest, CARs are an immunoglobulin binding domain fused to the zeta signaling chain of the T cell receptor ("IgTCR") that can redirect T cell killing against antibody-specified targets [1]. The versatility of this platform has spawned multiple options for their application. For the same target and CAR recognition domain, a diversity of signaling domains, co-expressed cytokines and anti-apoptotic genes may impact the survival and activity of the designer T cells, whereas other, adjunctive, procedures may support the stable engraftment of vast numbers of these effectors in vivo.

Time-honored methods of Phase I safety testing have relied on Dose Escalation of new drugs to protect patients while advancing therapeutic aims. However, these methods designed for short-acting inert agents are no longer sufficient with the advent of engineered cellular therapies that are "living drugs" with potential for lifelong exposures. Strategies applying different CARs and different means of their application may have different potentials for benefit, but which may also be paralleled in their potentials for harm. For these novel cellular agents, I propose a new concept to be added to the clinical trialist’s lexicon: Strategy Escalation.

Discussion
Designer T cells and safety
The application of adoptive cellular therapies in any format may have generic consequences with constitutional symptoms from cytokines released or co-administered. For the most part, these are manageable in experienced hands and present no new challenges. What is new is that specificities can be engineered into T cells in analogous fashion to monoclonal antibodies that have been adapted to target selected tumor antigens. These antigens are typically normal cell constituents that are enriched in tumors. From a T cell perspective, CARs allow bypassing of thymic editing that prevents normal T cells from high avidity reactions against self-tumor, but that primarily protects from such reactions against self-tissue ("tolerance").

This bypassing of normal tolerance means that some antigen targets may be unsafe for designer T cells. This was recently shown in a designer T cell trial against G250, a prominent renal cell carcinoma antigen [2]. Antibody
against G250 had been applied in humans without toxicity, but when this specificity was tested in designer T cell format, reaction occurred against low level G250 on biliary epithelium. This resulted in an intolerable hepatoxicity in two of three patients with low infused doses in the range of $10^9$ cells (100-fold below typical Surgery Branch TIL doses [3]), necessitating dose reductions and, in one case, systemic steroids for T cell suppression. When steroids were removed, the patient had no resurgence of liver attack - but also no tumor response.

This key study illustrated that designer T cells carried the potential for serious toxicity. The safety of comparable Phase I interventions against other antigens (folate binding protein [4], Tag72 [5], CEA [6], CD171 [7] and GD2 [8]) indicate that toxicity is a function of the target - with no obvious means to predict which. The G250 toxicity also demonstrated that safety of a target with antibody is no assurance of safety with designer T cells [2]. This latter conclusion is not surprising given the indirect means of antibody toxicity [9] in comparison with the direct cytotoxic potency of T cells that also brings far greater sensitivity, killing with just a few antigen molecules per cell, far below immunohistochemical detection thresholds [10].

This G250 agent was expertly managed via a dose escalation plan in a Phase I setting; the system worked: no one died. Instead, it is the evolution of more complex Strategies that raise the special concerns of this essay.

The Strategies

New Strategies evolved because several so-called 1st generation IgTCR designer T cells (above) had been tested in the clinic without major tumor regressions. Two contributing problems were identified. Firstly, the infused designer T cells initially distributed widely through the blood and tissues, but then they quickly perished in the host that is already replete with T cells. Secondly, the few T cells that trafficked into tumor could initially exhibit killing, but they ultimately disappeared via a process of activation-induced cell death (AICD) or passed to a resting, inactive state.

These two problems prompted two corresponding hypotheses for improving tumor responses:

1. Responses could be improved: if sufficient T cells were maintained systemically to sustain T cell percolation into tumor (although T cells survived for only a few days of tumor cell killing).
2. Responses could be improved: if T cells were to activate and proliferate on antigen contact in tumor (although T cells in tumor were few in starting number).

To address hypothesis #1, Dudley, Rosenberg and colleagues [11] applied "conditioning" to create a "hematologic space" with high dose chemotherapy and/or whole body irradiation prior to T cell infusion in their TIL studies in melanoma. With the burst of IL7 and IL15 that accompanies the lymphopenic state [12], the infused T cells rode the recovery with a homeostatic expansion, i.e., independent of antigen stimulation. As such, low doses of infused T cells could expand 100-fold in vivo to become a stable, "engrafted" component of the lymphoid compartment, in some instances >50% of the cells that would be the equivalent of $5 \times 10^{11}$ (0.5 kg!) tumor-specific T cells. This in turn led to dramatically improved tumor response rates with substantial numbers of durable remissions.

