Development of an anti-CD2/CD3/CD28 bead-based T-cell proliferation assay

Louisa Green¹,²,*

¹Clinical Laboratory Immunology, Oxford University Hospitals NHS Trust, Churchill Hospital, Old Road, Headington, Oxford OX3 7LJ, England
²Oxford Brookes University, Gypsy Lane, Headington, Oxford OX3 0BP, England

*Corresponding author: Tel: +44 07725748099. Email: lgreen2891@gmail.com

Supervisor: Dr Lisa Ayers, Clinical Laboratory Immunology, Oxford University Hospitals NHS Trust, Churchill Hospital, Old Road, Headington, Oxford OX3 7LJ, England. Email: lisa.ayers@nhs.net

Severe combined immunodeficiency (SCID) has many phenotypes, including those which result in the formation of T cells but render them incapable of an optimal level of proliferation following stimulation by an antigen. Phytohaemagglutinin (PHA) is a mitogen commonly used to assess T-cell proliferation, although anti-CD2/CD3/CD28-coated beads may provide a more physiological trigger. Some patients and healthy controls display poor T-cell proliferation (<45%) in response to PHA, but may be capable of an increased percentage proliferation when stimulated by another mitogen. This study aimed to develop an anti-CD2/CD3/CD28 bead-based T-cell proliferation assay and to compare T-cell proliferation generated by two mitogens both in healthy controls and in poor responders to PHA. A bead-based T-cell proliferation assay was optimized and validated. Peripheral blood mononuclear cells (PBMCs) were extracted from whole blood samples of 10 healthy controls and 3 poor PHA responders. PBMCs were incubated with carboxyfluorescin diacetate succinimidyl ester and three separate stimulants: PHA, Beads only and Beads plus IL-2. Flow cytometry was performed on Day 6, following fluorochrome staining with CD3 PerCP and CD4 APC, to identify CD4⁺ T cells. No significant difference was found between the results generated by PHA- or Bead stimulation in 10 healthy controls. A strong positive correlation was observed between the use of the Beads alone and the addition of IL-2. A varied response to the Beads was produced among the poor PHA responders. It has been concluded that the bead-based assay will be run alongside every PHA test in the Oxford Immunology diagnostic laboratory. The step to add IL-2 on Day 3 will not be included as this did not significantly impact T-cell proliferation. The Bead assay provides a more physiological addition to the current PHA test and may be useful in assessing T-cell proliferation in those patients who respond poorly to PHA. However, some individuals who respond poorly to PHA may also respond poorly to the anti-CD2/CD3/CD28 Beads.

Key words: PHA, anti-CD2/CD3/CD28 beads, T cell, proliferation, diagnosis, immunodeficiency

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Introduction

The immune system comprises a multitude of cells and pathways that work together to ultimately keep an organism safe from infectious disease and harm. However, a breakdown in this networking can have a direct and compromising effect on the successful functioning of the immune system, and the term used to cover and define such causative conditions is immunodeficiency. Primary immunodeficiencies arise due to intrinsic genetic defects resulting in the complete absence or impaired function of a cell type or types (Geha et al., 2007). In contrast, secondary immunodeficiencies are acquired in later life and can arise secondary to infection, such as with human immunodeficiency virus (HIV), severe malnutrition or as a result of immunosuppressive drug use (Hall and Yates, 2010). Whether the condition is congenital or acquired, both the innate and adaptive response types can be impaired (Geha et al., 2007), and there is often an overlap with both T and B
lymphocytes being affected, even if only one of those cell types is absent or does not function as it should.

An umbrella term used to cover many austere T-cell primary immunodeficiencies is severe combined immunodeficiency (SCID). The illness is termed severe because it is fatal and is described as combined because although it is often directly T lymphocytes which are affected, B lymphocytes cannot adequately function without the help of T cells and thus both cell-mediated and humoural immunity are lost (Tasher and Dalal, 2012). SCID is considered a paediatric emergency with patients appearing well at birth but after a few months, following the subsequent loss of maternal antibody, often present with diarrhoea, sepsis, otitis and an overall failure to thrive (Kelly et al., 2013). Typically, children with SCID have a reduced spleen size and a small thymus weighing <1 g (Buckley, 2004), although these abnormalities are not usually noted until it becomes apparent that the infant frequently falls ill and is not developing at a normal rate. Opportunistic infections, caused by viral pathogens including cytomegalovirus and herpes simplex virus, are another common medical concern for patients who have T-cell deficiencies, and SCID sufferers often have such compromised immunity that even an attenuated vaccine can result in fatality (Kelly et al., 2013).

