RESEARCH ARTICLE

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M2 Macrophage Prominently Distributed in the Rat's Colon of DMH-Induced Inflammation Associated Colorectal Cancer

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Abstract

Objective: The aim of this study is to examine the M1 and M2 macrophages distribution in the rat's colon of DMH-induced inflammation associated colorectal cancer. **Methods:** Colon tissue of three groups of 4 rats that induced using 1,2 dimethylhydrazine (DMH) at 30 mg/kg bw every week for 9, 11, and 13 weeks were used. The M1 and M2 distribution was examined by using antibody anti iNOS for M1 and anti-CD163 for M2 with immunohistochemistry method. The data was presents in figure and table in the form of percentage. **Result:** M1 macrophage was found in all groups in the low distribution level (25% - 50%), while M2 macrophage was observed in all groups with 100% distribution. In the longer period of DMH induction, M2 macrophages was distributed more abundant. **Conclusion:** All of the rat's colon showing chronic inflammation that led to the tumorigenesis.

Keywords: Colitis Associated- colorectal cancer- Inflammation- M2- Macrophage

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Introduction

Colorectal cancer (CRC) is the third most prevalent cancer and leading cause of cancer-related death worldwide [1,2]. More than 70% of CRC is classified as sporadic, which is influenced by genetic and environmental factors. Sporadic CRC showed a prominent inflammatory response or tumor-induced inflammation [2]. Patients with inflammatory bowel disease (IBD), especially ulcerative colitis, have higher risk to develop CRC. The incidence of CRC in IBD (i.e. colitis-associated colorectal cancer or CA-CRC) reaches more than 60% in the population [2].

Inflammation is one of the initial lesions in colorectal cancer pathogenesis and injury of colorectal mucosa caused by environmental substances. Chemical agents can cause chronic immune responses that lead to cell proliferation and regeneration. The failure of the immune response to resolve the injury causes an increased level of cytokines, growth factors and products of cellular respiration in the microenvironment that led to sustained inappropriate proliferation of genetic error cells. Several studies support the role of inflammation in the development of CRC [3].

One of chemical agents, 1.2 dimethylhydrazine (DMH) is often used to induce animal models, showing a similar feature of colorectal cancer to sporadic type in human CRC. DMH induction contributes to the malignant transformation from mild and moderate dysplastic into carcinoma in situ. DMH is an effective agent to promote the development of preneoplastic and inflammatory lesions until the advanced stages [4-7].

Inflammation prompts cancer growth and contributes to all stages of tumorigenesis. Initially, it is involved in host defense against infection and essential in tissue homeostasis regulation. Lately, the contribution of inflammation in cancer development, progression and therapy has become an interesting subject. However, the development of most cancers does not preface by long-term chronic inflammation. For example, even though IBD leads up to colitis-associated colorectal cancer (CA-CRC), only about 2% of CRC are preceded by intestinal inflammation [8].

Macrophages are the most important immune cells in maintaining homeostasis in the intestine, and also the key cells in the pro- and anti-inflammatory response, including in the microenvironment of malignant tumors [9, 10]. It produces various cytokines and soluble mediators that play a role in the epithelial progenitor proliferation and physiology of enteric neuron and endothelial

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cells. Furthermore, it prevents excessive inflammatory response due to the commensal microbiota and induces tolerance through the differentiation of Treg that mainly produce IL-10. However, genetic and environmental predispositions cause the impairment of the immune system that lead to chronic immune activation and pathogenesis of gastrointestinal tract disorder, including IBD [9].

