RESEARCH ARTICLE

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Necrosis Factor-α (TNF-α) and the Presence of Macrophage M2 and T Regulatory Cells in Nasopharyngeal Carcinoma

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Abstract

Objective: To investigate the correlation between TLR3 and pro-inflammatory cytokines (TNFα, IL6) expression with the distribution of macrophage M2 and Treg on Epstein Barr virus-encoded RNAs (EBER+) nasopharyngeal carcinoma (NPC) tissues. **Methods:** A total of 23 FFPE NPC tissue samples were obtained from patients in Dr. Sardjito General Hospital, Yogyakarta, Indonesia in 2008–2010, which expressed EBER was collected. The expressions of TLR3, TNFα, and IL6 were examined using immunofluorescence assay. The distribution of macrophage M2 and Treg were examined by immunohistochemistry with anti-CD163 and -FOXP3 antibodies, respectively. The quantification of fluorescence intensity was analyzed by the RGB space method using ImageJ software. The M2 interpretation was done by the eyeballing method and the M2 scores were divided into 0 (negative), 1 (scant), 2 (focal), 3 (abundant). The average number of Treg FOXP3+ cells in five high power fields was counted. The relationship between variables were tested by the Spearman correlation test, and the coefficient correlation was used to see the correlation between variables. **Results:** All EBER+ NPC specimens showed TLR3 expression intracellularly. The expression of TNFα could be observed in the cell membranes and secreted extracellularly, while IL6 was secreted to the extracellular area. The expression of TNFα was two times higher than IL6. Most specimens showed low M2 score (56.52%) and high Treg (52.17%). A positive correlation was found between TLR3 and IL6 (12.9%). TNFα was positively correlated with the M2 distribution of 13.7% and Treg distribution of 12.9%, while the rest were explained by other factors. **Conclusion:** TNFα has a positive correlation with M2 and Treg distribution,but mostly through a different mechanism other than EBER-TLR3 interaction. Possibly, other pro-inflammatory and anti-inflammatory cytokines are involved in the formation of the NPC microenvironment, especially related to the presence of M2 and Treg, which provide immunosuppressive effects in NPC tumors.

Keywords: Epstein Barr virus-encoded RNAs- TLR3- pro-inflammatory cytokines- M2- Treg

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Introduction

Nasopharyngeal cancer (NPC) is a malignancy with unique characteristics related to epidemiology, clinical manifestations, biological markers, risk factors, and prognosis compared to other types of head and neck cancers. NPC is endemic in southern China and Southeast Asia (Adham et al., 2012; Wang et al., 2017). NPC is the most common malignancy in the head and neck region and is one of the most common malignancies in Indonesia after breast, cervical, lung, and liver cancer. Approximately 98% of NPC cases in endemic areas are the non-keratinizing type and closely related to Epstein Barr virus (EBV) infection (Tsang et al., 2019).

Persistent EBV infection of the premalignant nasopharyngeal epithelium initiates the transformation of NPC tumor development (Tsao et al., 2017). EBV has several oncogenes and viral proteins; six EBV-encoded nuclear antigens (EBNA-1, EBNA-2, EBNA-3A, 3B, 3C, and EBNA-LP) and three latent membrane proteins

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(LMP1, LMP2A, and LMP2B), which are expressed at a different latency program, whereas two types of non-protein-coding RNA, EBV-encoded RNAs (EBER-1 and EBER-2) and long alternative splicing non-coding RNA at Bam H1 rightward transcripts (BARTs) are expressed in all latency types of EBV infection (Zeng at al., 2015).

EBV-encoded RNAs (EBER) is the most widely expressed viral transcript in almost all EBV-infected cells, therefore, it is often used as a marker of active EBV infection (Ahmed et al., 2014; Aaro et al., 2017). EBER is found in the serum of patients with active EBV infection and will induce type I interferon (IFN) and inflammatory cytokines through Toll-like receptor-3 (TLR3) signaling (Iwakiri et al., 2009). EBER contributes to oncogenesis by modulating innate immunity in patients with NPC (Takada, 2012). EBER induces an inflammatory response in NPC cells through TLR3, indicated by the increased production of inflammatory cytokines, especially tumor necrosis factor-alpha (TNFα) (Li et al., 2015).

