



NGS Evaluation of Colorectal Cancer Reveals Interferon Gamma Dependent Expression of Immune Checkpoint Genes and Identification of Novel IFN γ Induced Genes

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To evaluate the expression of immune checkpoint genes, their concordance with expression of IFN γ , and to identify potential novel ICP related genes (ICPRG) in colorectal cancer (CRC), the biological connectivity of six well documented (“classical”) ICPs (CTLA4, PD1, PDL1, Tim3, IDO1, and LAG3) with IFN γ and its co-expressed genes was examined by NGS in 79 CRC/healthy colon tissue pairs. Identification of novel IFN γ - induced molecules with potential ICP activity was also sought. In our study, the six classical ICPs were statistically upregulated and correlated with IFN γ , CD8A, CD8B, CD4, and 180 additional immunologically related genes in IFN γ positive (FPKM > 1) tumors. By ICP co-expression analysis, we also identified three IFN γ -induced genes [(IFN γ -inducible lysosomal thiol reductase (IFI30), guanylate binding protein1 (GBP1), and guanylate binding protein 4 (GBP4)] as potential novel ICPRGs. These three genes were upregulated in tumor compared to normal tissues in IFN γ positive tumors, co-expressed with CD8A and had relatively high abundance (average FPKM = 362, 51, and 25, respectively), compared to the abundance of the 5 well-defined ICPs (Tim3, LAG3, PDL1, CTLA4, PD1; average FPKM = 10, 9, 6, 6, and 2, respectively), although IDO1 is expressed at comparably high levels (FPKM = 39). We extended our evaluation by querying the TCGA database which revealed the commonality of IFN γ dependent expression of the three potential ICPRGs in 638 CRCs, 103 skin cutaneous melanomas (SKCM), 1105 breast cancers (BC), 184 esophageal cancers (ESC), 416 stomach cancers (STC), and 501 lung squamous carcinomas (LUSC). In terms of prognosis, based on Pathology Atlas data, correlation of GBP1 and GBP4, but not IFI30, with 5-year survival rate was favorable in CRC, BC, SKCM, and STC. Thus, further studies defining the role of IFI30, GBP1, and GBP4 in CRC are warranted.

Keywords: colorectal cancer, IFN γ gradient, immune checkpoint genes, co-expression network, novel immune checkpoint related genes

INTRODUCTION

CRC is the second leading cause of cancer-related mortality in the United States¹ (1) and, disturbingly, an increased incidence of CRC in patients <40 years of age has been reported (2). In recent years, immunotherapeutic approaches have opened important treatment options in a small subset of CRC patients with microsatellite instability high (MSI-H) tumors (3). Most CRC, however, are microsatellite stable (MSS) (4). In MSI-H CRC patients, the high response rate to the ICP blockade appears due to a higher tumor mutational burden, the presence of neoantigens and consequent infiltration by CD8⁺ (T_C, cytotoxic T lymphocyte) CTL and higher expression levels of ICPs (5). In this regard, IFN γ has been identified as the lynchpin factor in the induction and sustained expression of ICPs on tumor and infiltrating T cells in several tumor types and thus, qPCR detection of IFN γ has been considered a potential marker of response to ICP blockade in several cancer studies including in non-small cell lung cancer (NSCLC) and cutaneous melanoma (SKCM) (6–8). However, the role of IFN γ in establishing the immunological profile of CRC has not been thoroughly investigated. This prompted us to use NGS to evaluate expression of IFN- γ in CRC tumors, its link to known IFN γ -dependent ICPs, and to identify novel ICPRGs. In this study, we evaluated expression levels of six well-known immune checkpoint genes [six ICPs (CTLA4, PD1, PDL1, Tim3, IDO1, and LAG3)] as well as potential immune checkpoint related genes (ICPRGs) also induced by IFN γ by next generation sequencing (NGS) in 79 stringently collected and preserved primary human CRCs and their patient matched normal colonic tissues. Expression levels of six ICPs were evaluated as were their relationships to expression levels of IFN γ and other immunologically pertinent genes. Based on the ICP co-expression network, we searched for potential ICP related genes (ICPRGs) in IFN γ positive tumors that may function as novel ICPs and consequently identified IFI30, GBP1 and GBP4. Based on the identified literature (9–22), IFI30, GBP1, and GBP4 suppress mouse primary T cell activation *in vitro* and mouse innate immune response *in vivo* while IFI30 and GBP1 appear to increase cell proliferation in a glioma cell line and two breast cancer cell lines but diminish cell proliferation in a colon cancer cell line. Intriguingly, however, IFI30 RNA expression is associated with better patient survival in breast cancer (12) and diffuse large B cell lymphomas (DLBCL) (14) while GPB1 RNA is associated with better patient survival in melanoma (20) but poorer prognosis in human glioblastoma (21).

MATERIALS AND METHODS

Cohort

Seventy-nine paired-tissues (79 tumor and 79 normal controls, **Table S1**) of pretreatment CRCs were collected from 38 male and 41 female patients by Indivumed GmbH (Germany) for mRNA sequencing. The purchase of these de-identified samples was exempted by FDA IRB/RIHSC. To evaluate tumor content, hematoxylin and eosin stained microscopic slices were examined

by pathologists to determine the tumor cell and normal cell areas, respectively. Histologically, tumor samples had 50–70% content of cancer cells while normal samples had 0% content of cancer cells. Normal tissues were collected from a site at a minimum of 5 cm from the tumor margin. Ischemia time was 6–11 min. This short cold ischemia reduces post-surgical tissue processing artifacts (23). According to the medical pathology reports, tumors were classified as well, moderately, and poorly differentiated tumors following international guideline UICC TNM-classification (24). For the convenience of analysis, 26 stage I and II tumors were considered as low stage tumors (LSTs), while 53 stage III and IV tumors were considered as HSTs (25). In this study, a normal control adjacent to a low stage tumor is referred to as LSN. The ratio of high stage tumors vs. low stage tumors is 2–1. Among 26 low stage tumors, there were two either lymph node (LN) or lymphatic vessel (LV) positive tumors while among 53 high stage tumors, there were 28 either LN/LV positive tumors. For tumor grades, there were 17 well (low grade) differentiated, 36 moderately (medium grade) differentiated, and 26 poorly (high grade) differentiated tumors. Clinical and histopathological characteristics of the patients as well as tumor location are summarized in **Table S1**. Among these 80 tumor pairs, 79 pairs were sequenced (all except the T7/N7 pair). The information for the cohort of 50 CRC tumor pairs, 588 CRCs, 103 SKCMs, 1105 BCs, 184 ESCs, 416 STCs, and 501 LUSC for validation of six ICPs and three ICPRGs was extracted from TCGA_B38 through OncoLand (**Tables S2–S4**). As for tumor stage information of validating cohort, there were 57 LST and 82 HSTs (**Tables S1, S2**).

For protein and survival data, The Clinical Proteomic Tumor Analysis Consortium (CPTAC) (<https://cptac-data-portal.georgetown.edu/>) which contains Mass spectroscopy (MS) analyses of 95 CRCs (**Table S5**) and The Pathology Atlas (<https://www.proteinatlas.org/humanproteome/pathology>) were used.

mRNA Sequencing

RNA quality was assessed using the Agilent 2100 Bioanalyzer, with cellular RNA analyzed using the RNA 6000 Nano Kit (Agilent). Samples with an RNA Integrity Number (RIN) of 7 or higher were processed to generate libraries for mRNA sequencing following the Illumina[®] TruSeq Stranded mRNA Sample Preparation Guide. In this method, poly-A mRNAs were purified from 0.5 μ g total RNA, fragmented and reverse-transcribed into cDNAs. Double strand cDNAs were adenylated at the 3' ends and ligated to indexed sequencing adaptors, followed with amplification for 15 cycles. One femtomole of the sequencing libraries (median size \sim 260 nt) were denatured and loaded onto a flow cell for cluster generation using the Illumina cBot. Every six samples were loaded onto each lane of a rapid run flow cell. Paired-end sequencing was carried out on a HiSeq 2500 sequencer (Illumina, San Diego, CA, USA) for 100 \times 2 cycles (26). For each sample, we obtained \sim 50 million 100-bp reads that passed preset filtering parameters (27).

