1 Effect of a high and low dose of caffeine on human lymphocyte activation in response to

2 antigen stimulation

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1   **Abstract**

2   This study investigated the effect of caffeine on antigen-stimulated lymphocyte activation. 6

3   males rested for 3.5 h after ingesting 0 (PLA), 2 (2CAF) or 6 (6CAF) mg·kg^{-1} body mass of

4   caffeine. The number of antigen-stimulated NK CD69^{+} cells increased on 6CAF at 1h compared

5   with placebo (P=0.021). Caffeine did not influence the number of antigen-stimulated CD69^{+} T

6   cells or the GMFI expression of CD69 on antigen-stimulated lymphocytes, suggesting caffeine

7   has little effect on antigen-stimulated lymphocyte activation.

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9   Keywords: Caffeine, methylxanthine, immune, CD69, lymphocytes, T cell, NK cell.

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1 **Introduction**

2 Caffeine is a legal and socially acceptable drug and as such is commonly found in most people's

3 diets. Caffeine's effects have been determined in a number of physiological systems including

4 the cardiovascular, endocrine and central nervous systems (reviewed by Benowitz 1990).

5 However, little research has focused on how caffeine may affect various aspects of the immune

6 system (reviewed by Horrigan et al. 2006). Of the research that has been conducted, most have

7 used the rodent model and very few studies have used concentrations of caffeine considered

8 relevant to "normal" human consumption (~70 µM or less; Graham 2001). Even fewer have

9 used human participants (reviewed by Horrigan et al. 2006; Schiedel et al. 2013). It should also

10 be taken into account that there are significant differences in caffeine metabolism and the

11 plasma half-life of caffeine (rodents: 0.7-1.2 h vs. humans: 4-6 h) between rodents and humans

12 that may in fact limit extrapolation of findings from non-human models.

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14 At rest (unstimulated), small numbers of peripheral blood lymphocytes express low levels of

15 CD69 (Borrego et al. 1999; Testi et al. 1994). CD69 is one of the earliest cell surface

16 glycoproteins expressed on lymphocytes following activation (Ziegler et al. 1994). Although the

17 precise role of CD69 has not as yet been elucidated, many *in vitro* studies have demonstrated

18 that once expressed on lymphocytes, this antigen appears to be involved in triggering

19 subsequent cell proliferation, cytotoxicity and secretion of cytokines; such as tumour necrosis

20 factor-α and interferon-γ via its proposed actions as a co-stimulatory receptor (Borrego et al.

21 1999; Moretta et al. 1991; Mueller et al. 2002; Ziegler et al. 1994).

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1 Caffeine is a non-selective adenosine receptor antagonist and stimulates adrenaline release

2 from the adrenal medulla. T and NK cells express both β_2-adrenoreceptors and adenosine

3 receptors (T cells, A_{2A}, A_{2B} and A_3; NK cells, A_1, A_{2A} and A_{2B}), with the density of these receptors

4 increased following activation (Blay and Hoskin 2007; Shephard 2003). Both adenosine and

5 adrenaline have been shown to inhibit early lymphocyte activation (Lappas et al. 2005;

6 Shimamiya et al. 2003), via activation of adenylate cyclase and increased intracellular cAMP

7 (Raskovalova et al. 2006). Therefore, caffeine may have the potential to modulate lymphocyte

8 activation either directly via adenosine receptor antagonism or indirectly via stimulation of

9 catecholamine release.

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11 To the authors knowledge no study to date has investigated the effect of caffeine on human

12 lymphocyte activation in response to antigenic stimulation. Therefore, the aim of this study

13 was to investigate the effect of a high and low dose of caffeine on antigen-stimulated

14 lymphocyte (CD4$^+$, CD8$^+$ and CD3$^-$CD56$^+$) activation over a period of 3.5 h, as assessed by

15 expression of CD69.

