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Report for:

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Report 209-008. Immune Activation and Modulation of Inflammation In Vitro – Testing for cytokines and growth factors in 2-hour culture supernatants.

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Report 209-002. Immune Modulation In Vitro – Module 1.

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Report 209-008. Immune Activation and Modulation of Inflammation In Vitro – Testing for cytokines and growth factors in 2-hour culture supernatants.

1 Executive Summary

Mioty Voajanahary is a Madagascar-based grower and harvester of a unique aloe species, *Aloe macroclada.* The harvested material is used to produce a nutritional supplement, Alomac, through very gentle drying and processing methods.

Alomac was tested for its ability to activate immune cells and induce cytokine production. Two preparations of Alomac were evaluated: an aqueous extract (containing bioactive compounds readily available for absorption) and an in vitro digest (IVD) fraction (compounds from the solid fraction released after simulating gastrointestinal digestion).

Results – General

The cytokine levels in culture supernatants were tested by a multiplex Luminex assay covering pro-inflammatory, anti-inflammatory, regulatory cytokines, and growth factors. The testing reported here involves the early responses at 2 hours and are compared and contrasted to the previously reported results after 24 hours.

Both Alomac's aqueous and in vitro digested fractions triggered direct immune activation, with increased production of the majority of the cytokines tested, already at 2 hours.

In stark contrast, when immune cells were cultured under inflamed culture conditions, Alomac rapidly (within minutes) reprogrammed the immune cells to respond much less to an inflammatory insult.

When immune cells were treated with Alomac immediately prior to a viral mimetic challenge, there was a highly selective response involving six chemokines. Chemokines are a group of cytokines that induce directional movement of immune cells toward the

source of the chemokine secretion. Under a viral mimetic challenge, Alomac selectively modulated these chemokines differently at 2 hours compared to 24 hours. These results suggest a selective effect on immune cell recruitment into virally infected tissue during antiviral responses.

Conclusions

Alomac showed broad and potent immune-activating and -modulating effects. Alomac effectively activated innate immune cells and amplified anti-viral and immune-activating cytokines, suggesting enhanced immune support. The findings indicate that Alomac supports strong immune activation while at the same time reducing the immune response to inflammation.

Report 209-008. Immune Activation and Modulation of Inflammation In Vitro – Testing for cytokines and growth factors in 2-hour culture supernatants.

2 Purpose

To evaluate and compare the immune-activating and immunomodulatory effects of a natural product Alomac using human immune cell cultures in vitro under normal and challenged conditions. As part of a previous project, cultures of peripheral blood mononuclear cells (PBMC) were performed in the absence versus presence of an inflammatory insult, and the culture supernatants were collected after 2 and 24 hours. The 24-hour culture supernatants were tested for cytokine production using a comprehensive panel of 27 pro- and anti-inflammatory cytokines, anti-viral peptides, and regenerative growth factors. To complete this testing, the 2-hour culture supernatants were also tested, and the results are reported here.

3 Background

Natural products often aim to support the immune system. Alomac is such a product whose immunological effects are of interest. The immune system comprises a complex network of innate and adaptive cells working together to defend against pathogens. Many natural compounds can influence this system by activating immune cells or modulating inflammatory reactions. In particular, because a large portion of the body's immune tissue is associated with the gut, orally consumed products can interact with immune cells in the gastrointestinal tract, including antigen-presenting cells and lymphocytes, and thereby affect systemic immunity. An effective immunomodulating product should ideally bolster the body's ability to mount a rapid defense when challenged (i.e. trigger appropriate activation and cytokine release) but also prevent or resolve excessive inflammation in a timely manner.

In this context, three types of actions of a natural product are especially relevant:

- Direct effects on immune activation and modulation: The product may immediately influence immune cell activity in the absence of a pathogen.
- Priming for a bacterial challenge: The product may condition or "prime" immune cells so that they react differently (potentially more effectively or controlled) when subsequently exposed to a bacterial inflammatory stimulus.

- Priming for a viral challenge: Similarly, the product may prime immune responses to a subsequent viral-mimicking stimulus.

Understanding these actions for Alomac provides insight into how the product might perform in real-world use where the immune system is often challenged by infections. This study used in vitro human blood cell cultures to investigate those actions, which is a well-established model for assessing potential immune benefits of natural products.

4 Work Performed

4.1 Test Products

Table 1. Test products compared in this project.

Latin name	Source	Handling
Aloe macroclada	Mioty Voajanahary	Aqueous fraction
Aloe macroclada	Mioty Voajanahary	In vitro digested fraction
Buffer control	NIS Labs	Control for the in vitro digested fraction

4.2 Preparation of Aqueous and Post-Aqueous In Vitro Digest Fractions

4.2.1 Aqueous fraction

For aqueous handling, stock solutions of each test product were prepared using hot water to prepare a 100 g/L suspension. The powders were re-hydrated for 1 hour under gentle agitation at room temperature, during which time the hot water was allowed to cool to room temperature. Insoluble material was precipitated by centrifugation. On each lab testing day, serial dilutions of the aqueous fraction were prepared in physiological saline, so each test was done across a broad dose range.

4.2.2 In vitro digestion of post-aqueous solid fraction

The post-aqueous solid fraction of the powders went through an in vitro digest process to mimic the breakdown when products are consumed:

- Further removal of remaining aqueous compounds:
    - The post-aqueous solid fraction of each test product was resuspended in physiological saline, agitated, and precipitated by centrifugation, to further dilute and remove any remaining water-soluble components from the original powder.
- Digest:

  - The material was treated with alpha-amylase to mimic the enzymatic breakdown of polysaccharides that begin in the oral cavity.
  - Then the material was treated with pepsin under acidic conditions, to mimic the enzymatic and chemical breakdown of proteins that occurs in the stomach.
  - The in vitro digest fraction was treated with pancreatin under normal pH to mimic the breakdown in the upper part of the intestines, then the pancreatin was heat inactivated.
  - Remaining insoluble compounds were removed by centrifugation.
- Multiple portions were prepared and stored frozen at -20°C, such that one portion was thawed on each lab testing day.

Inactivation of the digestive enzymes prior to cell cultures: Alpha-amylase is neutralized by the acidic environment during pepsin treatment. Pepsin is inactivated by raising pH back to neutral at the end of the pepsin treatment. Pancreatin is inactivated by hot water bath exceeding 65C for 20 minutes. Therefore, there were little or no active digestive enzymes in the in vitro digested fraction. The in vitro digest method is based on existing and published protocols for in vitro digested fraction on aloe products, mushrooms, whole foods, and egg membrane.[1]

4.3 Tests performed

In the previous project, the immune-activating and -modulating properties of the test products were documented and compared in a select panel of lab assays to expand on our knowledge of their fundamental biological activities. As part of this work, cultures of peripheral blood mononuclear cells (PBMC) were performed in the absence versus presence of an inflammatory insult, and the culture supernatants were collected after 2 and 24 hours.

As part of the previous project, the 24-hour cultures supernatants were tested for cytokine production using a comprehensive panel of 27 pro- and anti-inflammatory cytokines, anti-viral peptides, and regenerative growth factors.

To complete this testing, the 2-hour culture supernatants were also tested, and the results are reported here.

Results from these reports will serve as a hub for further decision-making on which direction(s) may be most productive for Alomac. Further validation testing will be needed if this progresses to manuscript writing.

5 Cytokines and Growth Factors

5.1 General Introduction to the Cytokine Data

The culture supernatants from each culture are used for testing of a broad panel of pro- and anti-inflammatory cytokines, anti-viral peptides, and regenerative growth factors, using a 27-plex Luminex magnetic bead array and the MagPix® multiplexing system. The cytokine panel included**: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, eotaxin, basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF.** The panel contains biomarkers with multi-faceted roles and has generated results that may allow us to discuss pro-inflammatory immune-activating effects, anti-inflammatory effects, and restorative effects through growth factors.

For full names and descriptions, see tables 27-30 in Appendix B.

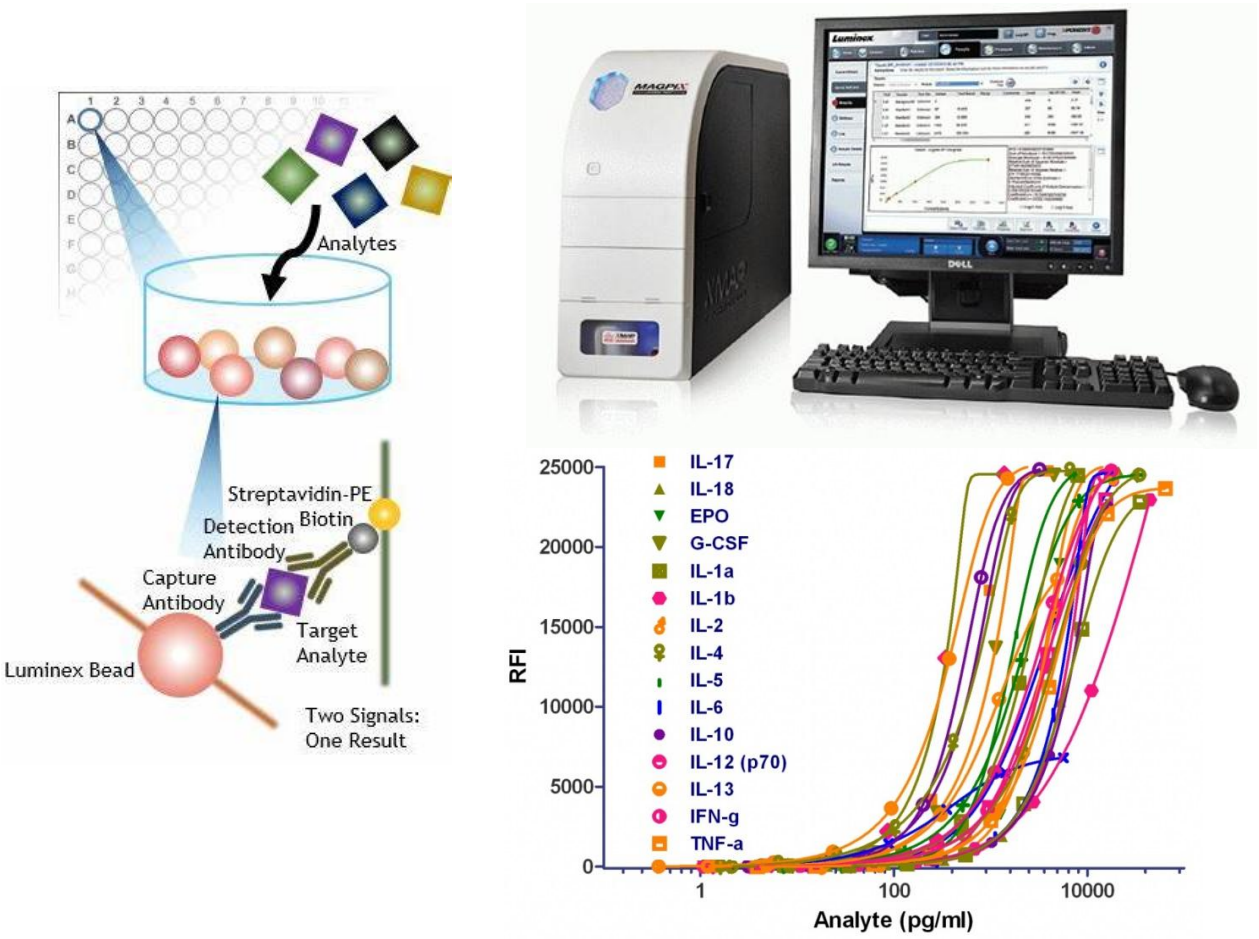


Figure 1. Luminex multiplex protein array using magnetic bead principles to capture and quantify multiple biomarkers in one small biological sample

5.2 Cytokine Levels – Direct Effects

In the absence of external immune stimulation, the aqueous and in vitro digest of Alomac directly affected levels of many of the 27 tested cytokines, **already after 2 hours of cell culture**.

Overview of changes to all detectable cytokines after 2 hours (Tables 2-9 below):

Several cytokines were affected by both the aqueous fraction and the in vitro digested fraction of Alomac, where 10 of the 15 pro-inflammatory cytokines were significantly increased following exposure to the test products, including IFN-γ, IL-1β, MCP-1, and TNF-α. Similarly, 4 of the 5 growth factors tested were significantly increased after exposure to the aqueous and in vitro digested fractions of Alomac. The in vitro digested fraction was able to increase the levels of the pro-inflammatory cytokines IL-17A, Eotaxin, and the growth factor PDGF-BB where the aqueous product produced no significant changes when compared to untreated cell cultures.

Two anti-inflammatory cytokines were tested, IL-1ra and IL-10. The aqueous fraction of Alomac did not cause significant changes to IL-1ra levels, but the in vitro digested fraction triggered a significant increase in cultures treated with lower doses when compared to untreated cultures except at the highest dose where a decrease was observed. Neither the aqueous fraction nor the in vitro digested fraction caused significant changes to IL-10.

Several cytokines with regulating properties were affected by both the aqueous and in vitro digested fractions of Alomac. The aqueous fraction significantly increased IL-2, IL-4, and IL-15, while decreasing IL-9. The in vitro digested fraction triggered increased levels of IL-2 and IL-15, while also triggering decreased levels of IL-2, as well as IL-4 and IL-9.

Selected cytokines as examples of rapid changes to cytokine levels already at 2 hours are described here, and shown in Figures 2-11 below:

- **IFN-γ (Interferon-gamma)** – An anti-viral peptide and activator of NK cells: The aqueous and in vitro digested fractions of Alomac significantly increased IFN-γ levels compared to untreated cultures. All 4 aqueous doses caused an increase, but the increase was only significant at 0.13g/L and 2g/L. The in vitro digested fraction triggered increases at the three lowest doses, with 1.56mL/L and 6.25mL/L causing significant increases. **This showed that IFN-γ production was initiated early, leading to the high levels at 24 hours.**
- **IL-1β (Interleukin-1-beta)** – An immune-activating pro-inflammatory cytokine: Both the aqueous and in vitro digested fractions of Alomac triggered increases of IL-1β when compared to untreated control cultures. The increase was stronger for the in vitro

digested fraction of Alomac. **This increase showed that IL-1β production was initiated early, leading to the high levels at 24 hours.**

- **MCP-1 (Monocyte chemoattractant protein-1, CCL2)** – A pro-inflammatory chemokine: MCP-1 is a chemokine that attracts inflammatory cells such as monocytes and macrophages towards the site of inflammation in order to mount immune responses. The aqueous and in vitro digested fractions of Alomac also triggered increased levels of MCP-1, with the increase being greater for the in vitro digest product. At 1.56mL/L MCP-1 levels were 400% higher than in cells not treated with Alomac. **This shows that even though MCP-1 production was initiated early.**
- **IL-10 (Interleukin-10)** – An anti-inflammatory cytokine: IL-10 levels were not significantly altered after cells were exposed to either the aqueous or in vitro digested fractions of Alomac when compared to untreated cultures. These 2-hour results suggest that Alomac stimulates broad immune-activating, pro-inflammatory responses rapidly, without impacting the anti-inflammatory cytokine IL-10 until later. **This shows that the robust production of IL-10 seen at 24 hours was initiated later, since changes were not detectable at 2 hours.**
- **G-CSF (Granulocyte-colony stimulating factor)** – A growth factor associated with stem cell mobilization: Both the aqueous and in vitro digested fraction of Alomac increased G-CSF production when compared to untreated cultures. **This shows that the production of G-CSF was triggered early in the process and continued to increase to reach the high levels seen after 24 hours of cell culture. This early production is promising and may be associated with the known effect of Alomac on stem cell surveillance.**[ii]

Table 2. Pro-inflammatory cytokines - direct effects: levels of significance

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IFN-γ	21		55	-4
IL-1β	118		222	-15
IL-5	58		73	-7
IL-6	384		686	-11
IL-8	479		831	-34
IL-12 (p70)	3	-3	6	-11
IL-13		-9	4	-9
IL-17A	66		100	-11
Eotaxin	12	-4	38	-13
IP-10	15		33	-11
MCP-1	287	-20	445	-59
MIP-1α	6,292		7,966	-24
MIP-1β	251		325	-8
RANTES	35	-68	19	-55
TNF-α	340		1,000	

Table 3. Anti-inflammatory cytokines - direct effects: levels of significance

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IL-1ra	59		84	-37
IL-10	7	-1	10	-8

Color Description	
	Increase
	Decrease

Color	P-valve	Description
	≥0.10	Not statistically significant
	<0.10	Statistical trend
	<0.05	Statistical significance
	<0.01	High level of significance

Table 4. Cytokines/chemokines with regulating properties - direct effects: levels of significance

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IL-2	117		147	-25
IL-4	92		156	-10
IL-7	32	-4	9	-12
IL-9		-61	23	-44
IL-15	42		74	-8

Table 5. Growth factors - direct effects: levels of significance

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
Basic FGF	44		125	-12
PDGF-BB	1	-4	25	-12
VEGF	27		67	-8
GM-CSF	29	-4	36	-9
G-CSF	388		505	-29

Color Description	
	Increase
	Decrease

Color	P-valve	Description
	≥0.10	Not statistically significant
	<0.10	Statistical trend
	<0.05	Statistical significance
	<0.01	High level of significance

Table 6. Pro-inflammatory cytokines - direct effects: levels of magnitude

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IFN-γ	63		86	-4
IL-1β	152		438	-15
IL-5	58		73	-7
IL-6	745		686	-11
IL-8	479		831	-34
IL-12 (p70)	3	-3	8	-11
IL-13		-9	10	-9
IL-17A	73		100	-11
Eotaxin	12	-4	38	-13
IP-10	40		37	-11
MCP-1	287	-20	445	-59
MIP-1α	6,488		8,221	-24
MIP-1β	251		325	-8
RANTES	35	-68	19	-55
TNF-α	1,282		1,000	

Table 7. Anti-inflammatory cytokines - direct effects: levels of magnitude

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IL-1ra	110		84	-37
IL-10	16	-1	17	-8

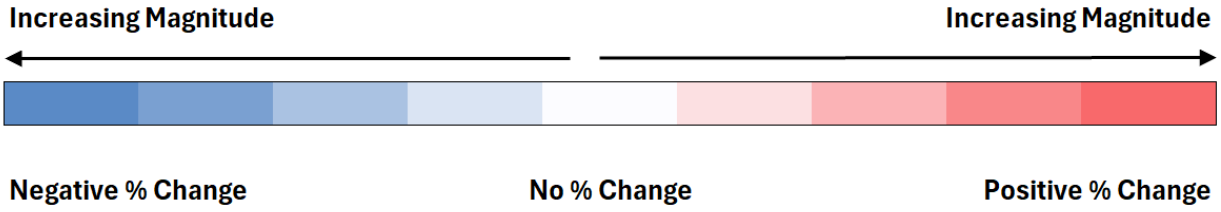
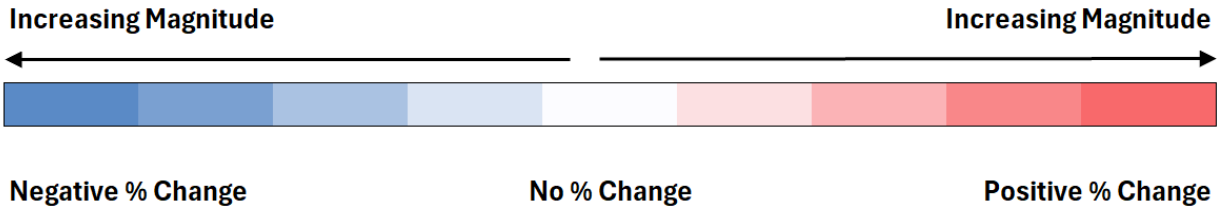


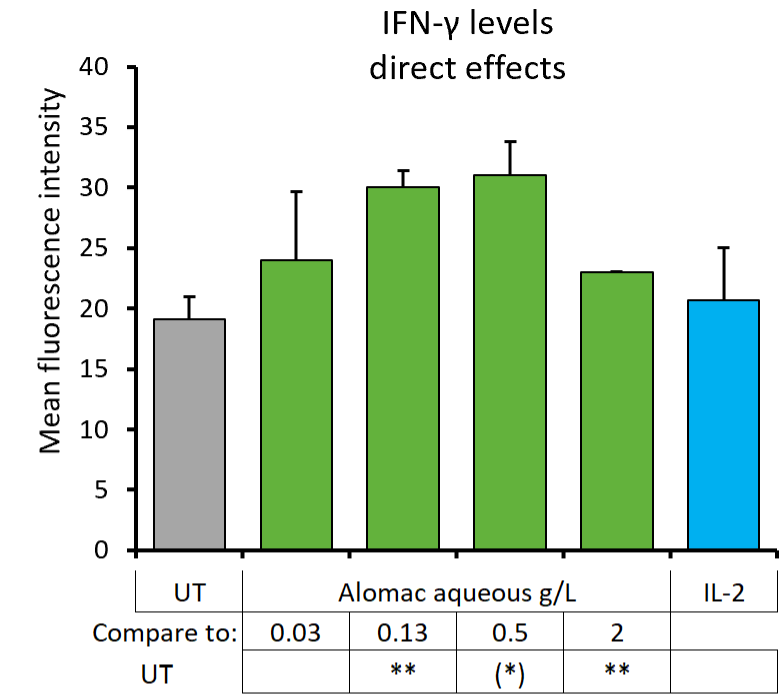
Table 8. Regulating cytokines - direct effects: levels of magnitude