To address hypothesis #2, so called 2nd generation "2-signal" CARs were created to improve their function [13]. To the basic TCRz signaling (Signal 1) of the IgTCR was added a co-stimulation Signal 2 via CD28 and/or other signaling domains, e.g., IgCD28TCRz. Signal 1 suffices for T cell killing, but Signal 1 + 2 engages the T cell proliferative capacity, avoiding AICD, and promotes T cell reactivation on antigen contact after passing to resting state. By this, even a few cells trafficking to tumor could activate and expand in situ to large numbers until tumor elimination, in the same way that virus-specific T cells respond to viral infections. Further, the added costimulation renders designer T cells resistant to regulatory T cell suppression [14]. The benefits of these modifications for improving therapy were enticing, and to many their combination appeared irresistible. With engraftment of 2-signal designer T cells, there would be huge numbers of effectors, and they would never lose their capacity to respond against the tumor threat - or against normal tissues, thereby motivating this essay.

With two independent approaches, however, it is not just their combination but a $2 \times 2$ array of four distinct Strategies that confronts the investigator in choosing safely how to treat his first patients with a new designer T cell agent: 1st generation or 2nd? Infuse or engraft? The philosophy of patient exposures during new drug testing is aimed at proceeding from low risk to higher risk in a regulated fashion. To order these Strategies for risk, therefore, it is instructive to perform a "What-if?" analysis to consider the consequences if G250 designer T cells [2] had had their initial patient exposures under one of these more advanced Strategies.

"What if...?"

"What if" G250 designer T cells were first applied via ...?

**Strategy 1.** 1st generation, infused [Actual]
In the least aggressive Strategy, infusion of 1st generation G250 designer T cells was seen to mediate significant toxicity. Steroids successfully suppressed the T cell reaction without reactivation after steroid withdrawal.
Strategy 2. 1st generation, engrafted

If the same T cells had been engrafted, their resulting vast numbers would likely induce a more severe and possibly lethal toxicity if left unchecked. However, intervention with steroids would again suppress the auto-immune attack. Once brought to resting state and steroids removed, these Signal 1-only designer T cells would be inert (anergic) on contact with antigen positive tissues, and the patient safe from resurgence of his symptoms. Toxicity under this Strategy should be manageable. (See endnote 1.)

Strategy 3. 2nd generation, infused

If G250 designer T cells were infused as before but in 2nd generation format, they also would induce toxicity and then respond to steroids. But with removal of steroids, these now-resting 2-signal designer T cells can reactivate on antigen contact with renewed toxicity. Importantly, at low initial exposures in the dose escalation, these infused designer T cells begin as a tiny fraction of the body’s T cell repertoire and undergo rapid systemic decline (e.g., 10⁹ cells infused vs 10¹² total T cells, or 0.1% at peak and lower thereafter). From the analogous clinical setting of donor lymphocyte infusion (DLI), we know that size (of dose) matters, and even with a fully competent allo-immune reaction, small numbers of allo-reactive T cells can be safely managed with a balance of GvH reaction and anti-tumor benefit [15,16]. Thus, toxicity under this Strategy should also be manageable.

Strategy 4. 2nd generation, engrafted

If 2nd generation T cells had instead been engrafted, G250-specific T cells would not only be capable of reactivation after steroids, but they would be vast in number. With up to 10% of the reconstituted T cell pool being antigen specific after the lowest injected dose (e.g., 10¹¹ cells expanding from 10⁹ injected) [17], these cells would be virtually impossible to control, like too high a dose in DLI settings. Maximal immune suppression would be required at all times, with infectious complications and a predictably fatal outcome. Had the initial patient exposure of G250 T cells been by Strategy 4, the consequences could have been dire.

Strategy Escalation

With these options, it can be seen that there are now choices, not just of dose levels as in typical Phase I drug studies, but of Strategies, with distinct consequences to each. With these Strategies available, how does one best advance the therapeutic aims while remaining faithful to principles of patient protection via an incremental exposure to risk? This brings us to the concept of Strategy Escalation. Strategy 1, simple infusion of 1st generation, is the most conservative; Strategies 2 and 3, engraftment OR 2nd generation, are intermediate in risk; Strategy 4, engraftment AND 2nd generation, is the most aggressive. To proceed from the untested state for a new target (“0”) to its most potent implementation, one could envision a Strategy Escalation path of 0 → 1 → (2 or 3) → 4.