If SCID or another primary immunodeficiency is suspected, there are a set of standard differential tests undertaken to achieve a diagnosis. Crucially, it is important to rule out a secondary immunodeficiency, particularly HIV, and providing this result is negative the next investigation should be the full blood count. The results of this test provide a breakdown of the amount of each cell type comprising the blood; however, the main one of immunological interest is the lymphocyte count; 1–5 × 10^9 l⁻¹ for young children is suggestive of an immunodeficiency. If this is the case, it should prompt a clinician to request analysis of specific immunoglobulin levels to rule out a B-cell immunodeficiency, and then lymphocyte phenotyping to attain an absolute count of lymphocyte subsets. In addition, the Oxford Immunology laboratory currently performs an assay that assesses the ability of T cells to proliferate following stimulation with a mitogen called Phytohaemagglutinin (PHA). During this assay, peripheral blood mononuclear cells (PBMCs) are first isolated from whole blood and incubated with carboxyfluorescin diacetate succinimidyl ester (CFSE), a stable, membrane-permanent dye that covalently couples to intracellular molecules. The T cells of healthy individuals would normally proliferate upon PHA stimulation, and flow cytometric analysis should reveal a decline in CFSE staining within the PHA-stimulated cells as the dye is divided equally with each cell division (Parish et al., 2009). However, this is not observed in those patients whose T-cell proliferation is impaired (Fig. 1); thus, the PHA investigation is often a supportive test that leads to a primary immunodeficiency diagnosis.

Some patients and healthy controls show a reduced PHA response (a proliferation of <45%), but may be capable of normal T-cell proliferation following stimulation by other mitogens. The overall aim of this research was to establish and develop a new test that uses anti-CD2/CD3/CD28-coated Anti-Biotin MACSiBead™ particles to stimulate T-cell proliferation. More specifically, we wanted to conduct the PHA and Bead assays on at least 10 healthy control samples, compare the results, identify any control samples that did not respond well to PHA and evaluate the T-cell proliferation using the Beads. Furthermore, we aimed to investigate the impact of IL-2 on T-cell proliferation. IL-2 plays a pivotal role in the immune response and is secreted by T cells following the binding of an antigen to the T-cell receptor (Cornish, Sinclair and Cantrell, 2006). The production of IL-2 upon antigen binding stimulates the up-regulation of the IL-2 receptor gene, thus facilitating increased interaction between the two (Beadling and Smith, 2002). Moreover, the engagement of the T-cell receptor CD3 complex on the surface of antigen-presenting cells with the co-stimulatory molecule CD28 leads to the activation of numerous tyrosine kinase pathways, which drive the transcription of the IL-2 gene. The interaction between IL-2 and its receptor stimulates the growth and

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**Figure 1.** Normal T-cell proliferation vs. impaired T-cell proliferation, and the effect of both on CFSE staining.
differentiation of antigen-specific CD4+ T cells and CD8+ T cells (Beadling and Smith, 2002). Our final aim was to establish a reference range of percentage proliferation values for the proposed Bead assay, with and without the addition of IL-2 on Day 3 of incubation.

**Methods**

**Subjects**

A cohort of subjects working in the Oxford Immunology laboratory was selected. All participants were over 18, and exclusion criterion was any known primary or secondary immunodeficiency. Demographics are shown in Table 1. Ethics approval was obtained (05/Q1605/88), and all participants gave informed consent prior to involvement.