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IL-2	144		192	-25
IL-4	109		156	-10
IL-7	32	-4	9	-12
IL-9	6	-61	23	-44
IL-15	45		74	-8

Table 9. Growth factors - direct effects: levels of magnitude

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
Basic FGF	103		171	-12
PDGF-BB	1	-4	25	-12
VEGF	52		67	-8
GM-CSF	38	-4	43	-9
G-CSF	446		632	-29





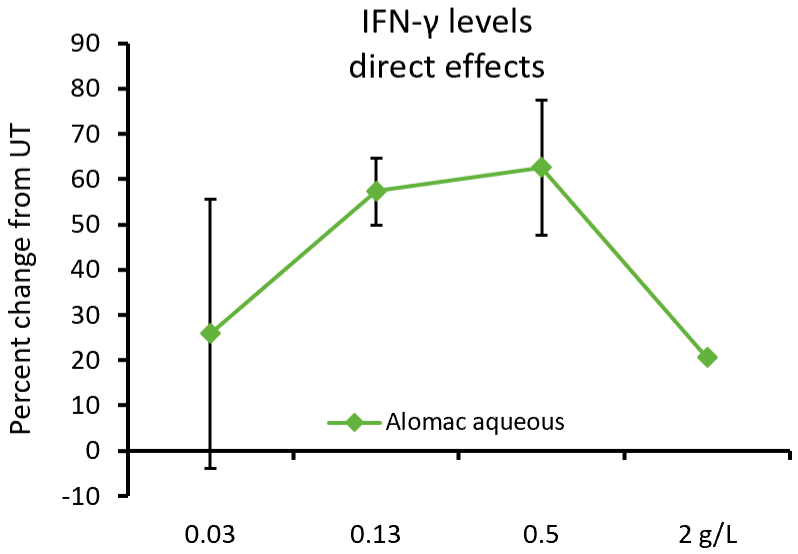


*Figure 2. Interferon-gamma (IFN-γ) levels when cells were treated with the aqueous fraction of Alomac, the untreated control (UT), or the positive control (IL-2). In the table below the graph, the levels of statistical significance compared to UT at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

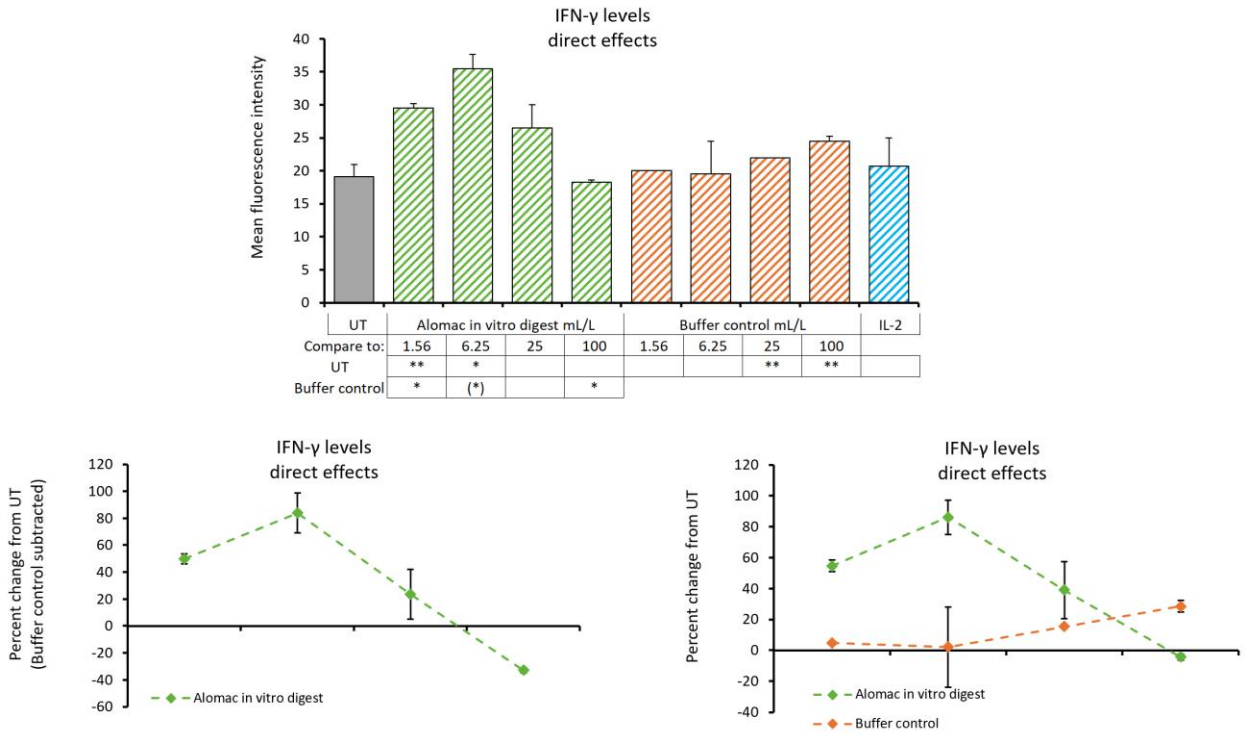
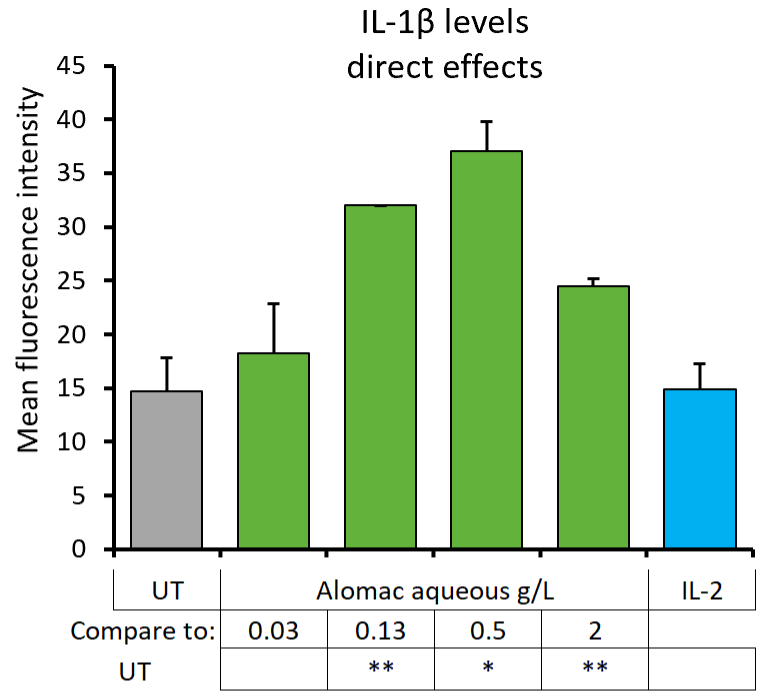


*Figure 3. Interferon-gamma (IFN-γ) levels when cells were treated with the in vitro digested fraction of Alomac, the untreated control (UT), the buffer control or the positive control (IL-2). In the table below the graph, the levels of statistical significance compared to UT or the buffer control at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*



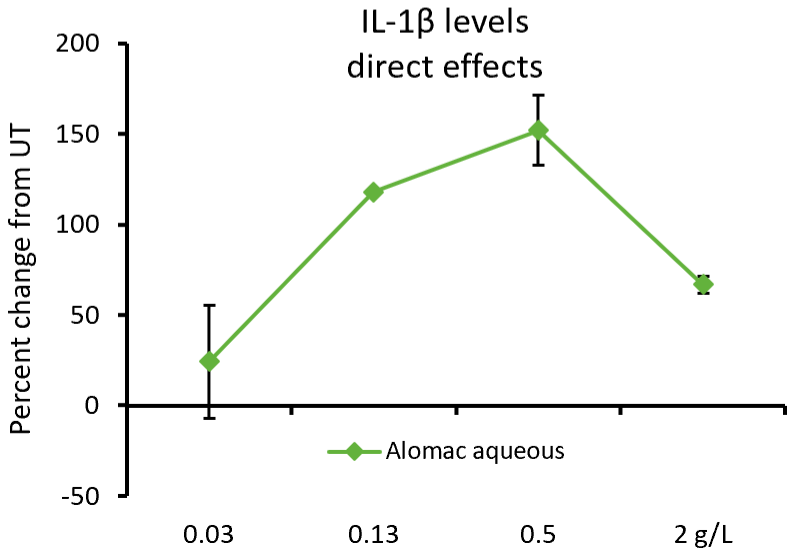


*Figure 4. Interleukin-1β (IL-1β) levels when cells were treated with the aqueous fraction of Alomac, the untreated control (UT), or the positive control (IL-2). In the table below the graph, the levels of statistical significance compared to UT at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

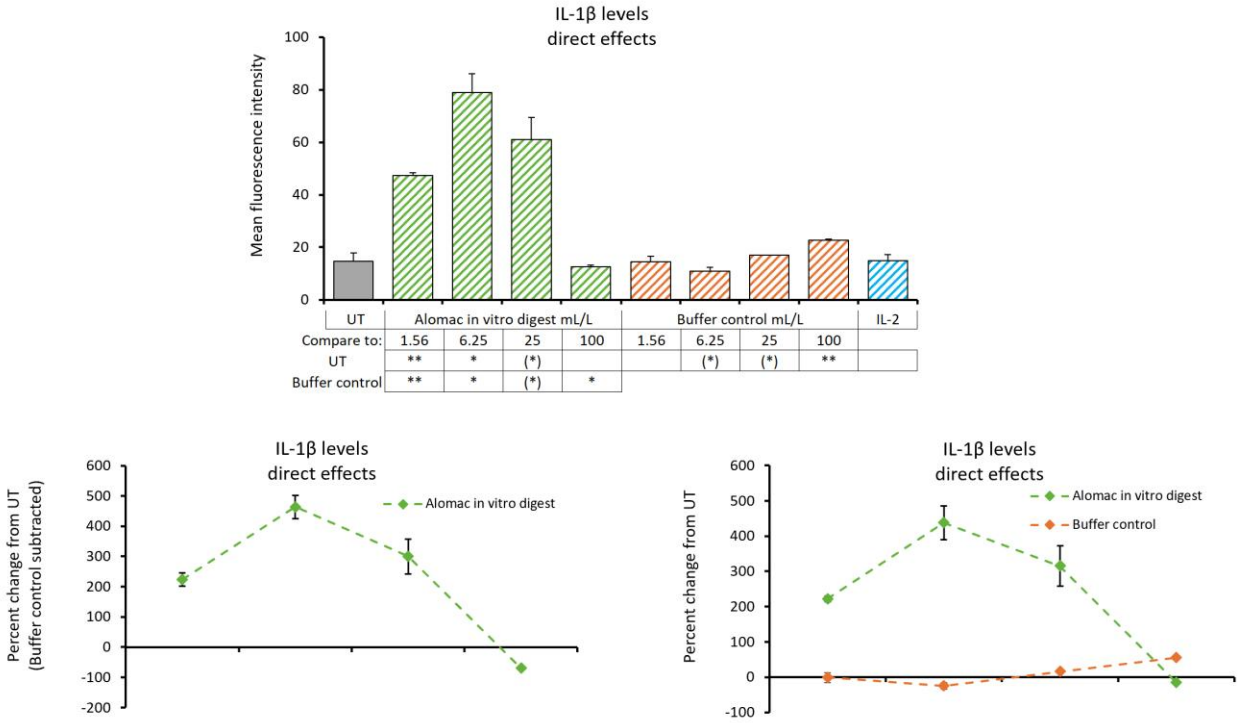


*Figure 5. Interleukin-1β (IL-1β) levels when cells were treated with the in vitro digested fraction of Alomac, the untreated control (UT), the buffer control or the positive control (IL-2). In the table below the graph, the levels of statistical significance compared to UT or the buffer control at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

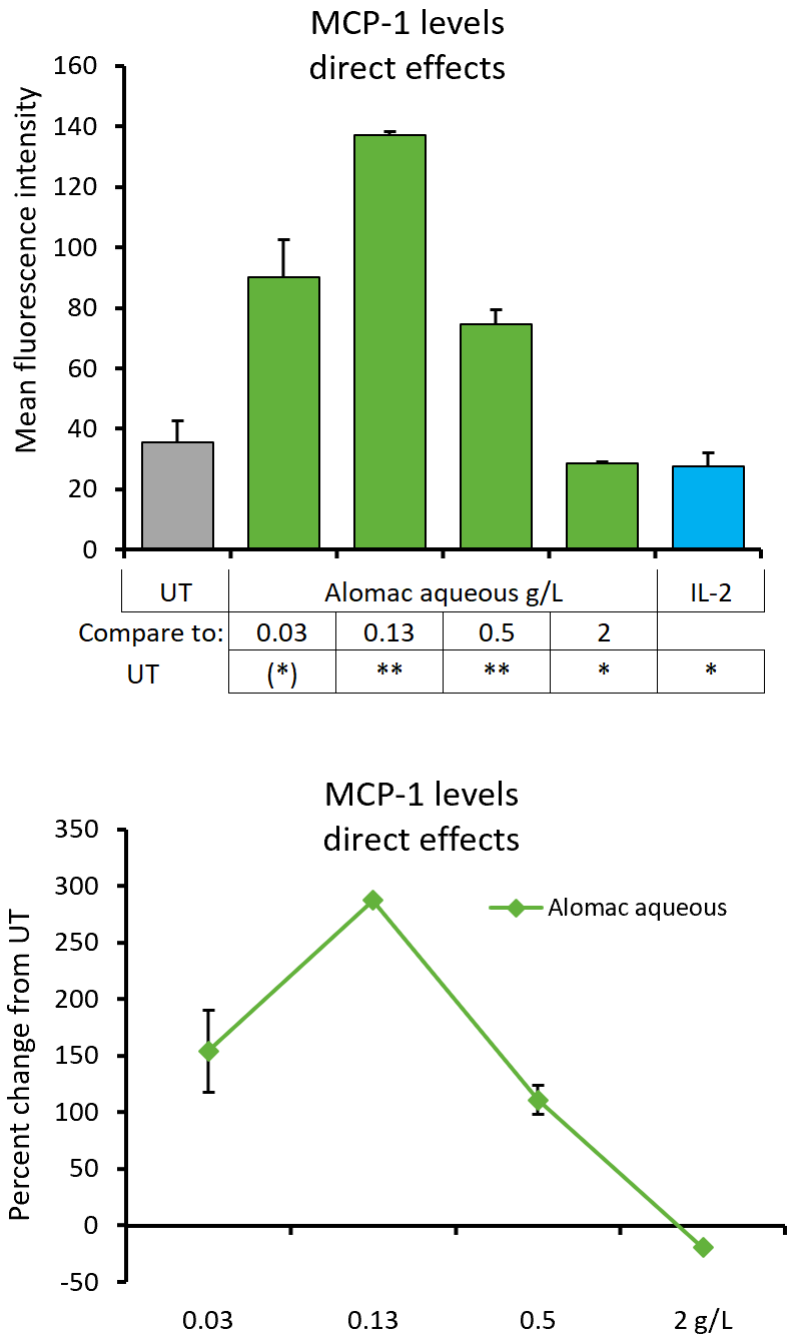


*Figure 6. Monocyte Chemoattractant Protein-1 (MCP-1) levels when cells were treated with the aqueous fraction of Alomac, the untreated control (UT), or the positive control (IL-2). In the table below the graph, the levels of statistical significance compared to UT at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

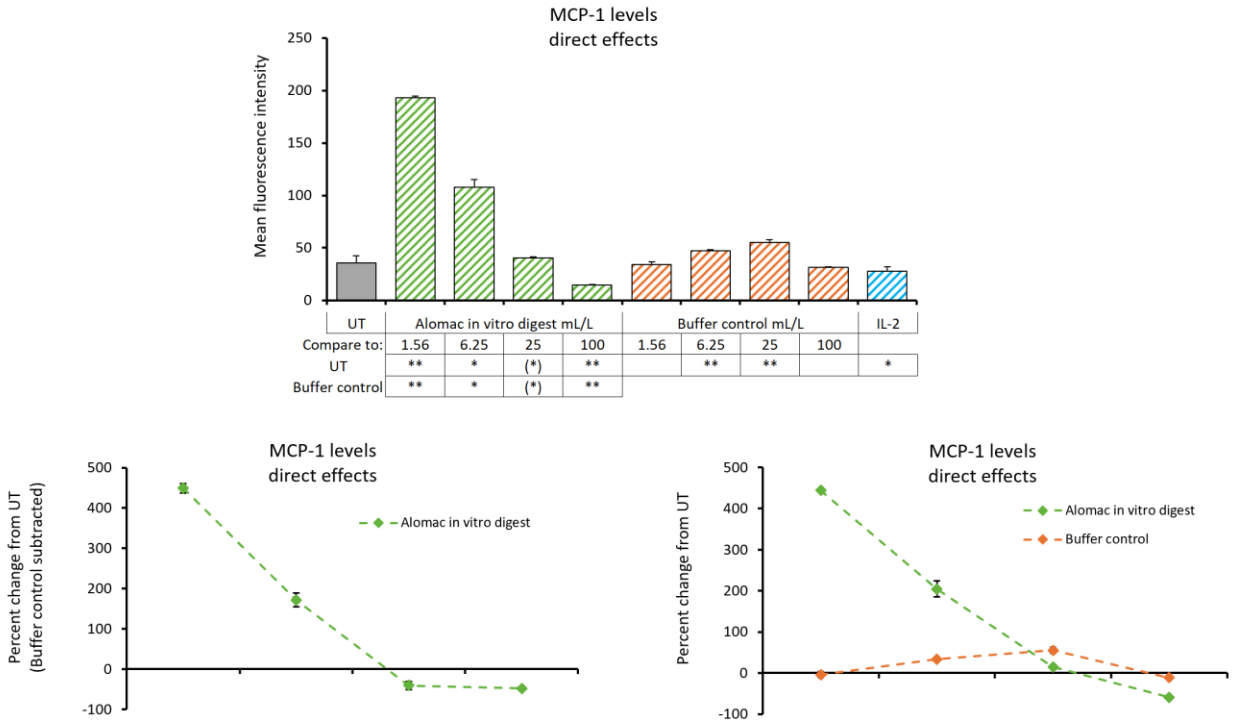


*Figure 7. Monocyte Chemoattractant Protein-1 (MCP-1) levels when cells were treated with the in vitro digested fraction of Alomac, the untreated control (UT), the buffer control or the positive control (IL-2). In the table below the graph, the levels of statistical significance compared to UT or the buffer control at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

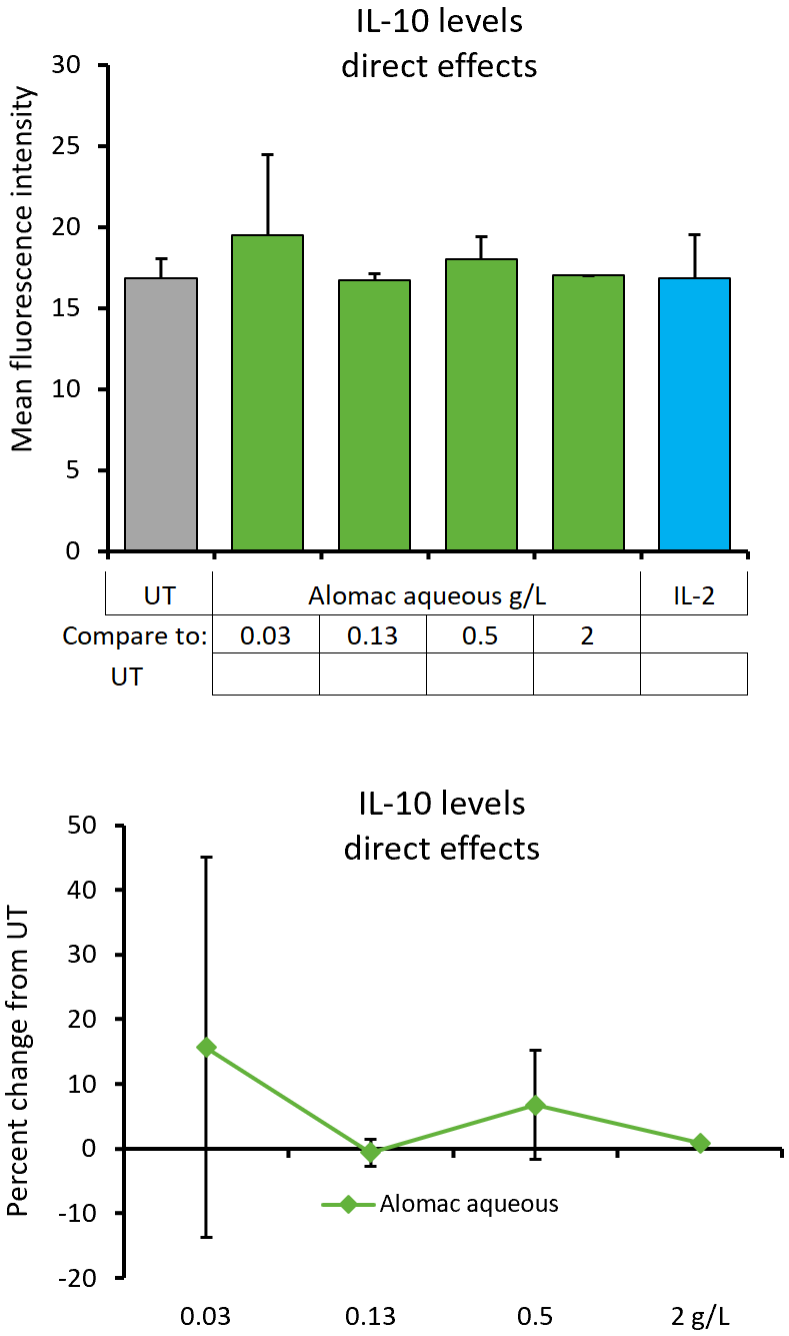


*Figure 8. Interleukin-10 (IL-10) levels when cells were treated with the aqueous fraction of Alomac, the untreated control (UT), or the positive control (IL-2). In the table below the graph, the levels of statistical significance compared to UT at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

