



# Inosine Acedoben Dimepranol promotes an early and sustained increase in the natural killer cell component of circulating lymphocytes: A clinical trial supporting anti-viral indications

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## ABSTRACT

Inosine Acedoben Dimepranol (IAD), licensed for the treatment of cell-mediated immune deficiencies associated with viral infections, has been reported to impact a variety of immune parameters both *in vitro* and *in vivo*. Here we report the results from a clinical trial where multiple lymphocyte subsets – CD19 + B cells, CD3 + T cells, CD4 + T-helper cells, FoxP3<sup>hi</sup>/CD25<sup>hi</sup>/CD127<sup>lo</sup> regulatory T cells (Tregs), CD3 –/CD56 + NK cells, and CD3 +/CD56 + NKT cells – were, together with serum immunoglobulins and IgG subclasses, followed during 14 days of IAD administration to ten healthy volunteers; these selected from 27 individuals pre-screened *in vitro* for their capacity to respond to IAD as gauged by increases in the percentage of Treg and/or NKT cells arising in PHA-stimulated cultures. While a transient spike and dip in Treg and T-helper fractions, respectively, was noted, the outstanding consequence of IAD administration (1 g po, qds) was an early and durable rise in NK cells. For half the cohort, NK cells increased as a percentage of total peripheral blood lymphocytes within 1.5 h of receiving drug. By Day 5, all but one of the volunteers displayed higher NK cell percentages, such elevation – effectively a doubling or greater – being maintained at termination of study. The IAD-induced populations were as replete in Granzyme A and Perforin as basal NK cells. The novel finding of IAD boosting phenotypically competent NK numbers in healthy individuals supports the drug's indicated benefit in conditions associated with viral infection and reinforces the potential for uplift where immune performance may be compromised.

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## 1. Introduction

Inosine Acedoben Dimepranol (IAD) is a synthetic purine derivative comprising the *p*-acetamidobenzoic acid salt of *N*, *N*-dimethyl-amino-2-propanol (DiP.PAcBA) and the β polymorph of the β anomer of inosine in a 3:1 M ratio. Also known by its tradenames Immunovir, Isoprinosine, Viruxan, Inosiplex, Methisoprinol and Inosine Pranobex, IAD, currently registered in 43 countries worldwide, has been licensed since 1971 for the treatment of cell-mediated immune deficiencies associated with various viral infections including human papillomavirus (HPV), herpes simplex virus (HSV), varicella-zoster virus (VZV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV) [1]. IAD is also

indicated for the treatment of measles virus-induced subacute sclerosing panencephalitis (SSPE) and post-viral chronic fatigue syndrome (CFS) [2,3].

Over the four decades since its introduction, numerous *in vitro* and *in vivo* studies have been undertaken on IAD's mode of action in both animals and humans. Depending on the models used, effects from IAD potentially compatible with its clinical indications have been assigned to a range of immune cell subtypes, functions, and effectors (e.g. cytokines, antibodies) [4–15]. Among these are studies demonstrating an ability of IAD to restore otherwise defective NK activity in various scenarios of immune compromise, including that associated with: HIV infection [16], uremia [17], chronic fatigue syndrome [3], and aging [18].

The present study was designed to appraise the outcome of IAD administration to a cohort of healthy subjects, focusing on several of the major immune players with respect to the control of viral infections including, for the first time, an assessment of drug action on Treg and NKT subsets. Any modulation of immune parameters resulting from administering IAD to the healthy population should likely extend to similar

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outcomes in individuals with those chronic viral conditions for which IAD is currently indicated. At the same time, studying IAD actions in normal healthy volunteers offers a steady (uniform) background on which to gauge any consensual change – irrespective of pathogenic state – that may result from its application while also having the potential to disclose benefits outside of and additional to current indications.

## 2. Materials and methods

### 2.1. Pre-screen of healthy volunteers for competence to respond to IAD *in vitro*

Venous blood from 27 healthy individuals was collected into K2 EDTA vacutainers® (BD, Oxford, UK). Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a layer of Ficoll Paque (GE Healthcare, Chalfont St Giles Buckinghamshire, UK). Cells were washed in RPMI media supplemented with antibiotics (Life technologies, Paisley, UK) (penicillin 100 U/ml, streptomycin 100 µg/ml) and resuspended at  $1 \times 10^6$  cells/ml in complete media supplemented with 10% heat-inactivated FCS (Life Technologies, Paisley, UK), L-glutamine (Life Technologies, Paisley, UK) and antibiotics before stimulation with PHA-L (phytohemagglutinin-L; 5 µg/ml (Sigma-Aldrich, Gillingham, Dorset, UK)) in either the absence or presence of IAD (600 µg/ml) and incubated for 4 days at 37 °C and 5% CO<sub>2</sub>. The percentage Treg and NKT cells present in the recovered population was determined by immunophenotyping as below.