Cytokines produced by tumor cells and T lymphocytes lead to macrophage infiltration into tumor tissue and direct its polarization to become an M2 phenotype. Polarization of M2 by tumor cells is continued with the recruitment of T regulatory cells (Treg) through conversion via transforming growth factor-beta (TGF-β) or interleukin-2 (IL-2). Recruitment of Tregs by M2 will provoke the evasion of cancer cells from the immune system so that it can worsen the progression of NPC (Wang et al., 2017).

The interaction between EBV, tumor cells, and the host cells form a unique tumor microenvironment (TME) that plays an important role in supporting tumor growth in patients and maintaining the stability of EBV infection in NPC cells (Tsang et al., 2019). The presence of EBER transcripts on carcinoma cells results in a worse prognosis for the patient (Aaro et al., 2017). This study was conducted to explore the relationship between the expression of TLR3 and pro-inflammatory cytokines (TNFα, IL6) with M2 and Treg distribution in the EBER+ NPC tissues.

Materials and Methods

Twenty-three (23) formalin-fixed paraffin-embedded (FFPE) specimens derived from NPC patients (2008-2010) in Dr. Sardjito General Hospital, Yogyakarta, Indonesia expressing EBER were used in this study. The inclusion criteria were: [1] clinically and histopathologically diagnosed, [2] EBV positive, proved by the presence of EBER expression, and [3] samples were taken before treatment. The study was approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine Universitas Gadjah Mada, Yogyakarta, Indonesia (No. KE/FK/0022/EC/2020). The data of this research were obtained from primary and secondary data. The primary data were the expression of TLR3, TNFα, and IL6 in all NPC tissues and 7 data for M2 and Treg distribution. Data collection on M2 and Treg distribution from 16 samples was performed by another researcher (Aliyah, unpublished). For each paraffin block, five adjacent tissue sections were stained with immunofluorescence assay (IFA) for the expression of TLR3, TNFα, and IL6 and immunohistochemistry (IHC) for M2 and Treg distribution.

Immunofluorescence Assay (IFA)

Tissue sections were deparaffinized and rehydrated. The expressions of TLR3, TNFα, and IL6 were examined using the IFA method with primary antibodies anti-TLR3 (ab62566), -TNFα (ab6671), -IL6 (ab9324) from ABCAM. After the priming with primary antibodies, the sections were incubated with antibodies labeled with fluorochrome Alexa Fluor 488 (ab150077) for TLR3 and TNFα, and Alexa Fluor 647 (ab150115) for IL6. The data obtained were the immunopositive color intensity of each variable using ImageJ software with the following steps: [1] The opening of image composite, [2] Splitting the channel (R, G, B), [3] Stacking the image, [4] Adjusting the threshold (red fluorochrome: 29-254, green fluorochrome: 112-255), [5] Region of Interest (ROI) selection, and [6] Intensity measurement (Durán and Arriazu, 2013). The observer specified 40 regions of interest (ROI) on each field of view to minimize the bias. The mean fluorescent intensity of each variable was categorized into low and high based on the median value (Mardhiyah, 2020).

Immunohistochemistry

Tissue sections were deparaffinized and rehydrated. Antibody anti-CD163 (ab182422, Abcam, Cambridge, UK) was used for macrophage M2 detection and anti-FOXP3 (ab20034, Abcam, Cambridge, UK) for Treg detection with Mouse and Rabbit Specific HRP/DAB IHC Detection Kit Micro-polymer (ab236466). The positive and negative control tissues were processed along with each procedure. Interpretation of M2 was done by looking at the whole sections directly under the light microscope with eyeballing method. The results were interpreted into four categories: 0 (negative), 1 (scant), 2 (focal), 3 (abundant). Furthermore, the results were grouped into low for score 0-1 and high for score 2-3 categories (13). The number of FOXP3+ cells was counted on five high-power fields (HPF), which were selected randomly at every corner and middle area. The data were expressed as the mean of all 5 fields (Wang et al., 2017). The median FOXP3+ value was used to categorize the data into high and low categories. The interpretation was done by two independent observers; the researcher and the pathologist to reduce subjectivity. Kappa and Cronbach alpha tests were done to assess reliability between two observers (Mardhiyah, 2020).