Macrophages can be activated through different stimuli. Interferon-γ (IFN-γ) stimulates pro-inflammatory macrophages with high capability in antigen presentation, bactericidal and tumoricidal activity. Other than stimulated by IFN-γ, macrophages can be stimulated by IL-4, IL-13, glucocorticoid, transforming growth factor β (TGF-β), immune complexes and IL-6. Such macrophages produce anti-inflammatory responses and express anti-inflammatory markers. Macrophages M1 and M2 terminology was initially introduced in 2000. M1 is pro-inflammatory macrophages and M2 is anti-inflammatory macrophages. IFN-γ-stimulated macrophages highly express inducible nitric oxide synthase (iNOS) or known as M1 macrophage. On the other hand, macrophages that abundantly found in the microenvironment of inflammation and tumorigenesis is CD163+ macrophages or M2 [10, 11].

Inducible nitric oxide (iNOS), one of three isoforms of NOS, plays a role in nitric oxide (NO) synthesis. NO has dual roles on immune-inflammation. NO can protect our body against viral infection, but also cause tissue damage to generate pathogenic effects and are involved in the development of certain diseases. Due to its capability in destroying and inactivating pathogens, M1 macrophage is involved in the pathogenesis of inflammatory diseases [11]. Macrophage M2, known as alternatively activated macrophage, which plays a role as anti-inflammatory and regulatory cells. Macrophage M2 poses angiogenic and immunosuppressive properties, by secreting IL-1RA, TGF- beta, IL-10 and VEGF. Therefore, macrophage M2 can promote tumor development [12].

In this study, we induce the rats with DMH which are expected to undergo inflammation in the beginning. However, with the long-term induction, the inflammation might be prolonged and will induce tumorigenesis. Therefore, we evaluate the distribution of M1 and M2 macrophage in the colon of DMH-induced rats in different inflammatory conditions.

Materials and Methods

Formalin-Fixed Paraffin Embedded Tissue Sample

Formalin-fixed paraffin embedded (FFPE) tissue samples of the colon of DMH-induced rat were derived from a previous study (Adrianto et al., in prep). The study was approved by ethical committee faculty of Medicine Universitas Diponegoro (No. 93/EC/H/FK-UNDIP/IX/2020).

Immunohistochemistry of M1 and M2

The macrophage were characterized by using the iNOS marker for M1 and CD163 marker for M2. The expression of iNOS and CD163 were performed by using immunohistochemistry method with antibody anti-iNOS (Abcam, ab 15323) and antibody anti-CD163 (Abcam, ab 182422), respectively. The immunohistochemistry was done following the protocol of Mouse and Rabbit Specific HRP/DAB IHC Detection Kit Micro-polymer (Abcam, ab236466). The iNOS immunopositive cell can be observed as cytoplasmic staining. Immunoreactive score (IRS) was used to interpret the positive result of iNOS expression. Score 0-4 interpreted from the percentage of positive cells, and staining intensity was scored from 0 - 3. The multiplication of the score of positive cells and staining intensity was calculated as the final score (Table 1) [13]. The positive result for CD163 expression was examined by eyeballing observation to the whole area. CD163 positive cells were observed as a granular cytoplasm or cytoplasmic and membrane staining. CD163 expression was classified into four grades (0-3), i.e. negative or no staining (0); scant represents a small amount of scattered staining (1), focal means concentrated staining with an irregular and non-continuous focus (2), abundant and concentrated staining represents an extensive and continuous focus (3) [14, 15]. All of the slide was observed by using a light microscope (OLYMPUS model CX21FS5) and the photo was captured by using Optilab Upgrade Advance; software Optilab Viewer 2.2.

Inflammation evaluation

The inflammatory responses were assessed qualitatively according to the amount and distribution of inflammatory cells found in the colon, by eyeballing observation. The response was divided into three categories, i.e. mild when the infiltrate represents 10-25% inflammatory cells including scattered neutrophils, moderate for 26-50% inflammatory cells and strong when >51% of inflammatory cells found in the colon. This method was modified from the one generated by Erben [16].

Data analysis

The data of M1 and M2 distribution were analysed in the form of percentage and represents as Figures and Table.