### 2.2. Clinical trial

The ten best responding donors from the pre-screen *in vitro* were entered into a Phase 1 Trial, receiving their first dose of IAD within 21 days of pre-screen. On Days 1 to 13 of the trial, each of the ten volunteers received a 1 g IAD (po, qds). On the morning of Day 14, subjects received a single 1 g oral dose. Samples for serum immunoglobulins and cellular immunophenotyping were taken prior to first dosing with IAD on Day 1 and following morning dosing on Days 1, 3, 5, 7, 10, and 14.

### 2.3. PBMC isolation and lymphocyte subset phenotyping

PBMC were isolated from whole blood by centrifugation on a layer of Ficoll Paque (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK). The plasma layer was removed and stored for immunoglobulin assessment (below). Cells were washed in RPMI media supplemented with antibiotics (Life Technologies, Paisley, UK) (penicillin 100 U/ml, streptomycin 100 µg/ml) before addition ( $1 \times 10^6$  cells/well) to a 96 well plate and washed twice in stain buffer (PBS supplemented with 2% FCS). For lymphocyte subset identification, cells were stained with the fluorochrome-conjugated antibodies indicated for 30 min on ice then washed and fixed using BD Cytotfix/CytoPerm™ Cell Fixation/Permeabilisation kit (according to manufacturer's instructions) (BD, Oxford, UK) and washed again before analysis on a flow cytometer. B cells: anti-CD19-PE (BD, Oxford, UK). T-cells: CD3-V500 (BD, Oxford, UK). T-helper cells: anti-CD3-V500 (BD, Oxford, UK), anti-CD4-PE/CY7 (Biolegend, London, UK). Tregs: anti-CD4-PE/CY7 (Biolegend, London, UK), anti-CD127-FITC (eBioscience, Hatfield, UK), anti-CD25-APC (Biolegend, London, UK), and anti-CD3-V500 (BD, Oxford, UK) as above followed by anti-FoxP3-PE (BD, Oxford, UK) for a further 30 mins on ice. CD4+ cells, that were CD25<sup>hi</sup>, CD127<sup>lo</sup> and FoxP3+ were deemed Tregs. NK and NKT cells: anti-CD3-FITC (BD, Oxford, UK) and anti-CD56-APC (BD, Oxford, UK). Cells that were CD56+ were deemed NK cells and cells that were both CD3+ and CD56+ deemed NKT cells.

### 2.4. Granzyme A and Perforin staining

Frozen PBMC samples from all 10 trial subjects isolated prior to first administration of IAD and on day 14 of trial were thawed and stained

with anti-CD3-V500 (BD, Oxford UK) and anti-CD56-APC (BD, Oxford UK) for 30 min on ice before fixing/permeabilising (as above) and then staining with anti-Granzyme A-PE (Biolegend, London, UK) and anti-Perforin-FITC (Biolegend, London, UK) for a further 30 min on ice prior to analysis by flow cytometry.

### 2.5. Multiparameter flow cytometry

Flow cytometry was performed using a CyAn™ ADP Analyzer (Beckman Coulter Ltd, High Wycombe, UK). Instrument setup and calibration were performed using BD Calibrite Beads™ (BD, Oxford, UK). Data analysis was done with FlowJo software.

### 2.6. Assessment of plasma immunoglobulin levels

Plasma was centrifuged ( $1000 \times g$ ) and the supernatant collected and stored ( $-80^\circ\text{C}$ ) until analysis for IgG (IgG SPAplus® kit), IgA (IgA SPAplus® kit), IgM (IgM SPAplus® kit), (Binding Site, Birmingham, UK) and subclasses of IgG (IgG1 (IgG1 SPAplus kit), 2 (IgG2 SPAplus kit), 3 (IgG3 SPAplus kit), and 4 (IgG4 SPAplus kit)) (Binding Site, Birmingham, UK). Methods were followed to manufacturer's instructions. Immunoglobulins were analysed using a Cobas® 6000 analyzer (Roche, Burgess Hill, UK).

### 2.7. Statistical analysis

Data were tested for normality by the Shapiro-Wilk test. To test whether granzyme or perforin levels were different from baseline values, the data were analysed using a two-tailed unpaired *t*-test and significance was taken to be  $p < 0.05$ . For the analysis of subset percentages, data were analysed by one way analysis of variance followed by a Dunnett's multiple comparison test comparing levels following treatment to baseline levels. Significance was taken to be  $p < 0.05$ .