Statistical Analysis

All variables were presented in categorical data and the relationships between variables were analyzed using the Spearman correlation test with the SPSS version 19 program (IBM Corp., Chicago). The coefficient correlation (r) was used to see the correlation between variables.

The study is part of an umbrella research on Tumor Microenvironment of Nasopharyngeal Carcinoma.

Results

Expressions of the TLR3, TNFα, and IL6 proteins

All parameters were examined on the tumor area. TLR3 protein was expressed on the membrane of the endosome and the endoplasmic reticulum. IL6 protein was secreted in the extracellular area, and TNFα was expressed on the cell membrane and secreted in the extracellular area. Positive staining for TLR3 and TNFα was indicated by a green fluorescent, while IL6 was indicated by red fluorescent (Figure 1). The expression of TLR3, TNFα, and IL6 proteins can be observed in each sample with different color intensities. Cronbach alpha test was done and the value was 0.99 (p<0.001), thus indicating good reliability between both observers (Watson and Petrie, 2010). The mean of TNFα fluorescent intensity was the highest (600635.10±24350.41) followed by TLR3 (543136.50±102761.06) and IL6 (241421.90±147280,85) (Figure 2). TNFα expression was more than two times higher compared to IL6.

Distribution of M2 and Treg

The distributions of M2 and Treg were examined by single staining IHC method, with anti-CD163 antibodies for M2 and anti-FOXP3 for Treg on adjacent tissue sections. The observations were done by two independent observers with a kappa value of 0.73 (p <0.001), indicating good reliability between the two observers. The expression of CD163 on M2 varied from low to high categories (Figure 3). The M2 score was assessed based on the predefined CD163 expression category. M2 immuno-positive cells were shown by brown color on the membrane and cytoplasm. Most of the samples (56.52%) had low M2 scores (Table 1), with the following distribution of M2 scores for the entire sample: score of 0 (17.39%), score 1 (39.13%), score 2 (34.78%), and score 3 (8.69%) (Figure 3F). The expression of FOXP3 on Treg was observed at various levels. Positive staining was seen in the nucleus (Figure 4). The median value was 5.2 and used to categorize FOXP3 expression into low and high. The distribution of the two categories was almost similar, with 11 specimens (47.83%) for the low Treg category and 12 specimens (52.17%) for the high category (Table 1).

Table 1. Distribution of Treg and M2 on the NPC Tissues

Variable	Description
Treg	5.2 (0.6-28.6)[a]
Low (<median)	11 (47.83%)
High (≥median)	12 (52.17%)
M2	
Low (0-1)	13 (56.52%)
High (2-3)	10 (43.48%)

[a], data are presented in median (minimum-maximum).

Relationship between TLR3 expression and pro-inflammatory cytokines (TNFα, IL6)

The mean fluorescent intensity of TLR3, TNFα, and IL6 from the two observers was divided into two categories based on the median value. The relationship between TLR3 with TNFα and IL6 was analyzed using the Spearman correlation test. The results show that the TLR3 expression has a negative correlation with the TNFα expression (r = -0.303), but has a positive correlation with IL6 (r = 0.129) (Table 2).

Table 2. Correlation between TLR3 Expression with TNFα and IL6 (n = 23)

	TNFα		IL6	
	r	*p*	r	*p*
TLR3	-0.303[a]	0.16	0.129a	0.558

Table 3. Correlation between TLR3, TNFα and IL6 expressions against M2 and Treg

	M2		Treg	
	r	*p*	r	*p*
TLR3	-0.137[a]	0.532	-0.129[a]	0.558
TNFα	0.137[a]	0.532	0.129[a]	0.558
IL6	-0.137[a]	0.532	-0.129[a]	0.558

[a], Spearman correlation test, p value <0.1 with a two-sided test was considered statistically significant.

