Multiplexed Analysis of Inflammatory Cytokines as Biomarkers in Moderate-Advanced Periodontal Disease and Systemic Conditions

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ABSTRACT

Immune responses to systemic and oral diseases are modulated by cytokine output of effector cells and driven by the nature of antigenic stimuli. Chronic inflammation in rheumatoid arthritis (RA) is marked by elevated T cell chemokines like TNFα and IL-17[1], while lower cytokine levels in cancer patients indicate tumor antigen mediated immune suppression[2]. This study compares cytokine signatures in dental patients displaying moderate-advanced periodontal disease with varying inflammatory and precancerous manifestations to those found within healthy, RA, and assorted cancer subjects, with the goal of identifying predictive biomarkers. Serum and saliva samples were collected from individuals (n=32) presenting with pre-cancerous lesions, localized- or generalized oral inflammation. A sandwich ELISA was utilized to measure IFNγ and IL-2 concentrations in these matrices, while multiplex cytokine analysis (Luminex) was performed on saliva from healthy donors, as well as on serum and cell-culture supernatants from RA and cancer patients (controls). Serum and salivary IL-2 concentrations were higher in the sampled oral disease states than in healthy subjects ($p<0.05$). Salivary IFNγ was notably lower in patients with localized- versus generalized-inflammation ($p=0.058$) and pre-cancerous lesions ($p=0.041$). Multiplex analysis of serum revealed raised IL-4 ($p<0.01$) and IL-15 ($p=0.049$) concentrations in cancer patients, and IL-7 to be the sole discerning factor between cancerous and rheumatic diseases ($p=0.032$). Peripheral blood derived supernatants from cancer and RA patients showed over 500-fold increases in IFNγ and IL-2, in addition to over 300-fold increases in IL-6 and TNFα in similar RA samples. Thus, elevated IL-2 may indicate oral pathogenesis while heightened salivary IFNγ suggests progression to advanced periodontitis and cancer. Future serum-based and *in vitro* studies should look for correlation of IL-4, IL-6, IL-7, IL-15, and TNFα concentrations and oral disease. In sum, monitoring the aforementioned cytokines could help track disease severity and serve as prognostic indication of treatment efficacy.

BACKGROUND

- In autoimmune diseases, chronic inflammation, or carcinogenesis levels of cytokines are observed to be elevated. IFN-γ along with, TNF-α, IL-1β are mostly pro inflammatory whereas other cytokines are pleiotropic in nature[3].
- Salivary cytokine levels indicate the presence of disease, epithelial behavior, the local inflammatory response, and carcinogenesis. For example, elevated levels of specific cytokines like IL-1α, IL-6, IL-8, VEGF-α, and TNF-α are prevalent in saliva of patients with tongue Squamous Cell Carcinoma (oral cancer) and pre cancerous lesions[3].
- The first mediators to have their role related to periodontal pathogenesis are innate immunity cytokines produced after microbial recognition, such as TNF-a, IL-1 and IL-6[4].
- Typically, there are two common diseases affecting the oral tissues and the supporting structures of a tooth. In gingivitis, inflammation is limited to the soft tissues, epithelium, and connective tissue; In periodontitis, inflammatory processes extend to the supporting tissues including the alveolar bone[5].
- Oral lichen planus (OLP), a chronic inflammation disease affecting the oral mucosa, is associated with upregulated IL-1, 2, 4, 5, 6, 8, 10, 12, 17, 18, TGF-β, IFN-γ and TNF-α, in lesions, saliva, serum and peripheral blood mononuclear cells from patients[6].
- IFN-γ, TNF-α, IL-4, IL-10 are involved in the susceptibility of oral chronic inflammatory disease states[6].