## 3. Results

### 3.1. *In vitro* pre-screen of healthy volunteers for competence to respond to IAD

A preliminary study on the capacity of IAD to impact the number and performance of specific lymphocyte subsets *in vitro* disclosed an ability of the drug to increase the percentage of Treg and/or NKT cells (including the minority iNKT cell subset as defined by reactivity with the monoclonal antibody 6B11) within stimulated cultures of PBMC from a majority of the healthy donors investigated: other subsets including total T and B cells, CD4+ and CD8+ T cells, and NK cells were neither substantively nor reproducibly impacted under these conditions (data not detailed). Correspondingly, the degree of change in these parameters on an *in vitro* pre-screen of healthy subjects' PBMC formed the criterion for volunteer inclusion into a clinical trial designed to capture the *in vivo* influence of IAD on defined lymphocyte subsets, together with serum immunoglobulins, over 14 days of continuous administration.

Table 1 details the influence of IAD (600 µg/ml) on the percentage of phenotypically defined CD4+ regulatory T cells (Treg) and NK-T cells (CD3+CD56+) arising in 4 day cultures of PHA-stimulated PBMC from 27 healthy volunteers. All showed an increased percentage of Treg cells in response to IAD with 18 of the 27 also increasing the proportion of NKT cells present.

The universal increase in Treg percentages, noted both in the present and our previous pre-clinical studies, established this parameter as the initial guiding factor to volunteer selection. An accompanying increase in the percentage of NKT cells appearing in culture was chosen as a secondary inclusion factor. Occasional samples needed to be discounted and volunteers excluded due to wbc counts laying outside of the normal range while others failed to progress due to self-withdrawal of volunteers from continuation to trial. Of the ten remaining best responding

**Table 1**  
Pre-screen of healthy volunteers by virtue of PBMC response to IAD *in vitro*.

Sample <sup>a</sup>	Treg IAD % change <sup>b</sup>	NKT IAD % change	Subject <sup>c</sup>
1	133	210	1
2	27	107	
3	177	66	
4	46	122	
6	61	1	
8	95	90	5
9	31	81	
12	53	–37	
15	48	33	
17	63	3	
18	22	–27	6
19	104	–9	
21	45	157	
22	80	0	
24	35	2	
25	142	–37	3
27	65	63	9
32	65	41	10
34	53	–40	8
36	60	54	
37	155	69	
38	83	354	
40	106	29	
42	64	128	4
43	81	17	
44	133	33	
45	11	58	

<sup>a</sup> Blood samples from 27 of 45 volunteers (18 of which were subsequently excluded on basis of selection criteria unrelated to data presented herein).

<sup>b</sup> Percentage change in the Treg or NKT subset (as total of all lymphocytes) due to the presence of IAD (600 µg/ml) during 4 day stimulation of PBMC with PHA *in vitro*.

<sup>c</sup> Subjects 1–10 selected for inclusion into trial.

volunteers based on Treg increases all but one had an accompanying increase in NKT cells and these ten were selected to enter into the Phase I Trial, receiving their first dose of IAD within 21 days of pre-screen.

### 3.2. Consequence of IAD administration on serum immunoglobulins

On Days 1 to 13 of the trial, each of the ten volunteers received a 1 g oral dose of IAD 4 times daily. On the morning of Day 14, subjects received a single 1 g oral dose. Samples for serum immunoglobulins and immunophenotyping were taken prior to first dosing with IAD on Day 1 and following morning dosing on Days 1, 3, 5, 7, 10, and 14.

Over the 14 days of the trial, serum levels of the three major immunoglobulins – IgG, IgA, and IgM – essentially remained constant across the subjects (Fig. 1). Pre-dosing and Day 14 serum samples were additionally measured for the four IgG isotypes, again showing IAD without impact on either IgG1, IgG2, IgG3, or IgG4 concentrations (Fig. 2).

### 3.3. Consequence of IAD administration on peripheral lymphocyte subsets

As can be seen from Fig. 3a–b, no substantive, durable pattern of change over baseline was observed during the 14 days of drug administration in either overall B- or T-cell compartments; neither in the CD4 + T-helper subset, apart from a brief dip at Day 5 (Fig. 3c). The same held true for phenotypically-defined Treg cells: with the exception of a transient spike at Day 3 (Fig. 3d).