**PBMC separation**

All works performed were conducted under aseptic conditions. Blood from each control subject was drawn into three 3 mL lithium heparin collection tubes (Becton Dickinson, UK) and diluted 1 : 2 with RPMI (Sigma-Aldrich, UK) in a sterile Falcon tube. The blood–RPMI solution of each control was carefully layered on the surface of an equal volume of Lymphoprep™ (Axis Shield, UK). The tubes were centrifuged (Sorvall, UK) at 350 g for 22 min. Care was taken not to disrupt the layers, and the small cloudy band of PBMCs was carefully removed using a sterile pastette (Sarstedt, UK) and added to a sterile 15 mL Falcon tube. The volume of PBMCs was made up to 10 mL with sterile pastette (Sarstedt, UK) and added to a sterile 15 mL Falcon tube. The blood–RPMI solution of each control was carefully layered on the surface of an equal volume of Lymphoprep™ (Axis Shield, UK). The tubes were centrifuged (Sorvall, UK) at 1000 g for 22 min. Care was taken not to disrupt the layers, and the small cloudy band of PBMCs was carefully removed using a sterile pastette (Sarstedt, UK) and added to a sterile 15 mL Falcon tube. The volume of PBMCs was made up to 10 mL with RPMI, and the tubes were centrifuged at 600 g for 7 min. The supernatant was discarded. The cell pellet of each control was resuspended in 10 mL RPMI, and the Falcon tubes were then centrifuged at 350 g for a further 7 min. The supernatant was again discarded and the pellets were resuspended in 1 mL RPMI.

**Cell counting**

Ten microlitres of cell suspension were diluted with 40 µL Trypan Blue (Sigma-Aldrich, UK) to give a 1:5 dilution. A cell count, using a 10-chamber Fast Read disposable counting chamber (Immune Systems Limited, UK), under a ×10 objective lens, was conducted.

**CFSE staining**

One microlitre of 2.25 µM CFSE stock (Sigma-Aldrich, UK) was added to the PBMC resuspensions. Tubes were wrapped in aluminium foil to reduce photosensitive CFSE reactions and placed into a water bath at 37°C for 15 min. Ten microlitres of ice-cold RPMI were added to prevent further proliferation and to ensure that all cells remained at the same point in the cell cycle. The tubes were centrifuged at 483 g for 5 min. The supernatant was discarded and the pellets were resuspended in 10 mL ice-cold RPMI. The tubes were again centrifuged at 483 g for a further 5 min and the supernatant was again discarded.

**Current protocol: PHA**

The volume of culture medium AIM-V (Oxford Immunotec, UK) required to achieve a cell concentration of 1 × 10^6 mL⁻¹ was calculated for each control. A sterile 96-well, round-bottomed plate (Sigma-Aldrich, UK) had wells marked for unstimulated, PHA-stimulated and two Bead-stimulated PBMC populations. One hundred and ninety microlitres of the cell suspensions were added to the marked wells. Nothing further was added to the unstimulated wells. Ten microlitres of a 1 : 2 PHA (Sigma-Aldrich, UK) dilution with RPMI were added to each of the PHA-stimulated wells. Ten microlitres of cell suspensions were added to the marked wells. Nothing further was added to the unstimulated wells. Ten microlitres of a 1 : 2 PHA (Sigma-Aldrich, UK) dilution with RPMI were added to each of the PHA-stimulated wells.

**Proposed protocol: Anti-Biotin MACSiBead™ particles**

The Anti-Biotin MACSiBead™ particles (Miltenyi Biotec, UK) were first coated with anti-CD2, -CD3 and -CD28. The Miltenyi Biotec kit included the following:

- 2 mL Anti-Biotin MACSiBead™ particles, corresponding to 4 × 10^8 particles
- 0.4 mL anti-CD2-biotin (100 µg/mL)
- 0.4 mL anti-CD3-biotin (100 µg/mL)
- 0.4 mL anti-CD28-biotin (100 µg/mL)

A phosphate-buffered saline (PBS) solution was prepared using 10 mL PBS, 0.05 g human serum albumin and 40 µL 2 mM EDTA. The solution was filtered using a sterile 0.1 µM filter (Millipore, UK) to remove any bacterial contamination. Five hundred microlitres of Beads and 100 µL each of anti-CD2-, anti-CD3- and anti-CD28-biotin were added to a sterile cryovial tube, along with 200 µL of the pre-prepared PBS. The cryovial was placed on a rotator in a 4°C cold room for 2 h. One hundred microlitres of the solution were added to 10 sterile tubes and all were stored at 4°C until use. The coated Beads may be stored for up to 3 months before expiry.