Sequencing Data Analysis

For mRNA sequencing, Tophat V.2.0.11 was used to align reads in fastq files to the UCSC human hg19 reference genome. Cufflinks V.2.2.1 was used to assemble the transcriptome based

¹<https://www.cancer.org/cancer/colon-rectal-cancer/about/key-statistics.html>

on the hg19 reference annotation, and Cuffquant/Cuffnorm (part of Cufflinks) were used in calculating relative abundance of each transcript reported as FPKM. Gene co-expression analyses were carried by Partek NGS & microarray data analysis software (25, 28, 29). Integrated Discovery (DAVID) v6.7 (<https://david.ncifcrf.gov/>) was used for biological pathway determination and Cytoscape (2.8.2) was used for gene co-expression networks construction.

Initial Expression Landscape of CRC

A total of 25,761 genes were detected. Because genes with higher FPKM values may have greater biological impact, we focused on genes with FPKM > 1 (25, 28). Ten thousand two hundred fifty-five genes (40% of total genes) had an average FPKM > 1 and differential expression between tumors and normal controls (False Discovery Rate (FDR) < 0.05 in ANOVA). A total of 3,893 genes (15% of total genes) with average FPKM > 1 showed no differential expression between tumor and normal controls with FDR (ANOVA) > 0.05 (25, 29).

NGS Evaluation of Immune Gene Expression

NGS is a technology that accurately quantifies gene expression and does not necessarily require further validation, as supported by the literature (25). To more fully establish NGS as a “stand alone” technology for gene quantification, we reasoned that NGS quantification of a critical hub gene should be reflected in the consequent up/downregulation of highly interconnected genes and thus examined the co-expression of IFN γ genes with T and NK cell specific genes based on the fact that IFN γ and granzymes are produced by T cells and NK cells (30). IFN γ was highly correlated ($cc > 0.80$) with 9 classical T and NK cell gene markers and two granzymes in IFN γ positive tumors, as assessed by NGS: CD8A [0.97], CD69 [0.93], CD52 [0.86], CD160 [0.85], CD3E [0.84], CD96 [0.83], CD8B [0.82], CD2 [0.82], CD7 [0.80], GZMA [0.80], and GZMM [0.80] (Table S6). These data indicate that NGS expression profiles of immune related genes do not necessarily require validation by other gene quantification technologies, especially for hypothesis-generating studies.

RESULTS

Upregulation of Six Established ICPs Associated With Higher Expression of IFN γ in CRC

Because IFN γ has been strongly implicated in the induction of PDL1 expression in tumors, and PD1 expression in tumor infiltrating T cells (6–8), we divided 79 CRCs into those with potentially significant IFN γ expression (abundance level of FPKM > 1; 32 CRCs), designated IFN γ^+ (positive), and those expressing lower levels of IFN γ expression (FPKM < 1; 47 CRCs), designated IFN γ^- (negative) (Figure 1A). The log₂ FPKM plot of tumor and normal showed that IFN γ and all six well-documented ICPs were significantly upregulated ($p < 0.01$) in IFN γ positive CRCs compared to their patient matched normal tissue controls [Figure 1B: IFN γ (24.1-fold),

IDO1 (7.8-fold), CTLA4 (2.9-fold), Tim3 (2.3-fold), PDL1 (3.0-fold), PD1 (2.1-fold) and LAG3 (1.6-fold)] while only 4 ICPs were significantly upregulated ($P < 0.05$) in IFN γ negative CRCs compared to their matched controls (Figure 1C): IFN γ (4.4-fold), IDO1 (1.4-fold), CTLA4 (1.7-fold), Tim3 (1.4-fold), PDL1 (1.3-fold), and PD1 (1.0-fold). Intriguingly, LAG3 (0.54-fold) was significantly downregulated ($p = 1.7E-0.5$) in IFN γ negative CRCs (Figure 1C). These data suggest that differential expression of ICPs, especially LAG3, may pertain to levels of IFN γ expression in CRCs. Regarding the quantitative relationship between IFN γ and the six ICPs, the expression levels of these six were 1.9 to 6.4 -fold higher in IFN γ positive vs. IFN γ negative tumors (Table S7), though even in the IFN γ positive tumors, these ICPs were expressed at relatively low abundance (average FPKM = 3–12) compared to oncogenes such as MYC, CDK4, and CCND1 (average FPKM = 105) (25), with the exception of IDO1 which is robustly upregulated (average FPKM = 89). These data suggest a positive effect of IFN γ with respect to consequent upregulation of ICPs but potentially at levels still insufficient to promote significant expression of ICP proteins on tumor and infiltrating T cells in CRC, supporting the lack of response of most of these tumors to ICP inhibitor therapeutics.

ICP Co-expression Profile in IFN γ Positive and Negative Tumors

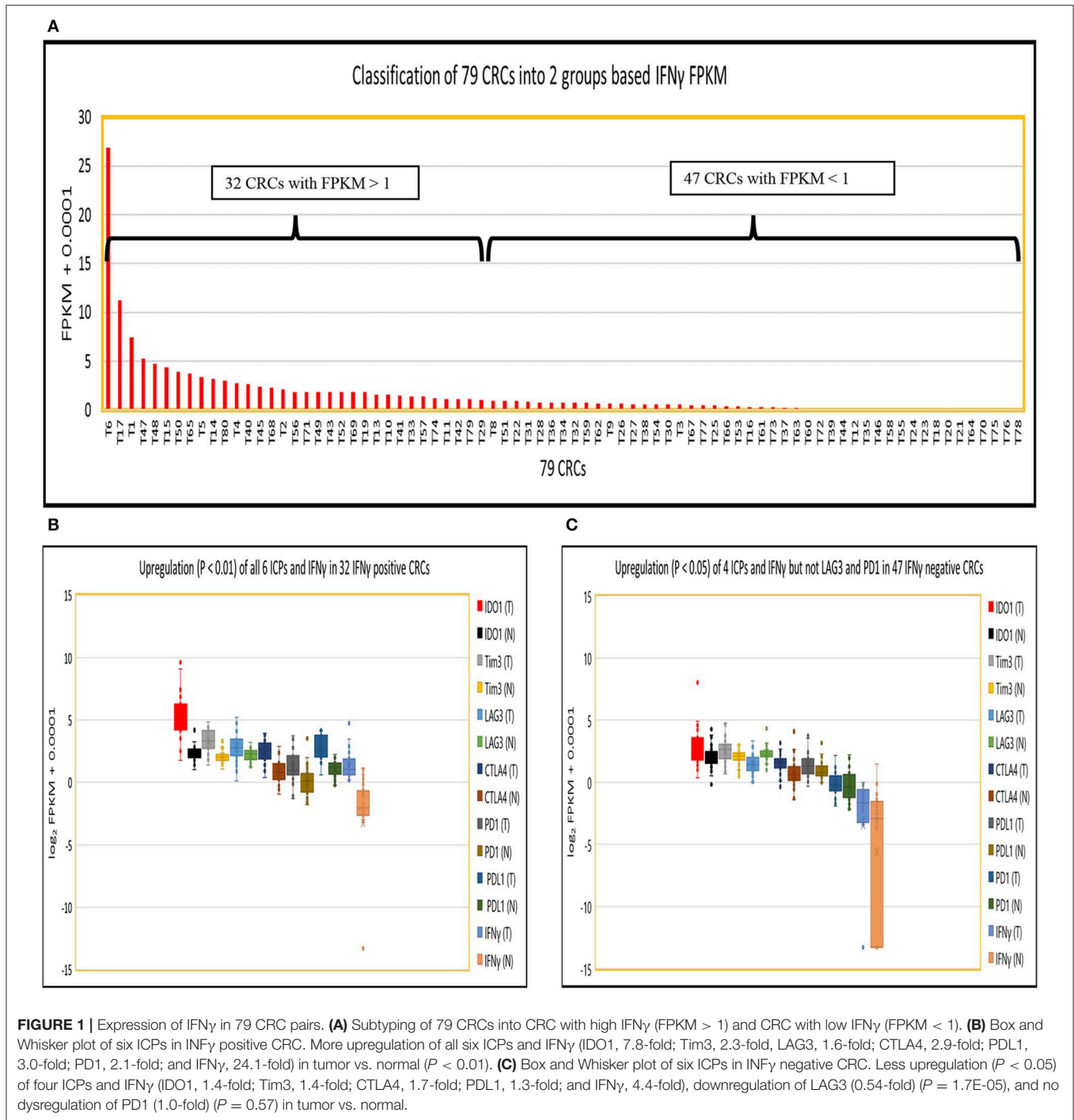
We then performed a Pearson correlation analysis to evaluate the co-expression profile of IFN γ and the six ICPs in IFN γ positive and negative CRC, as well as in normal controls using a stringent correlation coefficient ($cc > 0.8$). In IFN γ positive CRC, the following was observed: (i) IFN γ , CD8A, CD8B, and CD4 co-expression with all six ICPs genes within one network (190 genes); (ii) IFN γ and three ICPs (LAG3, Tim3, and IDO1) were defined as potential hub genes due to their substantial number of co-expressed genes ($n > 45$); and (iii) co-expression of IFN γ and six ICPs with 129 immune cell related genes (pale blue dots in Figure 2A) and 54 signaling genes (red dots in Figure 2A; Table S8).