The consistent, and by far the most compelling change, was an increase in the percentage of NK cells among the total lymphocyte population (Fig. 3e). The increase commenced early among the cohort: the majority between 1 and 3 days of IAD administration; with five subjects evidencing augmented NK cell percentages 1.5 h after first dosing. By Day 5, all but one volunteer had a higher proportion (over baseline) of their lymphocytes represented by NK cells: this effectively greater

than doubling of the NK contribution being maintained at Day 14 (trial termination).

Among the rarer NKT subset, there was a suggestion of change – primarily an increase. However, no clear or consistent pattern reaching statistical significance was discerned across the ten subjects (Fig. 3f).

Statistically significant increased NK cell percentages arising from IAD administration as judged by mean change across the ten subjects were registered at Day 5 (mean fold increase = 2.1,  $p < 0.05$ ), Day 10 (2.6,  $p < 0.01$ ), and Day 14 (2.1,  $p < 0.05$ ). With the exceptions of Treg cells on Day 3 (mean fold increase = 1.8,  $p < 0.05$ ) and T-helper cells on day 5 (mean fold decrease = 0.6,  $p < 0.01$ ) no other significant change in any of the subsets as a result of administering IAD was observed.

Next, the NK population was interrogated with respect to CD56<sup>bright</sup> versus CD56<sup>dim</sup> subsets: the former being abundant cytokine producers but only weakly cytotoxic before activation [19]. As expected, the CD56<sup>bright</sup> subset was the minor of the two populations in each of the subjects and throughout the course of the trial. Changes in the majority CD56<sup>dim</sup> subset mirrored those seen in the total NK population as might be anticipated (Fig. 4). Overall, the minority CD56<sup>bright</sup> subset showed less of a trend towards increasing in frequency than the CD56<sup>dim</sup> subset. Only at Day 10 was there a significantly ( $p < 0.05$ ) increased percentage of CD56<sup>bright</sup> NK cells compared to pre-administration of IAD.

### 3.4. IAD-augmented NK cells contain Granzyme A and Perforin

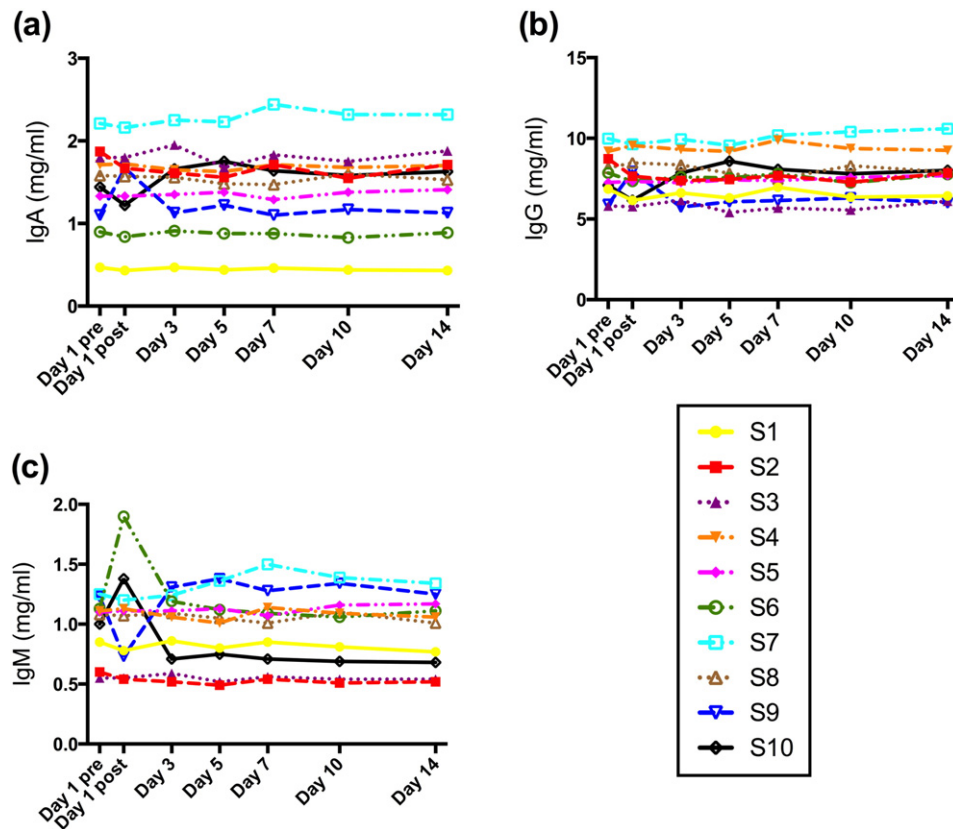
From the 10 subjects (S1–10) entered into the clinical trial, stored cells were revived from the Day 1 pre-drug administration and Day 14 post-administration time points. Each population was assessed for the percentage of CD3-CD56 + cells expressing Granzyme A (upper panel) or Perforin (lower panel), respectively.