The protocol provided with the Miltenyi Biotec kit is optimized for use with a PBMC concentration of 5 × 10^6 mL⁻¹. To enable this assay to be compared with the current PHA test, which requires a PBMC concentration of 1 × 10^6 mL⁻¹, the volumes were adjusted to allow for use with the lower

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| Table 1. Participant demographics | Healthy controls | Poor PHA responders |
|----------------------------------|-----------------|---------------------|
| Total number                     | 10              | 3                   |
| Age median                       | 29              | 27                  |
| Age range                        | 23–49           | 22–62               |
| Number of male subjects          | 4               | 1                   |
| Number of female subjects        | 6               | 2                   |

Poor responders were asymptomatic healthy control subjects within the laboratory without a known T-cell immunodeficiency, who gave a T-cell proliferation value of <45% (33.86, 18.42 and 29.45%) following stimulation by PHA.
PBMC concentration. Preliminary experiments were initially carried out on two control subjects using PBMC concentrations of both $1 \times 10^6$ and $5 \times 10^6$ mL$^{-1}$.

For use with a PBMC concentration of $1 \times 10^6$ mL$^{-1}$, 400 µL AIM-V was added to a Falcon tube containing 20 µL of the pre-prepared coated Beads and was vortexed thoroughly before centrifugation at 300 g for 5 min. The supernatant was carefully removed and a further 800 µL AIM-V was added and vortexed. Nineteen microlitres of the Bead suspension (to give a bead-to-cell ratio of 1 : 2 as determined by Miltenyi Biotec) were added to two Bead-stimulated PBMC populations of the round-bottomed plate. The plate was placed in the 5% CO$_2$ humidified 37°C incubator for 3 days. Thirty-eight microlitres of IL-2 at a concentration of 200 IU mL$^{-1}$ were added to the second of the two Bead-stimulated PBMC populations. The plate was then returned to the incubator for a further 3 days. The Miltenyi Biotec protocol is intended for T-cell expansion and recommends a 14-day assay, with fresh IL-2 advised to be added every 2–3 days. However, to enable our Bead assay to be run alongside the current assay and to determine whether or not IL-2 addition significantly impacts T-cell proliferation, IL-2 was added only on Day 3; halfway through the total 6-day incubation period.

Fluorochrome staining of PBMC and flow cytometry

After a 6-day incubation, the contents of each well were resuspended and like-wells were pooled together into sterile 5 mL Falcon tubes (Becton Dickinson, UK). The cells were washed in 1 mL PBS, vortexed and then centrifuged at 483 g for 5 min. The supernatant was decanted. Five microlitres of CD3 Perp (Becton Dickinson, UK) and 5 µL CD4 APC (Becton Dickinson, UK) were added and all tubes were vortexed and incubated at 4°C for 20 min. The cells were again washed in 1 mL PBS, vortexed and centrifuged at 483 g for 5 min. The supernatant of each tube was discarded and 400 µL one per cent formaldehyde (to fix the cells) was added. All tubes were vortexed again and stored at 4°C before flow cytometry was performed using the FACScalibur (Becton Dickinson, UK) following calibration with Calibrite beads (Becton Dickinson, UK). Fifty thousand CD3+ events were acquired.

Statistics

All statistics were performed using the GraphPad Prism software, version 6.04. A non-parametric, paired ANOVA (Friedman) test was used for the comparison of the higher and lower Bead and PBMC concentrations, and to analyse the data for the controls who responded poorly to the PHA assay. A non-parametric, paired T-test (Wilcoxon) was used to compare the percentage of T lymphocyte proliferation for the PHA and Bead assays. Correlations between the assays were determined by a Spearman test. Values of $p < 0.05$ were considered significant. The reference range of percentage proliferation for the Bead assay was determined by the 2.5th–97.5th percentiles as the data gathered were not normally distributed.

Results

Rounds of proliferation

A gating strategy was applied to select those cells within the PBMC populations that were the least granular and which also expressed CD3 and CD4, thus selecting CD4+ T cells. An example of the gating strategy for both unstimulated and PHA-stimulated PBMC populations is shown in Fig. 2A. A plot of side scatter vs. CD3 staining intensity was first applied and cells that were strongly CD3 positive and low in granularity were gated. This was to ensure that the CD3+ T lymphocytes were selected from the PBMC population (Fig. 2A). This gate was applied to a second plot to gather a population of CD3+ CD4+ helper T cells, and a gate was drawn around this population (Fig. 2B). The same principle as in Fig. 2A and B was applied to the PHA-stimulated population of PBMCs, although stimulated T cells can be more granular so a larger gate was drawn to include cells with an intermediate side scatter (Fig. 2C and D). The same principle was applied for all controls (healthy and poor PHA responders) with unstimulated, PHA-, Beads only- and Beads plus IL-2-stimulated PBMCs.