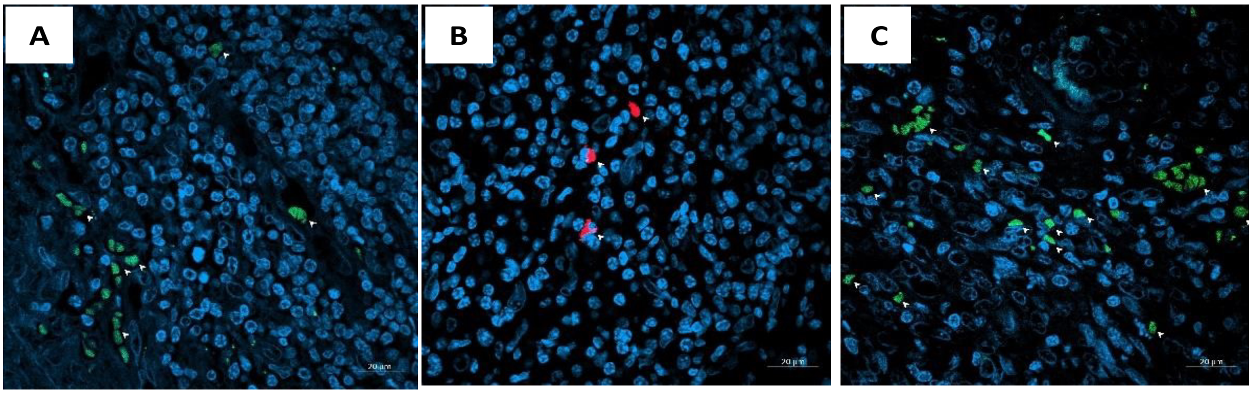

Figure 1. Protein Expression in the Intra-Tumor Area of the NPC Tissue is Indicated by White Arrows: (A), TLR3 expression was shown by green fluorescent staining on the endosomal membrane and endoplasmic reticulum; (B), Expression of IL6 was indicated by the presence of red fluorescent secreted in the extracellular area; (C) TNFα expression, shown by green fluorescent staining on the plasma membrane and secreted in the extracellular area. The image was taken with a 40x magnification.

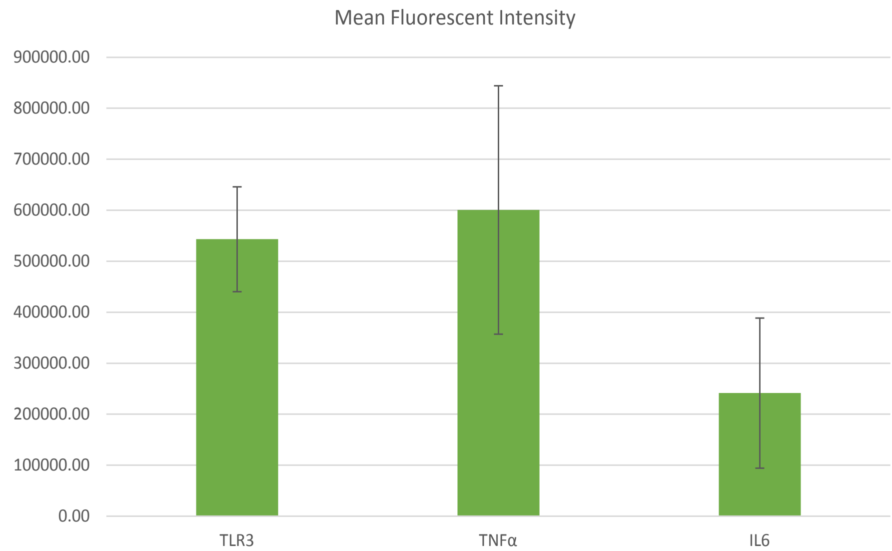

Figure 2. Fluorescent Intensity of TLR3, TNFα, and IL6 Protein Expressions.