In contrast, in IFN γ negative CRC, co-expression of Tim3, PDL1, and CD4 was found within one network comprised of 83 genes, but without linkage to IFN γ or other immune checkpoint genes including PD1, CTLA4, IDO1 and LAG3. Genes co-expressed with Tim3 and PDL1 consisted of 77 immune related genes (pale blue dots in Figure 2B) as well as six signaling genes (red dots in Figure 2B; Table S8).

In control normal colonic tissues, co-expression of two ICPs (CTLA4 and PD1) was observed but also was not linked to IFN γ and CD4, CD8A, and CD8B, or co-expression with PDL1, Tim3, LAG3, and IDO1. However, expression of CTLA4 and PD1 was noted within an 88-gene network including 63 immune related genes (pale blue dots in Figure 2C) as well as 25 signaling genes (red dots in Figure 2C; Table S8).

Identification of Three Novel ICPRG Genes in CRC

We next explored whether there were potential novel ICPRGs from the IFN γ co-expression network that potentially factored



into the refractoriness of CRC to immunotherapy. Three genes, IFI30, GBP1, and GBP4, were identified by two criteria: co-expressed ($cc > 0.8$) with known ICs and upregulated by a minimum 2-fold average over normal (T/N). In IFN γ positive tumors, IFI30, GBP1, and GBP4 were significantly upregulated ($p < 0.0001$) at 2.7-, 4.2-, and 6.2-fold, respectively (Figure 3A) while only IFI30 and GBP1 were upregulated ($p < 0.05$) at 1.4- and 1.2-fold, respectively in

IFN γ negative tumors compared to their matched normal controls (Figure 3B). Notably, the abundance of IFI30, GBP1 and GBP4 was substantially higher in IFN γ positive tumors (362, 51, 25 FPKM, respectively) than in IFN γ negative tumors (207, 18, 7 FPKM, respectively) (Table S9). These three genes have documented immune suppressive function and pro or anti-tumor roles in different types of cancers (9–23).

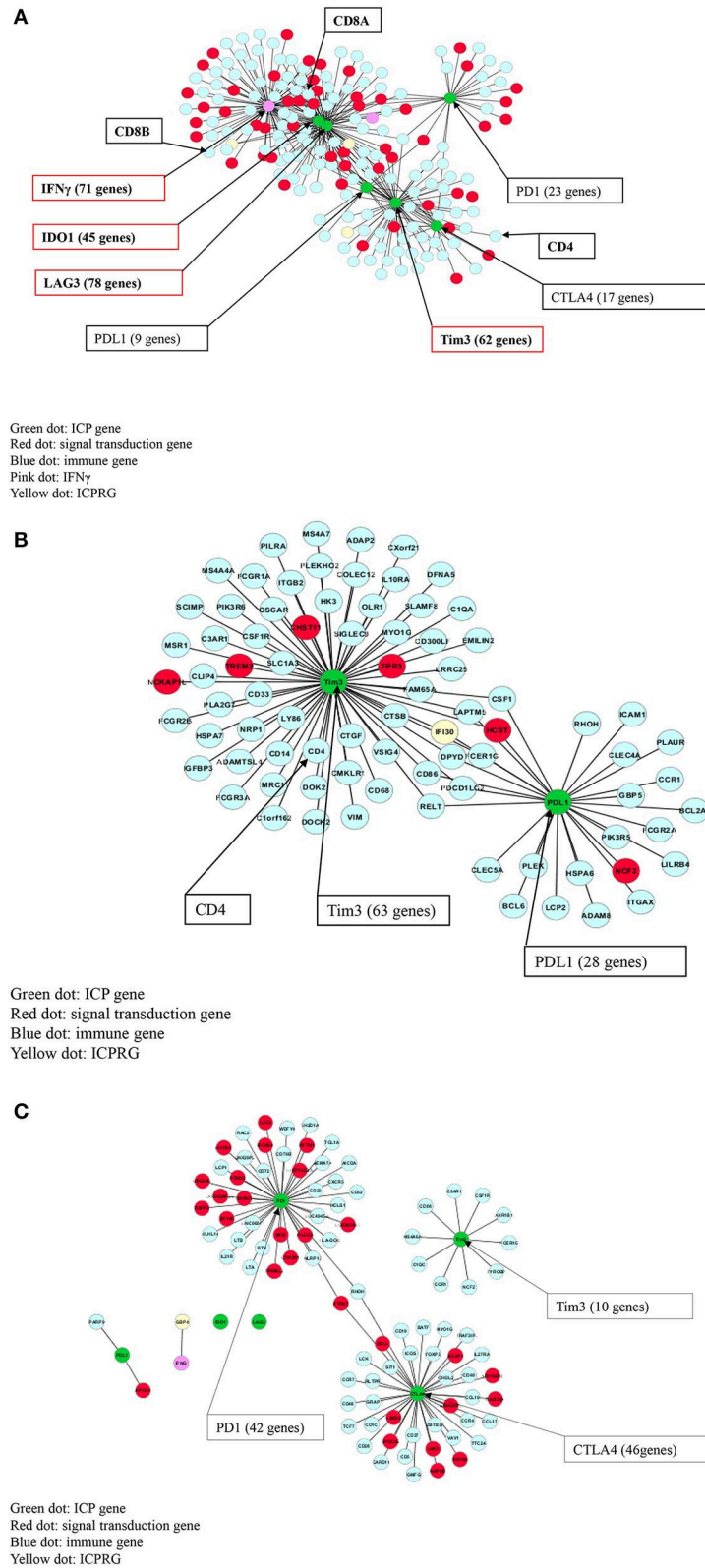


FIGURE 2 | Co-expression ($cc > 0.8$) of IFN γ with six ICs in CRC. **(A)** IFN γ and all six ICs co-expressed with CD8A/CD8B/CD4 within one 190 gene network in 32 IFN γ positive CRCs. **(B)** Tim3 and PDL1 co-expressed with CD4 within one 83 gene network in 47 IFN γ negative CRCs. **(C)** CTLA4 co-expressed with PD1 within one 88 gene expression network, but not Tim3, in normal colon without CD8/CD4.