The data for each PBMC population (unstimulated, PHA-stimulated, Beads only and Beads plus IL-2) for each control were then displayed using histograms (Fig. 3). Numerical analysis was obtained following the application of two gates, M1 and M2, enabling the percentage proliferation of each PBMC population to be calculated. Peaks, defined as a visible apex or spike within the green line outside of the blue unstimulated region, correspond to rounds of proliferation. Among the 10 healthy controls tested, the mean number of peaks, and therefore rounds of proliferation, produced with PHA (Fig. 3A) was 1.70, increasing to 2.50 when the Beads were used alone (Fig. 3B) and further increasing to 3.10 following the addition of IL-2 on Day 3 of incubation (Fig. 3C).

Choosing the Anti-Biotin MACSiBead™ particles concentration

Two healthy controls were set up using Bead and PBMCs at a concentration of both $1 \times 10^6$ and $5 \times 10^6$ mL$^{-1}$. The percentage proliferation yielded for both controls when the Beads were used alone and with the addition of IL-2 at Day 3 of incubation was not significantly different ($p > 0.05$) at the lower concentration than the higher concentration (Fig. 4). However, there was a trend for the lower concentration to give greater proliferation. The decision was made at this stage to only use PBMCs and Beads at the lower concentration, thus matching the PBMC concentration required for the current PHA assay, for the remaining control samples.

Healthy controls: PHA vs. Beads vs. Beads + IL-2

In order for conclusions on T-cell proliferation following stimulation from the different mitogens to be made,
proliferation was assessed by all three methods (PHA, the Beads alone and the Beads plus IL-2) (Fig. 5) for 11 healthy controls. However, after analysing the percentage proliferation values for all 11, it was found that 10 gave a PHA proliferation value above the normal range of 45% and one gave a PHA proliferation below 45%. This 11th control was therefore included in the statistical analysis for the poor PHA responders.

No significant difference \( (p > 0.05) \) was observed between the percentage proliferation when PHA and the Beads alone were used \( (p\)-value obtained by a Wilcoxon test) (Fig. 5A). An association was observed, but this was not significant \( (r\)-value obtained by a Spearman correlation) (Fig. 5B). There was also no significant difference \( (p > 0.05) \) between the percentage proliferation seen when PHA and the Beads plus IL-2 were used \( (p\)-value obtained by a Wilcoxon test) (Fig. 5C). Again, an association was observed, but this was not significant \( (r\)-value obtained by a Spearman correlation) (Fig. 5D). Interestingly however, one healthy control showed a marked increase in proliferation with the Beads and following the addition of IL-2 compared with PHA. No significant difference \( (p > 0.05) \) was observed between the percentage proliferation seen when the Beads were used alone and when IL-2 was added on Day 3 across all healthy controls \( (p\)-value obtained by a Wilcoxon test) (Fig. 5E). A strong positive correlation was observed (Fig. 5F).

**Poor responders to the current PHA assay**

The response to the Bead assay was varied among those controls which responded poorly to PHA. The first showed a decrease in proliferation with the Beads alone and then a further slight decrease when IL-2 was added, the second showed a slightly reduced proliferation when the Beads were used alone but an increase in proliferation following the addition of IL-2 and the third showed an increase in proliferation with the Beads alone and no change when IL-2 was added (Fig. 6).

**Establishing a percentage proliferation reference range for the Anti-Biotin MACSiBead™**

For a normally distributed (parametric) set of data, a reference range of values for diagnostic use is determined by calculating the mean value \( \pm 2 \) SD. However, as the data gathered for this research were not normally distributed (non-parametric), the 2.5th and 97.5th percentile values were used instead (Table 2).

**Discussion**

A new assay has been developed within the Oxford Immunology diagnostic laboratory to assess the ability of T cells to...
proliferate following stimulation with anti-CD2/CD3/CD28-coated Anti-Biotin MACSiBead™ particles.