Relationship between TLR3, TNFα, and IL6 expressions with M2 and Treg distributions

The relationship between TLR3 expression and pro-inflammatory cytokines (TNFα, IL6) with M2 and Treg distributions was analyzed using the Spearman correlation test. The TLR3 expression has a negative correlation with the M2 and Treg distributions with r values 0.137 and -0.129, respectively. The IL6 expression also had a negative correlation with the M2 distribution (r = 0.137) and Treg (r = -0.129), while the TNFα expression had a positive correlation with the M2 distribution (r = 0.137) and Treg (r = 0.129) (Table 3).

Discussion

All FFPE samples from EBV-positive NPC patients were confirmed by the presence of EBER expression, which was examined by using the in-situ hybridization (ISH) method (data not shown). The secondary structure of EBER was dsRNA that facilitates the binding with cellular proteins lupus erythematosis-associated antigen (La). The majority of EBER is released from EBV-infected cells in the form of the EBER-La complex in the exosome, so that EBER not only avoids nuclease degradation in the extracellular environment but also could be transported to other EBV uninfected cells (Iwakiri et al., 2009; Takada, 2012). Therefore, the presence of EBER is very stable and the transcript is mostly found in latent EBV infection.

This study revealed that all NPC EBER+ specimens showed TLR3 protein expression (Figure 1A). A previous study proved that EBER interacts with TLR3 and induces the production of various cytokines by tumor cells to recruit and activate macrophages (Li et al., 2015). Interaction between EBER and TLR3 in tumor tissue will

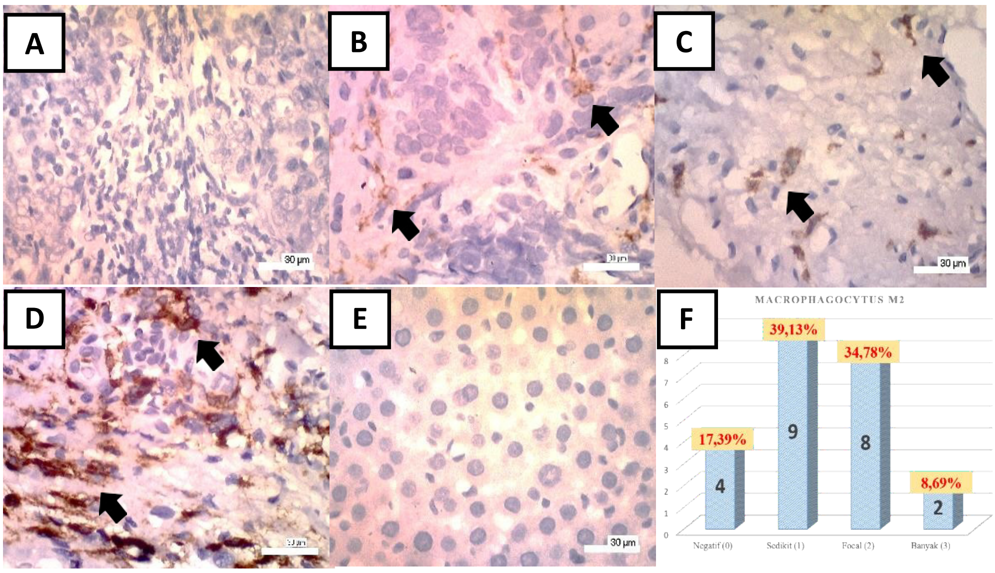

Figure 3. Distribution of M2 Macrophages on NPC Tissue Examined Using Anti-CD163 Antibody. Positive staining was shown on the membrane and cytoplasm (black arrow). Image taken with a 400x magnification. M2 score (A), negative; (B), scant; (C), focal; (D), abundant; (E), rat liver tissue without primary antibody as negative control; and (F), distribution of M2 scores.