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17 **Materials and methods**

18 *Participants and trial protocol*

19 Six healthy recreationally active participants [age 25 (2) years; weight 75 (7) kg] gave written

20 informed consent to take part in the study, which was approved by the local Ethical Advisory

21 Committee.

22 Participants completed an extensive health-screening questionnaire along with a physical

1   activity questionnaire during their initial visit to the laboratory to ascertain if they were suitable

2   for the study. Any participants that were currently on medication or had reported symptoms of

3   infection in the 4 weeks prior to the study were excluded. Participants' habitual caffeine

4   consumption was also recorded at the beginning of each study using a caffeine consumption

5   questionnaire to classify participants as low, moderate or high habitual caffeine users. On the

6   day of each main trial, a subsequent health-screening questionnaire was administered to

7   confirm that participants were not experiencing any symptoms of URTI and were happy to take

8   part in the study. Daily caffeine intake amongst participants was varied and ranged from 40 –

9   220 mg.day^{-1}, with an average intake of 120 ± 85 mg.day^{-1}. Two subjects were characterised as

10   as light users (< 50 mg. day^{-1}) and the other 4 as moderate users (50 – 250 mg. day^{-1}).

11

12   To standardize nutritional status, participants completed a 24 h food diary the day before the

13   first main trial and were asked to follow this during the 24 h preceding the second and third

14   main trial. Participants were randomly assigned to either the placebo (PLA), low caffeine dose

15   (2CAF) or high caffeine dose (6CAF) trial and acted as their own controls in a repeated-

16   repeated-measures, single-blind, cross-over design. Each trial was separated by 1 week.

17   Participants arrived at the laboratory at 08:20 h following an overnight fast of 12 h and 60 h

18   abstention from caffeine. After 10 min of resting quietly an initial (0 h) blood sample was

19   obtained from an antecubital forearm vein by venepuncture. Following the blood sample,

20   participants ingested 0, 2 or 6 mg·kg^{-1} body mass of caffeine powder (BDH Laboratory Supplies,

21   Poole, UK) taken in the form of cellulose capsules (G & G Food Supplies Ltd, West Sussex, UK)

22   with 300 ml plain water. For the PLA trial, participants ingested 6 mg·kg^{-1} body mass of

1   dextrose powder (BDH Laboratory Supplies, Poole, UK).   Participants then rested quietly in the

2   the laboratory for 3.5 h, during which time water was consumed *ad libitum*.   Further blood

3   samples were taken after 1 h (09:30 h), 2.5 h (11:00 h) and 3.5 h (12:00 h).

4

5   *Lymphocyte culture and flow cytometry*

6   Five ml of whole blood containing sodium heparin was immediately placed on ice after draw

7   and mixed for 20 min before setting up cultures and performing flow cytometry, as previously

8   described (Fletcher and Bishop 2012).

9

10   *Serum caffeine and plasma adrenaline*

11   Whole blood collected into a plain monovette (5.5 ml) was left to clot for 1 h before centrifuged

12   at 1500 g for 10 min at 4°C.   Heparinized plasma was obtained from blood collected into a

13   lithium heparin monovette (7.5 ml).   Samples were spun at 1500 g for 10 min at 4°C within 5

14   min of collection. Serum caffeine and plasma adrenaline concentration were measured as

15   previously described (Fletcher and Bishop 2012).

16

17   *Statistical analysis*

18   The data were examined using a two-factor (trial x time) analysis of variance with repeated

19   measures design.   If the data were not normally distributed, statistical analysis was carried out

20   on the logarithmic transformation of the data.   Assumptions of sphericity in the data were

21   checked, and adjustments in the degrees of freedom for the ANOVA were made using the

22   Huynh-Feldt method of correction where appropriate.   Any significant data were assessed using

1   using Student's paired *t*-tests with Holm-Bonferroni adjustments for multiple comparisons.

2   Statistical significance for this study was accepted at P<0.05. The observed powers of the

3   reported main and interaction effects are all >0.8.