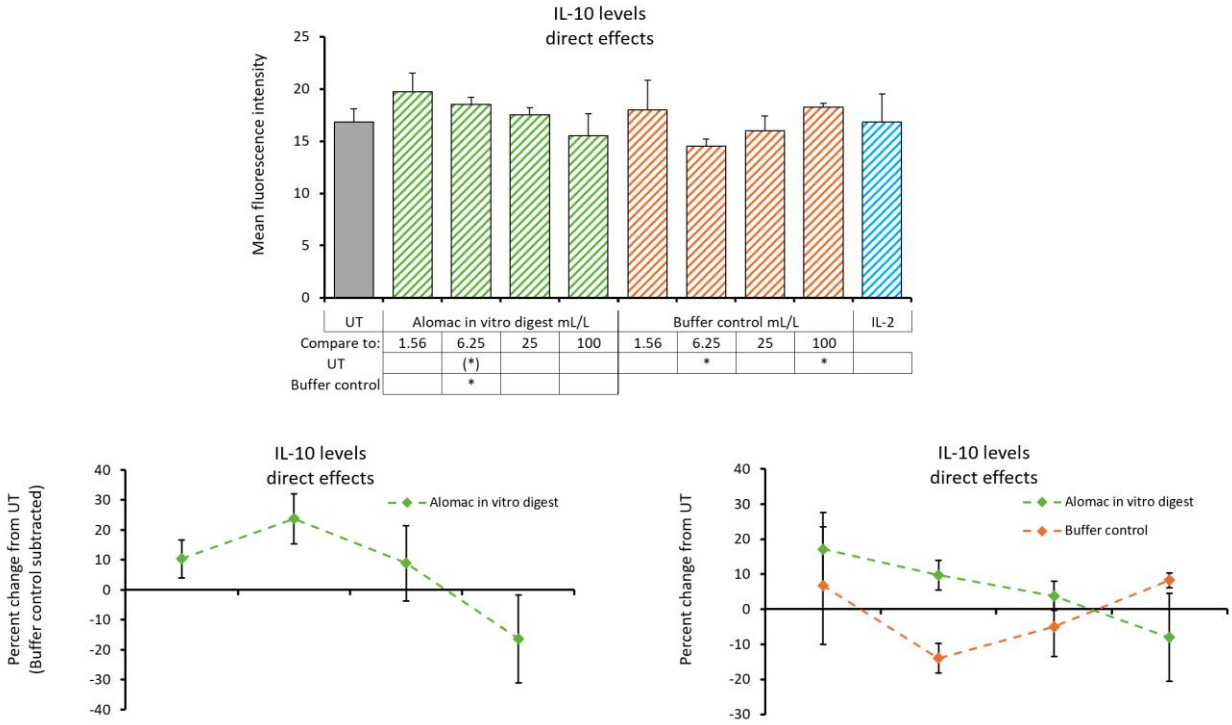


*Figure 9. Interleukin-10 (IL-10) levels when cells were treated with the in vitro digested fraction of Alomac, the untreated control (UT), the buffer control or the positive control (IL-2). In the table below the graph, the levels of statistical significance compared to UT or the buffer control at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

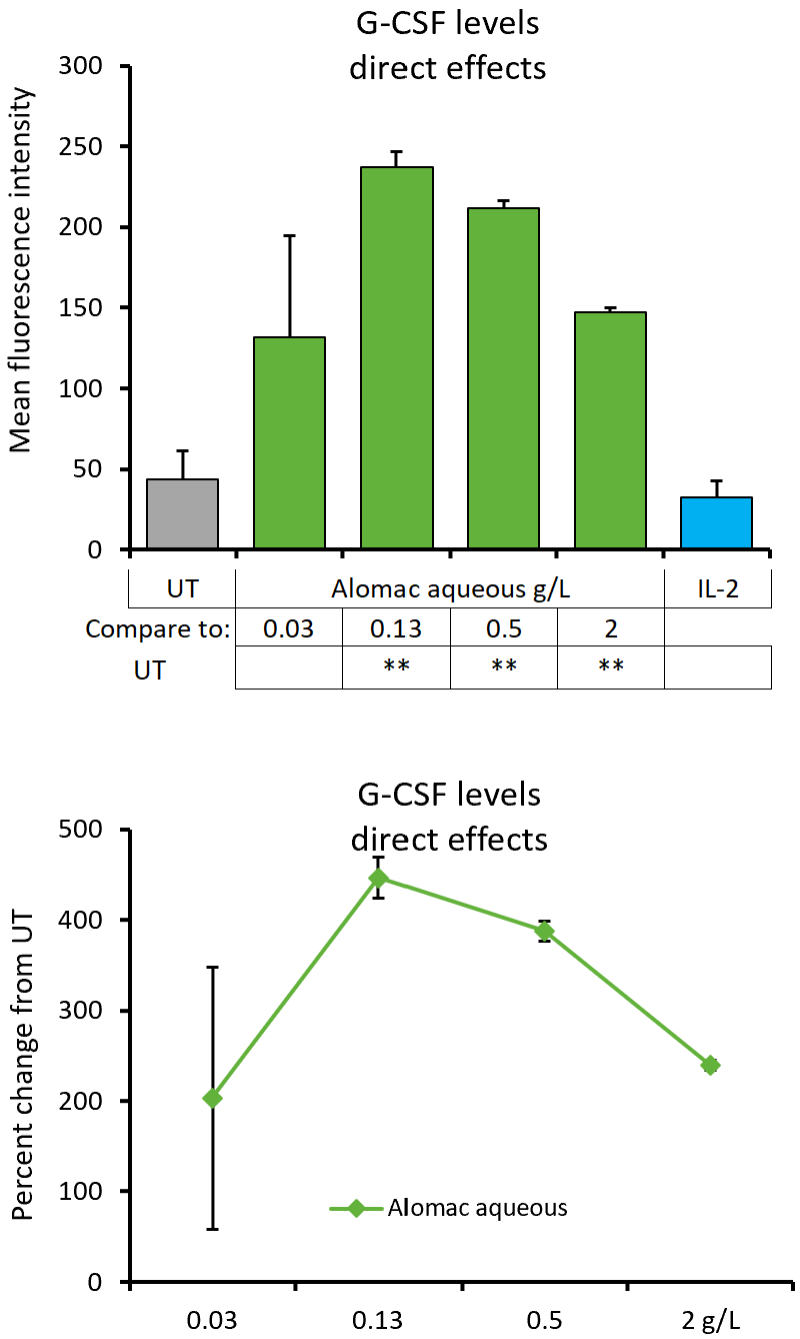


*Figure 10. Granulocyte colony-stimulating factor (G-CSF) levels when cells were treated with the aqueous fraction of Alomac, the untreated control (UT), or the positive control (IL-2). In the table below the graph, the levels of statistical significance compared to UT at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

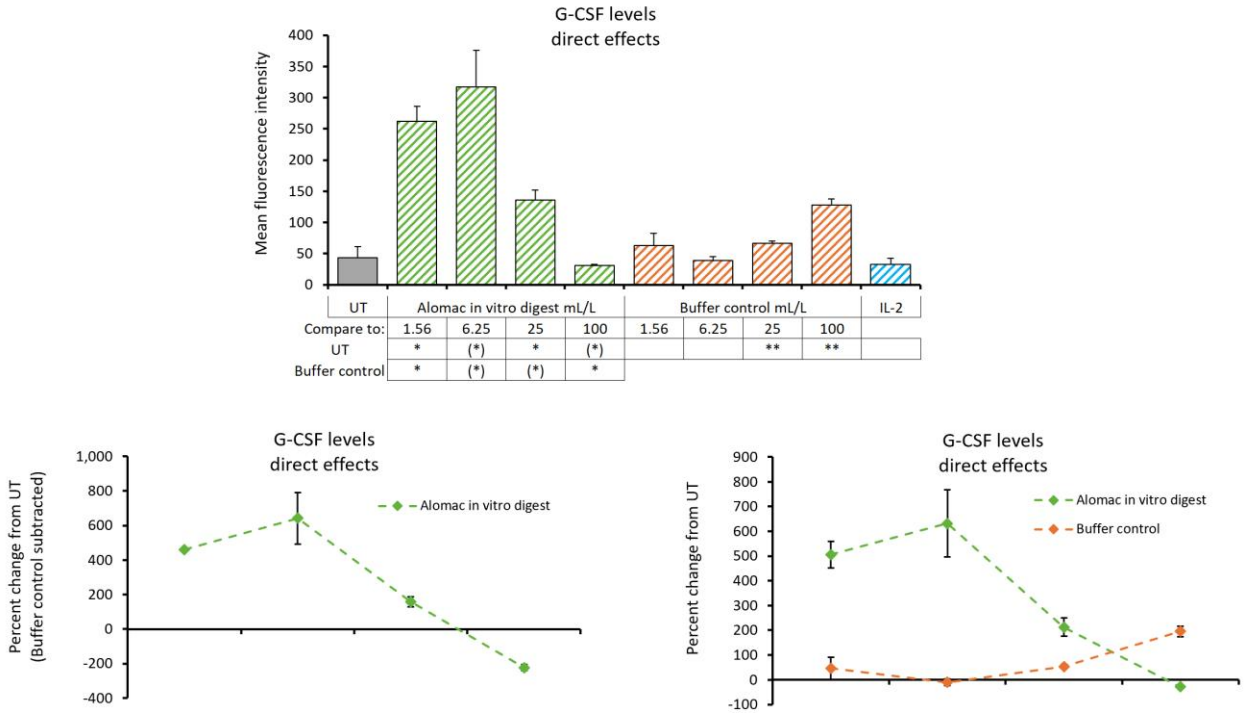


*Figure 11. Granulocyte colony-stimulating factor (G-CSF) levels when cells were treated with the in vitro digested fraction of Alomac, the untreated control (UT), the buffer control or the positive control (IL-2). In the table below the graph, the levels of statistical significance compared to UT or the buffer control at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

5.3 Cytokine Levels – Inflamed Conditions

To assess how Alomac influences an ongoing immune response under inflamed conditions, immune cells were pre-treated with Alomac aqueous and in vitro digested fractions before adding **LPS (lipopolysaccharide)**, a component of bacterial cell walls that provokes strong inflammation. LPS stimulates monocytes/macrophages to release a surge of cytokines (often termed a "cytokine storm" in severe cases) – a useful model of **acute bacterial infection or endotoxin exposure**.

Overview of changes to all detectable cytokines after 2 hours (Tables 10-17 below):

In the presence of the LPS challenge, both the aqueous and in vitro digested fractions affected the levels of all 27 cytokines tested. Most prominently, we observed significant decreases in all of the 27 cytokines tested when exposed to the aqueous fraction, reaching significance for 26 of the 27 cytokines, and no significant increases. We also observed significant decreases in 26 of the 27 cytokines tested when exposed to the in vitro digested fraction, with one exception of IL-7. The in vitro digested fraction also induced significant increases of RANTES and IL-9.

Selected cytokines as examples of rapid changes to cytokine levels already at 2 hours are described here, and shown in Figures 12-23 below:

- **IFN-γ** (**Interferon-gamma**), **IL-1β (Interleukin-1β)** and **TNF-α (Tumor necrosis factor alpha**) – The aqueous and in vitro digested fractions significantly decreased IFN-γ and IL-1β levels compared to cultures treated with LPS in the absence of Alomac. **This showed that LPS-induced IFN-γ, IL-1β, and TNF-α production were reduced early by Alomac, but in context of the previous report on 24-hour data, this suppression at 2 hours was temporary, since Alomac strongly amplified the LPS-induced IFN-γ, IL-1β, and TNF-α production after 24 hours.**
- **MCP-1 (Monocyte chemoattractant protein 1)** – The decrease in MCP-1 was robust, all doses of both the aqueous and in vitro digested fractions of Alomac significantly decreased MCP-1 levels relative to the LPS-treated control cultures. The highest and lowest doses of both fractions produced the strongest reductions in MCP-1 levels. **This shows that MCP-1 production was modulated early by Alomac but was not sustained since the levels after 24 hours were not much different than after 2 hours.**
- **IL-1ra (Interleukin-1 receptor antagonist)** – Both the aqueous and in vitro digested fractions caused highly significant decreases in IL-1ra levels. **This early modulation continued as this was also observed after 24 hours of cell culture.**

- **IL-10 (Interleukin-10)** – Alomac caused a small reduction in the LPS-induced IL-10 levels. **This early modulation continued as this was also observed after 24 hours of cell culture.**

Table 10. Pro-inflammatory cytokines - inflamed culture conditions: levels of significance

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IFN-γ		-9		-36
IL-1β		-44		-80
IL-5		-25		-51
IL-6		-62		-91
IL-8		-77		-85
IL-12 (p70)		-13		-13
IL-13		-15		-8
IL-17A		-36		-55
Eotaxin		-27		-30
IP-10		-22		-22
MCP-1		-42		-37
MIP-1α		-51		-97
MIP-1β		-57		-81
RANTES		-36	67	-20
TNF-α		-83		-95

Table 11. Anti-inflammatory cytokines - inflamed culture conditions: levels of significance

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IL-1ra		-21		-33
IL-10		-10		-10

Color Description	
	Increase
	Decrease

Color	P-valve	Description
	≥0.10	Not statistically significant
	<0.10	Statistical trend
	<0.05	Statistical significance
	<0.01	High level of significance

Table 12. Cytokines/chemokines with regulating properties - inflamed culture conditions: levels of significance

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IL-2		-64		-74
IL-4		-42		-59
IL-7		-14	7	
IL-9		-36	52	-18
IL-15		-27		-42

Table 13. Growth factors - inflamed culture conditions: levels of significance

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
Basic FGF		-24		-65
PDGF-BB		-18		-32
VEGF		-23		-40
GM-CSF		-38	2	-16
G-CSF	6	-33		-84

Color Description	
	Increase
	Decrease

Color	P-valve	Description
	≥0.10	Not statistically significant
	<0.10	Statistical trend
	<0.05	Statistical significance
	<0.01	High level of significance

Table 14. Pro-inflammatory cytokines - inflamed culture conditions: levels of magnitude

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IFN-γ		-31		-36
IL-1β		-62		-80
IL-5		-43		-51
IL-6		-66		-91
IL-8		-77		-85
IL-12 (p70)		-13		-13
IL-13		-15		-10
IL-17A		-36		-55
Eotaxin		-27		-30
IP-10		-22		-22
MCP-1		-42		-66
MIP-1α		-71		-97
MIP-1β		-72		-81
RANTES		-36	67	-20
TNF-α		-83		-95

Table 15. Anti-inflammatory cytokines - inflamed culture conditions: levels of magnitude

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IL-1ra		-40		-52
IL-10		-11		-12

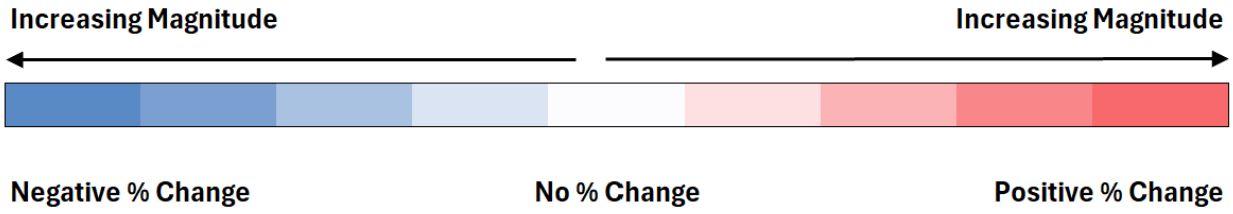
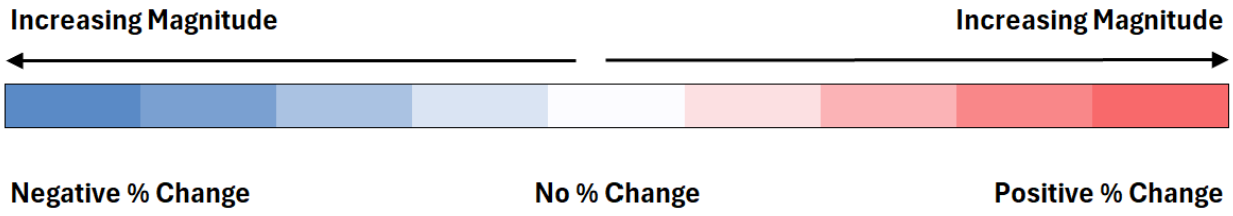


Table 16. Regulating cytokines - inflamed culture conditions: levels of magnitude

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IL-2		-64		-74
IL-4		-42		-59
IL-7		-14	7	
IL-9		-36	52	-19
IL-15		-27		-42

Table 17. Growth factors - inflamed culture conditions: levels of magnitude

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
Basic FGF		-47		-65
PDGF-BB		-19		-32
VEGF		-27		-40
GM-CSF		-38	2	-30
G-CSF	6	-52		-84



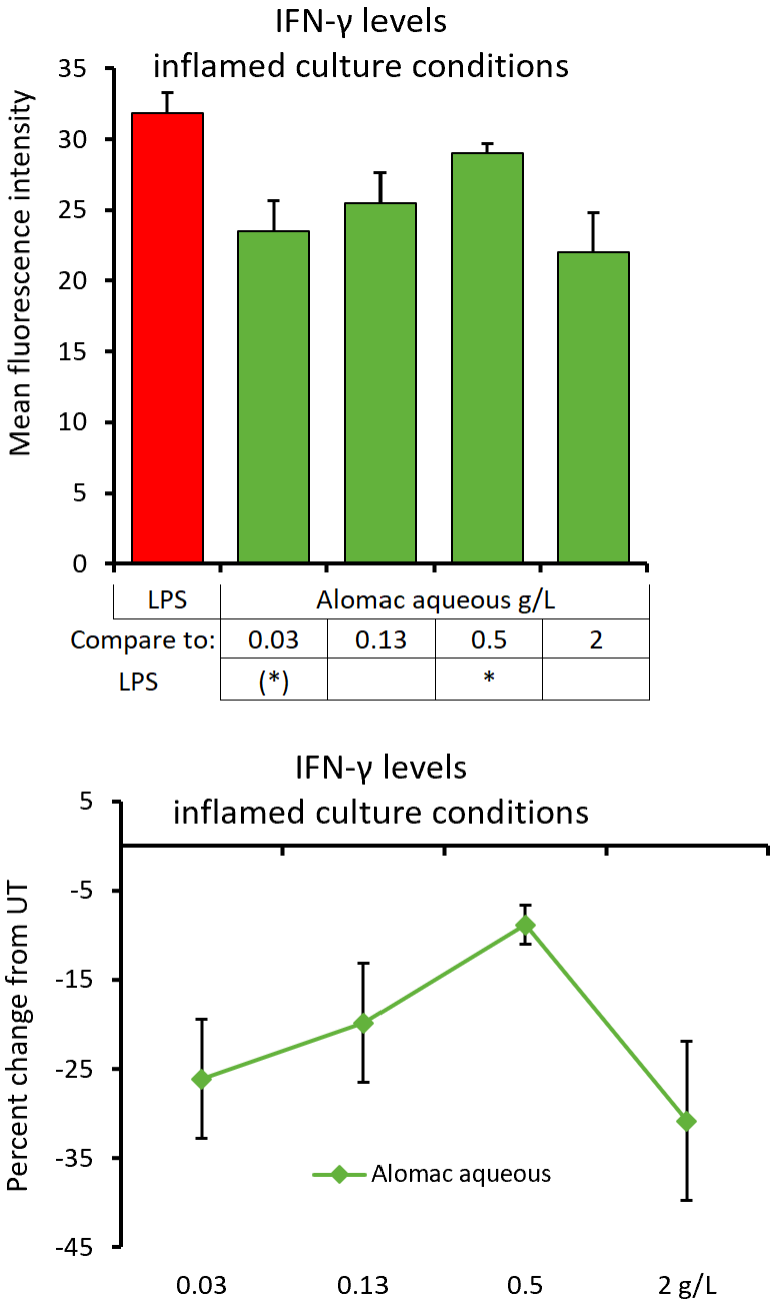


*Figure 12. Interferon-γ (IFN-γ) levels when cells were treated with the aqueous fraction of Alomac prior to treatment with Lipopolysaccharide (LPS). LPS (red bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to LPS at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