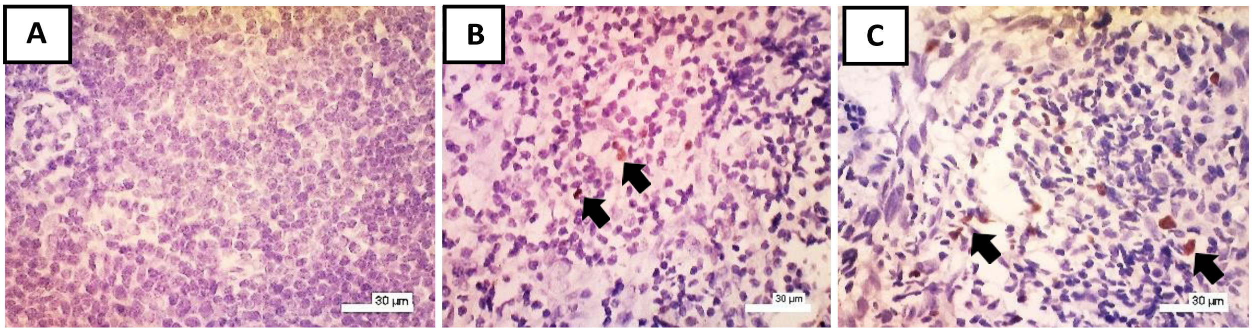

Figure 4. Distribution of Treg in NPC Tissue Examined by Immunohistochemical Method Using Anti-FOXP3 Antibody. Positive staining can be seen in the nucleus (black arrow). The image was taken with a 400x magnification. (A), human tonsillar tissue as negative control; (B), Tregs with scores below the median (low); (C), Tregs with scores above the median (high).

produce various bioactive molecules such as cytokines and other inflammatory mediators in the tumor area and its surroundings. These conditions also contribute to the formation of the TME that is favorable for NPC cells. However, this study did not perform colocalization between TLR3 and EBER, hence it is not known whether both of the molecules were expressed by the same cells.

The IFA method was chosen to visualize secreted proteins, such as IL6 and TNFα, and easier to quantify the result more objectively. IFA staining showed immune-positive for TLR3 in the intracellular compartment in green fluorescent. TLR3 in the intracellular vesicles will recognize nucleic acids that enter the intracellular compartment, in this case, is EBER. TLR3 is released from the endoplasmic reticulum and delivered to the endosome via the Golgi apparatus (Kawai and Akira, 2011). Once entering the endosome, the N-terminus region of TLR3 will be processed by various proteases and subsequently become a functional receptor that can produce further signaling (Ewald et al., 2011). IFA staining was also performed to visualize pro-inflammatory cytokines (TNFα and IL6) expression. The fluorescent intensity of TNFα was much higher than IL6 (Figure 2). In line with previous in vitro study, the exposure of high RNA of EBER from the serum of patients infected with chronic active EBV resulted in induction of IFN-β, IFN-γ, and TNFα expression (Iwakiri et al., 2009). Another research also found that TNFα was the most abundant pro-inflammatory cytokine compared to IL-6 and IL-1α in EBER expressing NPC cells (Li et al., 2015). However, IL6 is a potent activator of STAT3 signaling in immortal nasopharyngeal epithelium infected with EBV, thereby increasing NPC cell growth. In addition, in vitro IL6 stimulation can also increase the expression of matrix metalloproteinase-2 (MMP-2) and MMP-9, which influence the migration and invasion activities of the NPC cells (Zhang et al., 2013; Sun et al., 2014).