The CD3 – /CD56 + NK cell population was then assessed for the presence of two key NK effector molecules: Granzyme A and Perforin. Unlike above where cells were studied direct from volunteers, the measure of Granzyme A and Perforin was made retrospectively on previously frozen PBMC from the participants of the clinical trial. Notably, the augmented contribution from CD3 – /CD56 + cells to total PBMC as a consequence of IAD administration was as evident in frozen as in fresh cells. Again, all but one of the ten subjects demonstrated such an increase which at Day 14 averaged (over all subjects) 1.7-fold ( $p < 0.05$ ) compared to baseline.

Fig. 5 shows that among CD3-CD56 + cells, the percentage positive for Granzyme A or Perforin remained as high in the Day 14 IAD-impacted population as was observed pre-drug administration on Day 1.

## 4. Discussion

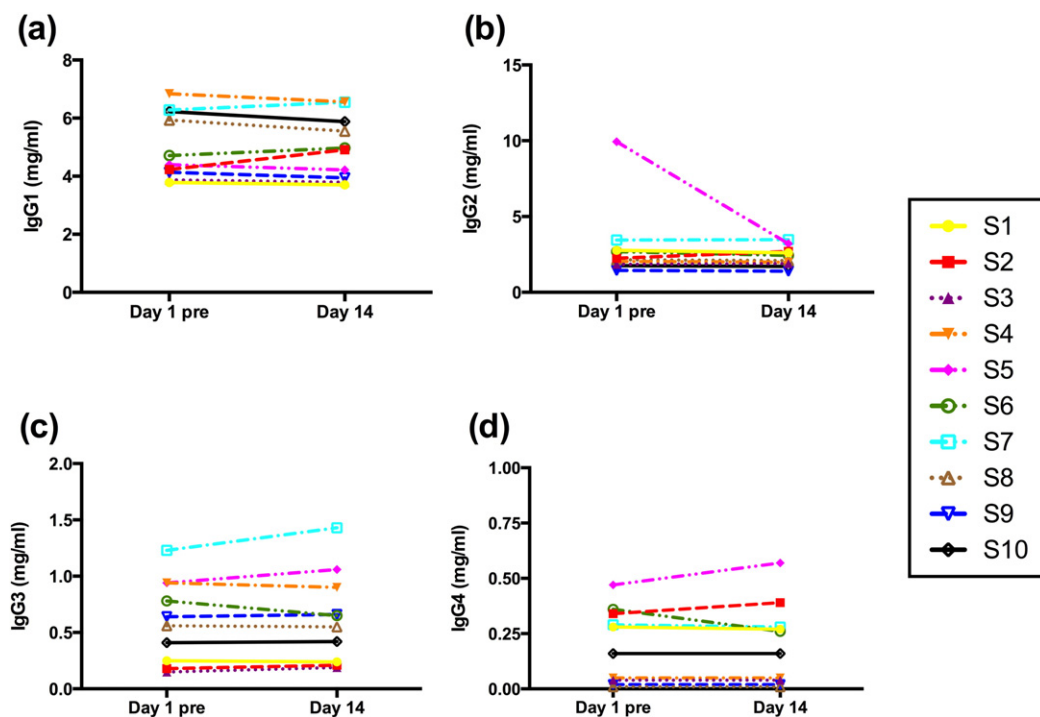
The impact of IAD on NK cells registered in the present study is compatible with and supports its current indication for the management of viral infections and associated complications. A beneficial influence on NK cells was shown to begin early, rise progressively, and persist throughout the 14 days of IAD administration to the participants of the clinical trial. Remarkably, all subjects showing this IAD-promoted NK uplift were healthy, without sign or manifestation of immune compromise. IAD has been used widely and studied intensely for four decades with no evidence of it promoting lymphopenia. The observed increase in percentage NK cells among total lymphocytes therefore likely results from a boost in absolute cell number. Indeed, on enumerating subsets within the PBMC fraction collected from Ficoll gradients during the trial, the NK population at Day 14, for example, now averaged 225% greater in number than at baseline (*i.e.* Day 1 pre-drug administration). While caveats must be placed around the accuracy of extrapolating these numbers directly to whole blood counts, such pattern of increased recovered NK cells was consistent over time while no other subset registered such magnitude of change by this measure.