4

5   **Results**

6   Serum caffeine concentration at 1 h, 2.5 h and 3.5 h after caffeine ingestion was higher on 6CAF

7   than 2CAF and PLA and higher on 2CAF than PLA (F=24.058, all P=0.000; Table 1). At 1 h and 2.5

8   h after caffeine ingestion, plasma adrenaline concentration was higher on 6CAF than PLA

9   (F=9.273, 1 h, P=0.038; 2.5 h P=0.003; Table 1). Neither caffeine ingestion nor time of day

10   affected the number of CD4$^+$ and CD8$^+$ cells within the circulating lymphocyte population (Table

11   1). However, the number of CD3$^-$CD56$^+$ cells was higher 1 h and 2.5 h after caffeine ingestion on

12   6CAF than on PLA and 2CAF (F=5.806, 1 h: 6CAF v PLA P=0.002, 6CAF v 2CAF P=0.042; 2.5 h:

13   6CAF v PLA P=0.046, 6CAF v 2CAF P=0.036; Table 1). There was no effect of caffeine on the

14   number of unstimulated CD4$^+$, CD8$^+$ and CD3$^-$CD56$^+$ cells or antigen-stimulated (both doses)

15   CD4$^+$ and CD8$^+$ cells expressing CD69 (unstimulated, data in Supplementary Table 1; antigen-

16   stimulated, Table 2). A high dose of caffeine increased the number of antigen-stimulated CD3$^-$

17   CD56$^+$ cells expressing CD69 1 h following caffeine ingestion compared with PLA (6CAF 1:4000 v

18   PLA 1:4000, F=2.536, P=0.021; Fig. 1). Neither caffeine ingestion nor time of day affected the

19   geometric mean fluorescence intensity (GMFI) of CD69 expression on unstimulated

20   (Supplementary Table 1) or antigen-stimulated CD4$^+$, CD8$^+$ and CD3$^-$ CD56$^+$ cells (Table 3).

22

1 **Discussion**

2 The main findings of this study suggest that (i) a high dose of caffeine increases the number of

3 antigen-stimulated $CD3^-CD56^+$ cells expressing CD69 1 h following caffeine ingestion, (ii)

4 ingesting high and low doses of caffeine has little influence on antigen-stimulated T ($CD4^+$ and

5 $CD8^+$) cell activation and (iii) caffeine ingestion has little effect on the density of CD69

6 expression on antigen-stimulated T and NK cells, as assessed by the GMFI expression of CD69.

7

8 In the present study both high and low doses of caffeine had little effect on the density of

9 expression of the CD69 receptor on antigen-stimulated T and NK cells. This is in contrast to

10 several reports that caffeine at physiological concentrations decreases other lymphocyte

11 functions such as mitogen/antigen stimulated lymphocyte proliferation, antibody production

12 and cytokine production (Kantamala et al. 1990; Rosenthal et al. 1992). However, these studies

13 studies used rodent models to investigate the possible effects of caffeine. Although the

14 present study in humans and the previous studies in rodents have not used exactly the same

15 measures of lymphocyte function, there is good evidence to suggest that CD69 expression is a

16 reliable measure of subsequent T cell proliferation and NK cell cytotoxicity *in vitro* (Green et al.

17 2003; McFarlin et al. 2004). Recently Schiedel et al. (2013) investigated the effects of caffeine

18 on both unstimulated and mitogen-stimulated human lymphocyte proliferation. These

19 reserachers found that caffeine at physiologically relevant concentrations (0.1-30µM) had no

20 effect on unstimulated or PHA-stimulated lymphocyte proliferation. The contrasting findings

21 between human and rodent studies itself raises issues of validity when comparing results, as

22 caffeine metabolism and plasma half-life between rodents and humans differs significantly

1   (Fredholm et al. 1999).

2

3   At physiological concentrations, it is thought that caffeine's main mechanism of action is via

4   adenosine receptor antagonism (Fredholm et al. 1999).   However, under physiological

5   conditions, caffeine will only actively compete as an adenosine receptor antagonist when the

6   receptors are engaged by endogenous adenosine (Yang et al. 2009). As such, it could be

7   suggested that in the present study caffeine (regardless of dose) had little effect on antigen-

8   stimulated lymphocyte activation (GMFI expression of CD69) in resting participants due to

9   plasma adenosine concentrations being too low to engage caffeine as an adenosine receptor

10 antagonist. It should be noted that plasma adenosine was not measured in this study and as

11 such this explanation is only speculation. However, support comes from the exercise literature

12 where prolonged intensive exercise, which is known to markedly increase plasma adenosine

13 (Vizi et al. 2002), increased the number of unstimulated $CD4^+$ and $CD8^+$ T cells expressing CD69

14 (Bishop et al. 2005). In contrast, plasma adrenaline concentration mirrored the effects of a high

15 dose of caffeine on the number of NK cells expressing CD69 in response to antigen stimulation.