But do I advocate that escalations for all new agents first pass through a Strategy 1 test, infusion 1st generation (0 → Strategy 1)? No, I do not. If the target was previously tested with a Strategy 1, it does provide more confidence of the safety or hazard for the more aggressive strategies. The G250 test by Strategy 1 showed it was unsafe as a target, from which one may forego all more advanced Strategies, thereby sparing patients from more serious injury. Ultimately, however, drugs must be tested for safety in a setting that reflects their potential utility. Sufficient evidence exists from diverse trials with infusion of 1st generation designer T cells to infer that none will be therapeutically successful by Strategy 1, and safety in this format becomes of mainly academic interest. If we instead start with a more advanced Strategy, what rationale could be invoked?

Strategy 2 with engraftment of 1st generation showed considerable benefit in the analogous setting of TILs where simple infusions had not yielded high response rates [12]. The promise of Strategy 3 with 2-signals to sustain an antitumor reaction in situ is an hypothesis based on encouraging preclinical data; clinical trials are just now underway. Both of these have a rationale for realistic benefit to patients where Strategy 1 no longer does. If we bypass Strategy 1 for initial human trials, there is more risk with first patient exposures via engraftment (0 → Strategy 2 test) OR 2nd generation (0 → Strategy 3 test), but there is also a rationale for controlling toxicities should they occur, as discussed above.

I would argue, however, that proceeding with an untested target (e.g., as was G250) to the most aggressive Strategy 4 (engraftment AND 2nd generation) is too much risk. A 0 → Strategy 4 test presumes much about the quality of our knowledge of the potential normal tissue targets and their susceptibility, and, of all Strategies, this one alone allows no exit strategy if we guess wrong. (See Appendix 1 for examples.) No one could foresee the hepatotoxicity of G250 designer T cells [2] or the cardio-toxicity of trastuzumab antibody (Herceptin®) [18] prior to the actual human trials. The graded exposures of their respective Phase I/II studies were essential to revealing toxicities before a Grade V event (death). After a target is shown to be safe by one Strategy, one may proceed with fair confidence to more aggressive Strategies, as shown in Figure 1.

More than safety

Although safer development drives the Strategy Escalation concept, the discipline of this structure can assist in finding more optimal development paths as well. For example, while a case can be made for safely escalating T
cells from a prior Strategy 1 or 2 to Strategy 4, these paths are not necessarily recommended (dotted in Figure 1). Three reasons unrelated to T cell safety may be considered for all paths instead passing through a full Strategy 3 test first:

1. Lower hazard: The NMA conditioning of Strategy 4 is routinely accompanied by infectious complications that can occasionally be fatal [19, 12]; see also Appendix 1: Designer T cell study deaths;
2. Lower cost: The clinical (non-manufacturing) costs in the real-world hospital setting are in the range of $4-$8,000 for simple infusion (Strategy 3) versus $60-$100,000 for engraftment protocols (Strategy 4), per our own experience [20-22]; and finally and importantly,
3. Better science: A direct 0 → Strategy 4 test with engraftment obscures any chance to test the core driving hypothesis of current research, e.g., that additional signals, as embodied in the advanced generation designer T cells, can promote a fully competent T cell response with in situ expansion until tumor elimination.

To this latter point, T cells do this quite efficiently in virus infections without conditioning, and when we have proven ourselves capable to bypass immunization and antigen-presenting cells via this technology, I expect we will prevail similarly with designer T cells against tumor. At the moment that we succeed with the right CARs, such engraftment strategies, with their attendant costs and hazard, will predictably be retired. Hence, in my opinion, engraftment should be viewed as an intervening measure, applied only until we get better at immunology, to compensate for our still-imperfect T cell engineering.

Further, when targeting a normal self antigen, a Strategy 3 infusion may allow "tuning" of the activity against tumor versus normal tissue by judicious dose exposures and a gradation of suppressive therapies (as needed) in the manner of DLI [15], where a Strategy 4 engraftment with its hard-to-control cell numbers may fail. That is, with each new product tested under Strategy 3, an appropriate dose escalation plan affords the best chance to define an optimal biologic dose (OBD) to establish proof-of-concept anti-tumor activity and conditions of safety to normal tissues.