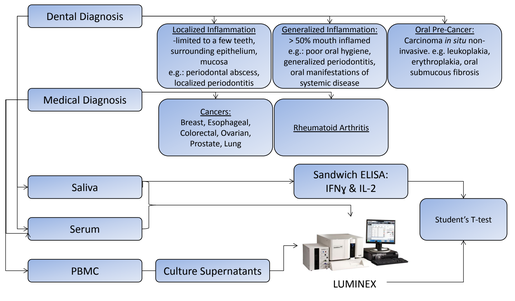
HYPOTHESES

If immune responses to oral pathogenesis parallel those in systemic disease, then

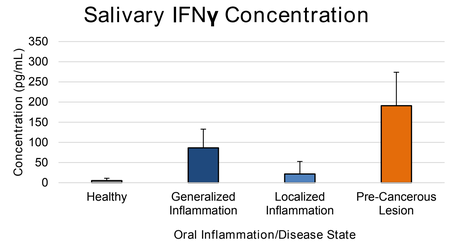
(1) concentrations of immunostimulatory IFNγ, IL-2, and other biomarkers should be higher in patients with generalized inflammation and oral pre-cancerous lesions than in subjects who are healthy or present with localized periodontitis.

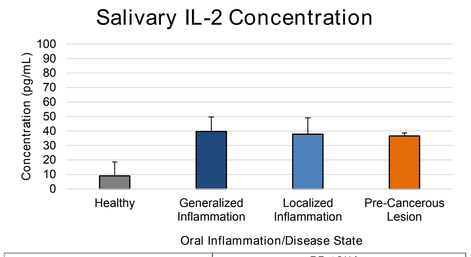
(2) saliva should be a better matrix for prognostic cytokine measurement than serum to differentiate between localized- and generalized inflammation / oral pre-cancer.

APPROACH



SALIVARY IFNγ & IL-2 as PERIODONTAL DISEASE BIOMARKERS

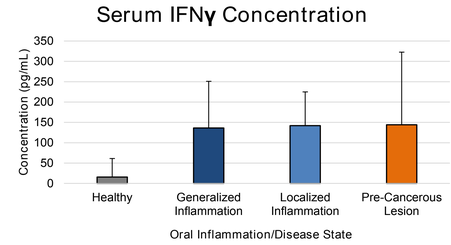


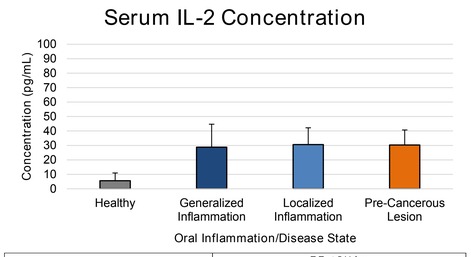


Saliva	T-Test *P* Value	
	IFNγ	IL-2
Generalized v Localized Inflammation	0.058	0.826
General Inflammation v Pre-Cancerous Lesion	0.300	0.547
Localized Inflammation v Pre-Cancerous Lesion	0.041*	0.875
Healthy v Generalized Inflammation	0.017*	0.001*
Healthy v Localized Inflammation	0.225	0.025*
Healthy v Pre-Cancerous Lesion	0.194	0.001*

Figure 1: Salivary Cytokines in Periodontal and Healthy Patients were measured via sandwich ELISA and Luminex, respectively. Average cytokine concentrations were graphed for IFNγ levels (top) and IL-2 (middle). Unpaired two tailed T-testing was performed on comparative data arrays in Microsoft Excel Version 14.0.7147.5001 (bottom).

SERUM IFNγ & IL-2 as PERIODONTAL DISEASE BIOMARKERS

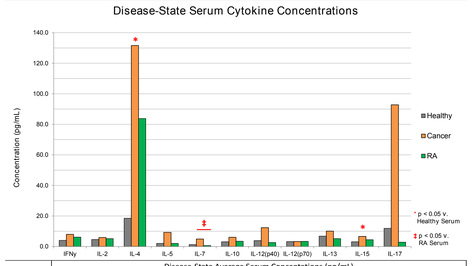




Serum	T-Test *P* Value	
	IFNγ	IL-2
Generalized v Localized Inflammation	0.875	0.732
General Inflammation v Pre-Cancerous Lesion	0.947	0.846
Localized Inflammation v Pre-Cancerous Lesion	0.987	0.969
Healthy v Generalized Inflammation	0.002*	0.000*
Healthy v Localized Inflammation	0.000*	0.000*
Healthy v Pre-Cancerous Lesion	0.339	0.048*