Results

Macrophage M1 Distribution

The distribution of M1 macrophages were defined according to the iNOS expression, and the immunopositive cells showed cytoplasmic staining from negative result, mild to strong positivity. The immunopositive cells mostly can be observed in between the epithelial cells and some of them in the lamina propria (Figure 1A). The colon from the 9th week and 11th week groups showed 75% negative results for iNOS expression and only 25% of strong iNOS expression, therefore only 25% M1 macrophage can be observed in 9- and 11- week DMH-induced rats. While in the 13th week group, 50% M1 macrophage can be found in with strong and moderate immunoreactive (IRS) score with equal distribution (Figure 1B).

Immunohistochemistry method using anti-iNOS to detect macrophages M1 was applied in all samples except the negative control. The scoring of the staining is based on the IRS method as mentioned in Table 1.

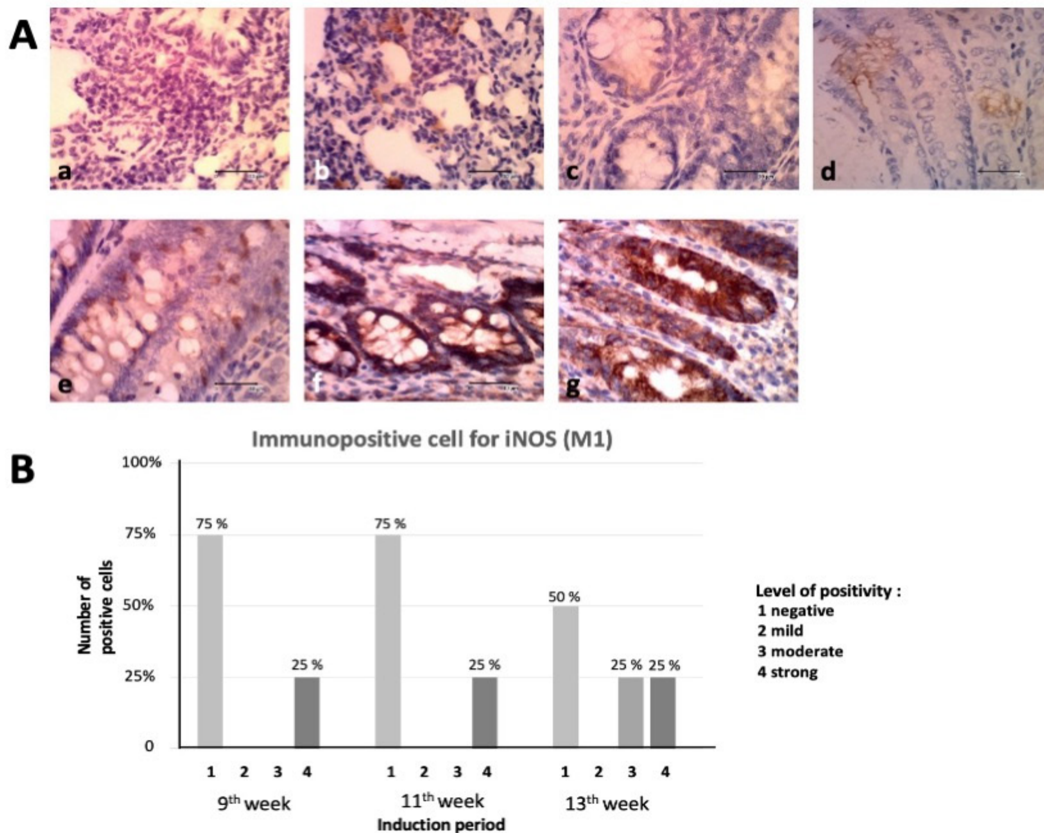

Figure 1. A) Immunohistochemistry staining for M1 macrophages based on iNOS expression. Immunohistochemistry method using anti-iNOS to detect macrophages M1 was applied in all samples except the negative control. The scoring of the staining based on IRS method as mentioned in Table 1. (a) Spleen tissue without antibody anti-iNOS incubation was used as negative control, showing unstained result; (b) Liver tissue with antibody incubation was used as positive control, showing moderate positive staining with score 4; (c) Colon of the 9- weeks DMH-induced rat showing negative staining staining with score 1; (d) Colon of the 9- weeks DMH-induced rat showing mild staining with score 2; (e) Colon of the 13- weeks DMH-induced rat showing moderate staining with score 4; (f) Colon of the 9- weeks DMH-induced rat showing strong staining with score 9; and (g) Colon of the 11- weeks DMH-induced rat showing strong staining with score 12. B) Number of iNOS positive cells.