At this point in time, however, the first studies with 2nd generation designer T cells under Strategy 3 (infused) are just coming on-line, and none has yet completed a full escalation with appropriate cytokine support (e.g., IL2). Thus, it is too early to infer sufficiency or deficiency of any of the existing 2nd generation reagents to eliminate tumors - without engraftment. But where these more advanced reagents are proven therapeutically inadequate (and safe) under Strategy 3 infusions, then engraftment via Strategy 4 with its higher cost and hazard is a justifiable next step in the Strategy Escalation.

Hence, for untested targets, it is my opinion that Strategy Escalations of 0 → 2 (1st generation, engrafted) or 0 → 3 (2nd generation, infused) are safe and acceptable for initial human exposures. For all targets, tested and untested, I believe for reasons of safety, science and cost that 2nd generation engrafted should instead have a full prior test of 2nd generation infused, i.e., a Strategy Escalation of (0 or 1 or 2) → 3 → 4. (See endnote 2.) This is represented in Figure 2.

Conclusions

It is recommended that every new immuno-gene therapy proposal be accompanied by a Strategy Escalation discussion that accounts for the molecular and biological features of the modified cells and the method of their proposed administration. This Commentary presents an example of such a discussion from the current state of the art for designer T cell therapies, counseling against the most intensive Strategies for untested antigen targets. If by an early Strategy, the patient can safely be treated, then one may reasonably advance to more potent Strategies with a rationale for safety. Further, it is clear that safety with an antibody is not the same as safety with a T cell; antibody studies therefore cannot substitute for directed designer T cell trials via a less than fully committed patient exposure. As a paradigm, Strategy Escalation is intended to be flexible and adaptive as new therapeutic opportunities are brought forward, e.g., anti-apoptotic genes, suicide genes, co-expressed cytokines, etc., as elaborated in Appendix 2: Future directions. Finally, the for-
malism of the Strategy Escalation discussion may ultimately find wider application, extending to other cellular therapies as their respective fields mature, e.g., as in stem cells where emerging concerns over options for their safe and incremental application were recently and cogently expressed [23].

Appendix 1: Designer T cell study deaths
In the past year, two patients died on Phase I designer T cell studies: one targeting CD19 in lymphoma [24,25] and the other targeting Her2/neu in breast cancer [26,27]. Both were previously untested targets for designer T cells. The patients in each case were treated with 2nd generation designer T cells incorporating costimulation, and the two deaths were the first patient in each case to undergo engraftment (Strategy 4). In the former, there was an initial exposure to designer T cells by infusion (Strategy 3) but only to low doses (~10^9 T cells) without toxicity, and then a death with the first patient to have engraftment of the same dose (0 → (3) → 4 test). (3 in parentheses because it was not a full dose-escalation test.) Was this death due to on-target toxicity (i.e., against CD19 on undefined normal tissue)? In that case, was the jump too big from 10^9 cells infused on Strategy 3 transiently present to 10^{11} stably engrafted on Strategy 4 (from 10^9 cells dose)? (See endnote 2.) Or was this death unrelated to any on-target toxicity, perhaps secondary to the conditioning? These questions could not be definitively answered. The study was ultimately allowed to proceed with the second patient treated at half-log lower dose without toxicity [24].

In the second case, targeting Her2/neu, the first patient exposure was a moderately high dose of 10^{10} designer T cells infused after conditioning. This was the first-in-human designer T cell test against this target (0 → Strategy 4 test). The patient experienced acute pulmonary edema within the first hour post infusion, and high dose steroids were initiated. The patient died after five days with cardiac arrest and hemorrhagic enteritis, the latter a recognized manifestation of severe GvHD. Her2/neu is known to be expressed on lung and bowel [28], and may be inferred at low levels in heart by the cardiotoxicity seen in a minority of patients treated with trastuzumab (Herceptin) [18]. This study is presently suspended.

