Immune response to JC virus T antigen in patients with and without colorectal neoplasia

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Abstract

JC virus (JCV) is a polyomavirus that infects approximately 75% of the population and encodes a T antigen (T-Ag) gene, which is oncogenic and inactivates the p53 and pRb/p107/p130 protein families. Previous work in our lab has identified the presence of T-Ag in colorectal neoplasms. While JCV remains in a latent state for the majority of those infected, we hypothesized that a disturbance in immunological control may permit JCV to reactivate, which may be involved in the development of colorectal neoplasia. Our aim was to determine the cell mediated immune response to JCV T-Ag, and determine if it is altered in patients with colorectal adenomatous polyps (AP) or cancers (CRC). Peripheral blood mononuclear cells (PBMCs) isolated from the blood of patients undergoing colonoscopy or colorectal surgery were stimulated by a peptide library covering the entire T-Ag protein of JCV. Cytokine production and T cell proliferation were evaluated following T-Ag stimulation using Luminex and flow cytometry assays. JCV T-Ag peptides stimulated secretion of IL-2, which induced T cell expansion in all three groups. However, stronger IL-10 and IL-13 production was seen in patients without colorectal neoplasms. IP-10 was produced at very high levels in all groups, but not significantly differently between groups. Most patients exhibited CD4$^+$ and CD8$^+$ T cells in response to stimulation by the T-Ag clusters. The combination of IL-2 and IP-10 secretion indicates the presence of T-Ag-specific Th1 cells in all patients, which is higher in patients without carcinoma.

Keywords

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Introduction

JC virus (JCV) is a polyomavirus that was first identified in 1971 as the causative agent of progressive multifocal leukoencephalopathy (PML) in a patient with Hodgkin lymphoma.[1] Infection with the virus is ubiquitous in humans and 75% of most adult populations have antibodies to the virus including—although at lower rates—remote native South American populations.[2,3] Transmission appears to take place within families and close community members.[4,5] Most children produce antibodies to JCV by their early teenage years.[6] The initial infection by JCV is asymptomatic and the virus is capable of entering a latent stage in several possible reservoirs in the body. JCV has been found in the kidneys, tonsils, bone marrow, and gut, indicating these as possible reservoirs for the latent virus.[7–9] How JCV interacts over the long-term with the reservoir cells, and what damage may be done to these cells (if any), is unknown. It is possible that latent JCV is able to interact with the cell and cause damage that may lead to permanent changes.

JCV encodes three proteins that are the structural capsid proteins: VP1, VP2, and VP3. JCV also encodes the T antigen proteins (i.e., Large T and multiple small t splice variants) and the agnoprotein, which is involved in the assembly of viral particles. T-Ag has several splice sites producing truncated proteins that are involved in regulating cell proliferation and viral transmission.[10] Multiple experimental animal models have demonstrated that JCV can cause brain tumors in rodents and nonhuman primates.[11–13] When T-Ag induces tumorigenesis, all of the tumors are aneuploid. T-Ag is capable of disrupting the function of tumor suppressor proteins p53 and members of the pRB family, which probably contributes to carcinogenesis in the animal models.[14,15] Previous work identified increased nuclear expression of the JCV T-Ag in colorectal neoplasia compared with normal mucosa.[16–19] CRC cell lines transfected with JCV develop chromosomal instability and features of increased migration and invasion.[20]

The presence of JCV has been identified throughout the digestive tract. JCV was detected in 85% of normal esophageal tissue samples and 100% of the tumors.[21] Almost half of gastric cancers sampled were positive for JCV.[22,23] A recent study evaluating the presence of JCV in anal cancer also evaluated the concomitant presence of human papillomavirus (HPV), since it is assumed that HPV causes anal cancer, as well as cervical cancer. Twenty two anal cancers were tested by PCR for JCV and all were positive, while 13 of the cases were positive for HPV.[24] These data demonstrate the presence of JCV throughout cancers of the digestive tract and suggest the possibility that JCV initiates cancers in these tissues.