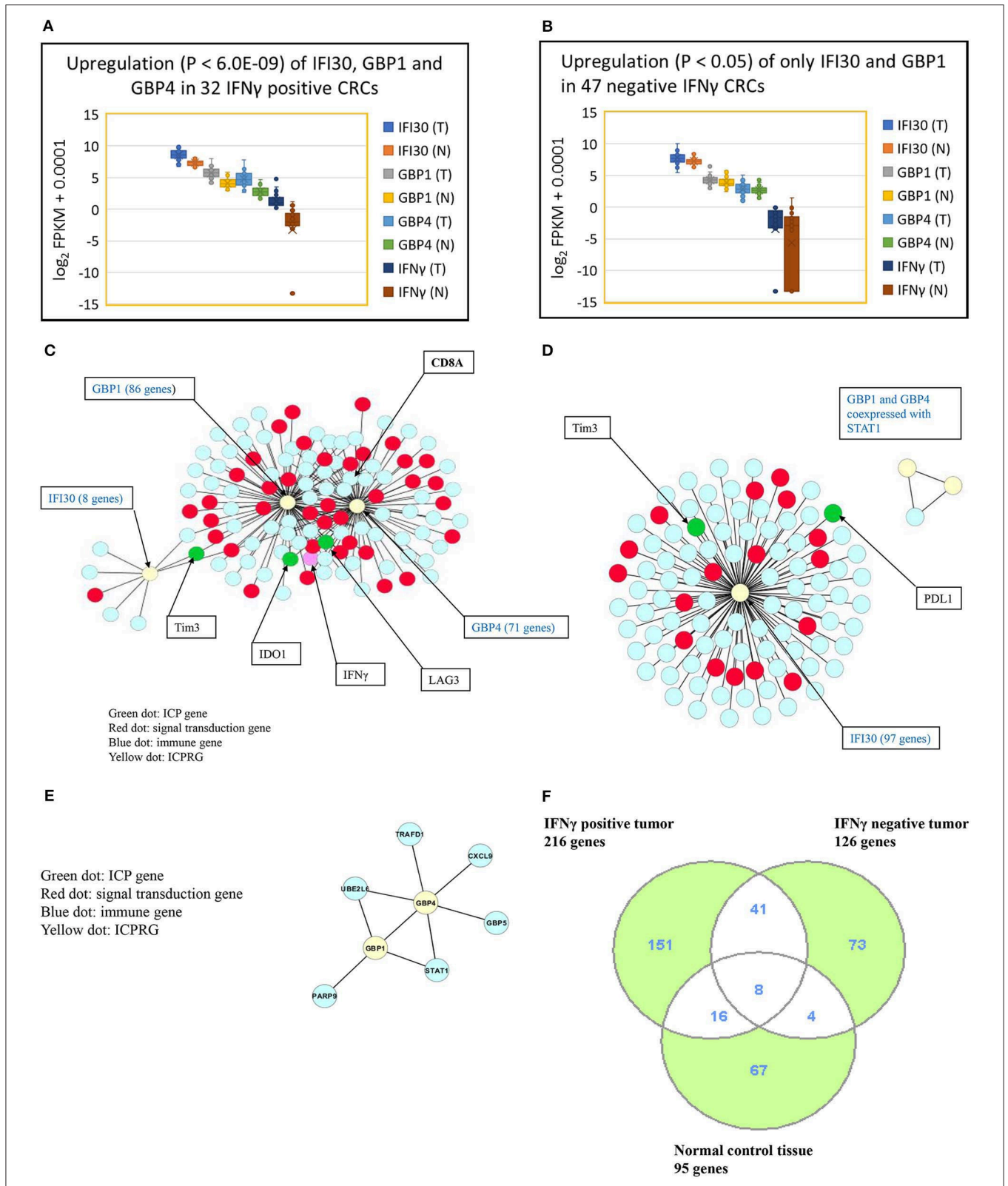


FIGURE 3 | Characterization of three ICPRGs in CRC. **(A)** Box and Whisker plot of three ICPRGs in IFN γ positive CRC. More upregulation ($P < 6.0E-09$) of all three ICPRGs (IFI30: 2.3-fold; GBP1: 3.1-fold, GBP4: 12.9-fold in tumor vs. normal). **(B)** Box and Whisker plot of three ICPRGs in IFN γ negative CRC. Less upregulation (Continued)

FIGURE 3 | ($P < 0.05$) of two ICPRGs (IFI30: 1.4-fold and GBP1: 1.2-fold) and GBP4 (1.1-fold) ($P = 0.23$) in tumor vs. normal. **(C)** Co-expression of IFI30, GBP1, and GBP4 with IFN γ , IDO1, Tim3, LAG3 and CD8A within a 119 gene network in IFN γ positive CRCs. **(D)** Co-expression of IFI30 with PDL1 and Tim3 within a 101 network without CD8/CD4 in IFN γ negative CRCs. **(E)** GBP1 co-expressed with GBP4 within an eight-gene network in normal colon. **(F)** Identification of unique genes co-expressed with 10 genes (IFN γ , six ICPs, and three ICPRGs) between IFN γ positive and negative tumors. There are 151 unique genes in IFN γ positive tumors, 73 unique genes in IFN γ negative tumors, and 67 unique genes in normal tissue.

We then employed a Pearson correlation analysis to study the co-expression of IFN γ with the three newly identified potential ICPRGs in IFN γ positive and negative tumors as well as in normal controls. In IFN γ positive tumors, all three novel ICPRGs were co-expressed with IFN γ /CD8A and three known ICPs (IDO1, Tim3, and LAG3) within one network (total 119 genes: GBP1 and GBP4 correlated with IDO1, LAG3, and CD8A; IFI30 correlated Tim3) (**Figure 3C**). Moreover, GBP1 and GBP4 were identified as potential hub genes due to their substantial number of co-expressed genes ($n > 70$). In IFN γ negative tumors, IFI30 was co-expressed with 97 genes including Tim3 and PDL1 (but not with CD8/CD4) while GBP1 and GBP4 were only co-expressed with STAT1 (signal transducer and activator of transcription 1) but not with IFI30 or with IFN γ (**Figure 3D**). In normal colonic tissue, GBP1 and GBP4, were co-expressed within an 8 gene network, including STAT1 and GBP5 but not in association with IFN γ or IFI30 (**Figure 3E**), though IFI30 is strongly expressed in normal tissue (**Table S9**).

To address whether protein expression correlates with quantification of identified genes, we took advantage of two critical databases: Clinical Proteomic Tumor Analysis Consortium and The Pathology Atlas. The Clinical Proteomic Tumor Analysis Consortium CPTAC (<https://cptac-data-portal.georgetown.edu/>) contains mass spectroscopy (MS) analyses of a cohort of 95 CRCs. By analysis of these data, we found that IFI30, GBP1, and GBP4 proteins are more abundantly expressed than are IDO1 and PD1 in this 95 CRC cohort (**Figure S1A**) (data pertaining to PDL1, Tim3, LAG3, CTLA4, and IFN γ are not available from this database) which is consistent with our findings that IFI30, GBP1, and GBP4 mRNAs (**Table S9**) were more abundant than six classical ICPs in our Indivumed and TCGA cohorts (**Table S7**).

Regarding the Pathology Atlas (<https://www.proteinatlas.org/humanproteome/pathology>), comprised of 5 million immunohistochemistry (IHC) images of different types of cancer, we compared the IHC data of the three novel ICP related genes and 5 classical checkpoint genes (ICPs) (CTLA4 is not available in this database) in CRC, breast cancer (BC), stomach cancer (STC) and skin cutaneous melanoma (SKCM). While 5 classical ICP proteins had low percentage staining (5–11%), the three ICP related proteins had high percentage staining (60–78%) in CRC and three other types of cancer (**Figure S1B**). This conclusion supports our findings that the proteins pertaining to the novel ICP related genes (**Table S9**), are not only markedly upregulated but are much more abundantly expressed than are the classical ICPs (**Table S7**) within tumor tissue, thus strongly supporting our gene expression data.

To gain insight into potential function of such genes in CRC, we sequenced six colon cancer cell lines (HCT15, SW480, SW620, SW116, HT29, HCT116, Colo205) and two normal colon cell

lines (CCD841, HCoEpiC) and found that all 8 cell lines (both tumor and normal) had low expression of these three ICP related genes, IFN γ and six classical ICPs compared to primary tumors and normal tissues in HCA and PCA analyses (**Figures S2A,B**). The data again emphasize key differences between cell lines and primary tumors and that future functional studies, such as RNA silencing or over-expression of IFI30, GBP1, and GBP4 should be performed in primary tumors to evaluate the function of such factors in the tumor microenvironment context.