**Fig. 1.** Influence of IAD administration on plasma immunoglobulins in healthy volunteers. Plasma immunoglobulins of ten subjects (S1–10) receiving a 1 g oral dose of IAD 4 times daily (with a single 1 g oral dose on the morning of Day 14) were assessed prior to first dosing Day 1 and following morning dosing on Days 1, 3, 5, 7, 10, and 14: (a) IgG; (b); IgM; (c) IgA.

Evidence for IAD impacting NK cells has been reported previously though never before documented as early and consistently, nor among healthy subjects, as here. Many of the historical studies focused on IAD restoring NK cell activity and/or number in individuals that were

otherwise deficient in this aspect. For example, Diaz-Motoma and colleagues [3] described how among CFS patients administered IAD over 12 weeks, those showing clinical improvement to drug correspondingly manifested increased NK number and function where before both were



**Fig. 2.** Plasma IgG subclasses before and after IAD administration. From the 10 subjects (S1–10) entered into the clinical trial, stored plasma was thawed from the Day 1 pre-drug administration and Day 14 post-administration time points and IgG isotype levels measured by subclass-specific ELISA: (a) IgG1; (b) IgG2; (c) IgG3; (d) IgG4.



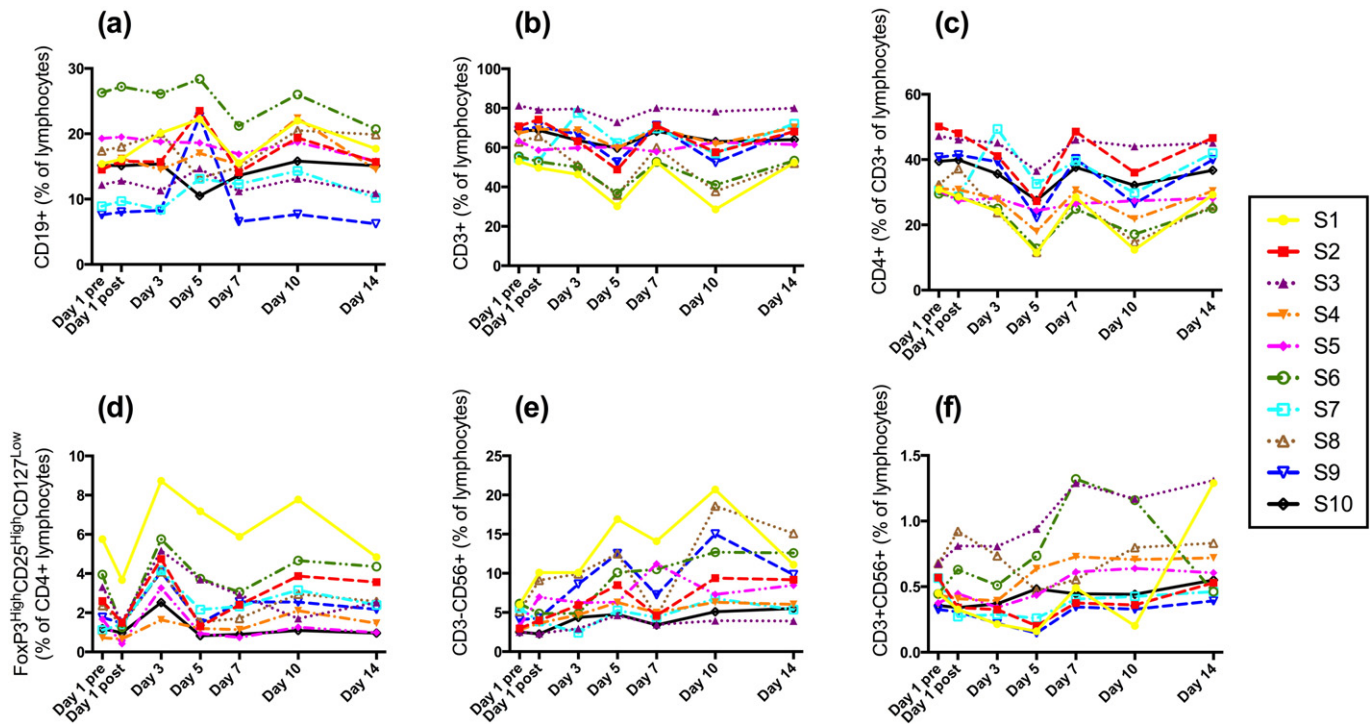


Fig. 3. Influence of IAD administration on lymphocyte subset distribution in healthy volunteers.

suppressed. Bekesi et al. [16] reported how 28 days of IAD administration 'normalized' otherwise depressed NK cell number and function in homosexual males at high risk of AIDS. The same group had earlier reported that both halfway through and a year on from a 28 day administration regime, immunodepressed patients with prolonged generalized lymphadenopathy showed improved NK activity [20]. A rare previous study on healthy individuals reported IAD administration augmenting NK activity among peripheral blood cells following drug cessation but did not interrogate NK numbers [21]. With respect to *in vitro* action, IAD has been reported as restoring otherwise depressed NK activity in the elderly [18] and in patients with uremia [17] with one study observing a boost in NK activity from the peripheral blood of healthy subjects [22].