PML was first diagnosed in patients with hematological malignancies. Now most cases are seen in human immunodeficiency virus (HIV)—infected patients or patients receiving treatment with immunomodulators such as natalizumab, rituximab, or efalizumab for diseases such as Crohn disease, multiple sclerosis, or psoriasis. All of these patient populations have reduced function of their immune systems which allows the virus to enter the lytic phase. We still do not understand how the state of viral latency is either maintained or broken for JCV.[25] Since these patients are at risk for development of PML, previous studies on these patient populations focused on the immune response to JCV.[21,26,27] More recent work is evaluating the immune response beyond those subsets.[18] There is a need to

clarify the immune response in patients with gastrointestinal diseases since JCV has been correlated with some cancers in this organ system. In order to characterize the immune response in these populations, PBMCs from patients with neoplasia-free colons, and individuals with colorectal adenomas or carcinomas, were stimulated with JCV T-Ag peptides and evaluated for CD4^{+} and CD8^{+} T cell proliferation and cytokine production. We demonstrated that the JCV T-Ag stimulates a robust Th1 response, which most individuals have evidence of prior exposure to JCV, but with the exception of a slightly diminished IL-2 response in the cancer patients, there is not a substantial difference in the response between those who have or do not have colorectal neoplasia.

Results

Patient population data

As shown in Table 1, the average age of the patients was 60.3 years. A majority of the patients enrolled were Caucasian (n = 48; 80%), there were 10 African Americans patients (17%), and 2 Hispanic patients (3%). Thirty five of the patients were male and 25 were female. Twenty patients were enrolled into each of the following groups: negative colonoscopy, presence of adenomatous polyp(s), or a diagnosis of colorectal cancer. Patients were categorized as having a negative colonoscopy even if hyperplastic polyps were found during colonoscopy, since they are not considered true neoplasms.

Cytokine response

In order to evaluate the type of immune response generated by the PBMCs when stimulated by T-Ag peptides in culture, cytokine concentrations were determined and the fold change compared with the negative control was calculated. Interleukins (IL)-2, -10, and -13 and interferon-γ inducible protein 10 (IP-10) were tested to determine the presence of a Th1 or Th2 response. Antigen-specific Th1 responses are detected by the induction of IL-2 and IP-10, while antigen-specific Th2 responses are detected by the induction of IL-13.[28–30]

IL-10 response

Supernatant was taken on day 2 of the culture protocol to determine the relative concentration of IL-10 in the supernatant of the PBMC culture (Fig. 1). A previous study on blood samples from melanoma patients shows that the induction of IL-10 in the culture supernatant reflects the presence of antigen-specific regulatory T cells.[29] Several patients did not produce IL-10 in response to the stimulation by JCV T-Ag peptide clusters as seen by the white areas on the heat map. Patients with negative colonoscopies produced a significantly higher fold change in IL-10 concentration compared with patients with APs or cancers ($P < 0.001$ and $P < 0.05$, respectively). The IL-10 concentration was below detection levels in more of the PBMC cultures from patients with APs or cancer compared with those with negative colonoscopies. For the majority of the patients producing IL-10, no more than 4 clusters stimulated a response.

IL-13 response

Supernatant was taken on day 2 of the culture protocol to determine the relative concentration of IL-13 in the supernatant of the PBMCs culture (Fig. 2), and there was no

significant difference between the three groups ($P = 0.976$). The concentration of IL-13 produced did not exceed 7 pg/mL (data not shown), indicating a minimal response even though there are some fold change compared with the negative control that appear significant.

IL-2 response

Supernatant was taken on day 2 of the culture protocol to determine the relative concentration of IL-2 in the supernatant of the PBMCs culture (Fig. 3), and all of the clusters stimulated IL-2 production in the majority of patients. The concentration of IL-2 produced was as high as 50 pg/mL for the strongly responding clusters (data not shown) which is considered a robust response. The N-terminal T-Ag clusters were the strongest stimulators, but the rest of the clusters also showed immunostimulatory activity. The patients with cancer produced the lowest levels of response ($P = 0.03$), and among the fewer clusters.

IP-10 response

Supernatant was taken on day 2 of the culture protocol to determine the relative concentration of IP-10 in the supernatant of the PBMC culture (Fig. 4), and all 3 groups produced high levels of IP-10 release with fold change values up 300. IP-10 concentration was over 2500 pg/mL in some cultures. There were no significant differences between the amount of IP-10 produced among the groups ($P = 0.17$). All clusters stimulated IP-10 production, although the N-terminal clusters seem to initiate a response higher than the others in the AP group.

Flow cytometry results

The PBMCs were stained for CD4 and CD8 on day 8 of the protocol in order to identify their presence, and a proliferative response. During the plating of the cells on day 0, the cells were stained with the dye CFSE, which passively enters cells, and half is passed onto each daughter cell during division, thus making it a marker of cell proliferation.[31] Therefore, analysis of the expression of CD4 and CD8 by the cell population that diluted CFSE permits the determination whether the peptides are recognized by CD4$^+$ or CD8$^+$ T cells.