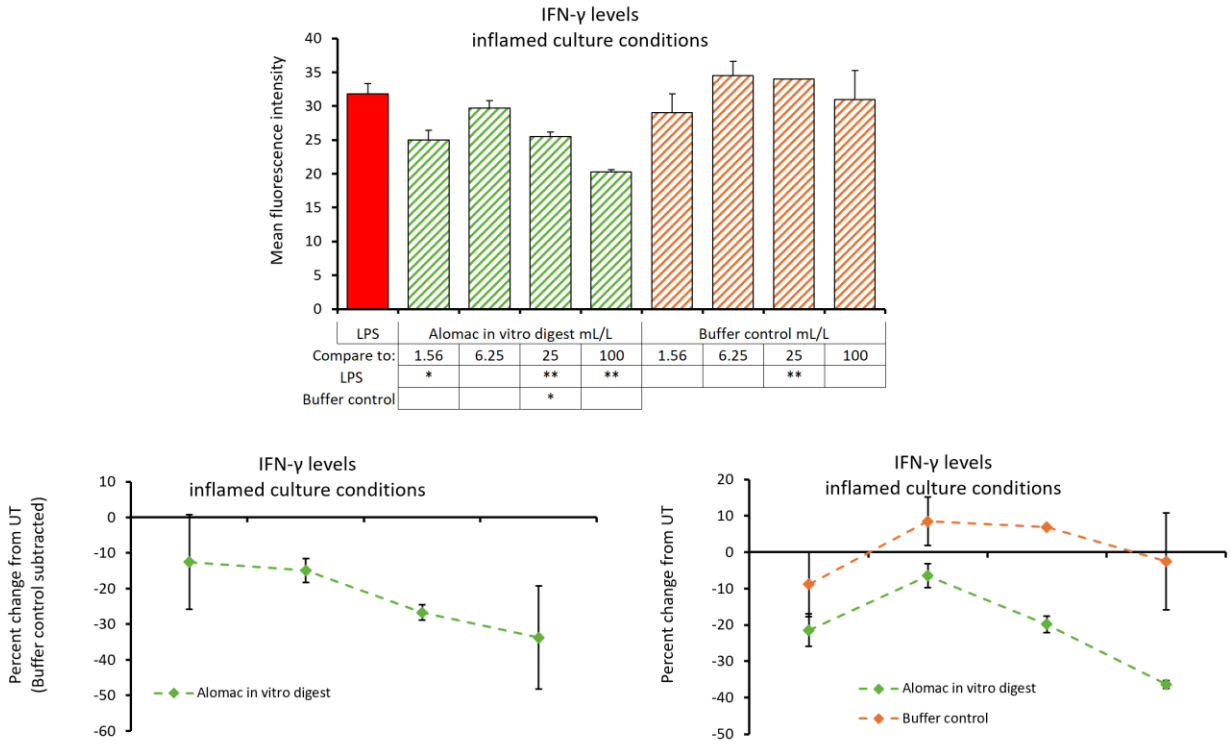


*Figure 13. Interferon-γ (IFN-γ) levels when cells were treated with the in vitro digested fraction of Alomac prior to treatment with Lipopolysaccharide (LPS). LPS (red bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to LPS or the buffer control at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

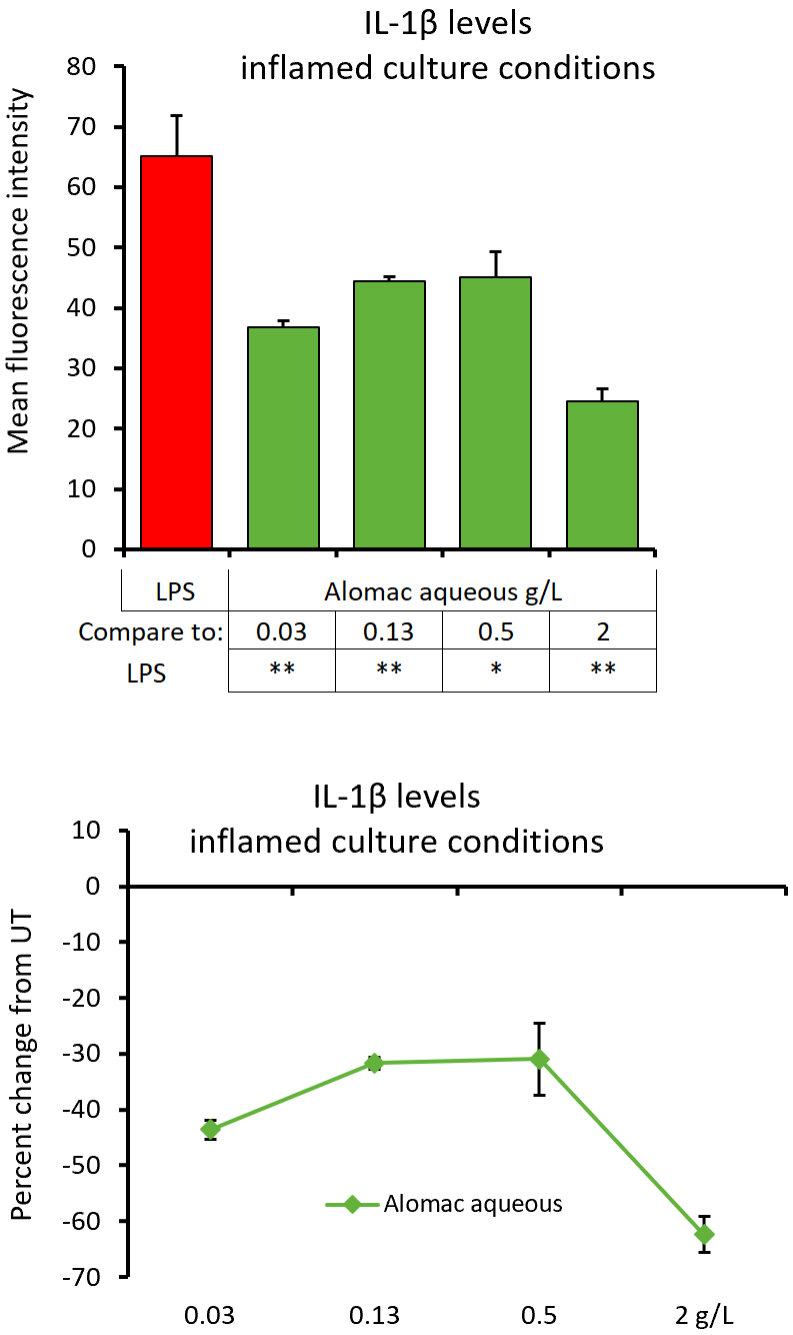


*Figure 14. Interleukin-1ϐ (IL-1ϐ) levels when cells were treated with the aqueous fraction of Alomac prior to treatment with Lipopolysaccharide (LPS). LPS (red bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to LPS at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

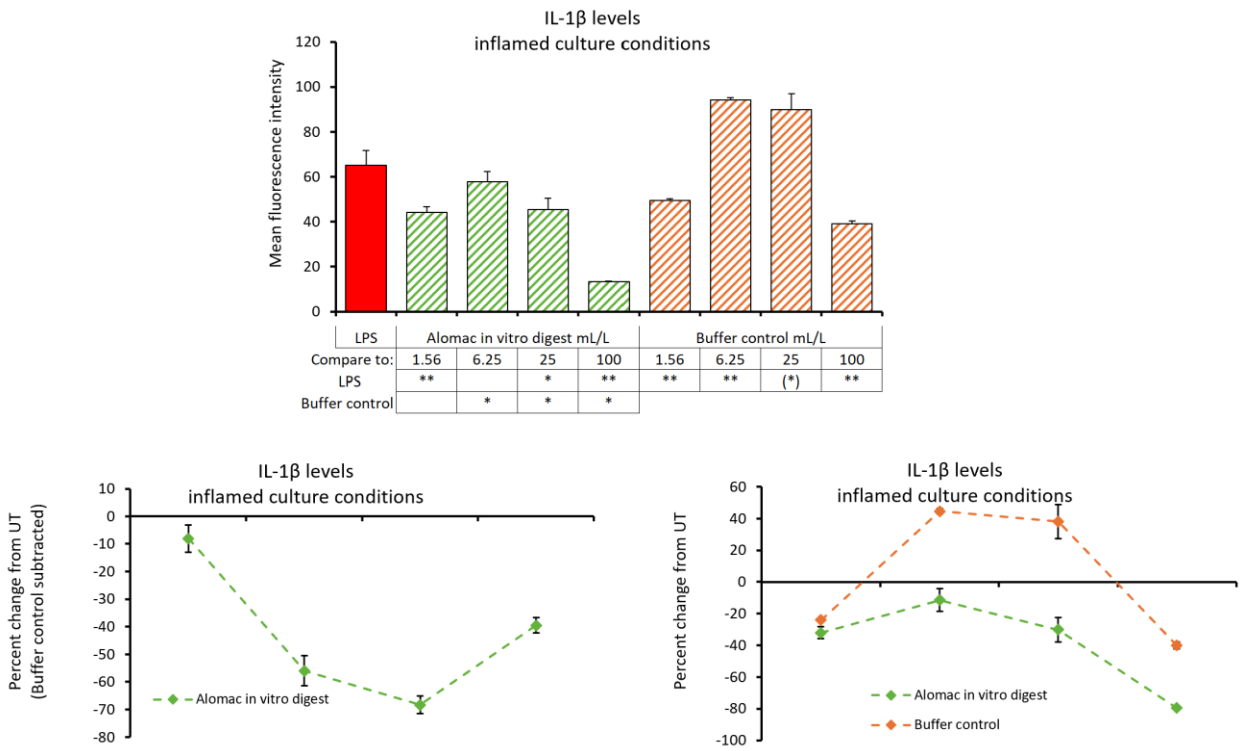


*Figure 15. Interleukin-1β (IL-1β) levels when cells were treated with the in vitro digested fraction of Alomac prior to treatment with Lipopolysaccharide (LPS). LPS (red bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to LPS or the buffer control at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

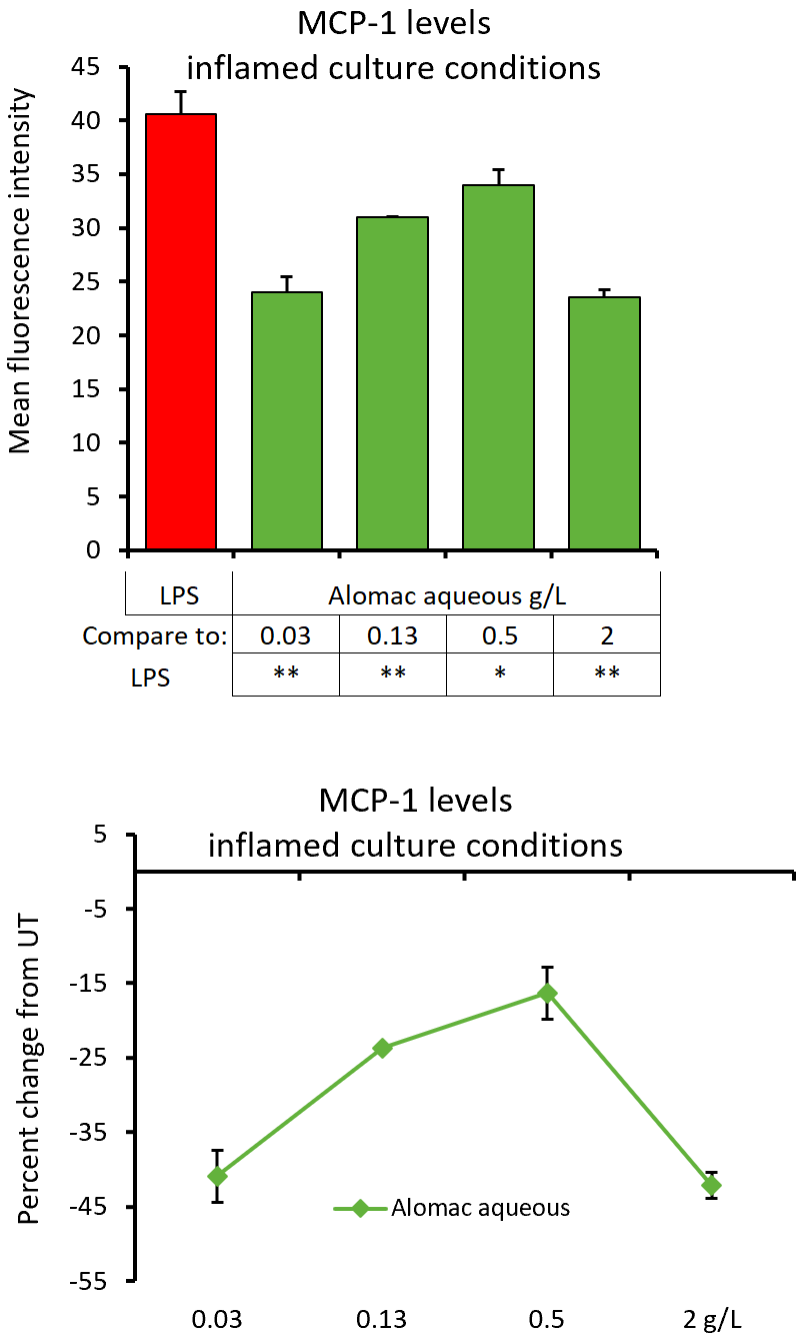


*Figure 16. Monocyte chemoattractant protein 1 (MCP-1) levels when cells were treated with the aqueous fraction of Alomac prior to treatment with Lipopolysaccharide (LPS). LPS (red bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to LPS at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

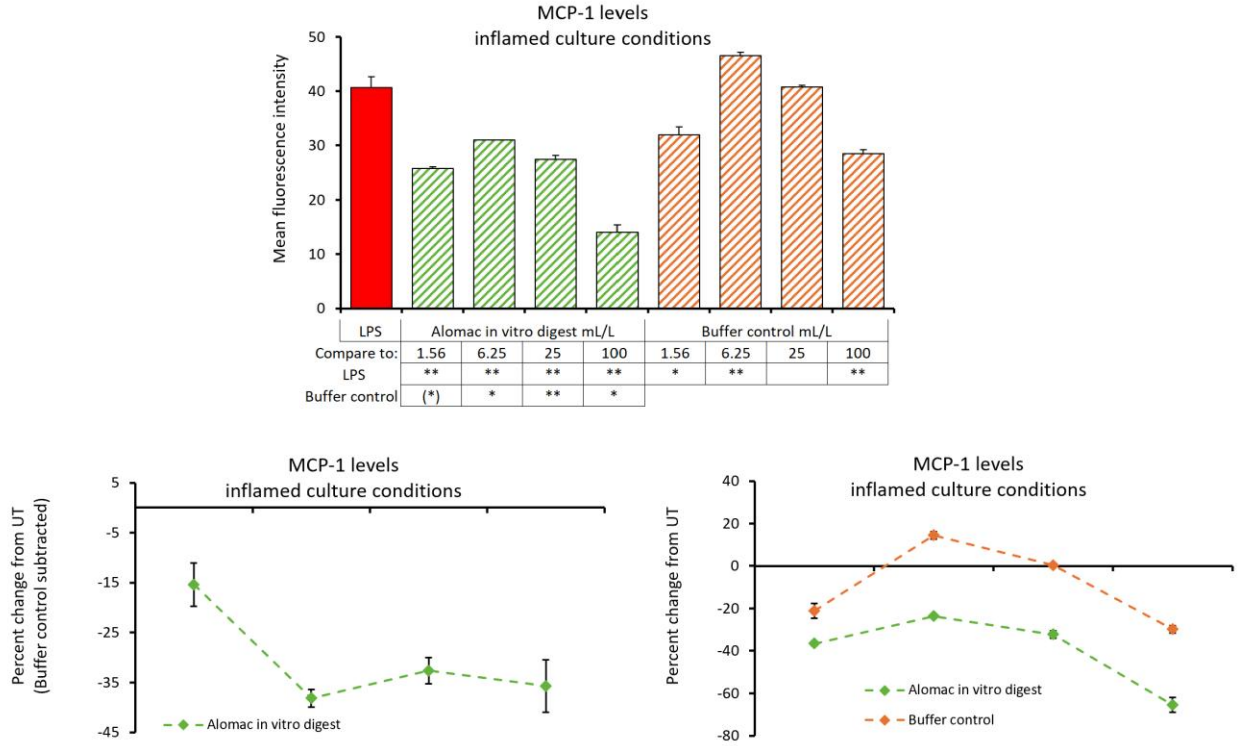


*Figure 17. Monocyte chemoattractant protein 1 (MCP-1) levels when cells were treated with the in vitro digested fraction of Alomac prior to treatment with Lipopolysaccharide (LPS). LPS (red bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to LPS or the buffer control at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

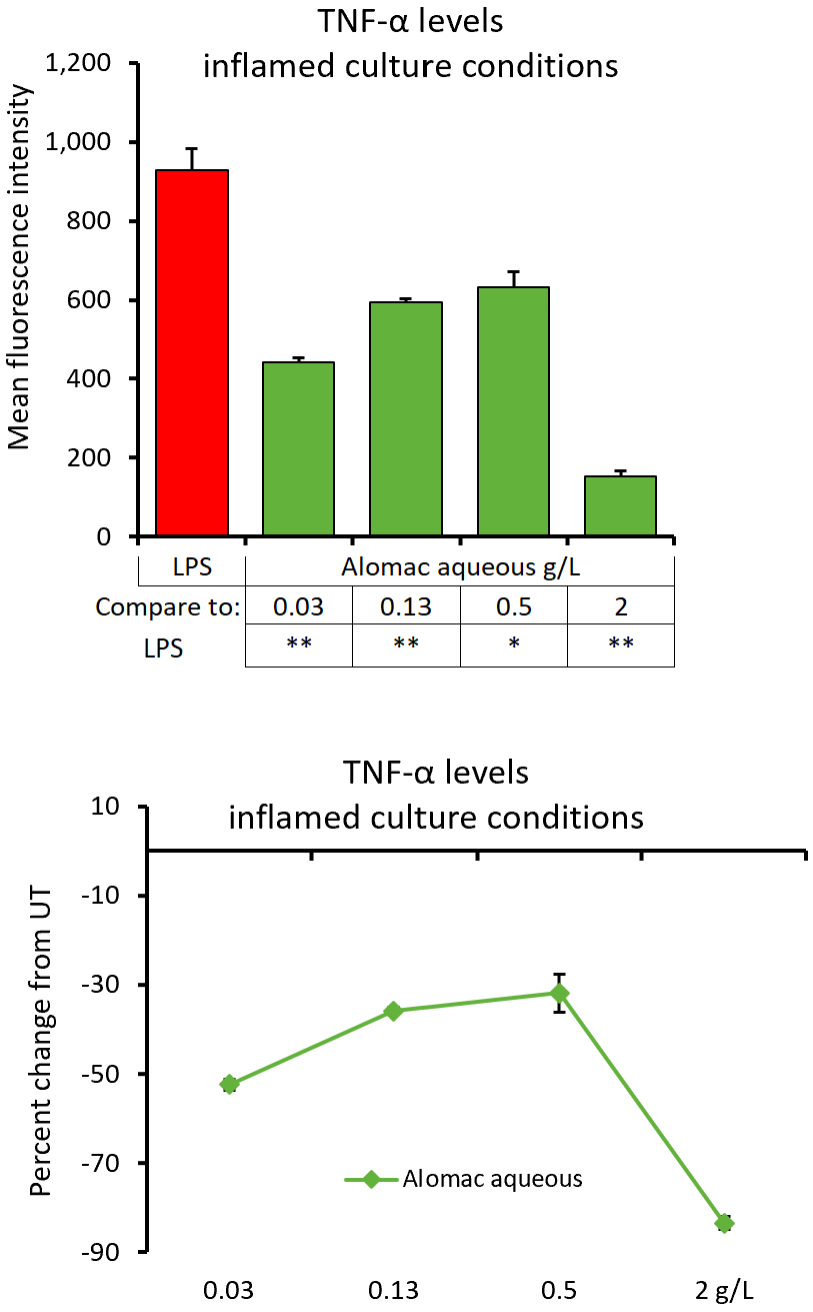


*Figure 18. Tumor necrosis factor (TNF-α) levels when cells were treated with the aqueous fraction of Alomac prior to treatment with Lipopolysaccharide (LPS). LPS (red bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to LPS at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