One may consider whether these are second and third examples of antibody therapy being relatively safe (i.e., anti-CD19 antibody [29] and trastuzumab [30]) but designer T cell therapy against the same target is toxic. From the details presented, the likelihood is the CD19 death was not due to T cell toxicity, but rather a complication of the conditioning regimen, a reminder that conditioning, integral to Strategies 2 and 4, is not a benign option. On the face of it, the Her2 death appears to be on-target toxicity in normal tissues, similar to the G250 study [2], but not reversible by steroids due to vast self-reactive T cell numbers in the Strategy 4 setting. An alternative in each case would have been to start with a full Strategy 3, escalating until 10^{11} cells infused, if tolerated, and then switch to Strategy 4, engrafting - but only if Strategy 3 is ineffective. In both instances, these deaths alert us to the potential for serious impact of our interventions, and that the choice of how we incrementally expose patients (i.e., Strategy) may be important to patient safety in a new therapy.

Appendix 2: Future directions
One may consider the structure of the 2 × 2 matrix for Strategy Escalation as deriving from inherent elements of T cell biology. One dimension is how many T cells there are (“quantity”, e.g., Strategy 1 → 2; T cells increased by engraftment) and the other dimension is how effective/potent they are (“quality”, e.g., Strategy 1 → 3; T cells more effective with costimulation). This matrix works well for the current state of the art represented in current clinical trials, but new permutations in these strategies are continually being invented. It is instructive to consider how these newer configurations may affect the application of this matrix.

The matter of when to assign a new intervention a new Strategy number (e.g., 5) comes down to whether an earlier trial needs to be performed before escalating to the new Strategy: e.g., to address safety concerns of a modifi-
cation or to serve better hypothesis testing. In most instances, however, it can be seen that these anticipated modifications are still covered under one of these four basic Strategies. That is, novel interventions may be conceptualized along these same two axes of number (quantity) and/or potency (quality), without dramatic changes in the risk implications for untested antigens. These can be annotated with + or - on a basic Strategy number (e.g., Strategy 1+ or 4-) when safety features are considered not to mandate a separate trial. Ultimately, whether a configuration is a Strategy 4+ or a Strategy 5 (needing a Strategy 4 trial first) can be a judgment call for the investigator, but the formalism of the Strategy Escalation discussion provides an explicit framework in which to support that assignment. In the end, however, the way the Strategies are numbered is less important than the structure that encourages their formal consideration as a strategy.

In the following, we consider several Strategy configurations that have been described in preclinical work that may find their way into the clinic.

Multiple co-stimulatory molecules
These include CD28, 4-1BB, OX40 and others. I have defined all of these constructs, single or multiple, as 2nd generation: they all make T cells more potent (quality), some more than others. The best co-stimulation combinations will make T cells quantitatively more able to mediate toxicity, possibly at lower starting cell exposures, but do not introduce qualitatively novel risks. Unrecognized toxicities against self-tissues should still be adequately covered via infusions (Strategy 3) under a dose-escalation plan with appropriately low starting doses, as in tuning donor lymphocyte infusions (DLI) [15]. Similarly, risks with engraftment (Strategy 4) are not qualitatively different among different 2nd generation constructs once proven safe in a Strategy 3 test.

Co-expressed cytokines
This falls into two categories: Growth factors (e.g., IL2, IL7, IL15) and Immune Modulators (e.g., IL12, IFNg). Growth factors constitutively expressed improve cell numbers (quantity) by prolonging T cell survival/expansion. Critically, none has been associated with T cell immortalization. For infusion protocols, the impact on quantity is incremental and manageable (versus the quantum changes for engraftment) and likely does not create new types of risks for 1st or 2nd generation when infused. (See endnote 3.) Immune modulators like IL12 make T cells more potent (quality) without affecting cell numbers. The anti-self potency can be managed by the same dose escalation as DLI protocols (above). By this Strategy discussion, it appears that there is no untoward risk by Strategy 1 or 3 infusions. Where these cytokines take on special significance, however, is in engraftment protocols. With 10^{11} or more cells post-recovery secreting cytokine, high systemic exposures may create a risk that is off-target and potentially life-long. With this qualitatively new risk, such a study might merit designation as a Strategy 5 protocol, to be conducted post Strategy 4, if ineffective. (However, see below, On-Off gene control.)

Reactivation modulators
Antigen-Fc molecules have been shown to stimulate designer T cells, 1st or 2nd generation, in the presence of monocytes that crosslink Ag-Fc and supply B7 for CD28 engagement and costimulation [31]. This molecule may in principle be used in vivo to reactivate and expand designer T cells in conjunction with any Strategy (1 and 3, post-infusion; 2 and 4, post-engraftment). The ability to control the dose and duration of Ag-Fc exposure allows assignment of Strategy 1+ or 4+, for example, without major risk increment.