No significant difference was seen between the percentage of T-cell proliferation when PHA and the Beads (with and without the addition of IL-2) were used as the stimulants for the healthy control samples. There was also no significant difference observed between the proliferation seen when IL-2 was added to the Bead assay on Day 3 of incubation compared with when the Beads were used alone.

Two known poor PHA responders and one healthy control gave a percentage proliferation below the normal range for PHA (<45%). A variable T-cell proliferation response was seen among the three subjects when the Beads were used in place of PHA. A reference range of T-cell percentage proliferation values has been established for the Bead assay.

Justifications for performing the Anti-Biotin MACSiBead™ particles assay with the lower bead and PBMC concentration

The decision to conduct the Bead assay with the lower PBMC concentration of $1 \times 10^6$ mL$^{-1}$ over a concentration of $5 \times 10^6$ mL$^{-1}$ was made given that there was no significant...
difference between the results for the Bead and PHA assays at the two PBMC and Bead concentrations (Fig. 4). Ideally, the Bead assay would have been performed at the two PBMC concentrations on a larger number of healthy control samples. This may have strengthened the statistical analysis, and perhaps a significant difference between the T-cell proliferation values at the two different concentrations would have been seen. However, conducting the assay at both concentrations requires eight 3 mL lithium–heparin samples from each participant. It was therefore not practical or realistic to conduct the Bead assay at the two concentrations on all healthy controls.

A second reason in favour of using the lower concentration is that a smaller volume of blood would be required from the patient, which is ideal given that the Bead assay would be used on younger patients.

Finally, the Bead assay would be used alongside the current PHA test that requires the lower PBMC concentration, and thus, it is more practical for both assays to use the same cell concentration.

Anti-Biotin MACSiBead™ particles induce more rounds of proliferation

A trend noted throughout was that those PBMC populations incubated with the Beads typically underwent more rounds of proliferation than when PHA was used (Fig. 3). All cell populations were incubated for the same length of time, and thus, it can be concluded that, generally, the Beads may facilitate more efficient T-cell proliferation. Moreover, a change in the colour of the culture medium from red to yellow, indicating nutrient utilization for proliferation, was seen typically 24–48 h sooner in those PBMC populations incubated with the Beads than with PHA. More specifically, in the presence of anti-CD3/CD28-coated beads, T cells have been known to begin proliferating 40–50 h following incubation (Li and Kurlander, 2010).

It was also noted that the cell populations incubated with the Beads took less time to acquire during flow cytometry; the more rounds of proliferation which occurred, the less time it took to reach the set number of 50 000 events. All T cells express CD3, and CD28 functions as a co-receptor and thus, one possible reason for the faster proliferation could be that, as the Beads are coated with both anti-CD3 and anti-CD28 simultaneously, a stronger proliferative signal is delivered through co-stimulation by these two molecules. This double signal transduction causes an increase in the number of rounds of proliferation without inducing premature cell death (Li and Kurlander, 2010). This contrasts with PHA, which induces T-cell activation by bypassing the early TCR/CD3 signal transduction (Pignata et al., 1996), and has previously been shown to lead to functional impairment of PBMCs in vitro (Duarte et al., 2002).

Anti-Biotin MACSiBead™ particles may increase the percentage of T-cell proliferation in some healthy controls compared with PHA

The difference between the percentage of T-cell proliferation in the 10 healthy controls when the Beads were used in place of PHA was not significant (Fig. 5A). One control showed a 37% increase in proliferation when the Beads were used compared with PHA, and three controls showed a slight decrease in proliferation. The same phenomenon was observed with the same controls following the addition of IL-2 on Day 3 compared with PHA. This shows that the Beads may increase T-cell proliferation in some individuals, but also highlights the possibility that those subjects who show a normal proliferative response to PHA may not produce a significantly different response when another mitogen is used.

Although there was no significant correlation between the use of the Beads alone and PHA with the healthy control subjects (Fig. 5B), there did appear to be a trend for a positive association. It may be that this study was underpowered by the limited number of healthy controls available.