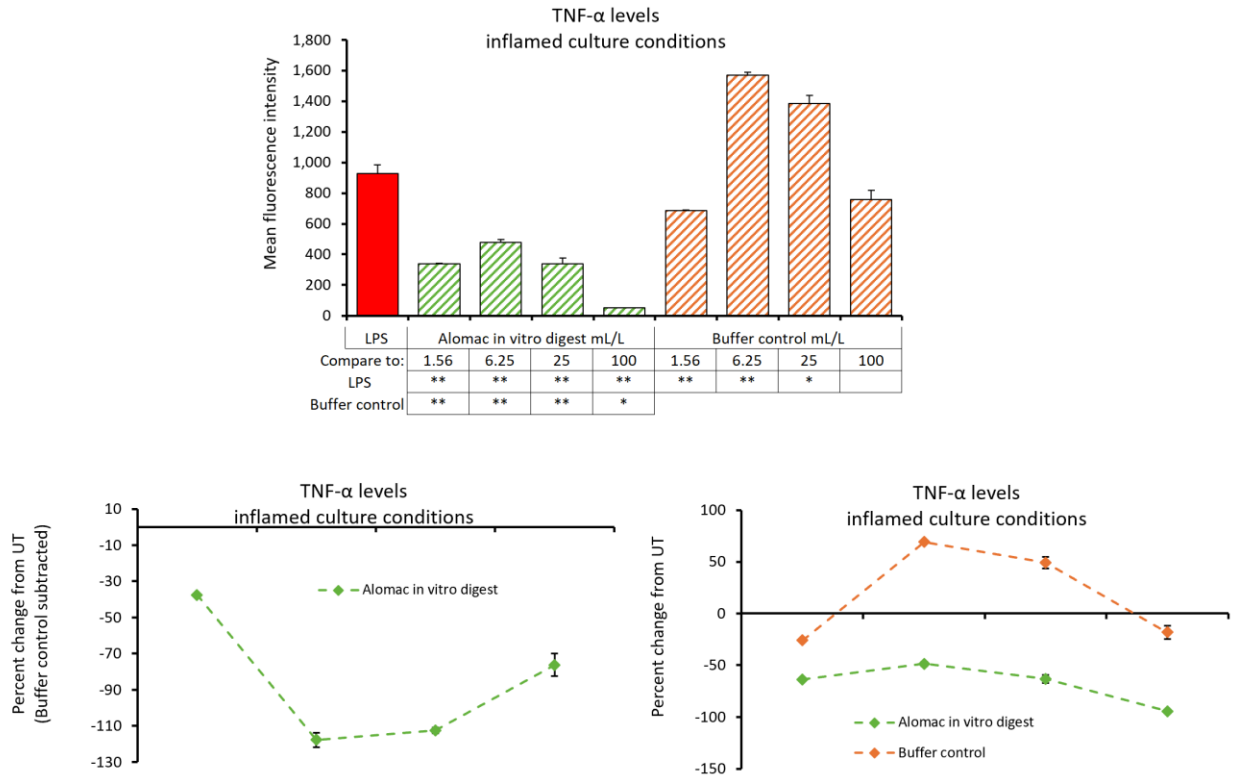


*Figure 19. Tumor necrosis factor (TNF-α) levels when cells were treated with the in vitro digested fraction of Alomac prior to treatment with Lipopolysaccharide (LPS). LPS (red bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to LPS or the buffer control at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

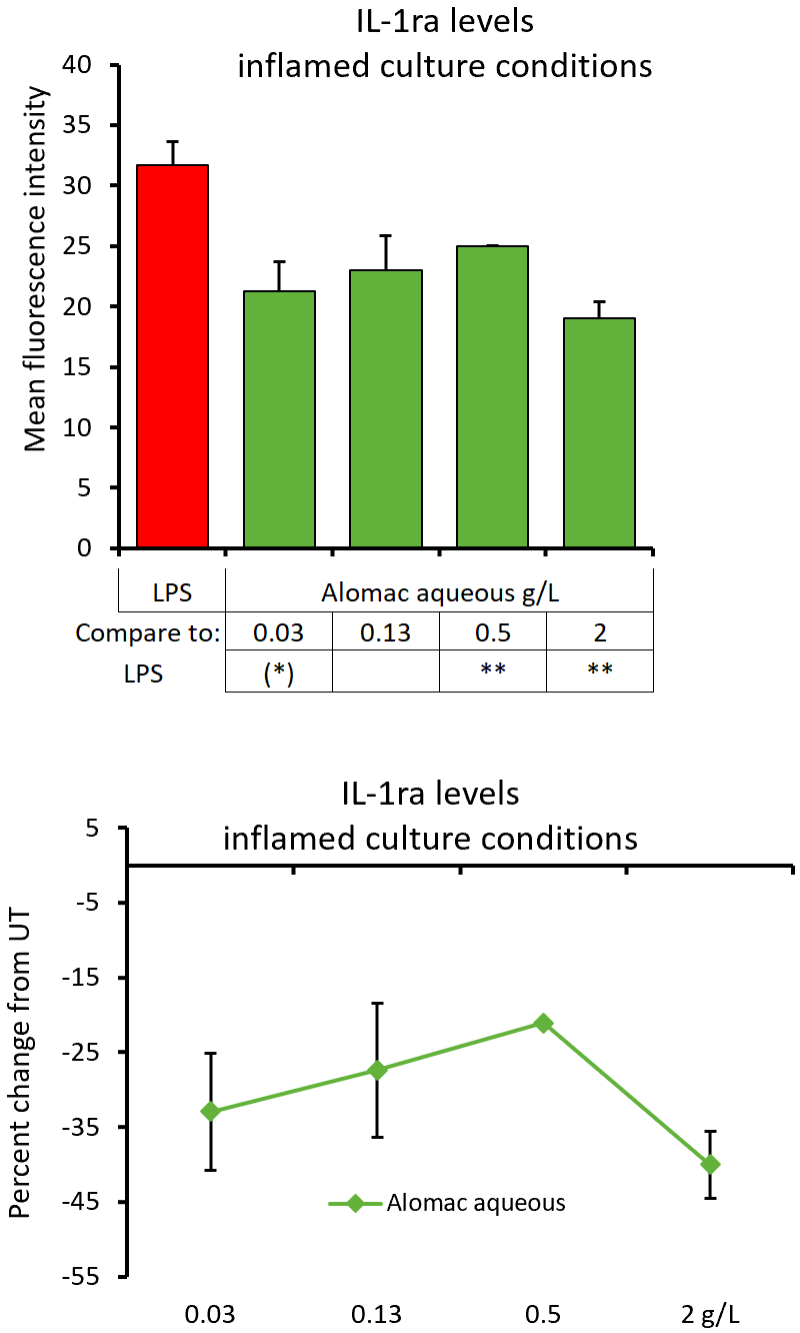


*Figure 20. Interleukin-1 receptor antagonist protein (IL-1ra) levels when cells were treated with the aqueous fraction of Alomac prior to treatment with Lipopolysaccharide (LPS). LPS (red bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to LPS at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

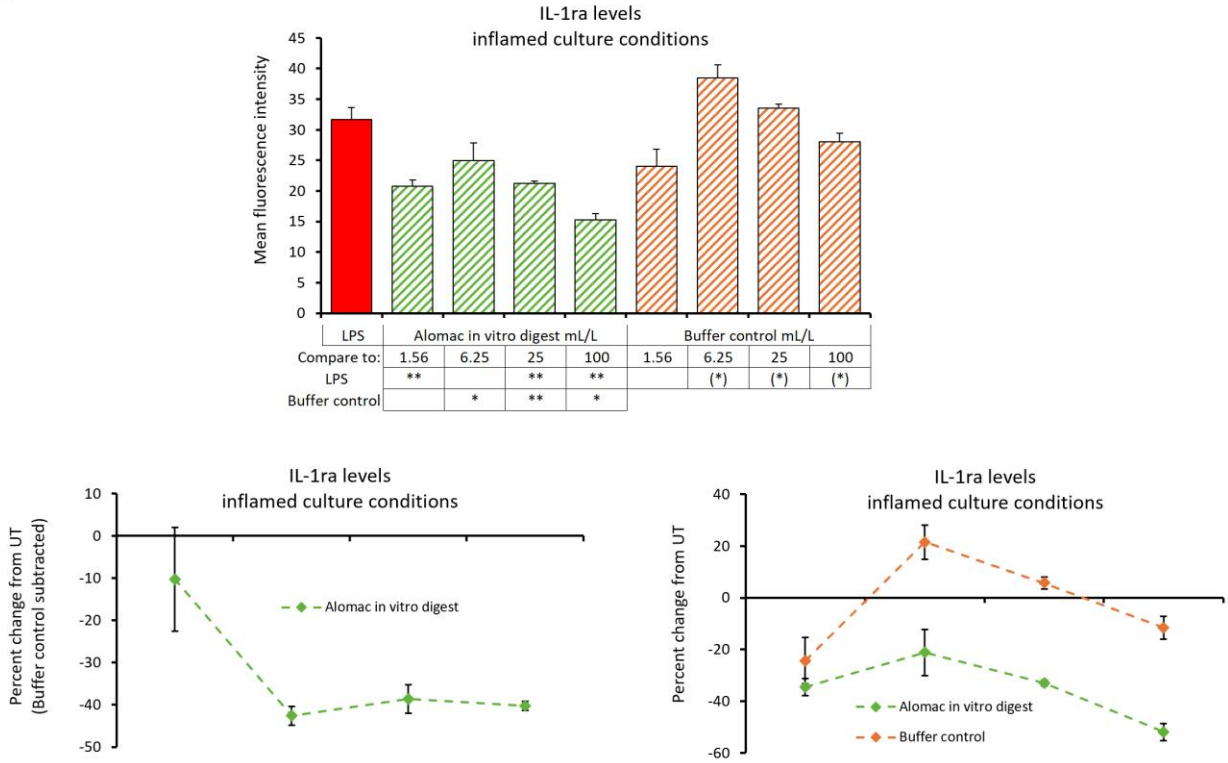


*Figure 21. Interleukin-1 receptor antagonist protein (IL-1ra) levels when cells were treated with the in vitro digested fraction of Alomac prior to treatment with Lipopolysaccharide (LPS). LPS (red bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to LPS or the buffer control at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

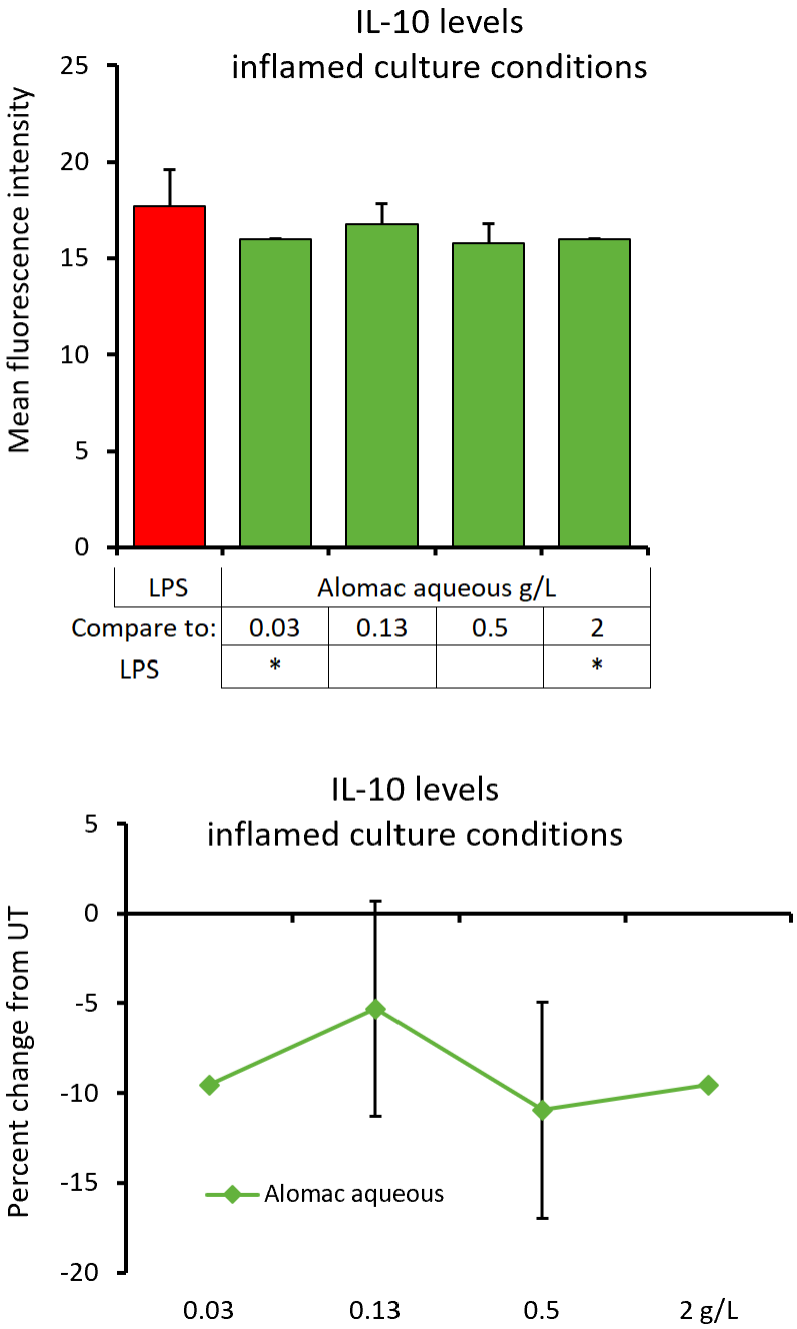


*Figure 22. Interleukin 10 (IL-10) levels when cells were treated with the aqueous fraction of Alomac prior to treatment with Lipopolysaccharide (LPS). LPS (red bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to LPS at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

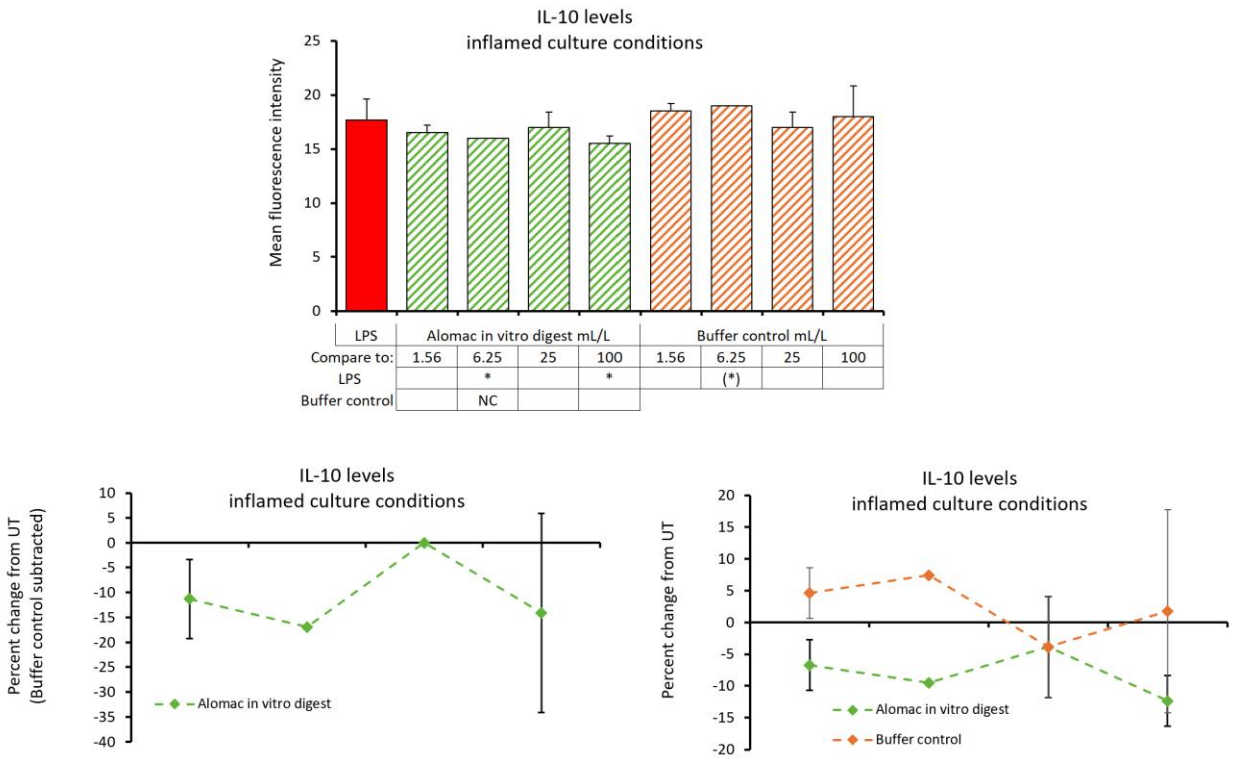


*Figure 23. Interleukin 10 (IL-10) levels when cells were treated with the in vitro digested fraction of Alomac prior to treatment with Lipopolysaccharide (LPS). LPS (red bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to LPS or the buffer control at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

5.4 Cytokine Levels – Viral Mimetic Challenge

Poly I:C (Polyinosinic:polycytidylic acid, abbreviated PIC here) is a synthetic analog of viral double-stranded RNA that triggers immune receptors (including TLR3, RIG-I/MDA5 pathways) to mimic a **viral infection**. It strongly induces type I interferons and related antiviral cytokines and peptides. To assess how Alomac influences an immune response in context of a viral mimetic challenge, immune cells were pre-treated with Alomac before adding PIC.

Overview of changes to all detectable cytokines after 2 hours (Tables 2-9 below):

In contrast to what we observed in context of LPS-induced inflammation, we observed significant **increases** to the majority of cytokine levels compared to the PIC-treated control cultures.

For the aqueous fraction, 22 of the 27 tested cytokines were significantly **increased**; exceptions were the pro-inflammatory cytokines IL-12 (p70), Eotaxin, RANTES, and the regulating cytokines IL-7 and IL-9. The aqueous fraction also caused decreases to two cytokines: RANTES and IL-9.

Similarly, for the in vitro digested fraction, we observed **increases** to the majority of cytokines, including 20 of the 27 cytokines tested; the exceptions being IL-12 (p70), IL-13, MCP-1, IL-10, IL-7, PDGF-BB, and GM-CSF. We did not observe significant decreases to any of the cytokines tested.