Figure 2: Serum Cytokines in Periodontal and Healthy Patients were measured via sandwich ELISA and Luminex, respectively. Average cytokine concentrations were graphed for IFNγ levels (top) and IL-2 (middle). Unpaired two tailed T-testing was performed on comparative data arrays in Microsoft Excel Version 14.0.7147.5001 (bottom).

SERUM IL-4, IL-7, & IL-15 as SYSTEMIC DISEASE BIOMARKERS



	Disease-State Average Serum Concentations (pg/mL)										
	IFNγ	IL-2	IL-4	IL-5	IL-7	IL-10	IL-12 (p40)	IL-12 (p70)	IL-13	IL-15	IL-17
Healthy	4.0	4.6	18.5	2.0	1.3	3.1	3.9	3.3	6.8	3.1	11.9
RA	6.1	5.1	83.8[a]	2.0	0.6	3.4	2.6	3.4	5.2	4.5[a]	2.8
Cancer	8.0	5.9	131.6	9.2	4.9[b]	6.0	12.3	3.3	10.1	6.6	92.8

[a] $p < 0.05$ v. Healthy Serum [b] $p < 0.05$ v. RA Serum

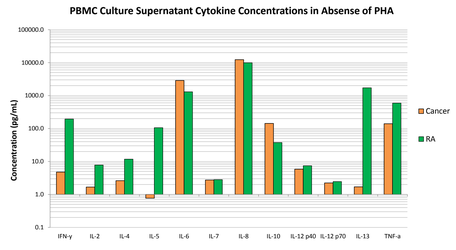
	Disease-State T-Test P Values										
	IFNγ	IL-2	IL-4	IL-5	IL-7	IL-10	IL-12 (p40)	IL-12 (p70)	IL-13	IL-15	IL-17
Healthy v RA	0.188	0.880	0.076	NA	0.115	0.797	0.539	0.246	0.556	0.672	0.329
Healthy v Cancer	0.191	0.437	0.001	0.319	0.066	0.172	0.113	0.520	0.434	0.049	0.306
RA v Cancer	0.544	0.824	0.202	0.319	0.032	0.248	0.060	0.327	0.199	0.527	0.254

Figure 3: Serum Cytokines in Cancer, Rheumatoid Arthritis (RA), and Healthy Patients were measured via 11-plex magnetic kit (EMD Millipore) using the Luminex platform (top). Average cytokine concentrations were calculated for each cytokine (middle). Unpaired two tailed T-testing was performed on comparative data arrays in Microsoft Excel Version 14.0.7147.5001 (bottom).

	Disease State Average Cell-Culture Supernatant Cytokine Concentration (pg/mL) – Pre-mitogen Challenge										
	IFN-γ	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12 p40	IL-12 p70	IL-13	TNF-a
RA	194.7	7.8	11.8	106.5	1303.7	2.8	9927.1	37.5	7.5	2.5	1723.6	591.9
Cancer	4.8	1.7	2.6	0.8	2883.4	2.8	12276.1	143.4	5.9	2.2	1.7	140.0
T-test P-value	0.335	0.467	0.696	0.792	0.389	0.110	0.318	0.387	0.647	0.965	0.391	0.481

	Disease State Average Stimulation Index – Post-mitogen Challenge											
	IFN-γ	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12 p40	IL-12 p70	IL-13	TNF-a
RA	1099.8	983.3	52.9	14.8	1245.4	3.5	1.8	70.1	228.6	34.4	26.6	431.1
Cancer	543.9	1263.8	29.6	13.1	61.8	3.7	1.0	16.9	50.7	12.8	51.4	210.1
T-test P-value	0.592	0.093	0.244	0.178	0.493	0.582	0.444	0.900	0.393	0.942	0.391	0.459