TNFα is produced in high quantity because it is one of the main inflammatory mediators, which can be induced by various pathogenic stimuli. TNFα induces other inflammatory mediators and proteases that regulate the inflammatory response. Tumor cells also produce TNFα, which acts as an endogenous tumor promoter. The role of TNFα is associated with all stages of tumorigenesis (Sethi et al., 2008). In addition, TNFα can cause phosphorylation of IκBα by IκB kinase (IKK). IκBα is an inhibitor of a major regulator of inflammation and immune response, NF-κB, in the cytoplasm. This phosphorylation causes IκBα to be degraded through the proteasome ubiquitin pathway, therefore NF-κB can be separated from the inhibitor and translocated into the nucleus and activates the transcription programs of various target genes (Napetschnig and Wu, 2013), which will induce the growth and development of NPC especially related to the inflammatory response.

The negative correlation between TLR3 and TNFα (r = -0.303) in this study (Table 2) is contradictory with the previous one, which suggested that the interaction of EBER with TLR3 will increase inflammatory response that was mainly indicated by the high production of TNFα (Li et al., 2015). It may be due to the ability of EBER to disable signaling of innate immunity in infected EBV cells and their surroundings, through EBER binding RNA-dependent protein kinase (PKR), thereby the anti-virus effect mediated by IFN will be inhibited (Ahmed et al., 2014; Iwakiri, 2014). The introduction of EBER will induce IFN type I, although IFN is not beneficial for viruses, EBV can maintain latent infection conditions due to resistance to IFN (Iwakiri, 2014).

The positive correlation of TLR3 expression and IL6 (r = 0.129) (Table 2) is consistent with previous in vitro study, which reported the decrease of IL6 expression in NPC TLR3-/- cell cultures with EBER exposure (Li et al., 2015). The low correlation between TLR3 and IL6 may be explained by the presence of other mechanisms, including the involvement of RIG-I, which may play a role in the induction of IL6 expression in the NPC EBER+ tissue. IL6 is an immunomodulator. An in vitro study showed that elevated levels of IL6 in NPC patients' serum were correlated with tumor recurrence, metastasis, and patient survival, therefore, IL6 can be used as a marker of disease progression and bad prognosis of NPC patients (Chow et al., 2003).

Another factor that affects the production of pro-inflammatory cytokines in EBV-infected cells is the viral oncogene latent membrane protein 1 (LMP1), which can also stimulate NF κB signaling that causes the unique inflammatory characteristics of EBV-infected cells (Li et al., 2015; Yi et al., 2017). LMP1 and EBER have a positive regulation in promoting the transition from inflammation to oncogenesis in EBV-infected epithelial cells (Li et al., 2015). Various NF-κB signaling, including p50/BCL3 and p50/RelB, will induce several survival genes and IL6 inflammatory cytokines (Tsao et al., 2017). The existence of other pathways that influence the production of these pro-inflammatory cytokines may explain the weak relationship between TLR3 and IL6 in this study.

The expression of TLR3 and IL6 in this study had a negative correlation with the M2 and Treg distributions, while on the contrary, TNFα expression showed a positive correlation with the M2 and Treg distributions with low r values, 0.137 and 0.129 for M2 and Treg, respectively (Table 3). Macrophages infiltrating tumor tissue are influenced by cytokines derived from tumor cells and T lymphocytes that induce macrophages to become M2 phenotype (Mantovani et al., 2002). NPC cells and viral products encoded by EBV play an important role in triggering the release of various pro-inflammatory cytokines including IL-18, TNFα, IL-6, CXCL-10, IFNγ, IL-12, IL-8, MIP-1α, and others, which will recruit monocytes and other immune cells, and subsequently cause the accumulation of macrophages in the tumor microenvironment (Huang et al., 2018). Anti-inflammatory molecules such as IL-4, IL-13, IL-10, apoptotic cells, and immune complexes are also found in the TME and induce M2 polarization, which has immunosuppressive properties and supports tumorigenesis (Biswas et al., 2008; Orecchioni et al., 2019). NPC cells can induce monocytes into the M2 phenotype through the production of TGF-β and IL-10 (Wang et al., 2017). The presence of pro-and anti-inflammatory cytokines that affects M2 polarization may explain the weak correlation between TNFα expression on M2 distribution in this study.