Macrophage M2 Distribution

The expression of CD163 was used to determine the distribution of M2 macrophages. Immunopositive result indicated by the staining in granular cytoplasmic, cytoplasm and/or membrane. M2 distribution on the colon of 9- weeks induced rats was relatively spread evenly from scant, focal till abundant. Twenty percent of rats in the 9th week group showed 25% of scant and abundant M2 distribution, and 50% of the rats showed focal distribution. In the 11th week group the rats have equal distribution of focal and abundant M2 distribution (50% each). In the 13th week group, most of the rats (75%) have abundant M2 distribution and 25% of them have focal distribution. It showed that the M2 distribution switched to more abundant in the longer period of DMH induction (Figure 1).

Distribution of M1 and M2 macrophages in different inflammatory condition

The inflammation conditions in the colon of the rats were examined in the colon section stained with haematoxylin eosin (HE). The heaviest inflammation was observed in the colon of the 9- week group, which showed the distribution of inflammation cells from epithelial layer till submucosal layer, and in one of the rats in the group showing large nodules of lymphocyte aggregates. The inflammation was gradually getting milder in the 11- and 13-week group respectively, the inflammatory cells were

Table 1. Immunoreactivity Score (IRS) Measurement (Modified from [14])

A (% of positive cells)	B (staining intensity)	Final IRS score (multiplication of A and B) (A x B) = 1-12
0 = no positive cells	0 = no color reaction	0-1 = negative
1 = <10% of positive cells	1 = mild	2-3 = mild
2 = 10-50% of positive cells	2 = moderate	4-8 = moderate
3 = 51-80% of positive cells	3 = intense	9-12 = strong positive
4 = >80% of positive cells		