Anti-apoptosis genes
Anti-apoptotic genes can replace growth factors (e.g., IL2) by blocking apoptosis from cytokine withdrawal, e.g., via Bcl-xl over-expression [[32]; Emtage & Junghans, unpublished data], impacting therapy along the cell number axis (quantity). This has the advantage of avoiding systemic cytokine exposures, whether exogenous or expressed in the T cells (above). However, the potential for transformation and immortalization with a Bcl family member [32] distinguishes this class from the expressed cytokines. This introduces a qualitatively new risk, meriting designation as a Strategy 5 protocol, to be tested (with appropriate rationale) only after failure of a prior Strategy 3 or 4.

Suicide genes
This measure would be unnecessary for most infusion protocols, where the dose escalation and suppressive measures provide adequate protection as discussed in the main text (an exception might be with anti-apoptosis genes). The fail-safe feature of incorporated suicide genes presents a potential escape from any toxicity, however it manifests [33]. In the most relevant clinical model, herpes TK (hTK) has been employed in allo-transplant, where it has successfully combated serious GvHD [34]. In the case of 2nd generation engraftment, a suicide gene could take a Strategy 4 down to a Strategy 4-. Yet, even here, the investigator will want to consider the rapidity and completeness of the suicide (for hTK, hours to days, depending on T cell cycling) versus the rapidity and intensity of onset of adverse effects. In the Her2 study, with a moderate (10^{10}) dose of T cells, the patient had respiratory distress by 15 minutes post-infusion, requiring intubation, and was dead in 5 days. (See Appendix 1: Designer T cell study deaths.) A suicide gene could not have prevented the initial event but perhaps the ensuing death. Thus, the option of suicide gene control of non-
hyperacute toxicities could take the designer T cells under Strategy 4 engraftment to a risk level approaching simple infusion (e.g., Strategy 3+) by reducing effector cell numbers (cell numbers being the essential difference between 3 and 4). However, it does nothing to improve safety or expense of conditioning, or to correct a muddled hypothesis test with the combined approach. The suicide gene ablation for serious toxicity in engraftment also loses the opportunity to "tune" the therapy in the manner of DLI, available to infusion protocols (e.g., Strategy 3), where a balance of anti-self and anti-tumor activity may be achieved with patient benefit [15]. Lastly, if fully tested under Strategy 3, where suicide genes are generally unneeded, a 2nd generation designer T cell does not require a suicide gene in a subsequent Strategy 4 because safety of the target was previously established.

On-Off gene control
In analogy to suicide genes, parallel descriptions could be made for control of genes desirable for expression (e.g., of cytokine) that is time-limited without terminating the T cells, allowing for resumption of activity at a later time if needed. Thus, an engrafted 2nd generation designer T cell with co-expressed cytokine under a Tet-On promoter [35], potentially termed Strategy 5 because of the added risk of systemic cytokine, is downgraded to a Strategy 4+ because of the potential to shut off growth factor on Tet withdrawal, thereby avoiding need for a prior Strategy 4 trial for patient safety.

Endnotes
1. This inference of toxicity manageable under Strategy 2 is consistent with observations in two non-designer T cell studies. TCR transfer engages CD3 Signal 1 on antigen contact, similar to 1st generation designer T cell CARs. Engraftment of T cells with MART1 specificity in a Strategy 2-like application had on-target toxicity that safely responded to steroids [35]. Engraftment with CEA specific TCR designer T cells also showed on-target normal tissue toxicity that was safely managed [37]. 1st and 2nd generation TCR-based CARs have been created [[38,39]; AJ Bais & RP Junghans, unpublished data] and will engender the same types of discussion as for the Ig-based CAR constructs.
2. Bearing in mind that there is a 100-fold expansion of T cells for the lowest useful doses in the engraftment protocols (e.g., 10^9 cells) [11,17], it is likely that a reasonable Strategy Escalation increment to a starting test with 10^9 T cell engrafted is not preceded by a test of 10^9 T cells infused, but by a test of 10^{11} T cells infused. In the latter case, one is comparing 10^{11} T cells transiently present by infusion versus 10^{11} T cells stably present by engraftment. By moderate increments in risk, the hope is that toxicities will be revealed at less than Grade V (death) on their first expression. See Appendix 1: Designer T cell study deaths.

Competing interests
The author declares that he has no competing interests.

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