There was variability in the T cell recognition of the clusters (Fig. 5A). Some of the clusters were better recognized by the T cells, such as cluster 1 (Fig. 5B). However there was no difference between patients with or without neoplasia ($P = 0.7$). As with the cytokine production, the N-terminal of the protein stimulated more T cell proliferation compared with the carboxy terminus. Cluster 15 near the carboxy terminus was not well recognized by the T cells resulting in little to no cell proliferation (Fig. 5C). Overall, the T-Ag peptides did induce proliferation of the T cells in all groups.

Discussion

Our aim was to characterize the immune response to JCV in normal subjects and individuals with colorectal neoplasia. Prior infection by the virus in the individuals would result in memory T cells that could be stimulated to quickly expand in culture to evaluate the immune response. In our initial experiments, we evaluated the immune response from patient PBMCs

to T-Ag stimulation. The T cell proliferation and cytokine production in response to the T-Ag stimulation is a starting point to understanding how the immune system responds to the ubiquitous presence of JCV.

One way to evaluate the immune response to a pathogen is to compare indicators of the Th1 and Th2 responses. CD4^{+} T cells are comprised of multiple subsets, including Th1 and Th2. The Th1 response is associated with a cell mediated response and aids CD8^{+} T cells, which is the primary response to most viral infections, as opposed to the humoral response of Th2. IL-10, initially identified in 1989, mediates an anti-inflammatory response, which helps suppress the Th1 response.[32] An important cytokine in the colon is IL-13, which is known to be active in immune responses against colonic pathogens, especially helminthic parasites.[33] Production of IL-13 is characteristic of a Th2 response.[34] To evaluate the Th1 response, we chose the markers IL-2 and IP-10. Activated T cells produce IL-2, which is capable of inducing T cells to produce interferon-γ (IFN-γ).[35,36] IFN-γ is characteristic of a classic Th1 cytokine response, and suppresses the Th2 response.[37] IFN-γ also induces the production of IP-10, which acts as a sensitive indicator of the activation of Th1 cells in an in vitro culture system.[28,30] The coordinated response of these four cytokines, IL-2, IL-10, IL-13, and IP-10, give us an indication of the type of immune response generated by a pathogen.

Flow cytometry demonstrated the T cell proliferation in the PBMC cultures, and we found equally strong CD4^{+} and CD8^{+} T cell proliferative responses in most patients although there was no significant difference between the cohorts.

The majority of our patients demonstrated a more robust Th1 response as indicated by an increased production of IL-2 and IP-10. This is expected in a viral immune response, as were the low levels of IL-10 and IL-13. High levels of Th1 cytokines from most clusters indicate a specific and strong Th1 response to T-Ag. Cancer patients did exhibit dampened IL-2 and IL-10 responses indicating some diminished functionality of this response in that population. The fact that most patients responded to T-Ag indicates prior exposure to virus, confirming previous reports of ubiquitous infection.

Cytokine secretion was induced by all clusters of the T Ag peptides and high levels of Th1 type cytokines were produced by most patients in response to the majority of the clusters. The N-terminal half of the T-Ag protein appears to stimulate a more robust IL-2 and IP-10 response than the carboxy-terminus, which may be a clue for the development of a vaccine, if that would be desired. Further investigation into what causes that part of the protein is more immunodominant will be useful for understanding the immune response to JCV. In order to do so, stimulation of PBMCs by the individual amino acids of the clusters would better discern the areas of T-Ag that contribute to the immune recognition of the protein. The lower production of these cytokines in cancer patients may be due to the presence of a tumor in the body; however, most patients still responded in a Th1-type manner, and to most of the peptides. This is in contrast to cervical cancer patients when PBMCs were cultured with HPV 16 E7 peptide clusters. Only 40% of the protein stimulated a high response.[38] This may indicate that the T-Ag of JCV is more immunogenic than E7 of HPV. Our initial hypothesis that patients with APs and cancers would have an altered immune response for

all the cytokines tested was not supported by our data. While there was a slightly diminished response compared with patients with a negative colonoscopy, it was not significantly different. These data do not demonstrate that an altered immune response to JCV T-Ag in patients with colorectal neoplasia is an indicator of viral involvement in the cancer.

Patients and Materials and Methods

Patients

Sixty patients undergoing colonoscopy or colorectal surgery provided informed consent for blood collection prior to the procedure. Blood was provided prior to the procedure and used to isolate PBMCs. Patients ranged from 17 to 94 years of age and potential subjects were excluded for previous chemotherapy, radiation therapy, or chronic infection, such as HIV or hepatitis B virus. Patients were grouped according to colonoscopy results: negative or hyperplastic polyp present, adenomatous polyp present, or adenocarcinoma present. Any adenomatous polyps removed during surgery where confirmed to be adenomatous polyps and not adenocarcinoma. The protocol for human sample collection and use was approved the Baylor University Medical Center's Institutional Review Board.