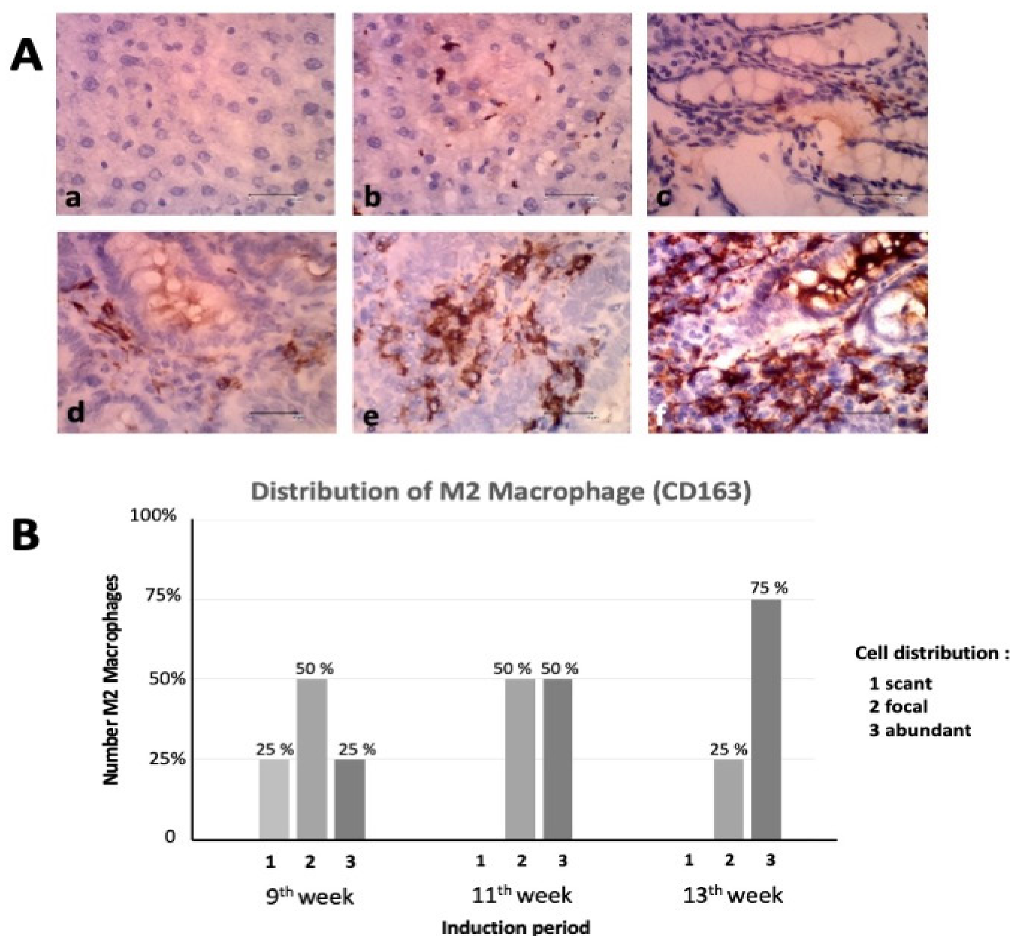

Figure 2. A) Distribution of M2 macrophages based on CD163 expression Immunohistochemistry method using anti-CD163 to detect macrophages M2 was applied in all samples except the negative control. (a) Liver tissue without antibody anti-CD163 incubation was used as negative control, showing unstained result; (b) Liver tissue with antibody anti-CD163 incubation was used as positive control, showing focal staining; (c & d) Colon of normal rats and the 9-weeks DMH-induced rat showing scant staining with score 1, respectively; (e) Colon of the 9- weeks DMH-induced rat showing focal staining with score 2; (f) Colon of the 9- weeks DMH-induced rat showing abundant staining with score 3. B) Number of M2 macrophage

found in lamina propria and submucosal layer, and the number of the cells were also getting fewer (Adrianto et al., in prep).

Table 2. M1 and M2 Distribution in Different Inflammation Conditions

M1 and M2 distribution (M1 = iNOS expression; M2 = CD163 expression)	Inflammation		
	Week 9 Strong (3)	Week 11 Moderate (2)	Week 13 Mild (1)
M1			
1 (Negative)	75%	75%	50%
2 (Mild)	0	0	0
3 (Moderate)	0	0	25%
4 (Strong)	25%	25%	25%
	100%	100%	100%
M2			
1 (Scant)	25%	0	0
2 (Focal)	50%	50%	25%
3 (Abundant)	25%	50%	75%
	100%	100%	100%

Immunohistochemistry method using anti-CD163 to detect macrophages M2 was applied in all samples except the negative control. (a) Liver tissue without antibody anti-CD163 incubation was used as negative control, showing unstained result; (b) Liver tissue with antibody anti-CD163 incubation was used as positive control, showing focal staining; (c & d) Colon of normal rats and the 9- weeks DMH-induced rat showing scant staining with score 1, respectively; (e) Colon of the 9- weeks DMH-induced rat showing focal staining with score 2; (f) Colon of the 9- weeks DMH-induced rat showing abundant staining with score 3. B) The distribution of M1 and M2 macrophage in different inflammation conditions is represented in Table 2. In the 9- and 11- week group which showed strong and moderate inflammation, only 25% M1 macrophage can be found with strong expression. In the 13- week group with mild inflammation, 50% of M1 macrophage can be found in the moderate and strong expression evenly. In general, M2 macrophages can be found in all groups or in all different inflammation conditions, but with different distributions. In the 9-week group which has strong inflammation, the M2 distribution is relatively equal, with 25% scant, 50% focal and

25% abundant distribution. In the 11-week group with moderate inflammation condition, the M2 distribution switches to focal and moderate distribution with equal amount (50% each). In the 13-week group, 75% of M2 macrophages were distributed abundantly, and 25% were distributed focally.