Addition of IL-2 did not significantly impact T-cell proliferation

No significant difference was found between the percentage proliferation yielded when the Beads were used alone and when IL-2 was added on Day 3 (Fig. 5E). However, in all but one of the 10 healthy controls, those cell populations incubated with IL-2 at Day 3 gave a slightly higher percentage proliferation. A strong positive correlation was observed between the use of the Beads only and the addition of IL-2 at Day 3 (Fig. 5F). This indicates that both assays give comparable results and therefore suggests that the addition of IL-2 is not necessary. Furthermore, the addition of IL-2 on Day 3 limits the number of days that the assay can be performed so...
as to allow for the work on Day 3 to be carried out during the routine laboratory hours.

**Poor responders to PHA may also respond poorly to Anti-Biotin MACSiBead™ particles**

The response of the three poor PHA responders to the Bead assay was varied (Fig. 6). Poor Responder 1 gave a percentage proliferation value of 33.86% with PHA. This dropped to 14.79% when the Beads were used alone, and the value fell by a further 0.72% in the PBMC population to which IL-2 was added on Day 3. Poor Responder 2 gave a percentage proliferation of 18.42% with PHA, and this value dropped to 17.08% when the Beads were used alone. However, the addition of IL-2 on Day 3 increased the proliferation to 30.08%. Poor Responder 3 (the 11th healthy control that gave an unexpected poor PHA response) yielded a T-cell proliferation of 29.45% with PHA, 40.55% when
the Beads were used alone and 40.72% when IL-2 was added on Day 3.

Given that no significant difference between the results for the poor PHA responders across the two assays was found, a definitive conclusion as to whether the Bead assay yields a different T-cell proliferation percentage in those who respond poorly to PHA cannot be made.

**Significance of the Anti-Biotin MACSiBead™ particles reference range in a diagnostic setting**

A reference range is a range of values where 95% of the results in healthy individuals fall within, thus excluding the upper and lower 2.5% (Ahmed, 2011). A reference range of values for any given diagnostic test can help a clinician to decide whether or not a result is abnormal and therefore a cause for concern; however, the whole clinical picture should always be taken into account. If a test result falls outside the reference range, it does not necessarily mean that the patient is unwell; 95% of the results are covered by the range, and therefore there is a 1 in 20 chance that a result for a healthy individual will fall outside this interval. This can be illustrated by the three poor PHA responders analysed who gave a T-cell percentage proliferation of <45% and therefore fell outside the normal range set by the clinical Immunology laboratory. None of these subjects have a known primary or secondary immunodeficiency and so would appear to simply be healthy individuals who happen to not fall within the 95% included in the PHA reference range.

Following the calculation of reference range values for the Bead assay both with and without the addition of IL-2 on Day 3 (Table 2), it can be concluded that only the proliferation yielded for Poor Responder 3 when the Beads were used alone fell into the established reference range. None of the poor responders fell within the reference interval for the Beads plus IL-2, and Poor Responders 1 and 2 did not fall into either reference interval.

**Limitations and further work**

Perhaps the most significant limitation of the practical work carried out was that all subjects used were adults. Primary immunodeficiencies are typically diagnosed at a young age, and therefore, the reference ranges calculated for the Bead assay may not be reflective of a younger cohort. Unfortunately, the ethical implications of recruiting healthy children for a continuation of this research are great.

Secondly, only 10 healthy controls were available. To strengthen the results and to validate the reference range, 100 healthy controls would ideally be recruited but this was simply not feasible.

To enhance the likelihood of attaining a significant difference in the T-cell percentage proliferation between poor PHA responders and the proposed Bead assay, a larger number of poor PHA responders should be recruited. However, this would be challenging in practical terms as most healthy individuals respond well to PHA.

To further investigate the capability of T-cell proliferation in healthy individuals who show a poor response to PHA, other stimuli could be used and compared with the Beads. *Phytolacca Americana*, more commonly known as Pokeweed, is a perennial plant found in North and South America, East Asia and New Zealand. When extracted from the roots with saline, pokeweed has been shown to have mitogenic properties such that it can stimulate the proliferation of both T and B lymphocytes (Bodger et al., 1979). Concanavalin A is a second mitogen capable of inducing T-cell proliferation (McCole et al., 1998). These mitogens may show a significant difference in T-cell proliferation compared with PHA.