Sample collection and cell culture

Immediately after the blood draw, PBMCs were isolated by Ficoll (Amersham Biosciences, 17144002) separation and stained with 5,-6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, V12883) and resuspended in RPMI 1640 (Gibco, 21870) supplemented with HEPES (Gibco, 15630), nonessential amino acids (Sigma-Aldrich, M7145), penicillin and/or streptomycin (Sigma-Aldrich, P4458), L-glutamine (Sigma-Aldrich, G7513), sodium pyruvate (Sigma-Aldrich, S8636), and 2-mercaptoethanol (Sigma-Aldrich, M7522). Subsequently, 5×10^5 PBMCs per well were plated in triplicate in a 96-deep well plate (Nunc, 260251) with T-Ag clusters, Staphylococcal Enterotoxin B (Sigma-Aldrich, 50812; positive control), or 50% acetonitrile (Sigma-Aldrich, 271004; negative control). The cells were incubated at 37 °C, 5% CO_2 for eight days with fresh media added on day two.

JCV T antigen clusters

A commercially (Mimotopes, custom) prepared library of JCV T-Ag peptides was used to stimulate the PBMCs in culture. The library contains 170 amino acid peptides with 10 peptides in each cluster that overlapped the neighboring clusters by 4 peptides (Table 2). Peptides were resuspended in 50% acetonitrile to a concentration of 10 mM and stored at −80 °C until use.

Cytokine analysis

On day 2 of the PBMC culture, media from the wells was collected without disturbing the cells. The supernatant was incubated with beads containing immobilized antibodies for the following cytokines: IL-2, IL-10, IL-13, and IP-10 (Luminex). Evaluation of cytokine expression level was determined by a Bio-Plex Luminex 200. The concentrations were calculated using Bio-Plex Manager software. Circumstances beyond our control necessitated a change in the standard lots after 15 patients were enrolled. The resulting concentration

values did not change significantly but data are reported as fold difference in order to combine the two data sets.

Flow cytometry

On day eight of the PBMC culture, the cells were collected for evaluation by flow cytometry. The cells were stained with antibodies against CD3, CD4, CD8 (eBioscience, 45-0037-42, 17-0049-42, 12-0088-42), and CD45 (Molecular Probes, MHCD4530) to identify T cells. Cells were acquired on a FACS Canto II (BD Biosciences) and analyzed with FlowJo software (version 8.8.6, TreeStar).

Statistical analysis

All concentration and fold difference values were entered into GraphPad Prism for statistical analysis. The Kruskal-Wallis test with post hoc Dunn's multiple comparisons were used to evaluate the differences between the three patient populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

|	
AP	adenomatous polyps
CFSE	5,-6-carboxyfluorescein diacetate succinimidyl ester
CRC	colorectal cancer
HIV	human immunodeficiency virus
HPV	human papillomavirus
IL	interleukin
IFN-γ	interferon γ
JCV	JC virus
PBMCs	peripheral blood mononuclear cells
PML	progressive multifocal leukoencephalopathy
T-Ag	T antigen