Selected cytokines as examples of rapid changes to cytokine levels already at 2 hours are described here, and shown in Figures 24-35 below:

- **IFN-γ** (**Interferon-gamma)** and **IL-1β (Interleukin-1β)** – Unlike the Alomac-modulation of LPS-induced immune reactions, we observed increases in IFN-γ and IL-1β in context of the PIC viral mimetic challenge for both the aqueous and in vitro digested fractions. This showed that **under viral challenge Alomac rapidly enhances the virally induced IFN-γ and IL-1β production already at 2 hours.**
- **MCP-1 (Monocyte chemoattractant protein-1, CCL2)** – Both the aqueous and in vitro digested fraction increased the production of the chemokine MCP-1 for all but the highest dose. This was in contrast to the reduced levels of MCP-1 we previously observed after 24 hours. **This shows that MCP-1 production was an early event, likely supporting the recruitment of monocytes in the early phase on immune responsiveness, but did not continue to increase over time.**
- **MIP-1α (Macrophage Inflammatory protein 1 alpha, CCL3)** – The production of MIP-1a was strongly increased already at 2 hours. This sets the stage for the previously reported dramatic increase in MIP-1a production after 24 hours of a viral mimetic

challenge. **The highly selective increase in this chemokine suggests that Alomac supports the release of this chemokine to selectively attract natural killer cells and T cells which are important cell types for anti-viral immune defense activity.**

- **IL-1ra (Interleukin-1 receptor antagonist)** – The aqueous fraction of Alomac significantly increased IL-1ra levels. In contrast, the in vitro digested fraction triggered a mild decrease when compared to the buffer control. This early modulation by the aqueous fraction continued, as we previously reported high levels of IL-1ra after 24 hours. The slight suppression of IL-1ra seen at 2 hours was later reversed since we also observed robust increases in IL-1ra levels after 24 hours exposure to the in vitro digested fraction of Alomac in context of the viral mimetic challenge.
- **G-CSF (Granulocyte-colony stimulating factor)** – In context of a viral mimetic challenge, both the aqueous and in vitro digested fraction of Alomac increased G-CSF production. **This shows that not only in the absence of an immune challenge, but also in context of a viral mimetic challenge, the production of G-CSF is triggered early in the process and continued to increase to reach the high levels seen after 24 hours of cell culture. This early production is promising and may be associated with the known effect of Alomac on stem cell surveillance.**

Table 18. Pro-inflammatory cytokines - viral mimetic challenge: levels of significance

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IFN-γ	26	-1	36	-5
IL-1β	190		126	
IL-5	45		64	-2
IL-6	1,054		15	
IL-8	110		868	-20
IL-12 (p70)	20		5	-1
IL-13	7		10	-2
IL-17A	53		51	-5
Eotaxin	54		42	
IP-10	37		42	
MCP-1	67		118	-30
MIP-1α	5,917		4,782	
MIP-1β	506		303	-16
RANTES		-30	19	-5
TNF-α	362		408	

Table 19. Anti-inflammatory cytokines - viral mimetic challenge: levels of significance

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IL-1ra	41	-7	20	0
IL-10	16	-1	6	-7

Color Description	
	Increase
	Decrease

Color	P-valve	Description
	≥0.10	Not statistically significant
	<0.10	Statistical trend
	<0.05	Statistical significance
	<0.01	High level of significance

Table 20. Cytokines/chemokines with regulating properties - viral mimetic challenge: levels of significance

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IL-2	167		36	-7
IL-4	120		84	
IL-7	23	-3	5	-7
IL-9	2	-27	22	-4
IL-15	52		30	-7

Table 21. Growth factors - viral mimetic challenge: levels of significance

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
Basic FGF	137		140	-3
PDGF-BB	18		16	3
VEGF	73		75	-1
GM-CSF	29	-1	32	-8
G-CSF	657		637	

Color Description	
	Increase
	Decrease

Color	P-valve	Description
	≥0.10	Not statistically significant
	<0.10	Statistical trend
	<0.05	Statistical significance
	<0.01	High level of significance

Table 22. Pro-inflammatory cytokines - viral mimetic challenge: levels of magnitude

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IFN-γ	54	-1	36	-5
IL-1β	190		281	
IL-5	60		64	-2
IL-6	1,054		688	
IL-8	752		868	-20
IL-12 (p70)	20		5	-1
IL-13	25		10	-2
IL-17A	89		77	-5
Eotaxin	54		42	
IP-10	37		42	
MCP-1	263		118	-30
MIP-1α	11,623		8,874	
MIP-1β	506		303	-16
RANTES		-30	23	-5
TNF-α	1,808		926	

Table 23. Anti-inflammatory cytokines - viral mimetic challenge: levels of magnitude

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IL-1ra	41	-7	20	0
IL-10	16	-1	6	-7

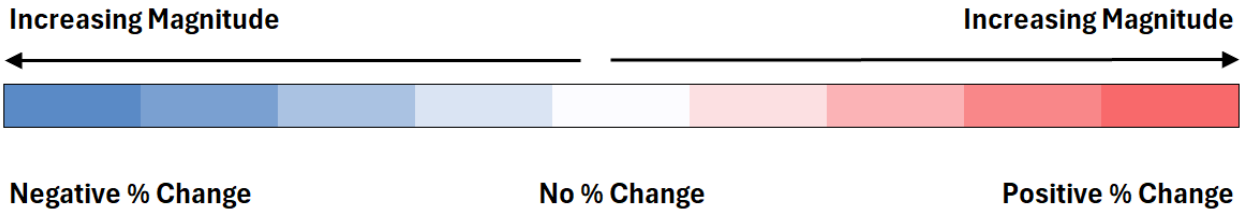
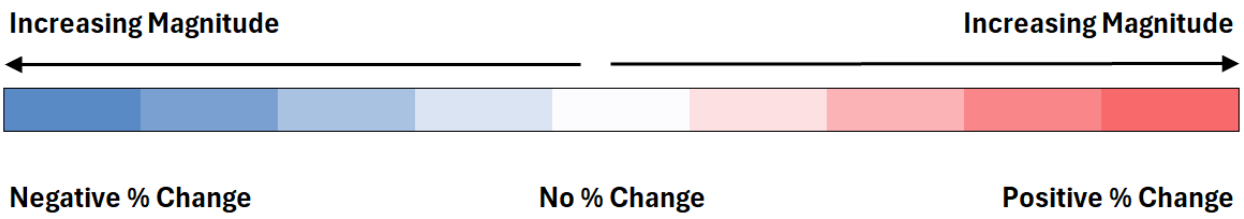


Table 24. Regulating cytokines - viral mimetic challenge: levels of magnitude

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
IL-2	167		164	-7
IL-4	120		84	
IL-7	23	-3	5	-7
IL-9	2	-27	22	-4
IL-15	52		49	-7

Table 25. Growth factors - viral mimetic challenge: levels of magnitude

	Alomac-AQ		Alomac IVD	
	% Increase	% Decrease	% Increase	% Decrease
Basic FGF	137		140	-3
PDGF-BB	18		16	-3
VEGF	73		75	-1
GM-CSF	29	-1	32	-8
G-CSF	657		637	



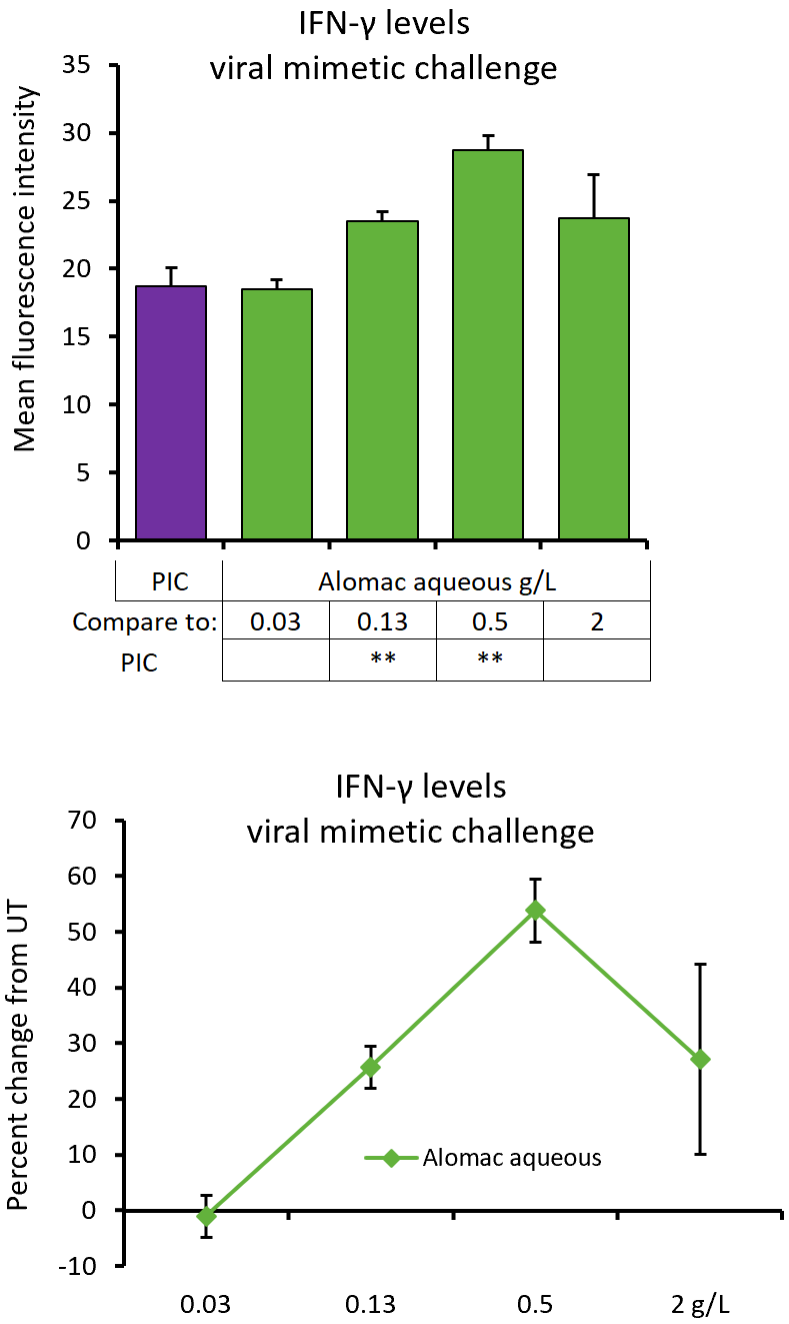


*Figure 24. Interferon-γ (IFN-γ) levels when cells were treated with the aqueous fraction of Alomac prior to treatment with Polyinosinic:polycytidylic acid (PIC). PIC (purple bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to PIC at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

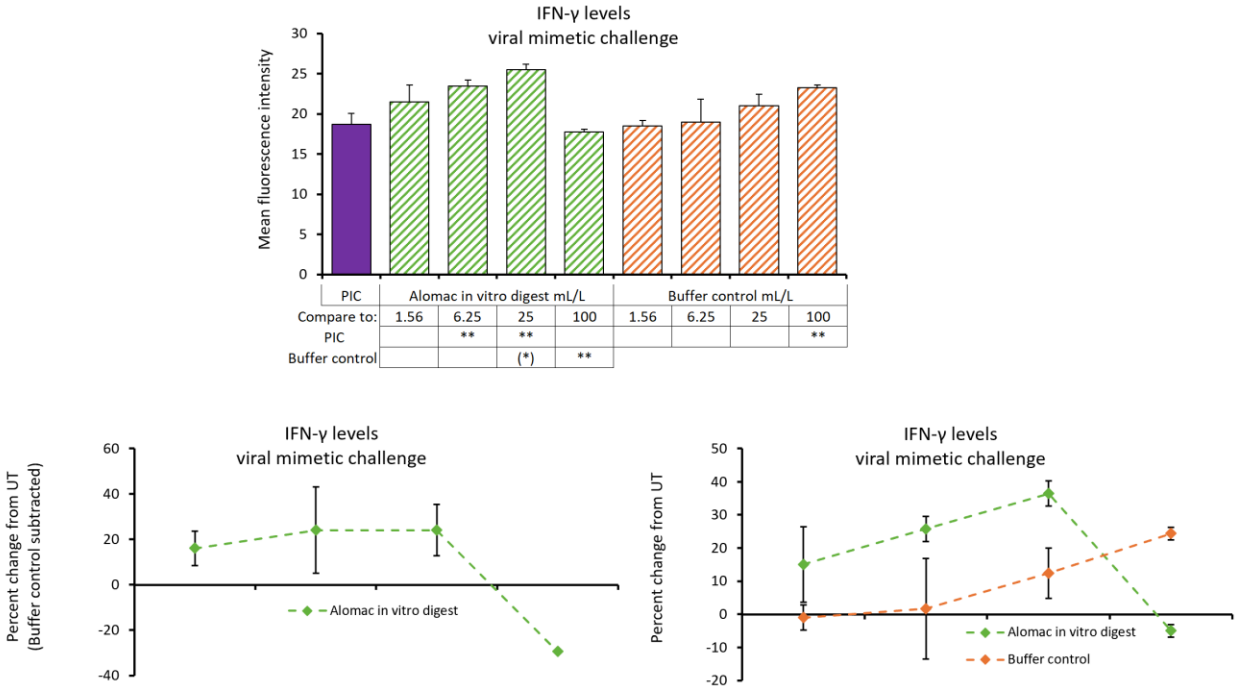
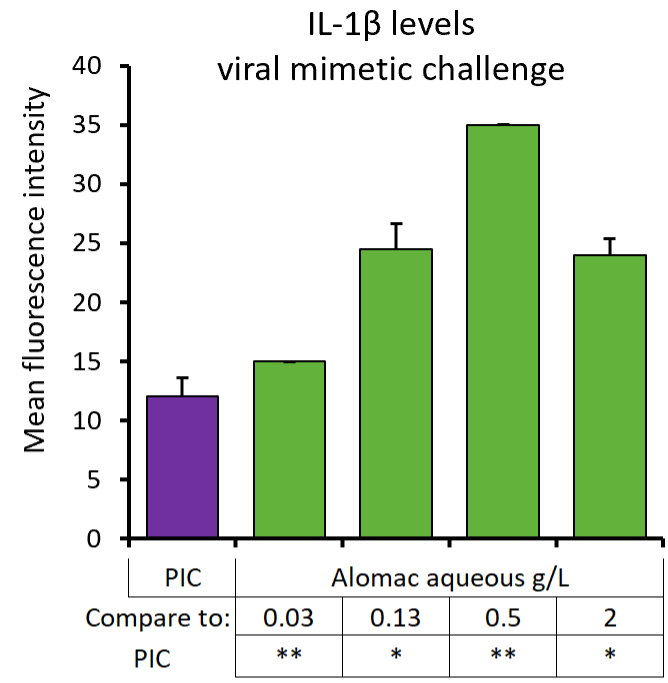


*Figure 25. Interferon-γ (IFN-γ) levels when cells were treated with the in vitro digested fraction of Alomac prior to treatment with Polyinosinic:polycytidylic acid (PIC). PIC (purple bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to PIC or the buffer control at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*



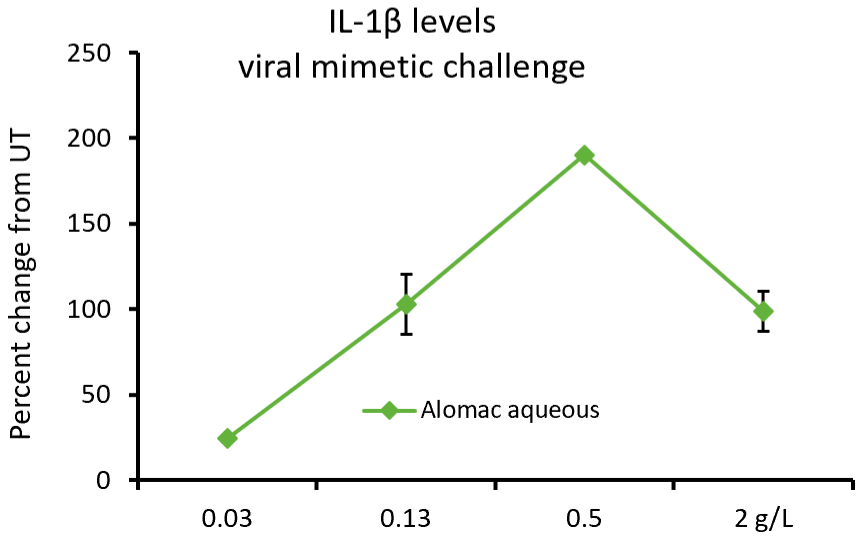


*Figure 26. Interleukin-1β (IL-1β) levels when cells were treated with the aqueous fraction of Alomac prior to treatment with Polyinosinic:polycytidylic acid (PIC). PIC (purple bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to PIC at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

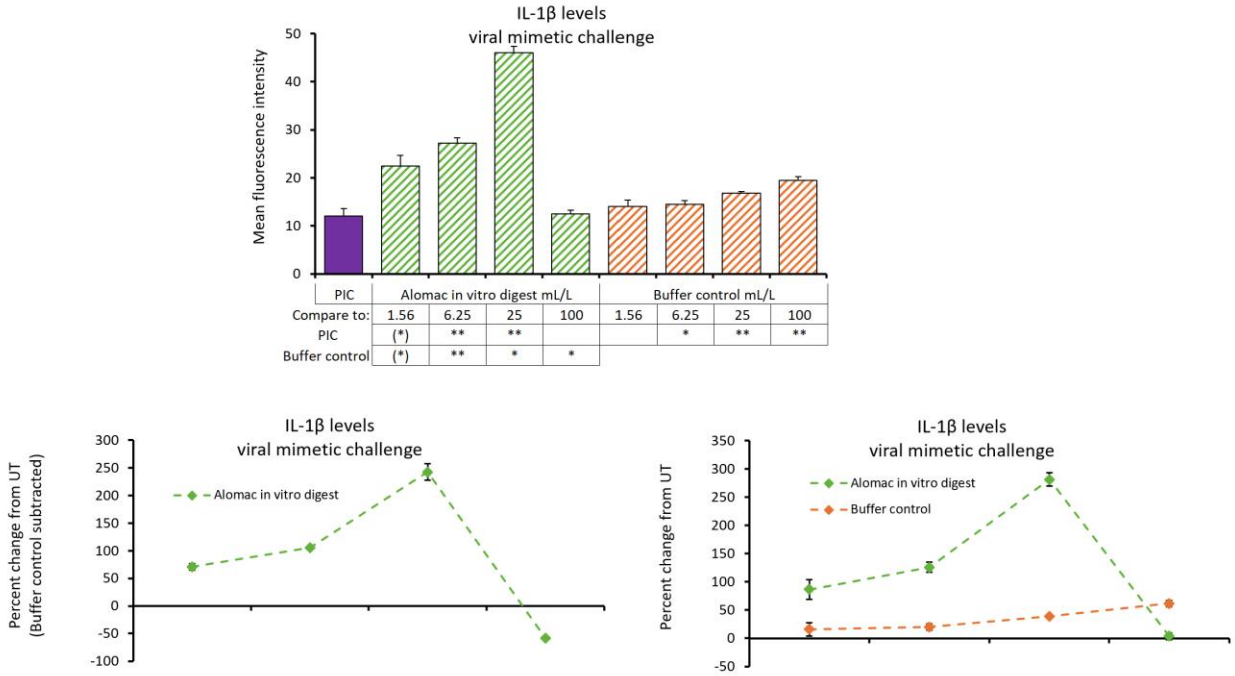
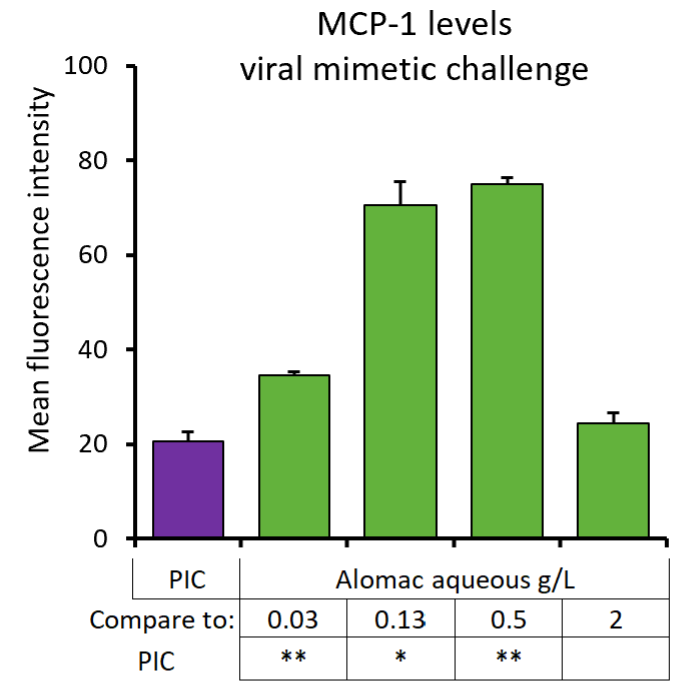


*Figure 27. Interleukin-1β (IL-1β) levels when cells were treated with the in vitro digested fraction of Alomac prior to treatment with Polyinosinic:polycytidylic acid (PIC). PIC (purple bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to PIC or the buffer control at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*



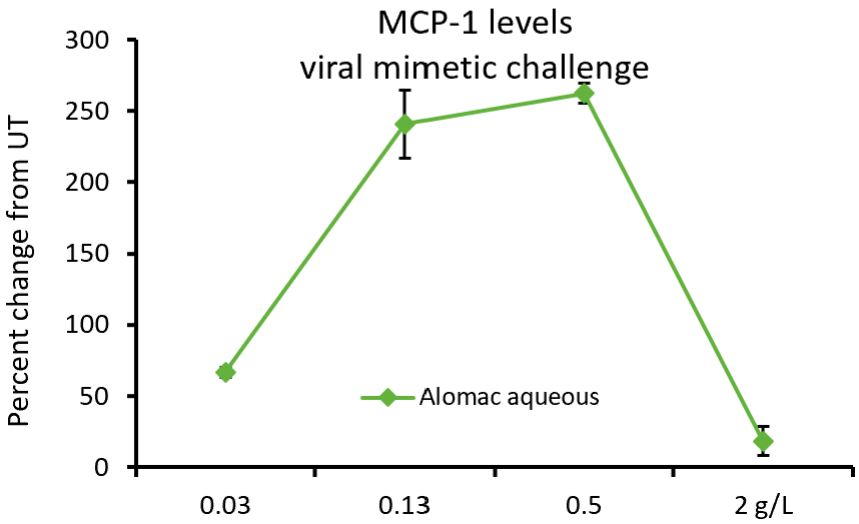


*Figure 28. Monocyte chemoattractant protein-1 (MCP-1) levels when cells were treated with the aqueous fraction of Alomac prior to treatment with Polyinosinic:polycytidylic acid (PIC). PIC (purple bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to PIC at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