Discussion

Inflammation is one of the hallmarks of cancer and can be influenced by many factors, including infection, chemical agent and other environmental carcinogen, and intrinsic factors sucs as obesity. In colitis associated-CRC (CA-CRC), inflammation is an important mechanism in the tumorigenesis [2]. Approximately 15-20% of all cancer begins with infection and chronic inflammation at the same organ site. Inflammation that precedes cancer occurs long before the tumor formation. IBD is one of the most prominent examples [8].

The chemical induction such as DMH cause inflammation as the immune responses in colorectal mucosa and the characteristics in early lesions of DMH induced colorectal cancer pathogenesis [3, 4]. In this study, high levels of inflammation can be seen at the 9th week group, and the inflammation level was gradually lower in the 11th and 13th week group (Table 1). Chronic inflammation causes the immune response to function improperly for immune surveillance and leads to the microenvironment that inhibits anti-tumor response. This condition generates tolerogenic DC and Treg infiltration that support tumor cell growth [17].

The most prominent infiltrate in the microenvironment that supports the tumor growth is M2 macrophages, which also contributes in angiogenesis and metastasis [11]. In this study M2 macrphages were identified by the expression of CD163, and according to previous study [10]. CD163 expression was induced by anti-inflammatory cytokines, such as IL-10 [10]. The macrophage M2 was observed in the colon of all groups, which indicates that the rat's colon of all groups were in the chronic inflammation condition. In the longer period of DMH-induction, the distribution of M2 macrophages is getting higher, i.e. 75% of M2 was observed in abundant distribution in the 13 week induction period (Figure 2b, Table 2). High M2 distribution is observed in ulcerative colitis (UC) and Cohrn's disease (CH) [18], and also in the case of inflammation that progresses to malignant carcinoma stages, including colitis-associated colorectal cancer (CA-CRC). M2 macrophages compose tumor microenvironment to facilitate invasion and metastasis [19].

In this study, M1 that represents pro-inflammatory macrophages was observed at a low distribution level in all of the groups (Figure 1 and Table 1). Proinflammatory macrophages are the third major type of intestinal macrophages, observed specifically in lamina propria and the external muscular layer of the gut. It can be derived from either embryonic precursor or bone marrow [20]. These macrophages play a role in the maintenance of gut homeostasis. During inflammation, monocytes differentiate into proinflammatory macrophages, which produce proinflammatory cytokines such as tissue necrosis factor-α (TNF-α), interleukin 1b (IL1b), and IL6 [21]. In the tumor microenvironment, M1 macrophages have dual anti- and pro-tumoral functions. M1 macrophages have intrinsic anti-tumor functions, such as trapping, phagocytosis and lysis tumor cells. However, M1 macrophages are also being used by tumor stem cell as a natural filter to avoid being destroyed the immune system and for the survival to the next stage [22].

Author Contribution Statement

WAO: immunohistochemistry, data analysis, drafting manuscript; SS: immunohistochemistry, interpretation IHC result, data analysis, drafting manuscript; AAA: animal induction and maintenance, data analysis; YAP: drafting manuscript, design the work, revising intellectual content; DKP: design the work, revising intellectual content, drafting manuscript, data analysis, final approval of the published data and manuscript

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General

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Data Availability

The data can be requested to the corresponding author.

Ethical Declaration

The study was approved by ethical committee faculty of Medicine Universitas Diponegoro (No. 93/EC/H/FK-UNDIP/IX/2020).

Conflict of Interest

The authors declare that there is no conflict of interest in this study.

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