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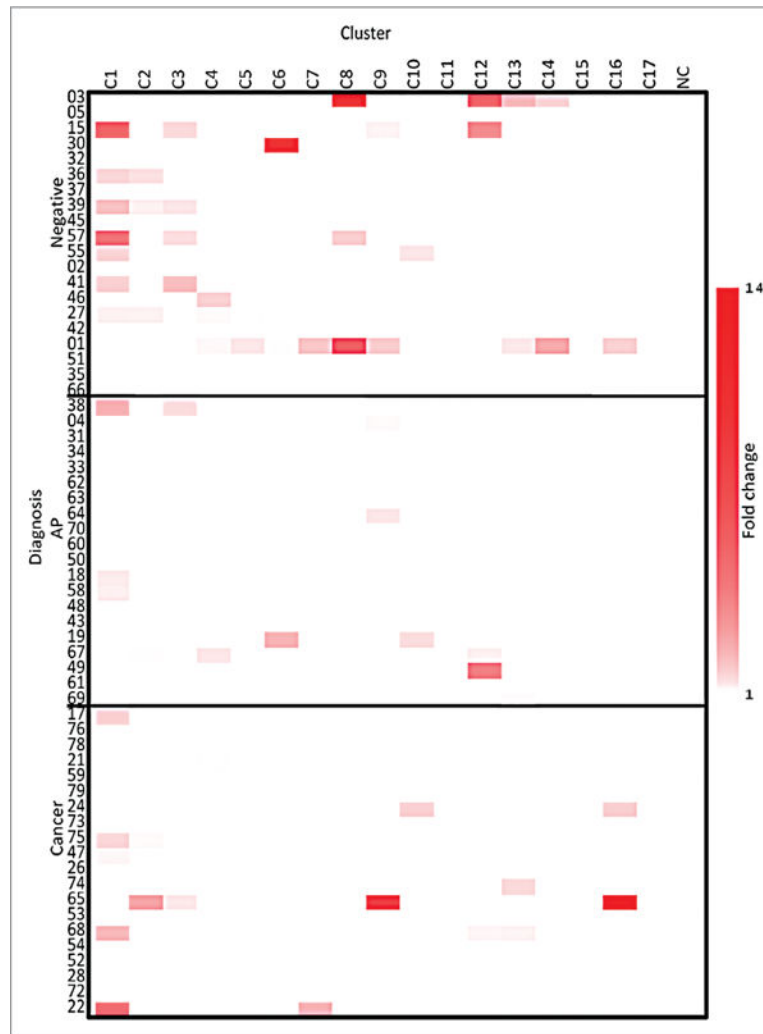
Figure 1.
IL-10-fold change heatmap. The fold change of the supernatant levels of IL-10 from the three groups compared with the NC, as evaluated on Day 2, NC is a negative control. The clusters are listed across the top and the three cohorts are listed on the y axis.
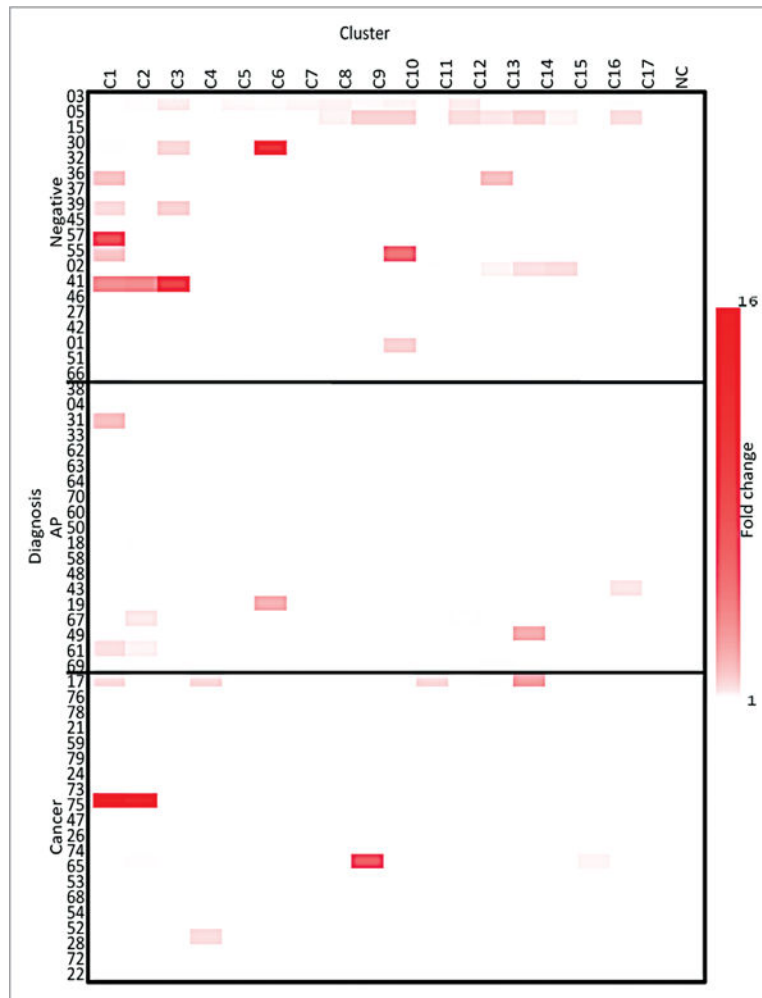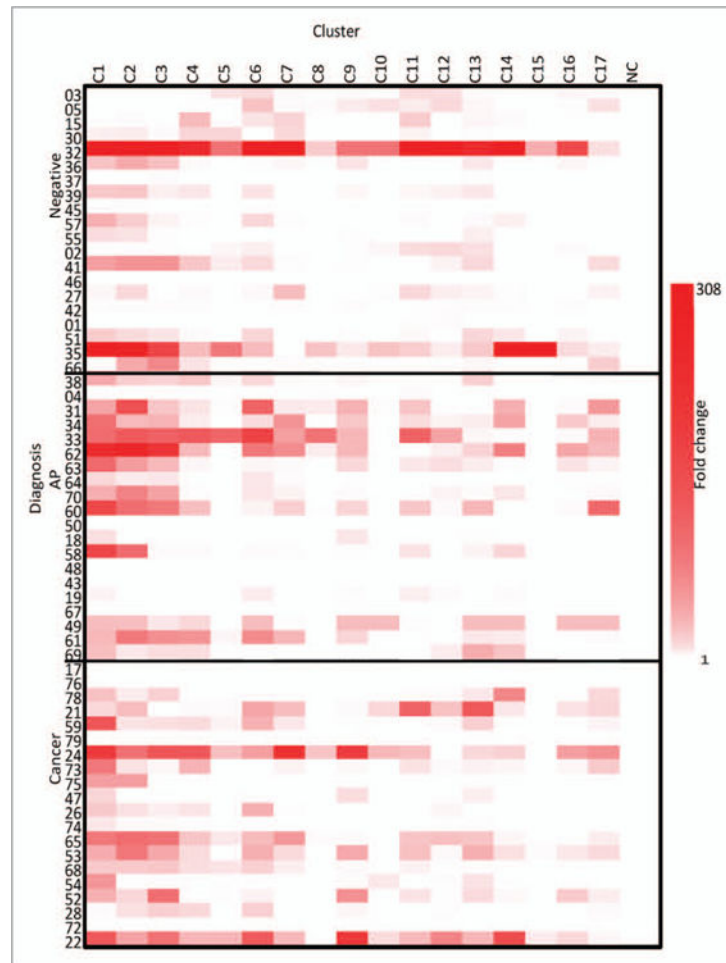