Identification of Uniquely Co-expressed Genes in IFN γ Positive vs Negative Tumors as Well as Normal Control Tissues

Because IFN γ , the six ICPs and the three ICPRGs were co-expressed with different numbers of genes in IFN γ positive and negative tumors, as well as in normal controls, we identified non-overlapping as well as overlapping genes among these groups (**Figures 2A–C, 3C–F**) and consequently determined their related pathways with David Bioinformatics (25, 29). The results demonstrate that (i) in IFN γ positive tumors, 10 ICP and ICPRG genes (IFN γ , six ICPs and three ICPRGs) were uniquely co-expressed with 151 genes mainly related to CD8 T cell activation, inactivation, cytotoxicity, co-stimulation, response to vitamin A (T_H differentiation) and Wnt/ β -catenin signaling (T cell development); (ii) in IFN γ negative tumors, three ICP and ICPRG genes (PDL1, Tim3, and IFI30) were uniquely co-expressed with 73 genes mainly related to B cell and macrophage activation, B cell antigen presentation, B cell response to lipopolysaccharide, wound healing/B cell maturation and EGFR signaling/B cell differentiation; (iii) in normal controls, 5 ICP and ICPRG genes (PD1, CTLA4, Tim3, GBP1, and GBP4) were uniquely co-expressed with 67 genes mainly related to T cell inhibition/MDSC2, lymph node development, induction of apoptosis, B cell proliferation and endoplasmic reticulum signaling/cell cycle (**Tables 1A–C, Table S8**). These data suggest the possible presence of distinct ICP/ICPRG involved in pathological and physiological pathways among IFN γ positive and negative tumors (two CRC subtypes) as well as in normal colonic tissues.

Close Association of IFN γ With T Cell Gene Expression in IFN γ Positive CRC

As indicated in our evaluation of NGS for gene quantification (see Methods above), in IFN γ positive tumors, higher expression levels of IFN γ correlated specifically with higher expression levels of T cell related genes including CD8 α (3.5-fold), CD3 ϵ (2.0-fold), CD4 (1.3-fold), and FOXP3 (1.5-fold) compared to IFN γ negative tumors ($p < 0.05$) but not with expression levels of genes pertaining to other immune cells ($p > 0.05$),

TABLE 1 | IFN γ dosage dependent immune checkpoint gene related pathways.

3 different groups	Total genes	Immune genes	Signaling genes
(A) NUMBER OF UNIQUELY CO-EXPRESSED GENES WITH SIX ICPs AND THREE ICPRGs IN IFNγ POSITIVE TUMOR, NEGATIVE TUMOR, AND NORMAL CONTROL			
32 IFN γ positive CRCs	151	87	64
47 IFN γ negative CRCs	73	62	11
79 normal controls	67	52	15
4 main pathways identified from 87 unique immune genes co-expressed with six ICPs and three ICPRGs in IFNγ positive CRC		Pathway related genes	
(B) IMMUNE PATHWAYS AMONG IFNγ POSITIVE TUMOR, NEGATIVE TUMOR, AND NORMAL CONTROL			
CD8 T cell activation and inactivation (17 genes)		IFN γ , IDO1, LAG3, ITGAL, MICB, CD3G, CD3D, CD8A, CD8B, CD3E, SLA2, IL15, ADA, NLRC3, CD2, SPN, CD7	
cytolysis (7 genes)/T cell		DNASE2, GZMM, IL2RA, GZMA, GPR65, BIRC3, SRGN	
T cell co-stimulation (2 genes)		TNFSF13B, SPN	
response to vitamin A (3 genes)/TH differentiation		CD38, MICB, MAP1B	
4 main pathways identified from 62 unique immune genes co-expressed with six ICPs and three ICPRGs in IFNγ negative CRC		Pathway related genes	
B cell and macrophage activation (7 genes)		ICAM1, PLEK, OLR1, CTGF, ITGA5, CD209, ADAM8	
B cell antigen presentation (5 genes)		HCK, FCGR1A, FCER1G, COLEC12, CD14	
B cell response to lipopolysaccharide (2 genes)		SLC11A1, PTAFR	
wound healing (6 genes)/B cell maturation		SLC11A1, PLEK, ITGA5, ANXA5, PLAUI, PLAUR	
4 main pathways identified from 52 unique immune genes co-expressed with six ICPs and three ICPRGs in normal control		Pathway related genes	
T cell inhibition/MDSC2 (8 genes)		CD48, ZBTB32, CARD11, LCK, FOXP3, VAV1, LCP1, CD28, CCR7	
lymph node development (3 genes)/B cell		CXCR5, LTB, LTA	
induction of apoptosis (2 genes)/B cell		VAV1, CD5	
B cell proliferation (2 genes)		CARD11, CD40, CD19, CD79B	
4 main pathways identified from 64 unique signaling genes co-expressed with six ICPs and three ICPRGs in IFNγ positive CRC		Pathway related genes	
(C) SIGNALING TRANSDUCTION PATHWAYS AMONG IFNγ POSITIVE TUMOR, NEGATIVE TUMOR, AND NORMAL CONTROL			
Wnt/ β -catenin signaling (3 genes)/T cell development		NMI, RNF213, RNF31	
GTPase signaling (6 genes)/T cell activation		GNGT2, GPR171, GPR174, GPR18, NCF1, SMAP2	
nuclear receptor signaling (13 genes)/T cell response		ATXN7, BTN3A2, CEP170, CSTF2, CTRL, FAM78A, GTF2H4, NPL, RFX5, SFMBT2, SMCHD1, SNTB2, SNX20	
phosphorylation (8 genes)/T cell activation		EVL, GSG2, HSPA1A, PPP1R16B, PTPN22, TBC1D10C, USF1, ZAP70	
4 main pathways identified from 11 unique signaling genes co-expressed with six ICPs and three ICPRGs in IFNγ negative CRC		Pathway related genes	
EGFR signaling (1 genes)/B cell differentiation		EMP3	
cell-cell recognition (1 genes)/B cell receptor		ST3GAL6	
phosphorylation (3 genes)/B cell receptor		ETV5, FGR, KIFC3	
Ca ²⁺ signaling (1 genes)		ITPRIP	
4 main pathways identified from 15 unique signaling genes co-expressed with six ICPs and three ICPRGs in normal control		Pathway related genes	
ER signaling (1 genes)/cell cycle		UBQLN3	
phospholipase (1 genes)/B cell receptor		PLCG2	
relaxin-3/RXFP3 signaling (1 genes)		RXFP3	
TREM2/DAP12 signaling (1 genes) myeloid cell		TREM2	

including B cells (CD19), neutrophils (CD11b), M1 macrophages (ARG1 and ARG2), and M2 macrophages (ARG2 and CCR7) (Figure 4A). In contrast, there were no differences ($p > 0.05$) in expression of the above 9 immune cell specific genes in normal tissues from patients with IFN γ positive vs. negative CRC (Figure 4B). As for T cell related cytotoxins, essential in tumor killing, stacked FPKM of 8 genes (PRF1, GZMM, GZMK, GZMH, GZMB, GZMA, FASLG, and FAS) demonstrated that these genes were more highly expressed 1.7-fold ($P = 0.037$) in IFN γ positive tumors compared to IFN γ negative tumors (Figure S3A). Eleven co-stimulatory genes (C10orf54 [B7-H5], BTLA4, CD86, CD80, ICOS, CD28, CD27, CD40, TNFRSF9 [4-1BB], TNFRSF18 [GITR], TNFRSF4 [OX40]) (30, 31) were expressed at higher (1.5-fold) levels in IFN γ positive vs. IFN γ negative CRC, but this did not reach statistical significance ($p = 0.30$) (Figure S3B). Moreover, stacked FPKM of 9 caspases (CASP2 to CASP10) (Figure S3C) and 9 cell cycle related genes (PRKDC, H2AFX, FANCD2, BRCA2, BRCA1, CHEK1, ATR, ATM) (Figure S3D) demonstrated that these genes were not significantly upregulated in IFN γ positive vs. negative tumors. These findings suggest that although IFN γ may upregulate CTL-associated proteins, it does not directly affect expression of co-stimulatory molecules (required for full T cell activation), caspases and cell cycle related genes, critical factors contributing to T cell activation and function.