SUPERNATANT IFNγ, IL-2, IL-6, & TNFA as SYSTEMIC DISEASE BIOMARKERS



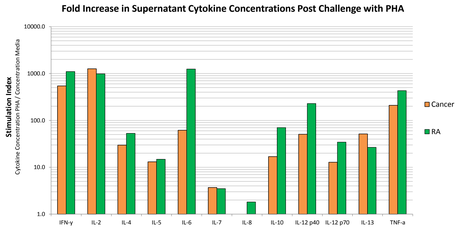


Figure 4: Peripheral Blood Derived Cell-Culture Cytokines in Cancer and Rheumatoid Arthritis (RA) Patients were obtained by incubating PBMCs in growth media (90% RPMI/9% FBS/1% Antibiotic) for 1 week in the presence or absence of the mitogenic stimulus phytohaemaglutinnin (PHA). Cytokines were measured via 12-plex magnetic bead kit (EMD Millipore) using the Luminex platform. Average cytokine concentrations were calculated and graphed for each cytokine (top, left-top). Stimulation indexes were calculated by dividing each donor's cytokine response to PHA by media background (bottom, left-bottom). Unpaired two tailed T-testing was performed on comparative data arrays in Microsoft Excel Version 14.0.7147.5001 (left).

CONCLUSIONS

- Serum is a better matrix than saliva for diagnosing periodontitis, showing significant increases in both IFNγ and IL-2 versus in healthy subject ($p < 0.05$).
- Salivary IFNγ concentration is useful for distinguishing generalized oral inflammation ($p = 0.058$) and pre-cancerous lesions ($p = 0.041$) from localized inflammation.
- Salivary IL-2 concentrations elevated above those of healthy donors correlates with moderate-to-advanced periodontal disease.
- Luminex revealed IL-4 and IL-15 levels significantly greater in cancer patients ($p < 0.05$), and noticeable increases in IL-5, IL-12 (p40), and IL-17 ($p > 0.05$).
- Unexpectedly no changes were seen in IL-2 and IFNγ concentrations in RA/cancer versus healthy serum.
- High levels of IFNγ (194.7 pg/mL) and IL-2 (7.8 pg/mL) observed in RA cell-culture supernatants prior to mitogenic challenge with PHA.
- High levels of IL-6 and IL8 (> 2000 pg/mL) observed in cancer cell-culture supernatants prior to mitogenic challenge with PHA.
- Lymphocyte potential to respond to antigens seen via > 500-fold increase in IFNγ and IL-2 in RA and Cancer, and > 300 –fold increase in IL-6 and TNFα in RA post-PHA challenge.
- Significant differences in the concentrations of the aforementioned cytokines in serum and saliva matrices suggests their usefulness as biomarkers in diagnosis.
- PBMC cell-culture experiment showed qualitative upregulation of immune stimulatory cytokines, indicative of patients' ability to fight disease, thereby demonstrating utility of multiplexing in disease-prognosis and predicting response to immunotherapies.

FUTURE DIRECTIONS

- Expand number of test subjects and samples to enhance statistical relevance.
- Perform additional Luminex assays with all matrices (saliva, serum, and cell-culture supernatant) on same plate to account for inter-assay variability.
- Add healthy donor subset to in vitro PBMC prediction assay to understand the context of immunostimulatory cytokine upregulation.
- Repeat saliva ELISA/multiplex analysis to look for increased concentrations of biomarkers identified in this project- IL-4, IL-6, IL-7, IL-15, and TNFα.- in patients with periodontal disease.
- Test for additional chemokines such as Macrophage Inflammatory Proteins (MIP), Tumor Growth Factors, and IL-1β in serum of patients with advanced periodontitis and pre-cancerous lesions.
- Add oral-cancer sample subsets for comparative studies.

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