The steady longitudinal *in vivo* action of IAD on NK cells was not matched across other immune parameters investigated. This was despite the clinical trial being predicated on a pre-clinical study that disclosed an unexpected property of IAD: its ability to increase the proportion of Treg and NKT cells contained within PBMC populations. Indeed, efficacy in this regard provided the basis of the pre-screen of volunteers for recruitment into the trial here. Notwithstanding, within the trial there was a suggestion/trend of IAD positively modulating NKT percentages and while there was no overt durable increase in the Treg fraction, a statistically significant doubling of Tregs as a consequence of IAD administration was observed at Day 3.

It should be noted that in the pre-clinical study, the action of IAD was followed against PBMC undergoing stimulation (PHA): here, individual

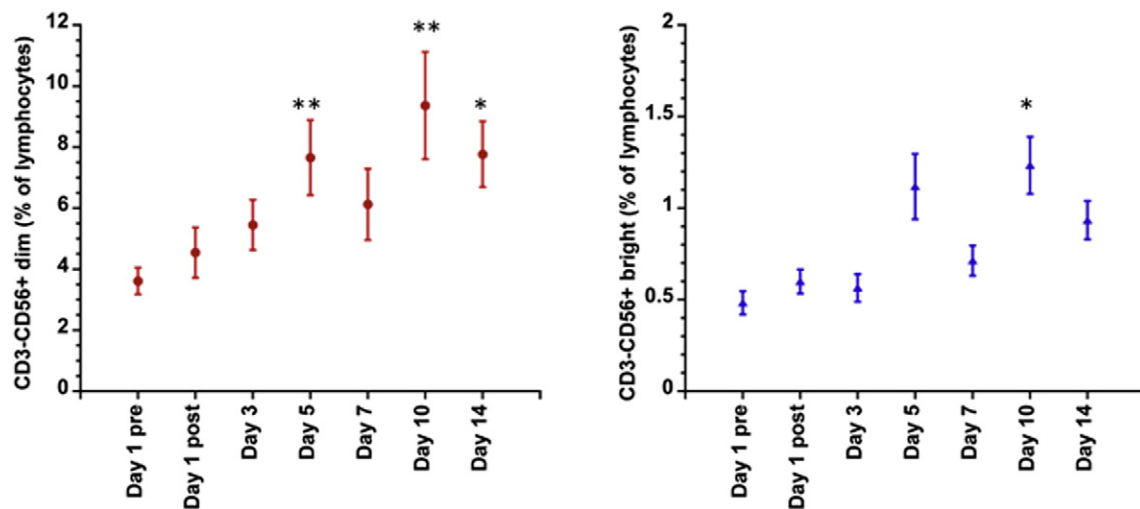


Fig. 4. Influence of IAD administration on NK cells subsets in healthy volunteers. As for Fig. 3 but with CD3-CD56 + NK cells gated by virtue of CD56<sup>dim</sup> vs. CD56<sup>bright</sup> expression and mean values  $\pm$  SEM from the ten subjects plotted (one-way ANOVA followed by Dunnett's multiple comparison test). \* $p < 0.05$  compared to Day 1 pre-admin; \*\* $p < 0.01$  compared to Day 1 pre-admin.