In exploring further correlates of immune activation in tumors, we examined the relationship between IFN γ expression and thirteen DNA mismatch repair enzymes (MMR) (PMS2P5, PMS2P4, PMS2P3, PMS2P1, PMS2CL, PMS2, PMS1, MSH6,

MSH4, MSH3, MSH2, MLH3, and MLH1), because the loss of DNA MMR function is associated with increased expression of neoantigens, immune cell recruitment and induction of ICPs (32). We did not find a difference between IFN γ expression and expression of 13 DNA MMRs (1.0-fold) ($p = 0.86$) in IFN γ positive vs. negative tumors (Figure S3E). Although the MSS/MSI status was available for only 7 CRCs in our cohort, 5 MSS CRCs, and two MSI CRC (Table S1), we found that all 10 genes (IFN γ , six ICPs, and three ICPRGs) were upregulated in MSI CRC compared to MSS CRC but this did not reach statistical significance ($P = 0.31$) in this small sample size (Figure S3F). Thus, the relationship between IFN γ upregulation and the presence or loss of the MMRs and MSI/MSS status needs to be studied further.

Validation of IFN γ Dependent Expression of Six Classical ICPs and Three ICPRGs in a Larger CRC Cohort

To further define the dosage impact of IFN γ on the expression of six ICPs and three ICPRGs, we generated six IFN γ expression level gradients¹ IFN γ : FPKM > 5 (4 CRCs); (1) IFN γ : FPKM = 4.9–2 (20 CRCs); (2) IFN γ : FPKM = 1.99–1 (44 CRCs); (3) IFN γ : FPKM = 0.99–0.5 (73 CRCs); (4) IFN γ : FPKM = 0.49–0.01 (467 CRC); and (5) IFN γ : FPKM < 0.009 (107 CRCs) in 716 CRCs (Indivumed [79 CRCs] and TCGA [637 CRCs]) (Figure 5A) and examined the impact of the levels on expression of the ICPs and ICPRGs examined in our more limited cohort. We found that the expression level of IFN γ was highly co-related

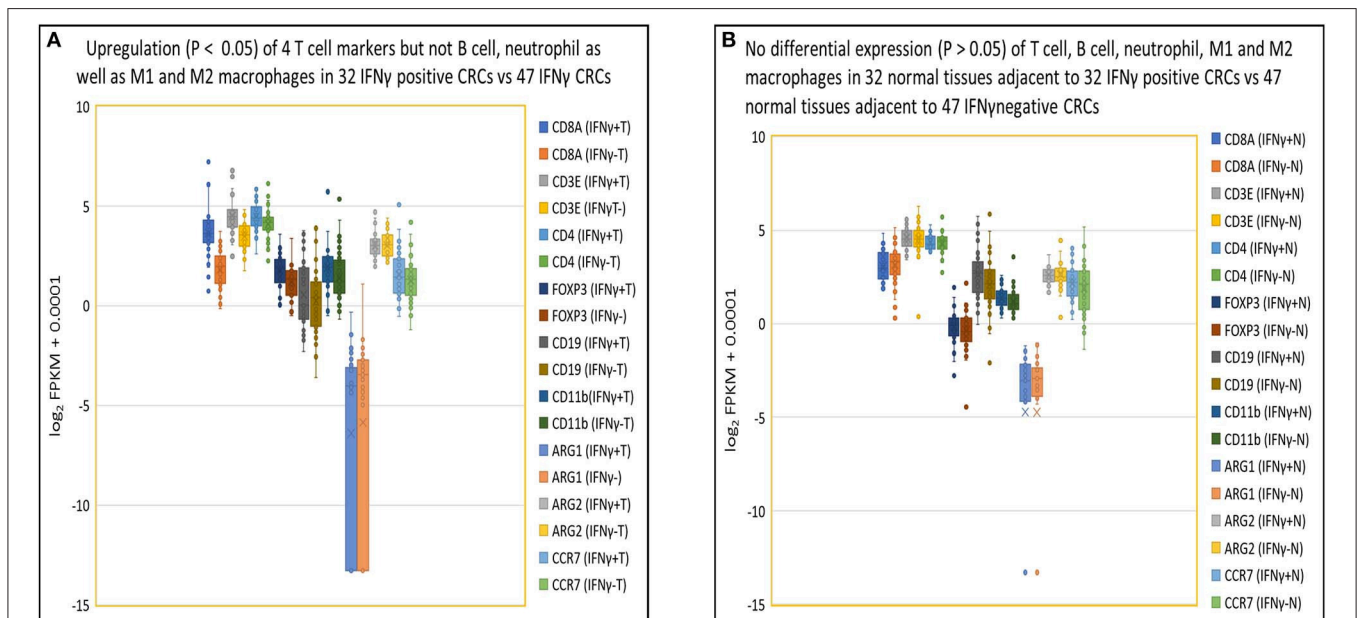


FIGURE 4 | Close association of IFN γ with T cells in tumor but not in normal tissues. **(A)** Box and Whisker plot of immune cell genes in IFN γ positive CRCs. Upregulation ($P < 0.05$) of T cells (CD8A, 3.5-fold; CD3E, 2.0-fold; CD4, 1.3-fold; FOXP3, 1.5-fold) but not B cell (CD19) (1.2-fold, $P = 0.38$), neutrophil (CD11b) (1.4-fold, $P = 0.079$), M1 (ARG1) (0.68-fold, $P = 0.62$), (ARG2) (1.0-fold, $P = 0.87$), or M2 (ARG2, CCR7) (1.2-fold, $P = 0.30$) leukocyte-related genes in IFN γ positive CRC vs. negative CRC. **(B)** Box and Whisker plot of immune cell genes in normal colon tissue. No upregulation ($P > 0.05$) of T cells (CD8A, 0.93-fold; CD3E, 1.0-fold; CD4, 1.0-fold; FOXP3, 1.1-fold), B cell (CD19) (1.4-fold), neutrophil (CD11b) (1.1-fold), MDSC1 (ARG1) (1.0-fold), (ARG2) (0.97-fold), or MDSC2 (ARG2, CCR7) (1.2-fold) leukocytes related genes in tissues adjacent to IFN γ positive and negative CRCs.