**Conclusions**

An anti-CD2/CD3/CD28-coated Anti-Biotin MACSiBead™ particles assay has been established. The Bead and PBMC
concentration has been optimized for diagnostic use. The Bead assay was successfully performed, both with and without the addition of IL-2, on 10 healthy control subjects. The assay was conducted on three controls who respond poorly to PHA and comparisons between the two assays were made. Following the calculation of a reference range for the new assay, it was found that two out of the three individuals who responded poorly to PHA also responded poorly to the Beads, and one poor responder to PHA fell into the reference range for the Bead assay.

After taking the results into account, the proposed Bead assay will now be offered as a diagnostic test, alongside the current PHA assay, within the routine Oxford Immunology laboratory. The newly developed assay will not include the addition of IL-2 on Day 3.

The Bead assay provides a more physiological addition to the current PHA test and may be useful in determining the ability of T cells to proliferate in some patients who respond poorly to PHA.

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Author Biography
Louisa Green studied for her BSc (Hons) Biomedical Science degree at Oxford Brookes University over 4 years whilst working as a Trainee Biomedical Scientist at the Oxford University Hospitals NHS Trust. After spending the first 2 and a half years of her course rotating through each laboratory discipline for 6 months at a time, she chose to spend the final 18 months of her employment specializing in Immunology. Here, Louisa completed both her undergraduate research project and generic registration Portfolio for the Institute of Biomedical Science, and she is now a fully qualified Biomedical Scientist. She graduated from Oxford Brookes University in June 2014 winning the Best in Year project prize, and within the next year plans to apply for a 4-year integrated MRes/PhD in Infection and Immunity.

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References
Ahmed, N. (2011) Clinical Biochemistry, Oxford University Press, Oxford.
Beadling, C. and Smith, K. A. (2002) DNA array analysis of interleukin-2-regulated immediate/early genes, Medical Immunology, 1 (2), 1–14.
Bodger, M. P., McGiven, A. R. and Fitzgerald, P. H. (1979) Mitogenic proteins of pokeweed, Immunology, 37, 785–792.
Buckley, R. H. (2004) Molecular defects in human severe combined immunodeficiency and approaches to immune reconstitution, Annual Review of Immunology, 22, 625–655.
Cornish, G. H., Sinclair, L. V. and Cantrell, D. A. (2006) Differential regulation of T-cell growth by IL-2 and IL-15, Blood, 108 (2), 600–608.
Duarte, R. F., Chen, F. E., Lowdell, M. W. et al. (2002) Functional impairment of human T-lymphocytes following PHA-induced expansion and retroviral transduction: implications for gene therapy, Gene Therapy, 9 (20), 1359–1368.
Geha, R. S., Notarangelo, L. D., Casanova, J.–L. et al. (2007) Primary immunodeficiency diseases: an update from the International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee, The Journal of Allergy and Clinical Immunology, 120 (4), 776–794.
Hall, A. and Yates, C. (2010) Immunology, Oxford University Press, Oxford.
Kelly, B. T., Tam, J. S., Verbsky, J. W. et al. (2013) Screening for severe combined immunodeficiency in neonates, Clinical Epidemiology, 5, 363–369.
Li, Y. and Kurlander, R. J. (2010) Comparison of anti-CD3 and anti-CD28-coated beads with soluble anti-CD3 for expanding human T cells: differing impact on CD8T cell phenotype and responsiveness to restimulation, Journal of Translational Medicine, 8 (104), 1–15.
McCole, D. F., Doherty, M. L., Baird, A. W. et al. (1998) Concanavalin A-stimulated proliferation of T cell subset-depleted lymphocyte populations isolated from Fasciola hepatica-infected cattle, Veterinary Immunology and Immunopathology, 66 (3–4), 289–300.
Parish, C. R., Glidden, M. H., Quah, B. J. C. et al. (2009) Use of the intracellular fluorescent dye CFSE to monitor lymphocyte migration and proliferation, Current Protocols in Immunology, 84, 4.9.1–4.9.10.
Pignata, C., Sanghera, J. S., Soiffer, R. J. et al. (1996) Defective activation of mitogen-activated protein kinase after allogeneic bone marrow transplantation. Blood, 88 (6), 2334–2341.
Tasher, D. and Dalal, I. (2012) The genetic basis of severe combined immunodeficiency and its variants, Journal of the Application of Clinical Genetics, 5, 67–80.