16 Given adrenaline's known role in lymphocyte mobilization (Shephard 2003) this may suggest an

17 adrenaline-mediated mobilization of this specific population of cells.

18

19 In summary, this is the first study in humans to determine that a high (6 mg·kg^{-1}) but not a low

20 (2 mg·kg^{-1}) dose of caffeine increases the number of NK cells expressing the early activation

21 marker CD69 in response to antigen stimulation 1 h following caffeine ingestion. This may be

22 related to increases in adrenaline and subsequent mobilization of this specific population of

1   cells. In contrast, caffeine ingestion (regardless of dose) has little effect on the density of the

2   expression of CD69 on antigen-stimulated T and NK cells in humans. These findings are in

3   contrast to previously published studies in the animal model and as such suggest that perhaps

4   rodents are not a reliable model with which to investigate the effects of caffeine on human

5   lymphocyte functions.

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	0 h	1 h	2.5 h	3.5 h
Table 1: Serum caffeine, plasma adrenaline concentrations, total lymphocyte count and number of circulating $CD4^+$, $CD8^+$ and $CD3^-CD56^+$ cells during PLA, 2CAF and 6CAF trials.				
Serum caffeine concentration (μmol·L^{-1})				
PLA	0 (0)	0 (0)	0 (0)	0 (0)
2CAF	0 (0)	16 (8) *‡	17 (3) *‡	15 (3) *‡
6CAF	0 (0)	48 (11) †‡	47 (11) †‡	43 (9) †‡
Plasma adrenaline concentration (nmol·L^{-1})				
PLA	0.10 (0.04)	0.09 (0.05)	0.11 (0.07)	-
2CAF	0.13 (0.07)	0.12 (0.05)	0.18 (0.08)	-
6CAF	0.08 (0.06)	0.15 (0.07) **‡‡	0.26 (0.10) *‡	-
Total lymphocytes (x10^9 cells·L^{-1})				
PLA	1.90 (0.30)	1.70 (0.10)	1.80 (0.20)	1.80 (0.30)
2CAF	1.80 (0.40)	1.60 (0.40)	1.70 (0.30)	1.80 (0.03)
6CAF	1.90 (0.30)	2.00 (0.30)	2.00 (0.30)	1.80 (0.10)
$CD4^+$ cells (x10^9 cells·L^{-1})				
PLA	0.77 (0.23)	0.70 (0.11)	0.68 (0.13)	0.67 (0.11)
2CAF	0.66 (0.19)	0.62 (0.16)	0.62 (0.15)	0.71 (0.14)
6CAF	0.66 (0.15)	0.62 (0.13)	0.58 (0.19)	0.64 (0.08)
$CD8^+$ cells (x10^9 cells·L^{-1})				
PLA	0.58 (0.10)	0.50 (0.09)	0.51 (0.09)	0.52 (0.13)
2CAF	0.54 (0.13)	0.50 (0.14)	0.50 (0.11)	0.54 (0.10)
6CAF	0.50 (0.10)	0.60 (0.25)	0.50 (0.23)	0.49 (0.13)
$CD3^-CD56^+$ cells (x10^9 cells·L^{-1})				
PLA	0.30 (0.12)	0.22 (0.07)	0.28 (0.11)	0.30 (0.16)
2CAF	0.22 (0.09)	0.21 (0.07)	0.20 (0.10)	0.30 (0.13)
6CAF	0.29 (0.11)	0.41 (0.17) ††‡	0.37 (0.07) ††‡‡	0.30 (0.06)

Values are mean (SD).

*$P<0.01$, ** $P<0.05$: Significantly higher than PLA

† $P<0.01$, †† $P<0.05$: Significantly higher than PLA and 2CAF

‡ $P<0.01$, ‡‡ $P<0.05$: Significantly higher than 0 h within trial

	0 h	1 h	2.5 h	3.5 h	
1	**Table 2**: Number of antigen-stimulated CD4^{+}, CD8^{+} and CD3^{-}CD56^{+} cells expressing CD69 during				
2	PLA, 2CAF and 6CAF trials.				