Figure 2.
IL-13-fold change heatmap. The fold change of the supernatant levels of IL-13 from the three groups compared with the NC, as evaluated on Day 2, NC is a negative control. The clusters are listed across the top and the three cohorts are listed on the y axis.
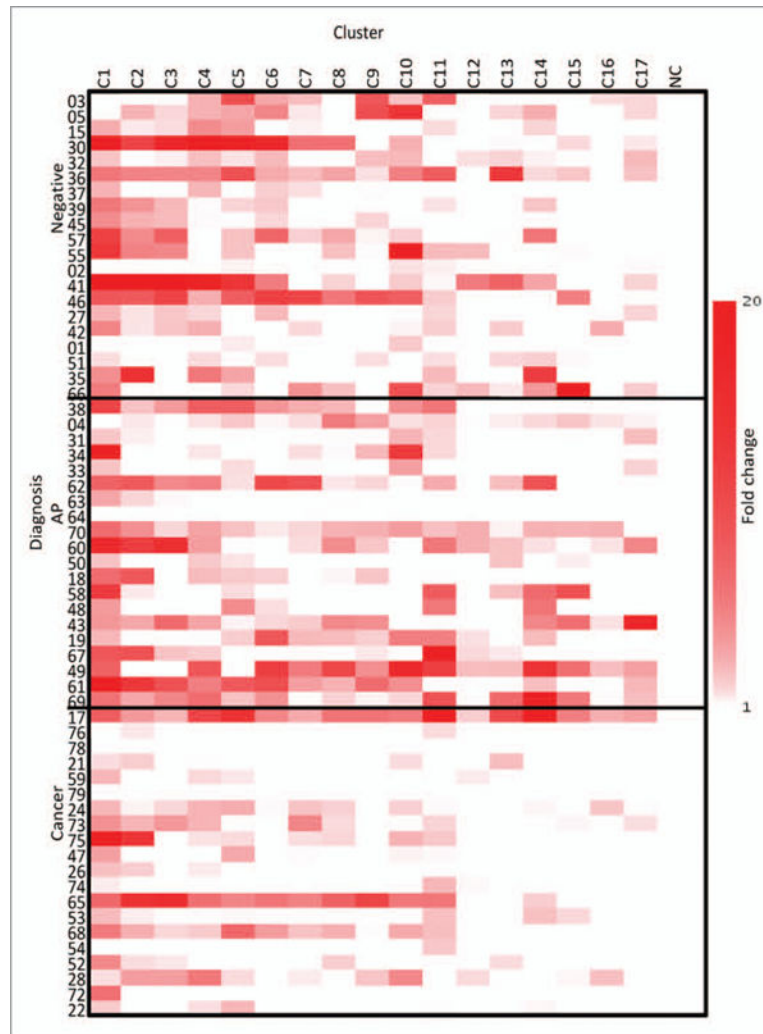
Figure 3.
IL-2-fold change heatmap. The fold change of the supernatant levels of IL-2 from the three groups compared with the NC, as