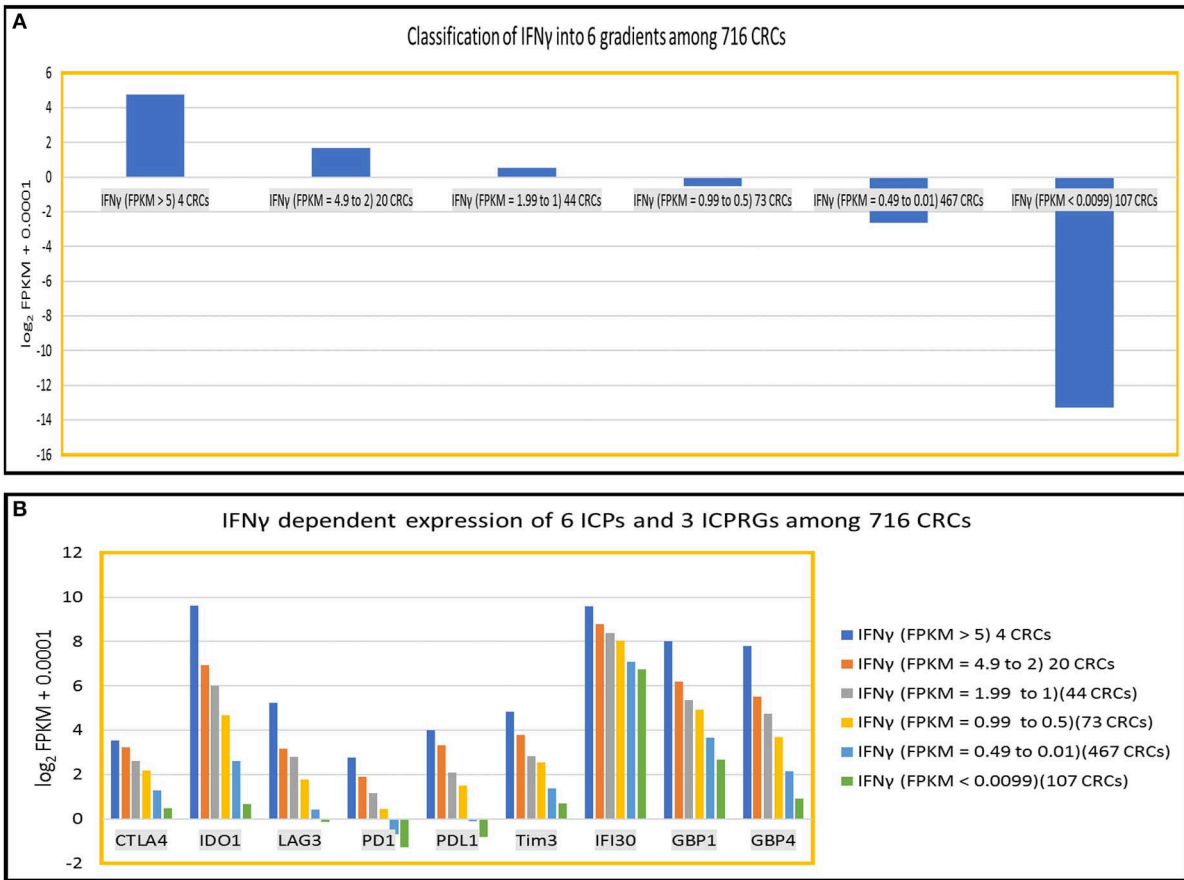


FIGURE 5 | IFN γ dependent expression of six ICPs (PDI, PDL1, CTLA4, IDO1, LAG3, Tim3) and three ICPRGs (IFI30, GBP1, GBP4) in 716 CRCs. **(A)** Classification of IFN γ into six expression gradients in 716 CRCs. **(B)** IFN γ dosage dependent expression positive correlation ($cc > 0.94$) with six ICPs and three ICPRGs across six IFN γ expression level gradients (six CRC subsets).

($cc > 0.94$) with expression levels of the six ICPs and three ICPRGs across the six IFN γ gradients (**Figure 5B**).

Regarding expression of ICPs and ICPRGs in matched normal control tissues, we found no differential expression of IFN γ , six ICPs and three ICPRGs between normal tissues adjacent to IFN γ positive vs. negative tumors (**Tables S10, S11**).

Further Confirmation of IFN γ Dependent Expression of Six Classical ICPs and Three ICPRGs Among Five Other Solid Cancer Types

To evaluate whether our findings regarding IFN γ -associated expression of six classical ICPs and three ICPRGs in CRC also applied to distinct tumor types, we compared the overall stacked \log_2 expression of the six ICP genes and three ICPRGs in the following tumor types: 103 skin cutaneous melanomas (SKCMs); 1,105 breast cancers (BCs); 184 esophageal cancers (ESCs); 416 stomach cancers (STCs); and 501 lung squamous carcinomas (LUSC) all from the TCGA database. Similar to the CRC findings, the overall stacked \log_2 FPKM expression levels of six classical

ICPs [CRC: (1.73-fold, $p = 0.58$), SKCM: (3.2-fold/ $p = 0.10$), BC: (27.8-fold/ $p = 0.051$), ESC: (2.1-fold/ $p = 0.39$), STC: (1.9-fold/ $p = 0.51$) and LUSC: (4.4-fold/ $p = 0.10$)] (**Figure S4A**) and three ICPRGs [CRC: (4.3-fold/ $p = 0.29$), SKCM: (5.4-fold/ $p = 0.27$), BC: (3.6-fold/ $p = 0.33$), ESC: (2.8-fold/ $p = 0.36$), STC: (3.1-fold/ $p = 0.36$) and LUSC: (2.9-fold/ $p = 0.47$)] (**Figure S4B**) were increased (1.7 to 27.8-fold) in IFN γ positive (FPKM > 1) tumors vs. IFN γ negative (FPKM < 1) tumors across these cancers but without statistical significance.

Because these three ICPRGs have the potential to be novel actionable targets in cancer therapy, we further examined these individual genes by Box and Whisker plots of \log_2 FPKM expression levels in IFN γ positive and negative tumors of CRC, SKCM, BC, ESC, STC, and LUSC. IFI30, GBP1 and GBP4 were upregulated 2-8-fold ($p < 0.0001$) in IFN γ positive tumors vs. IFN γ negative tumors (**Figure 6**) in each of the six tumor types. Among these six cancers, only STC had a higher abundance (average FPKM) of IFI30, GBP1, and GBP4 (469, 78, 54) than did CRC (334, 47, 25) in the IFN γ positive tumors (**Table S12**). These three ICPRGs were also 24-fold more abundant than six ICPs in 5 types of normal tissues adjacent to BC, STC, LUSC, ESC,

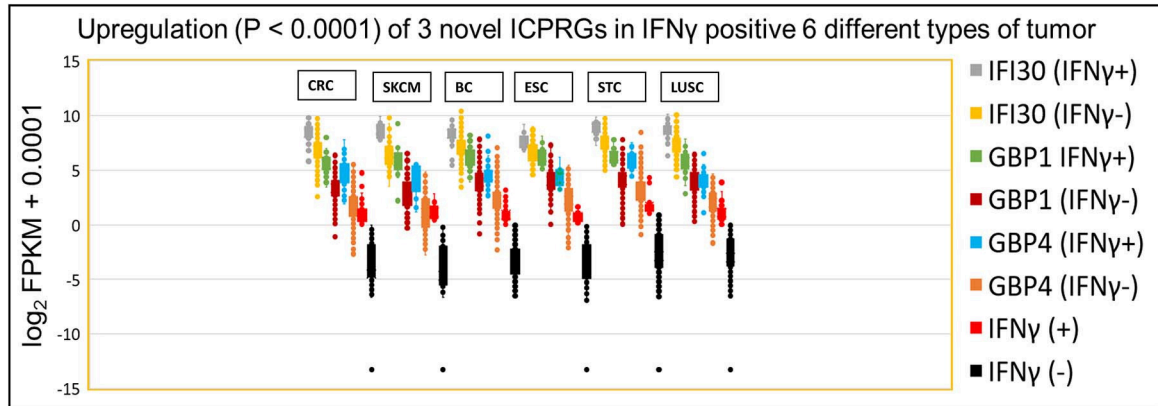


FIGURE 6 | IFN γ dependent expression of three ICPRGs among five other solid cancers in Indivmed and TCGA cohort. Box and Whisker analysis of three ICPRGs in six types of cancer. Higher three ICPRGs and IFN γ expression in IFN γ (+) 69 CRCs (IFI30, 2.9-fold; GBP1, 4.6-fold; GBP4, 7.9-fold; and IFN γ , 44-fold), 13 SKCMs (IFI30, 4.6-fold; GBP1, 8.5-fold; GBP4, 8.3-fold; and IFN γ , 55-fold), 85 BCs (IFI30, 2.3-fold; GBP1, 4.9-fold; GBP4, 4.5-fold; and IFN γ , 19-fold), 13 ESCs (IFI30, 2.0-fold; GBP1, 4.5-fold; GBP4, 4.1-fold; and IFN γ 28-fold), 71 STCs (IFI30, 2.5-fold; GBP1, 4.5-fold; GBP4, 6.2-fold; and IFN γ 23-fold), and 68 LUSCs (IFI30, 2.6-fold; GBP1, 3.4-fold; GBP4, 4.1-fold; and IFN γ 17-fold), vs. IFN γ (-) 647 CRCs, 90 SKCMs, 1,020 BCs, 171 ESCs, 345 STCs, and 433 LUSCs.

and CRC (Table S13) again raising the issue of whether these three genes are involved in a universal tolerance mechanism for normal tissues.

Strikingly in LUSC, all 68 IFN γ positive tumors had high expression of PD1 (FPKM > 1) while all 434 IFN γ negative tumors had low expression of PD1 (FPKM < 1) with a statistically significant 4.8-fold difference (Figure S5). These data suggest a dosage effect of IFN γ with respect to consequent expression of the six ICPs as well as for the three ICPRGs in six solid tumor types.