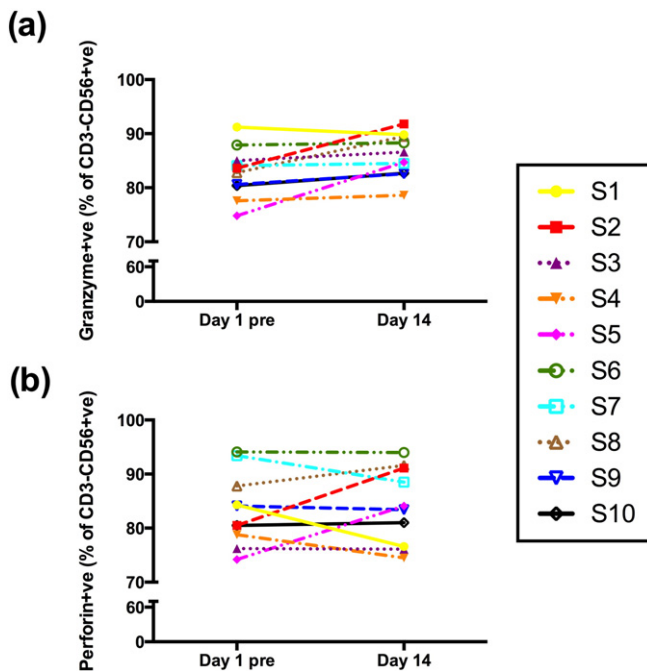


Fig. 5. Percentage of NK cells positive for Granzyme A and Perforin before and after IAD administration.

subsets have the potential to expand and/or die selectively. The immune status of the healthy subjects within the trial would instead be 'resting', basal. These differences could, potentially, account for the greater impact of IAD on Treg and NKT fractions *in vitro* as compared to what was observed *in vivo*. Conversely, the clear NK increase observed during the trial was not predicted from the prior *in vitro* study of IAD impact on PHA-stimulated PBMC (nor indeed against unstimulated PBMC; Gardiner et al., School of Biochemistry & Immunology, TCD, unpublished observations). This being the case, then a conceivable mechanism behind the observed NK increase *in vivo* – underscored by it starting early – is one of IAD promoting mobilization of cells from tissue reservoirs to blood. While NK cells are actively recruited from blood to sites of viral infection [23], in order to accumulate in effective numbers in the target organ, NK cells must first of all mobilize from spleen and bone marrow stores to peripheral blood [24]. Thus agents – seemingly like IAD here – that facilitate the latter would be considered beneficial in such a scenario. Within this context, it is of note that the minority CD56<sup>bright</sup> NK subset showed a less impressive increase during the trial than the majority CD56<sup>dim</sup> population. Though the CD56<sup>bright</sup> subset appears to dominate in selected body tissues, the opposite holds true for spleen and bone marrow where CD56<sup>dim</sup> NK cells prevail [25].

The present study confirms and extends the rationale for IAD being considered as a useful medicament for disease modification in the context of viral infections. Moreover, the lack of any major impact on overall B- or T-cell number, or on serum immunoglobulins underscores the drug's established safety profile. That the IAD-induced blood NK cells were replete in Granzyme A and Perforin supports earlier clinical studies describing augmented NK activity among isolated PBMC following IAD administration [3,21,26]. While the limitation on blood sample size (and correspondingly cell numbers available) during the present Phase I trial precluded the ability to assess NK cytotoxicity directly, the earlier studies demonstrating IAD augmenting NK function [3,21,26] together with the observed increase here in the percentage of cells carrying the two cytotoxic effectors are each compatible with IAD increasing functionally competent NK cells in healthy individuals. Moreover, a separate *in vitro* study revealed IAD increasing the percentage of Granzyme B (GrB)-positive NK cells in donors deplete in basal GrB (Gardiner et al., School of Biochemistry & Immunology, TCD, unpublished observations).

The NK axis has a vital role in several key aspects of host defence and immune surveillance: its importance in combating and protecting from multiple classes of virus is undisputed. As an example, a recent review details the central contribution of NK cells to the control of: influenza virus, CMV, HIV-1, and hepatitis C virus [27]. With regards to influenza, it has been suggested that NK lymphopenia may correlate with increased disease severity [28]. Of note, in this context, is a recent independent Phase 4 trial in a large patient cohort with confirmed acute respiratory viral infections where IAD was deemed not only safe but also effective in non-obese subjects <50 years of age with clinically diagnosed influenza-like illnesses in terms of time to resolution of associated disease symptoms [29].

Through a capacity to enrich blood in phenotypically adept NK cells – as shown here – IAD gains the potential to offer benefit against viral infection in a variety of settings including otherwise healthy individuals and perhaps, importantly, our growing elderly population where NK performance is known to be compromised and where its underperformance may be a key contributor to the increased rates of viral infection associated with immunosenescence [18,30,31].

### Authorship contributions

JG, NMB, SJC supervised the project and wrote the paper. JO'D and SD designed the study. SRA and ASN carried out the laboratory studies. GG and CAB supported the laboratory studies and the preparation of the paper.

### Compliance with ethical standards

The experiments on this study were carried out after written informed consent had been obtained and according to the conditions of favourable ethical opinion from the Research Ethics Committee of Northern Ireland (REC reference 13/NI/0036).

### Conflict of interests

Celentyx Ltd and Immcell Ltd partnered on this project.

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