evaluated on Day 2, NC is a negative control. The clusters are listed across the top and the three cohorts are listed on the y axis.
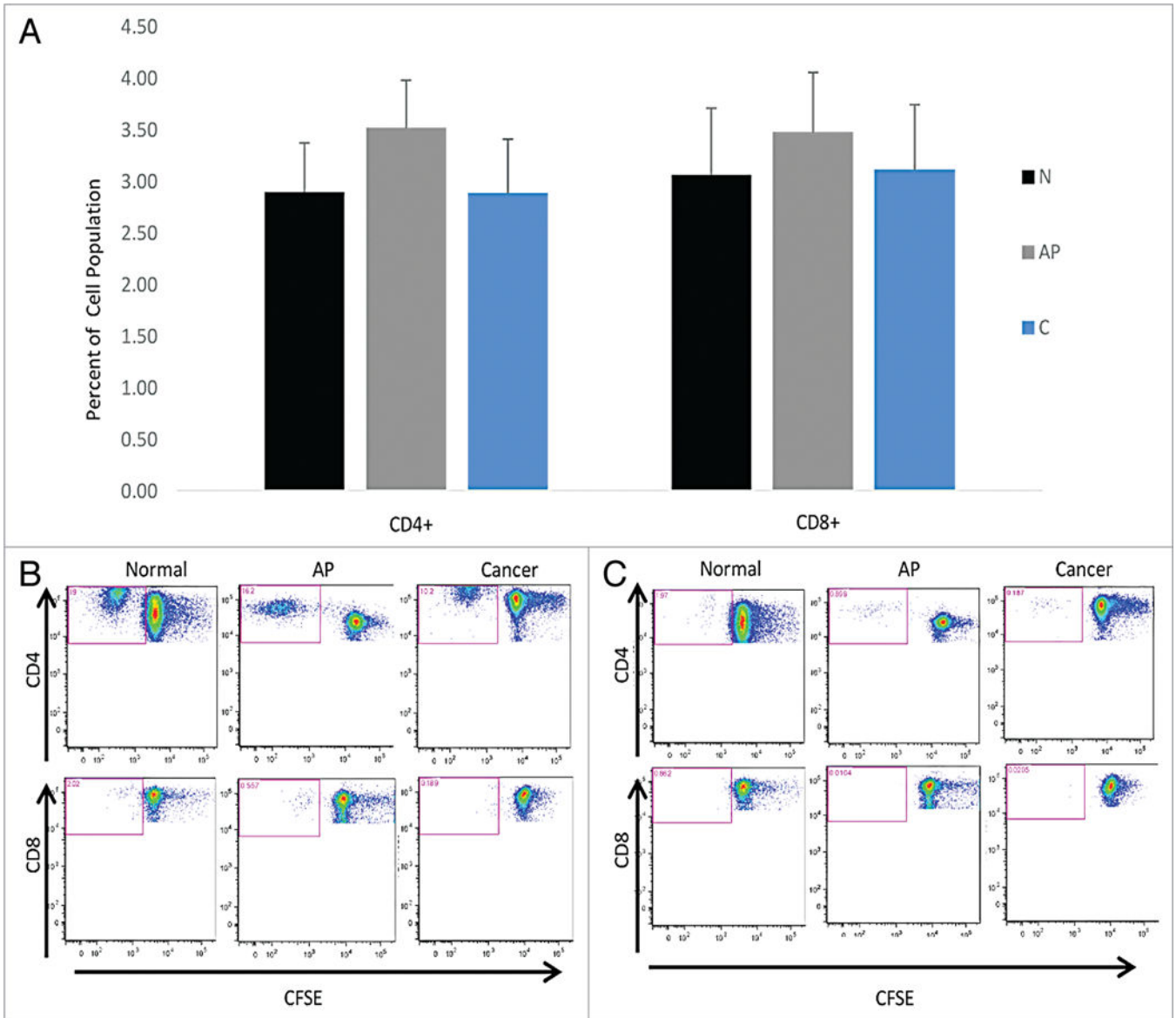
Figure 4.
IP-10-fold change heatmap. The fold change of the supernatant levels of IP-10 from the three groups compared with the NC, as evaluated on Day 2. NC is a negative control. The clusters are listed across the top and the three cohorts are listed on the y axis.