	0 h	1 h	2.5 h	3.5 h
CD4$^+$ CD69$^+$ cells (x10^6 cells·L^{-1}; 1 :4000)				
PLA	25 (21)	28 (19)	32 (20)	41 (23)
2CAF	25 (15)	30 (15)	43 (30)	44 (30)
6CAF	28 (20)	34 (25)	34 (21)	35 (21)
CD4$^+$ CD69$^+$ (x10^6 cells·L^{-1}; 1 :8000)				
PLA	15 (8)	22 (14)	24 (15)	37 (24)
2CAF	19 (10)	20 (13)	34 (21)	40 (31)
6CAF	22 (17)	28 (20)	35 (27)	34 (26)
CD8$^+$ CD69$^+$ (x10^6 cells·L^{-1}; 1 :4000)				
PLA	45 (37)	33 (20)	45 (24)	50 (27)
2CAF	26 (18)	32 (20)	38 (23)	45 (26)
6CAF	29 (17)	50 (42)	41 (26)	37 (23)
CD8$^+$ CD69$^+$ (x10^6 cells·L^{-1}; 1 :8000)				
PLA	28 (16)	30 (19)	34 (25)	52 (33)
2CAF	19 (14)	24 (14)	31 (20)	41 (29)
6CAF	23 (15)	40 (35)	44 (37)	36 (28)
CD3$^-$CD56$^+$CD69$^+$ (x10^6 cells·L^{-1}; 1 :8000)				
PLA	39 (25)	42 (35)	43 (22)	57 (33)
2CAF	33 (29)	30 (23)	30 (29)	62 (60)
6CAF	51 (61)	47 (29)	61 (43) ‡‡	55 (21)

Values are mean (SD).

‡‡ *P*<0.05: Significantly higher than 0 h within trial

Table 3: GMFI expression of CD69 on antigen-stimulated CD4$^+$, CD8$^+$ and CD3$^-$CD56$^+$ cells during PLA, 2CAF and 6CAF trials.

	0 h	1 h	2.5 h	3.5 h
CD4$^+$ CD69$^+$ GMFI (1:4000)				
PLA	100 (0)	101 (15)	102 (17)	89 (11)
2CAF	100 (0)	119 (11)	130 (50)	122 (30)
6CAF	100 (0)	110 (35)	106 (39)	111 (38)
CD4$^+$ CD69$^+$ GMFI (1:8000)				
PLA	100 (0)	109 (36)	108 (26)	112 (33)
2CAF	100 (0)	126 (14)	126 (33)	134 (27)
6CAF	100 (0)	118 (25)	137 (34)	113 (14)
CD8$^+$ CD69$^+$ GMFI (1:4000)				
PLA	100 (0)	106 (14)	108 (33)	111 (20)
2CAF	100 (0)	113 (21)	125 (19)	125 (22)
6CAF	100 (0)	98 (29)	100 (35)	115 (32)
CD8$^+$ CD69$^+$ GMFI (1:8000)				
PLA	100 (0)	120 (26)	118 (33)	129 (35)
2CAF	100 (0)	120 (12)	113 (31)	129 (38)
6CAF	100 (0)	102 (18)	109 (22)	110 (23)
CD3$^-$CD56$^+$CD69$^+$ GMFI (1:4000)				
PLA	100 (0)	107 (14)	119 (17)	119 (28)
2CAF	100 (0)	100 (16)	101 (18)	115 (21)
6CAF	100 (0)	105 (9)	111 (20)	121 (34)
CD3$^-$CD56$^+$ CD69$^+$ GMFI (1:8000)				
PLA	100 (0)	116 (36)	120 (28)	132 (55)
2CAF	100 (0)	107 (12)	109 (16)	113 (12)
6CAF	100 (0)	107 (13)	113 (17)	116 (29)

Values are mean (SD). The GMFI expressions are expressed as a percentage relative to the 0 h value.

1 **Figure Caption**

2 **Fig. 1.** Number of antigen-stimulated (1:4000) CD3⁻CD56⁺ NK cells expressing CD69 within the

3 circulating lymphocyte population during PLA, 2CAF and 6CAF trials. * significantly higher on

4 6CAF than PLA, P=0.021; † significantly higher than 0 h within trial (P<0.05). Values are means +

5 SD.