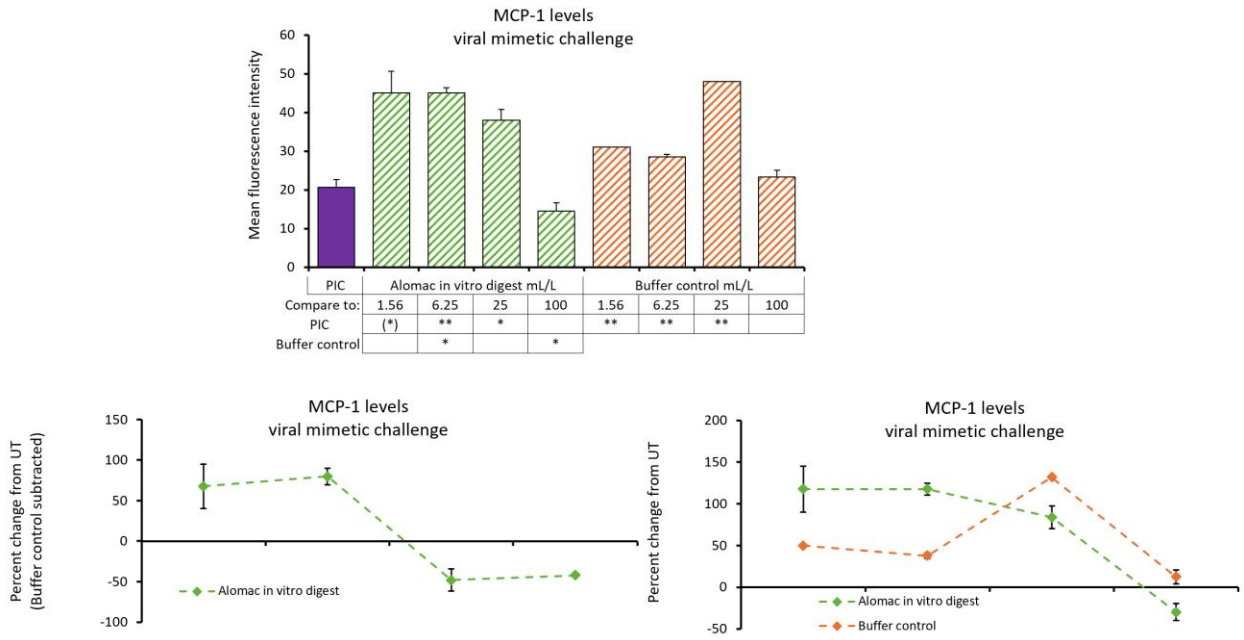
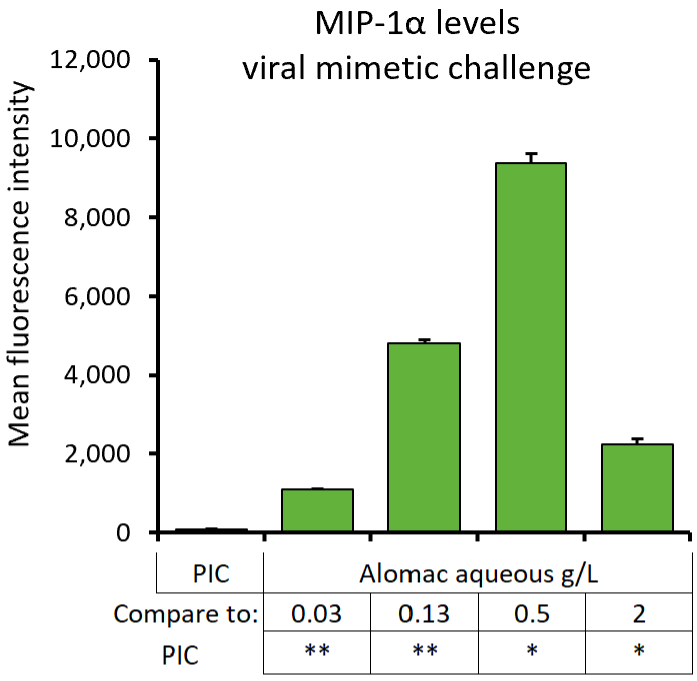


*Figure 29. Monocyte chemoattractant protein-1 (MCP-1) levels when cells were treated with the in vitro digested fraction of Alomac prior to treatment with Polyinosinic:polycytidylic acid (PIC). PIC (purple bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to PIC or the buffer control at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*



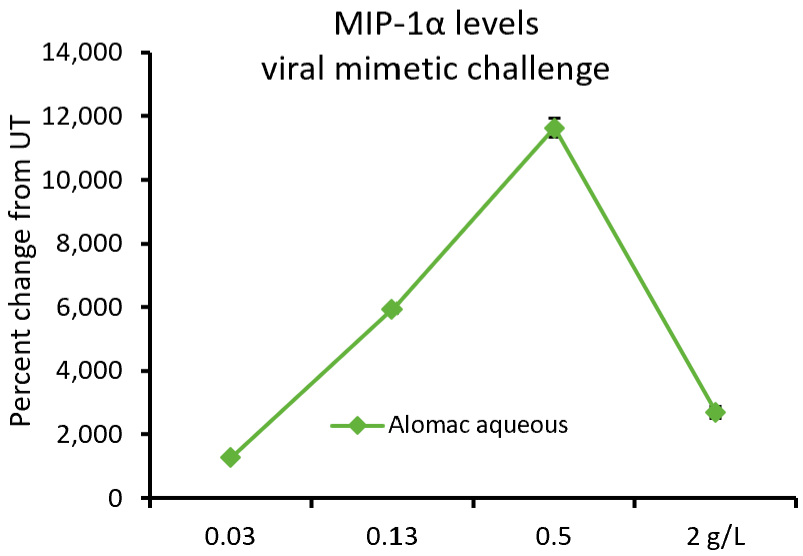


*Figure 30. Macrophage inflammatory protein-1 alpha (MIP-1α) levels when cells were treated with the aqueous fraction of Alomac prior to treatment with Polyinosinic:polycytidylic acid (PIC). PIC (purple bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to PIC at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

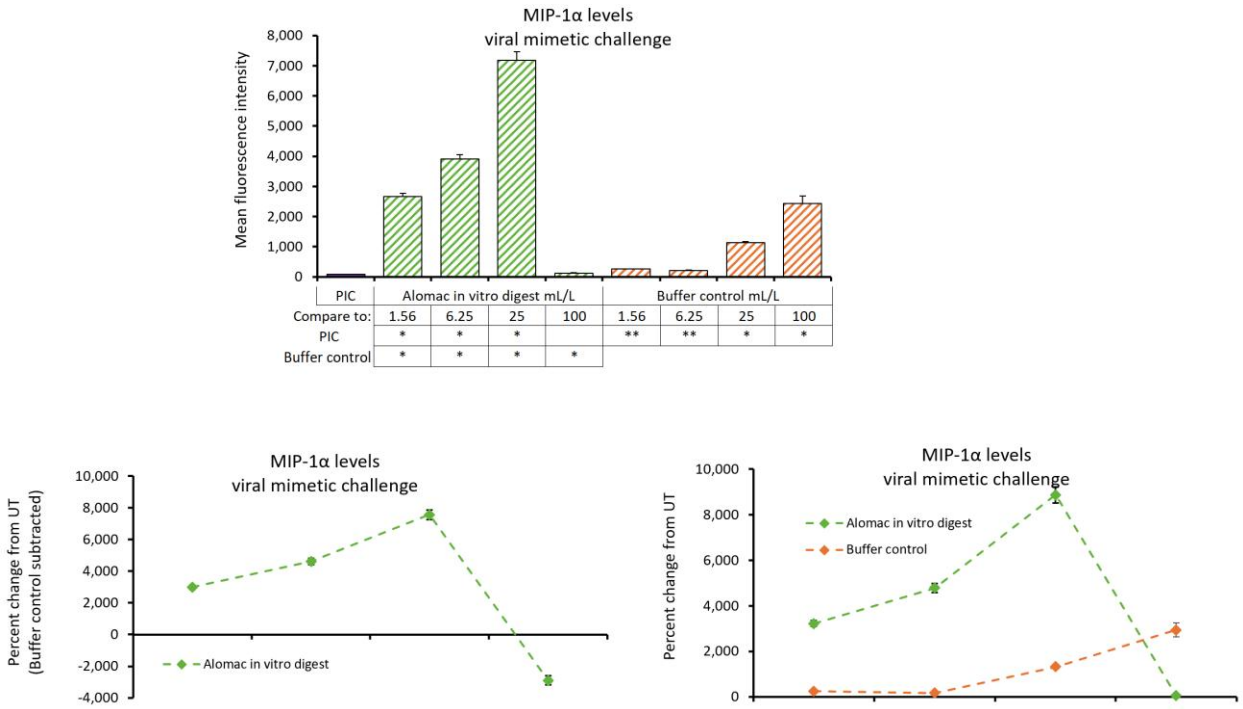
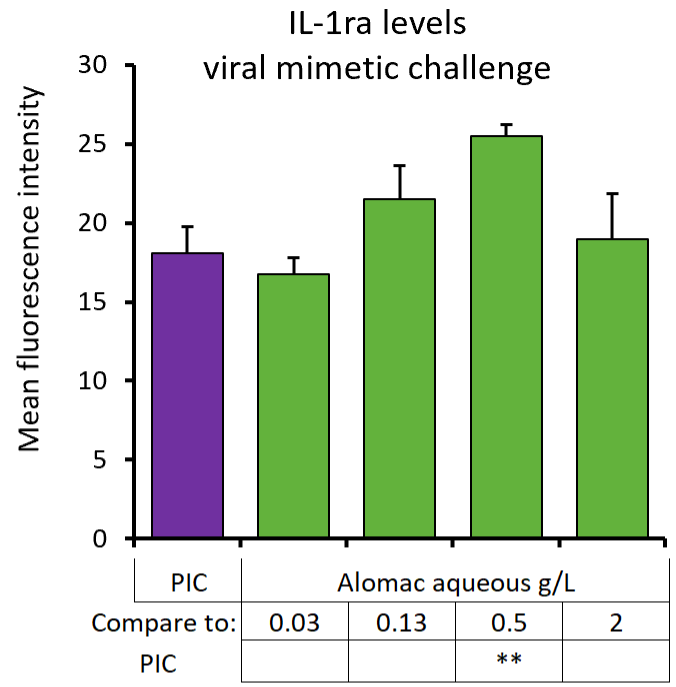


*Figure 31. Macrophage inflammatory protein-1 alpha (MIP-1α) levels when cells were treated with the in vitro digested fraction of Alomac prior to treatment with Polyinosinic:polycytidylic acid (PIC). PIC (purple bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to PIC or the buffer control at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*



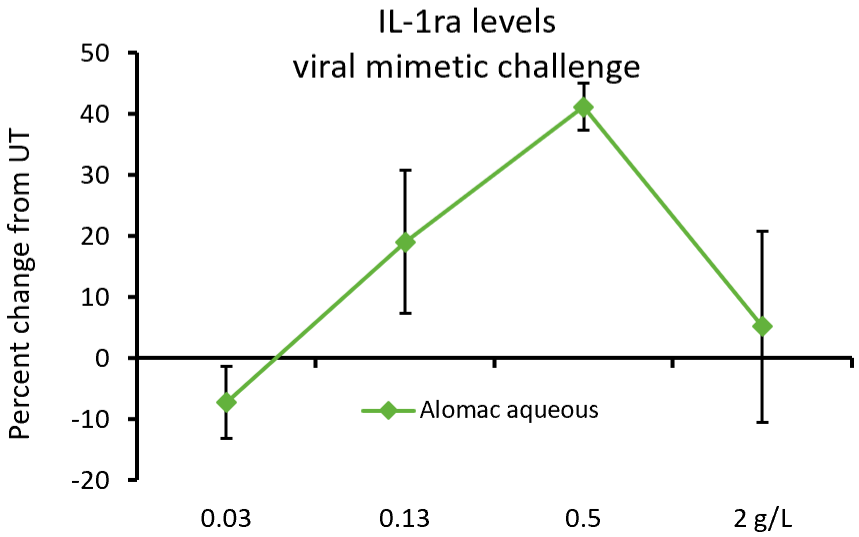


*Figure 32. Interleukin-1 receptor antagonist protein (IL-1ra) levels when cells were treated with the aqueous fraction of Alomac prior to treatment with Polyinosinic:polycytidylic acid (PIC). PIC (purple bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to PIC at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

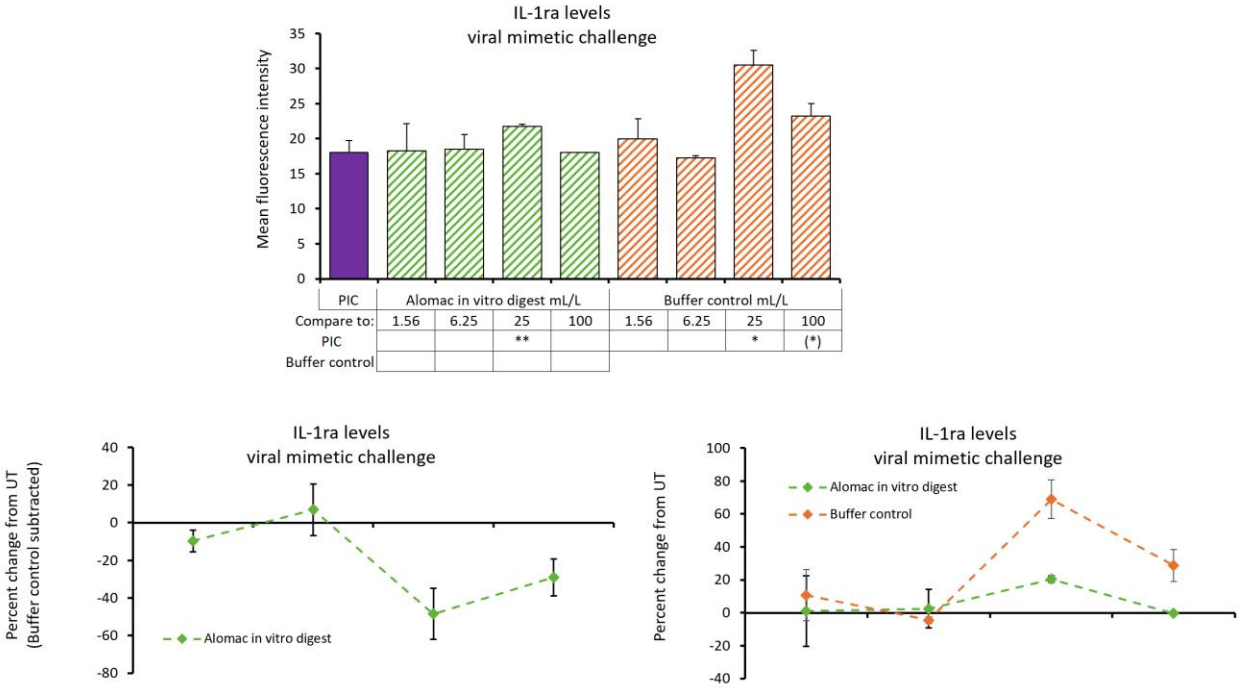
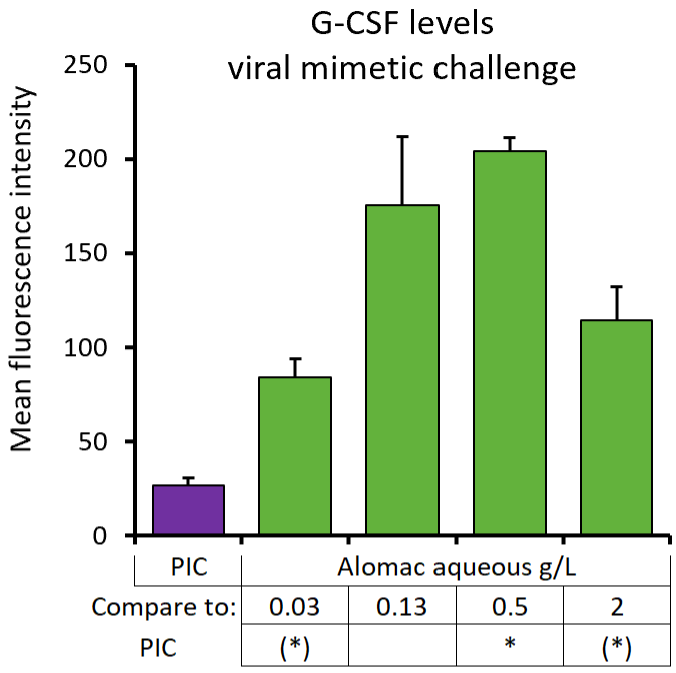


Figure 33. *Interleukin-1 receptor antagonist protein (IL-1ra) levels when cells were treated with the in vitro digested fraction of Alomac prior to treatment with Polyinosinic:polycytidylic acid (PIC). PIC (purple bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to PIC or the buffer control at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*



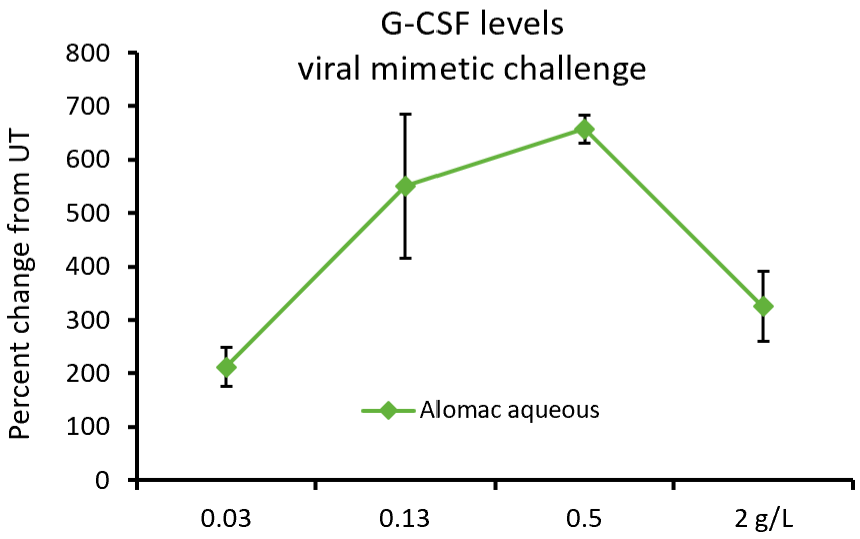


*Figure 34. Granulocyte-colony stimulating factor (G-CSF) levels when cells were treated with the aqueous fraction of Alomac prior to treatment with Polyinosinic:polycytidylic acid (PIC). PIC (purple bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to PIC at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

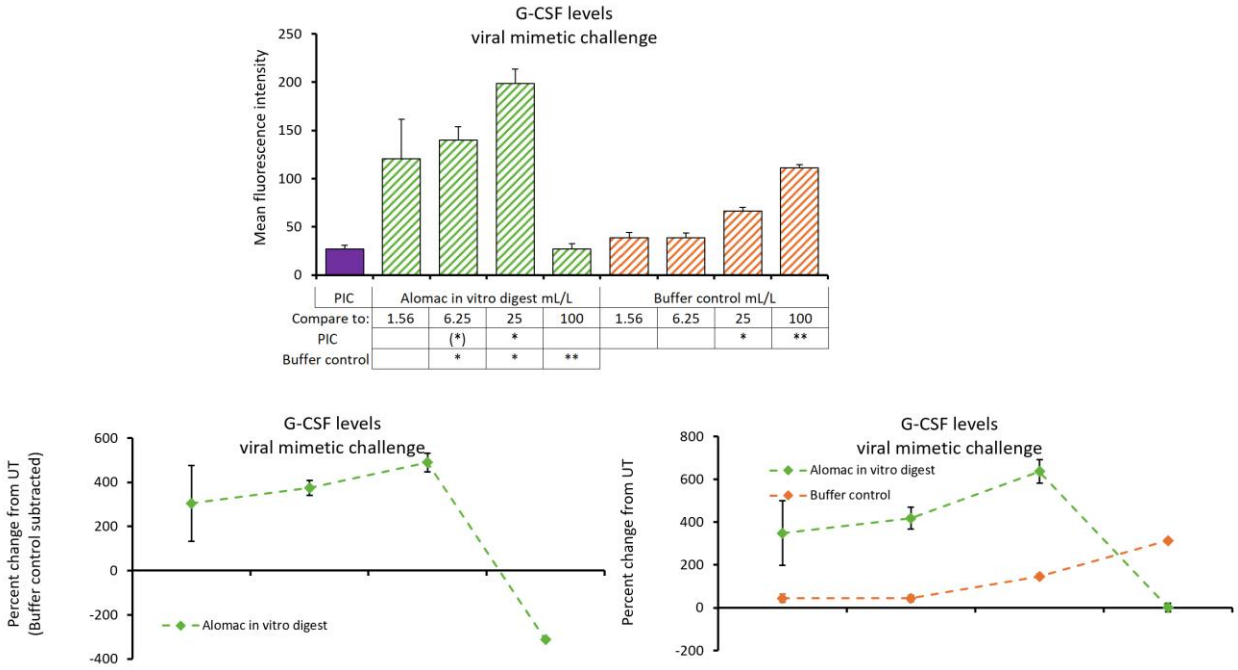


*Figure 35. Granulocyte-colony stimulating factor (G-CSF) levels when cells were treated with the in vitro digested fraction of Alomac prior to treatment with Polyinosinic:polycytidylic acid (PIC). PIC (purple bar) served as the positive control. In the table below the bar graph, the levels of statistical significance compared to PIC or the buffer control at different doses are indicated by asterisks, when P<0.10: (*), P<0.05: *, P<0.01: ** and NC when not calculated.*

6 Conclusion

While many natural products do not trigger cytokine induction or modulation already at 2 hours of immune cell culture, Alomac showed many direct effects, as well as modulating effects under inflamed and virally challenged cultures. Both the aqueous and in vitro digested fractions contributed to the 2-hour effects.