The distribution of M2 macrophage was almost similar in all NPC tissue, but the majority (56.52%) of the samples showed low M2 scores (Figure 5; Table 7). The low M2 differentiation in EBV-positive NPC tissue may be influenced by the presence of EBV protein BamHI-A rightward frame 1 (BARF1). The BARF1 protein is frequently expressed in tissues of various EBV-related epithelium malignancies. The BARF1 transcript and protein can be detected in most NPC tissue (Takada, 2012). BARF1 protein could inhibit macrophage colony-stimulating factor, thus affecting M2 differentiation (Hoebe et al., 2013).

The negative correlation of IL6 expression and Treg distribution (r = -0.129) can be explained by the findings of Ahmed et al. (2014) that IL6 secreted by dendrocytes will inhibit the proliferation and function of Treg (Li et al., 2015). Dendrocytes are found in the TME and some of them have been found to suppress T cell responses at the tumor site (Balkwill et al., 2012). On the other hand, the positive correlation between TNFα and Treg (r = 0.129) supports the premise that TNF induces the proliferation of Treg cells at tumor sites via TNFR2. Treg cells that expressed TNFR2 enhanced the escape of tumor cells from immune surveillance (Salomon et al., 2018; Jung et al., 2019).

In this study, Treg distribution in the high category was slightly higher (52.17%) than low category (Table 1). EBV involvement can affect the function and population of the Treg (Li et al., 2011). EBV LMP-1 protein can induce Treg to secrete interleukin-10 (IL-10) which is known as a cytokine inhibiting factor and plays an important role in immunosuppression (Marshall et al., 2003; Tsao et al., 2017). One of the cytokines inhibited by IL-10 is interleukin-2 (IL-2), which plays a role in the proliferation and differentiation of T lymphocytes. Therefore, decreased IL-2 expression will affect the differentiation and proliferation of T lymphocytes (Han et al., 2015), and further affect the composition of T lymphocytes that infiltrate the NPC tumor area.

The existence of Treg and tumor-associated macrophage (TAM) is associated with poor prognosis in NPC (Ooft et al., 2017; Wang et al., 2017). High CD163 expression is significantly associated with a poor prognosis in various types of cancer (Yu et al., 2018). The combination of M2 and Treg will worsen the condition of NPC patients. The various mechanisms affecting both cells need to be studied further. The understanding of M2 and Treg in NPC tumorigenesis is important in their evaluation as prognostic markers or target therapy. Treg is an independent prognostic factor for better overall survival on NPC (Ooft et al., 2017). Differentiation, proliferation, and function of Tregs that infiltrate tumors are also affected by metabolic reprogramming along with changes in the physical and chemical properties of the TME (Wang et al., 2018).

Several limitations in this study arise in the use of limited parameters that only included two cytokines and 1 PRR, whereas, many cytokine pathways affect the formation of the tumor microenvironment. All samples were derived from the advanced stage NPC (III and IV) and no survival data were available, making it difficult to relate to clinical parameters, even though such research has the potential to better study prognostic markers and target therapy. The small sample size may also influence the results.

In conclusion, the expression of TLR3 had a positive correlation with IL6 expression, and TNFα expression also had a positive correlation with M2 and Treg distributions. Eventually, the presence of macrophage M2 and Treg in NPC tumor cells was affected by TNFα, but mostly through a different mechanism other than EBER-TLR3 interaction. Possibly, other cytokines, both anti-inflammatory (IL3, IL4, IL10, TGFβ) and pro-inflammatory (IL2, IL-1β), are involved in the mechanism of the formation of NPC microenvironment, especially related to the presence of M2 and Treg, which provides immunosuppressive effect in NPC tumor.

Author Contribution Statement

Design of the study: D.K.P., J.F., and E.K.D.; acquiring and analysis of the data: I.M., Y.N.A., S.H.A., C.H., E.C.S., F.A., and S.; interpretation of the results, drafting and revision of the manuscript, and decision to submit: all authors.

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Conflict of interest

The authors declare no potential conflict of interest.

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