Correlation of Immune Genes With Clinical Parameters in CRC

To address this issue, we separated by stage the 129 CRC pairs (79 CRC pairs as well as 50 CRC pairs/TCGA_38 cohort) into 57 low stage tumors (TNM stage I/II) and 82 high stage tumors (TNM stage III/IV) and found that there was no significant difference ($p = 0.81$) in the expression of IFI30, GBP1, GBP4, PD1, PDL1, CTLA4, Tim3, LAG3, IFN γ , and IDO1 between low and high stage CRCs (Figure S6). Because the tumor genetic profile may impact survival, we analyzed the Pathology Atlas data and found that (i) higher expression of IFI30, GBP1 and GBP4 was associated with better 5-year survival rate in breast cancer as well as in skin cutaneous melanoma, (ii) higher expression of GBP1 and GBP4 was associated with better 5-year survival rate in colorectal and stomach cancer, and (iii) higher expression of IFI30 was associated with worse 5-year survival rate in colorectal and stomach cancer (Table S14). These data suggest that any impact of IFI30, GBP1, and GBP4 on tumor response to diverse therapeutics is likely tumor type and context- dependent, clearly warranting further study.

DISCUSSION

Although IFN γ secreted by immune cells promotes growth arrest of tumors by augmenting MHC class I expression, contributing

to the recruitment of effector cells, mediating Treg fragility and coordinating innate and adaptive antitumor responses (33, 34), IFN γ signaling can also compromise antitumor immunity by blocking these activities through the induction of immune checkpoint inhibitory molecules on T and tumor cells (35). The overall balance and timing of IFN γ expression over the course of tumor development and the downstream consequences likely critically determine an effective vs. suppressive immune response and an immunologic profile of consideration for immunotherapeutic approaches to treatment (36). In this study, we first demonstrated the specific upregulation and co-expression of six ICPs associated with higher expression of IFN γ in CRC. These data provide the molecular basis of using more than one ICP blocker in CRC with higher IFN γ expression but not lower IFN γ expression. Then, by analysis of genes co-expressed with IFN γ , we discovered three IFN γ associated ICPRGs. These three ICPRGs are expressed at higher abundance in CRC compared to the classical ICPs (except IDO1). Furthermore, there was differential co-expression of IFN γ with other immunologically pertinent genes between IFN γ positive and negative CRCs. IFN γ , the six ICPs, and the three ICPRGs were mainly co-expressed with T cell genes related to T cell activation, cytolysis and co-stimulation in IFN γ positive tumors while 2 ICPs and one ICPRG, but not IFN γ , were mainly co-expressed with B cell genes related to B cell activation, antigen presentation and response to lipopolysaccharide in IFN γ negative tumors. These data indicate dosage dependence of IFN γ on immune regulatory mechanisms in CRC. Finally, the co-upregulation of IFN γ with six ICPs as well as three ICPRGs was strongly supported by findings in the TCGA cohorts of melanoma, colon, breast, esophageal, stomach, and lung cancer. Thus, in addition to factors such as microsatellite stability status, tumor mutational burden, and expression of checkpoint inhibitory molecules, high IFN γ expression levels could potentially be investigated as a predictive biomarker for the potential for immune responsiveness of a tumor.

In our evaluation of the six classical ICPs, LAG3 appears to be a critical hub gene with the greatest number of co-expressed genes and though upregulated in IFN γ positive tumors, was downregulated and lacking co-expressed genes in IFN γ negative tumors. Thus, LAG3 may be a marker of biologically meaningful expression levels of IFN γ and an important drug target for CRC therapy in IFN γ positive CRC. The molecular mechanisms of LAG3 immune suppression have not been extensively defined. An additional ICP expressed at higher abundance compared to the other well-known ICP was IDO1, a rate-limiting metabolic enzyme that converts tryptophan into immune suppressive kynurenines (37). IDO1 is highly expressed in multiple types of human cancer (38) and studies indicate that while single-agent treatment with IDO1 enzyme inhibitor may not substantially decrease the established cancer burden, approaches combining select therapies with IDO1 blockade may have additive or synergistic effects, as shown in animal studies (39).

Based on their co-expression with six classical ICPs and with T cell markers, it is likely that the newly identified IFN γ related proteins, IFI30, GBP1, and GBP4 are immunomodulatory and may serve, in some tumors, as ICPs. That GBP1 and GBP4 are directly co-expressed with CD8A suggests the correlation of the three ICPRGs with a higher basal level of CD8A related infiltration in IFN γ positive CRC. It is well known that CD8⁺ T-cell infiltrates predict favorable prognosis in the majority of cancer types (40). In fact, GBP1 and GBP4 were associated with a favorable prognosis in 4 types of cancer (CRC, SKCM, BC, and STC) according to the Pathology Atlas analysis. These data are consistent with the evidence [KEYNOTE-001 trial/pembrolizumab (anti-PDL1) treatment] (41, 42) that high expression of PDL1, a classical immune suppressive check point molecule was associated with better survival among pembrolizumab-treated NSCLC and melanoma patients.

The immune and tumor related nature of these three genes are supported by the following published data: (i) IFI30 suppresses mouse primary T cell reactivity *in vitro* and mouse autoimmunity through cellular redox chemistry and ERK1/2 phosphorylation *in vivo*, promotes cell proliferation of a glioma cell line, but IFI30 RNA has been associated, with better patient survival rate in breast cancer and diffuse large B cell lymphoma (DLBCL) (9–14), (ii) GBP1 suppresses TCR signaling through lymphocyte cell-specific protein-tyrosine kinase and IL2 production in a human T cell line promotes cell proliferation/anti-apoptosis of a glioblastoma and two breast cancer cell lines, but inhibits cell proliferation of a colon cancer line. Furthermore, GBP1 reduces radioresistance of two human oral and liver cancer cell lines and correlates with better prognosis in melanoma but with poorer prognosis in human glioblastoma (15–21), (iii) GBP4 inhibits innate responses to viral infection (22) but lacks known tumor related functions to date. Thus, both knock-down and overexpression of these three genes should be tested in the future experiments to define the exact roles of these proteins within specific contexts. Additionally, there is the potential that inhibiting or stimulating them could change responses to infection and autoimmunity given their abundant expression in normal colonic tissues.

The co-expression of CTLA4 and PD1 with predominantly B cell markers and the co-expression of GBP1/GBP4 with six genes mainly related to anti-viral and microbial infection in normal intestinal epithelium [CXCL9, GBP5, STAT1, PARP9 (Poly ADP-Ribose Polymerase 9), TRAFD1 (Type Zinc Finger Domain Containing 1), and UBE2L6 (ubiquitin conjugating enzyme E2 L6)] suggest that maintenance of homeostasis, challenged by commensal bacteria, food antigens and potential autoantigens, may be maintained by B regulatory cell induction of ICPs (43–45). The relationship of these factors to mechanisms of intestinal tolerance and immunity clearly requires further study.

In summary, by applying NGS to study the expression of six classical ICPs and their co-expression networks, we found not only the well-established connection between IFN γ and the expression of ICPs in CRC, a relatively immunotherapy-refractory tumor type, but also, a novel set of ICPRGs as well as potential new hub genes which may be potential therapeutic targets. This study also provides comprehensive ICP co-expression information and fortifies the importance of NGS profiling in CRC and other tumors. The expression of higher abundance and novel ICPRG genes, including IFI30, GBP1, and GBP4, requires further evaluation of protein expression levels and immune inhibitory function in tumors.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

RW, WW, J-NP, C-TL, and R-FS carried out experiments. RW, HM, L-HW, EP, W-LA, and LX performed data analysis. AR, LX, RW, LP, HM, EP, and HJ designed experiments and interpreted results. AR, RW, HM, LP, and LX wrote the manuscript, and all authors edited it. AR was the principal investigator of this study. All authors reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.00224/full#supplementary-material>

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Conflict of Interest: HJ was employed by the company Indivumed GMBH.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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