This testing of 2-hour cytokine production, put into context of the previous report on 24-hour cytokine production, showed many interesting findings.

Direct effects (in the absence of an immune challenge): Alomac's aqueous and in vitro digested fractions directly triggered increased production of multiple immune-activation pro-inflammatory cytokines, and several growth factors, already at 2 hours. To summarize the most impactful observations, in context of the previous report:

- IFN-γ (Interferon-gamma) and IL-1β (Interleukin-1β) production were initiated already at 2 hours, leading to the high levels at 24 hours. This shows that the direct immune activating effect of Alomac started rapidly and involved these two key cytokines helping to orchestrate an immune response. IL-1β also triggers the release of other inflammatory mediators such as IL-6, TNF-α, and chemokines.
- MCP-1 (Monocyte chemoattractant protein 1) production was initiated early, showing that this chemokine played a role in the early phase of Alomac's immune activating effects. This suggests that MCP-1 plays a role in the rapid induction of an immune response with an initial burst of chemokine attraction of monocytes.
- IL-10 (Interleukin-10) production was not increased at 2 hours, showing that the robust production of IL-10 seen at 24 hours must have been initiated later, since changes were not detectable at 2 hours.
- G-CSF (Granulocyte-colony stimulating factor) production was triggered early in the process, showing increases at 2 hours, and continued to increase to reach the high levels seen after 24 hours of cell culture. This early production is promising and may be associated with the known effect of Alomac on stem cell surveillance seen 2 hours after consuming a single dose of Alomac.

This rapid direct immune cell activation by Alomac seen already at 2 hours and affecting the majority of cytokines tested, shows that Alomac has broad immune activating properties.

Under an inflammatory challenge (LPS exposure): In context of an inflammatory insult, Alomac triggered rapid reduction of the inflammatory reaction, showing highly statistically significant reductions in almost all cytokines tested. This was seen for both the aqueous and in vitro

digested fractions. To summarize the most impactful observations, in context of the previous report:

- LPS-induced IFN-γ (Interferon-gamma), IL-1β (Interleukin-1 beta), and TNF-α (tumor necrosis factor alpha) production were reduced early by Alomac, but in context of the previous report on 24-hour data, this suppression at 2 hours was temporary, since Alomac strongly amplified the LPS-induced IFN-γ, IL-1β, and TNF-α production after 24 hours. This unique time-pattern deserves further research.
- A suppression of the LPS-induced production of MCP-1 (Monocyte chemoattractant protein 1) production was initiated early, showing that this cytokine was down-regulated in the early phase of Alomac's anti-inflammatory activating effects. MCP-1 did not continue to regulate the downstream effects, since the levels after 24 hours were rather similar to the levels after 2 hours. This suggests that Alomac helps reduce inflammation induced recruitment of monocytes to the affected tissue.
- IL-1ra (interleukin-1 receptor antagonist) production was highly significantly decreased by Alomac at 2 hours; this early modulation continued as this reduction was even stronger after 24 hours of cell culture. It is possible that the broad and robust anti-inflammatory effect of Alomac under inflamed conditions reduced the need to produce IL-1ra to balance the inflammation.
- IL-10 (interleukin-10) was mildly reduced at 2 hours compared to the LPS-treated control cultures. This early modulation continued as this was also observed after 24 hours of cell culture. It is interesting in light of the lack of measurable effects of Alomac on IL-10 levels in the absence of inflammation.

The rapid anti-inflammatory effects of Alomac are noteworthy. Alomac was added to the immune cells within 5 minutes of adding the inflammatory insult LPS. Within these few minutes, Alomac reprogrammed the immune cells so they responded remarkably differently to inflammation, compared to control cultures where LPS was added without Alomac's protective effects. This reprogramming was evident both at 2 and 24 hours of cell culture.

Under a viral mimic challenge (PIC exposure): In context of a viral mimetic immune challenge, Alomac triggered rapid modulation of the immune reaction, showing highly statistically significant increases in a majority of the cytokines tested. This was seen for both the aqueous and in vitro digested fractions. To summarize the most impactful observations, in context of the previous report:

- Under the viral mimetic challenge Alomac rapidly enhanced the virally induced IFN-γ (Interferon-gamma) and IL-1β (Interleukin-1 beta) production already at 2 hours. This

was opposite to the effects under LPS-induced inflammatory culture conditions, demonstrating the selective effect of Alomac under different types of immune challenges.

- MCP-1 (Monocyte chemoattractant protein 1) levels increased in the presence of Alomac. This was in contrast to the reduced levels of MCP-1 we previously observed after 24 hours. This shows that even though MCP-1 production was increased by Alomac early in the immune response to a viral challenge, it later suppressed the MCP-1 production. This suggests an initial burst of chemokine attraction of monocytes, followed by a later reduced recruitment of monocytes.
- MIP-1α (Macrophage Inflammatory protein 1 alpha, CCL3) – The production of MIP-1a was strongly increased already at 2 hours, which continued to increase after 24 hours. The highly selective increase in this chemokine suggests that Alomac supports the release of this chemokine to selectively attract natural killer cells and T cells which are important cell types for anti-viral immune defense activity.
- G-CSF (Granulocyte-colony stimulating factor) levels were rapidly increased at 2 hours, showing that both in the absence of an immune challenge, and also in context of a viral mimetic challenge, the production of G-CSF was initiated early in the process and continued to increase to reach the high levels seen after 24 hours of cell culture. This early production is promising and may be associated with the known effect of Alomac on stem cell surveillance.

The rapid immune-modulating effects of Alomac in context of a viral challenge are important and differs from the impact in context of LPS-induced inflammation. For the viral mimetic challenge, Alomac was added to the immune cells within 5 minutes before adding the viral mimetic PIC. Within these few minutes, Alomac reprogrammed the immune cells so they responded remarkably differently to PIC, compared to control cultures where PIC was added without Alomac's protective effects. This reprogramming was evident both at 2 and 24 hours of cell culture.

7 Further work

Further work has been discussed and may include:

- Immune modulation in vitro – validation module in preparation for manuscript writing.
- Immune modulation in vitro – Effects on additional innate immune functions.
- Effects on cellular senescence in vitro.

- Manuscript writing on the combined results from in vitro testing.
- Stem cell and immune cell surveillance – Expanded panel of stem cell types, and also testing immune cell surveillance, testing Mioty's current Alomac product.
- Manuscript writing on the new clinical results.

In addition, based on the results presented here, further research may be considered:

- Investigate which specific components in Alomac are driving the IFN-γ and selective chemokine increases. Is there a polysaccharide or glycoprotein in Alomac particularly responsible? If identified, that could become a proprietary highlight.
- Clinical trials or in vivo studies to confirm that these in vitro immune markers translate to real-world outcomes (e.g., improved resistance to a common cold virus, or better vaccine antibody titers when taken as a supplement). Having direct clinical evidence will strengthen Alomac's position as a scientifically backed product.
- Monitor safety markers: Ensure that Alomac's stronger pro-inflammatory effect does not cause any adverse inflammatory reactions in vivo. The in vitro data did not show any uncontrolled cytokine release (no excessive TNF-α or IL-1β beyond what a normal immune response would be, which is reassuring. Still, documenting tolerance in humans will be important for consumer trust.

8 Appendix A. Cytokine Standards

Below is a table that shows the mean fluorescence intensity for each cytokine standard, added at a known amount indicated in picograms/milliliter (pg/mL). The testing used the Bio-Plex human cytokine/chemokine 27-plex cytokine protein array (Bio-Rad, Hercules, California, USA).

Table 26. Mean fluorescence intensity for the cytokine standards at a known dose.

	Mean Fluorescence Intensity	pg/mL
Pro-inflammatory cytokines		
IFN-γ	15,671	32,873
IL-1β	14,122	8,630
IL-5	9,858	175,664
IL-6	13,418	7,023
IL-8	10,061	11,828
IL-12 (p70)	14,315	35,294
IL-13	20,791	4,631
IL-17A	24,685	60,959
Eotaxin	15,238	2,411
IP-10	16,966	15,690
MCP-1	19,739	8,808
MIP-1α	29,397	857
MIP-1β	14,536	6,737
RANTES	8,399	28,181
TNF-α	14,660	143,425
Anti-inflammatory cytokines		
IL-1ra	13,564	156,832
IL-10	11,560	29,333
Regulating cytokines		
IL-2	14,371	45,753
IL-4	17,344	3,454
IL-7	11,691	59,322
IL-9	8,324	79,136
IL-15	15,279	349,512
Growth factors		
Basic FGF	8,018	50,124
G-CSF	17,158	147,139
GM-CSF	24,792	6,993
PDGF-BB	4,880	53,744
VEGF	15,493	162,418

9 Appendix B. Cytokine Descriptions

Table 27. Pro-inflammatory cytokines.

Title	Abbreviation	Definition
Interferon gamma	IFN-γ	Important cytokine for innate and adaptive immunity against viral and some bacterial infections. Inhibits viral replication, due to its immunostimulatory and immunomodulatory effects. Produced by natural killer cells and natural killer T cells. Lesser quantities produced by T cells and innate lymphoid cells. IFN-Y treatment has shown significant anti-viral effects against the herpes simplex virus and may be an effective treatment in individuals with low T cells.
Interleukin-1 beta	IL-1β	Interleukin-1 beta (IL-1β) is a pro-inflammatory cytokine; crucial for host-defenses against infection and injury. IL-1β affects cell proliferation, differentiation, and apoptosis. IL-1β is a pivotal communication link between the immune system and the central nervous system, including fever induction. IL-1β is produced by activated monocytes and macrophages. Elevated levels of IL-1β may lead to inflammatory disorders in the gastrointestinal tract, bones, and other regions of the body.
Interleukin-5	IL-5	Stimulates B cell growth and increases immunoglobulin secretion, primarily IgA. IL-5 is an activator and chemoattractant of eosinophils. Produced by Type-2 T Helper cells and Mast cells. Elevated levels of IL-5 have been associated with inflammatory disorders, such as allergic rhinitis and eosinophilic esophagitis.
Interleukin-6	IL-6	Acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine, produced in response to infections and tissue injuries. IL-6 stimulates Acute Phase Response, hematopoiesis, and immune reactions. IL-6 expression is strictly controlled by transcription and post-transcriptional mechanisms. IL-6 is produced during acute inflammation by monocytes, macrophages, and endothelial cells. Elevated levels of IL-6 have been associated with inflammatory and auto-immune diseases, such as multiple sclerosis, diabetes, prostate cancer, and rheumatoid arthritis. Anti-IL-6 agents, such as tocilizumab, have been developed as a treatment for inflammatory diseases such as arthritis.
Interleukin-8	IL-8	Neutrophil chemotactic factor. Induces migration toward site of infection and stimulates phagocytosis. In a typical infection, macrophages see an antigen first, and are the first cells to release IL-8 to recruit other cells. Induces a series of physiological responses required for migration and phagocytosis, such as increases in intracellular Ca2+, exocytosis (e.g., histamine release), and respiratory burst. Elevated serum levels of IL-8 have been associated with the pathogenesis of cystic fibrosis.
Interleukin-12 (protein 70)	IL-12p70	Involved in the differentiation of naïve T cells into Th1 cells. Stimulates the production of IFN-γ and TNF-α from T cells and natural killer cells. Produced by activated antigen-presenting cells, such as dendritic cells, macrophages, neutrophils, and B-Lymphoblastoid cells. Elevated levels of IL-12 have been associated with the pathogenesis of autoimmune diseases.
Interleukin-13	IL-13	Central regulator of IgE synthesis, goblet cell hyperplasia, airway hyperresponsiveness, and mucus secretion. Mediator of allergic inflammation. Secreted by Type-2 T helper cells, CD4 cells, NKT cells, mast cells, basophils, and eosinophils. Dysregulated levels of IL-13 have been implicated in the pathogenesis of autoimmune diseases, such as systemic lupus and rheumatoid arthritis.

Interleukin-17A	IL-17A	Interleukin-17A (IL-17A) is a proinflammatory cytokine that plays a key role in the induction of innate immune defenses. IL-17A is important for host defense against infections caused by extracellular bacteria and fungi. IL-17A is produced by CD4+ T cells (Th17 cells), when activated by IL-23. High levels of IL-17A are associated with several chronic inflammatory disease including rheumatoid arthritis, psoriasis, and multiple sclerosis.
Eosinophil chemotactic protein (CCL11)	Eotaxin	Eotaxins are a CC chemokine subfamily of eosinophil chemotactic proteins. In humans, there are three types of Eotaxins: CCL11, CCL24, and CCL26. Eotaxins selectively recruit eosinophils via chemotaxis. Produced by activated endothelial cells, eosinophils, monocytes, and dermal fibroblasts. Higher blood plasma concentrations are seen in patients with schizophrenia, asthma, and those who suffered from an allergic response. Elevated levels of Eotaxins have been found in cannabis users.
Interferon gamma-induced protein 10 (CXCL10)	IP-10	Chemoattractant for monocytes, macrophages, T and NK cells, and dendritic cells. Involved in the promotion of T cell adhesion to endothelial cells, antitumor activity, and inhibition of bone marrow colony formation and angiogenesis. Secreted by endothelial cells, monocytes, and fibroblasts in response to binding of IFN-y.
Monocyte chemotactic protein 1 (CCL2)	MCP-1	Released at sites of inflammation due to injury or infection. Regulates the migration and infiltration of monocytes and macrophages. Produced by endothelial, fibroblasts, epithelial, and microglial cells. Elevated levels of MCP-1 are implicated in the pathogenesis of several diseases characterized by monocyte infiltrates, such as psoriasis, atherosclerosis, and rheumatoid arthritis.
Macrophage Inflammatory protein 1 alpha (CCL3)	MIP-1α	Proinflammatory cytokine crucial for immune response to infection and inflammation. Activates neutrophils and induces the release of pro-inflammatory cytokines. Produced by macrophages and monocytes following stimulation with bacterial endotoxins.
Macrophage Inflammatory protein 1 beta (CCL4)	MIP-1β	Proinflammatory cytokine crucial for immune response to infection and inflammation. Activates neutrophils and induces the release of pro-inflammatory cytokines. Produced by macrophages and monocytes following stimulation with bacterial endotoxins.
Regulated on Activation, Normal T cell Expressed and Secreted (CCL5)	RANTES	Proinflammatory chemokine responsible for recruiting leukocytes to sites of inflammation. Chemotactic for T cells, eosinophils, basophils, monocytes, natural killer cells, dendritic cells, and mastocytes. Induces the proliferation and activation of NK cells. Produced by T cells and Monocytes. Elevated levels of RANTES is associated with viral infections and cancer.
Tumor necrosis factor alpha	TNF-α	Cytokine and adipokine, responsible for regulating immune cells. TNF-α can induce fever, apoptotic cell death, weight loss, inflammation, inhibit tumorigenesis and viral replication, and respond to sepsis via IL-1 and IL-6-producing cells. Produced by activated macrophages, T lymphocytes, and Natural Killer cells. Dysregulation of TNF-α production is implicated in Alzheimer's disease, cancer, major depression, psoriasis, and inflammatory bowel disease.

Table 28. Anti-inflammatory cytokines.

Title	Abbreviation(s)	Definition
Interleukin-1 receptor antagonist	IL-1ra	Natural inhibitor of the pro-inflammatory effects of IL-1a and IL-1β. Secreted by various types of cells, including epithelial cells, adipocytes, and immune cells. A polymorphism of the IL-1ra gene is associated with an increased risk of osteoporotic fractures and gastric cancer.
Interleukin-10	IL-10	Interleukin 10 (IL-10) is an anti-inflammatory cytokine that helps contain a pro-inflammatory immune defense reaction towards pathogens, prevents tissue damage, and maintains normal tissue homeostasis. IL-10 enhances B cell survival, proliferation, and antibody production, and can block NF-kB activity. IL-10 is produced by T helper cells, monocytes, macrophages, and dendritic cells. Recombinant IL-10 is used in treatment of chronic gut inflammatory illnesses, demonstrating the importance of IL-10 for counteracting a hyperactive immune response in the human body.

Table 29. Regulating cytokines.

Title	Abbreviation(s)	Definition
Interleukin -2	IL-2	Signaling cytokine necessary for the growth, proliferation, and differentiation of T cells. Regulates the activity of leukocytes and lymphocytes and plays a part in the body's natural response to microbial infections through the discrimination between self and foreign molecules. Released by CD4+ T cells and activated CD8+ T cells. Elevated levels of IL-2 may be associated with itchy psoriasis.
Interleukin -4	IL-4	Induces differentiation of naive helper T cells (Th0 cells) to Th2 cells, which subsequently produce additional IL-4 in a positive feedback loop. IL-4 is produced primarily by mast cells, Th2 cells, eosinophils and basophils. IL-4 stimulates proliferation of activated B and T cells, and differentiation of B cells into plasma cells. IL-4 induces B cell class switching to IgE.
Interleukin -7	IL-7	Cytokine responsible for stimulating the differentiation of multipotent hemopoietic stem cells into lymphoid progenitor cells. IL-7 also stimulates the proliferation of cells in the lymphoid lineage (T cells, NK cells, and B cells). Secreted by stromal cells in the bone marrow and thymus. Elevated levels of IL-7 have been associated with patients with viral infections, such as HIV. IL-7 is currently being studied in clinical trials as an immunotherapy agent for treating HIV infections.
Interleukin -9	IL-9	Stimulates cell proliferation and prevents apoptosis in hematopoietic cells. Signal transducer and activator of the STAT proteins, specifically STAT1, STAT3, and STAT5. Produced by mast cells, natural killer T cells and CD4 T helper cells. Elevated levels of IL-9 have been associated with Crohn's disease.
Interleukin -15	IL-15	Regulates the activation and proliferation of T and natural killer cells. Secreted by mononuclear phagocytes following infection by virus(es). Suppression of IL-15 may be a potential treatment for celiac disease.

Table 30. Growth Factors.

Title	Abbreviation	Definition
Basic Fibroblast Growth Factor	Basic FGF	Growth factor and signaling protein, with broad mitogenic and cell survival activities. Involved in angiogenesis, morphogenesis, tissue repair, embryonic development, and tumor growth and invasion. In normal tissue, Basic FGF is present in basement membranes and in the subendothelial extracellular matrix of blood vessels.
Granulocyte-Colony Stimulating Factor	G-CSF	Stimulates bone marrow to produce granulocytes and stem cells for release into the bloodstream. Stimulates the proliferation, survival, and differentiation of neutrophil precursors and mature neutrophils. Functionally, G-CSF, is both a cytokine and hormone. Produced by endothelium, macrophages, and several other immune cells. G-CSF injections are an FDA approved treatment used to increase stem cells in blood circulation after trauma
Granulocyte-Macrophage Colony Stimulating Factor	GM-CSF	Stimulates stem cells to produce granulocytes, such as neutrophils, eosinophils, basophils, and monocytes. Protective role against intestinal infection. Stimulates the proliferation of intestinal mucosal myeloid cells in response to bacterial invasion. Produced by T cells, macrophages, endothelial cells, intestinal epithelial cells, and fibroblasts. Part of the immune/inflammatory cascade, leading to further immune activation and GM-CSF release. Elevated levels of GM-CSF have been associated with autoimmune inflammatory diseases, such as arthritis and encephalitis.
Platelet-Derived Growth Factor subunit Beta	PDGF-BB	Growth factor involved in angiogenesis and is a potent mitogen for cells of mesenchymal origin. Important for wound healing. PDGF is synthesized and stored in the alpha granules of platelets and is released upon platelet activation. Produced by activated macrophages, smooth muscle cells, and endothelial cells.
Vascular Endothelial Growth Factor	VEGF	Growth factor protein that stimulates vasculogenesis and angiogenesis. Stimulates new blood vessel growth during embryonic development, after vessel injury, and to bypass blocked vessels. Produced by macrophages, platelets, keratinocyte, renal mesangial cells, and tumors. Elevated serum levels of VEGF are seen in patients with bronchial asthma and diabetes mellitus.

10 References

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