Figure 5.

CD4$^+$ and CD8$^+$ T cell proliferation. (**A**) CD4$^+$ and CD8$^+$ T cell proliferation. The CD4$^+$ T cell proliferation is the first set of 3 for the cohorts and the CD8$^+$ T cell proliferation is the second set of the three cohorts. T cells are represented as the percentage of total cells. Standard error of the mean is represented above each column. (**B**) CD4$^+$ and CD8$^+$ T cell proliferation from a strong stimulating cluster. Representative patients for T cell proliferation from the three groups for cluster 1, a cluster stimulating a strong response. CFSE decreases in replicating cells, indicated by a population shift to the left. The CD4$^+$ and CD8$^+$ staining is indicated on the y axis and the CFSE staining is on the $\times$ axis. (**C**) CD4$^+$ and CD8$^+$ T cell proliferation from a poorly stimulating cluster. Representative patients for T cell proliferation from the three groups for cluster 15, representing a weak response. CFSE

decreases in replicating cells, indicated by a population shift to the left. The CD4$^+$ and CD8$^+$ staining is indicated on the y axis and the CFSE staining is on the x axis.

Table 1

Summary of the patients enrolled in the study

		Patient Population		
		Number	Mean age (SD)	P-Value
Total		60	60.3 (11.9)	–
Gender	Female	25	61.6 (11.9)	0.24
	Male	35	59.3 (12.0)	
Ethnicity	African American	10	61.3 (9.4)	0.87
	Caucasian	48	60.2 (12.6)	
	Hispanic	2	56.5 (7.8)	
Diagnosis	Adenocarcinoma	20	64.7 (12.5)	0.17
	Adenomatous Polyp	20	59.0 (13.8)	
	Hyperplastic Polyp	8	58.3 (8.0)	
	Without Neoplasia	12	55.5 (8.1)	

Table 2

The amino acids in the peptide clusters for T Antigen stimulation of PBMCs

| Cluster | AA | JCV T Antigen Peptide Clusters | | Target | |
		Sequence			
C1	1–51	mdkvlnrees melmdllgld rsawgnipvm rkaylkkcke lhpdkggded k	POL α		
C2	41 – 91	lhpdkggded kmkrmmflyk kmeqgvkvah qpdfgtwnss evptygtdew e	POL α		
C3	81 – 131	evptygtdew eswwntfnek wdedlfchee mfasddentg sqhstppkkk k	pRB	p107	Phosphorylation site
C4	121 – 171	sqhstppkkk kkvedpkdfp vdlhaflsqa vfsnrtvasf avyttkekaq i		Helicase	DNA binding NLS
C5	161 – 211	avyttkekaq ilykklmeky svtfisrhgf gghmlflflt phrhrvsain n		Helicase	DNA binding
C6	201 – 251	phrhrvsain nycqklctfs flickgvnke ylfysalcrq pyavveesiq		Helicase	DNA binding
C7	241 – 291	pyavveesiq gglkehdfnp eepeetkqvs wklvtqyale tkcedvfllm g	POL α	Helicase	DNA binding Zinc finger
C8	281 – 331	tkcedvfllm gmyldfqenp qqckkcekkd qpnhfnhhek hyynaqifad s	POL α	Helicase	Zinc finger
C9	321 – 371	hyynaqifad sknqksicqq avdtvaakqr vdsihmtree mlverfnfll d	POL α	Helicase	Zinc finger
C10	361 – 411	mlverfnfll dkmdllifgah gnavleqyma gyawihcllp qmdtviydfl k	POL α	Helicase	
C11	401 – 451	qmdtviydfl kcivlnipkk rywlfkgpid sgkttlaaal ldlcggksln v	POL α	Helicase	ATP binding. ATPase
C12	441 – 491	ldlcggksln vnmplerlnf elgygidqfm vvfedvkgtg aesrdlpsgh g	POL α	Helicase	ATP binding. ATPase
C13	481 – 531	aesrdlpsgh gisnldclrd yldgsvkvnl erkhqpnkrq vfppgivtmn e	POL α	Helicase	ATP binding. ATPase
C14	521 – 571	vfppgivtmn eysvprtlqa rfvrqidfrp kaylrkslsc seyllekril q		Helicase; p53	ATP binding. ATPase
C15	561 – 611	seyllekril qsgmtlllll iwfrpvadfa aaiherivqw kerldleism y		Helicase; p53	ATP binding. ATPase
C16	601 – 651	kerldleism ytfstmkanv gmgrpildfp reedseaeds ghgsstesqs q		Helicase; p53	ATP binding. ATPase
C17	641 – 688	ghgsstesqs qcfsqvseas gadtqenctf hickgfqcfk kpktpppk	p53	Host range	Phosphorylation site

AA, amino acid; POL α, DNA polymerase α; NLS, nuclear localization signal.