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Research paper

Respiratory macrophages regulate CD4 T memory responses to mucosal immunization with recombinant adenovirus-based vaccines

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ABSTRACT

Respiratory immunization is an attractive way to generate systemic and mucosal protective memory responses that are required for preventing mucosally transmitted infections. However, the molecular and cellular mechanisms for controlling memory T cell responses remain incompletely understood. In this study, we investigated the role of respiratory macrophage (MΦ) in regulating CD4 T cell responses to recombinant adenovirus-based (rAd) vaccines. We demonstrated that rAd intranasal (i.n.) vaccination induced migration and accumulation of respiratory MΦ and circulatory monocytes in the mediastinal lymph nodes and lung parenchyma. Under the influence of respiratory MΦ CD4 T cells exhibited slow proliferation kinetics and an increased tendency of generating central memory, as opposed to effector memory, CD4 T cell responses *in vitro* and *in vivo*. Correspondingly, depletion of MΦ using clodronate-containing liposome prior to i.n. immunization significantly enhanced CD4 T cell proliferation and increased the frequency of CD4 memory T cells in the airway lumen, demonstrating that MΦ initially serve as a negative regulator in limiting generation of mucosal tissue-resident memory CD4 T cells. However, clodronate-containing liposome delivery following i.n. immunization markedly reduced the frequencies of memory CD4 T cells in the airway lumen and spleen, indicating that respiratory MΦ and potentially circulating monocytes are critically required for maintaining long-term memory CD4 T cells. Collectively, our data demonstrate that rAd-induced mucosal CD4 T memory responses are regulated by respiratory MΦ and/or monocytes at multiple stages.

1. Introduction

Mucosal surfaces of respiratory, genital and gastrointestinal tracts are the major gateways of infectious agents, and hence, the development of mucosal vaccines to stimulate protective humoral and/or cellular mucosal immunity has been one of the major focuses of vaccine research for decades [1–3]. However, the development of mucosal vaccines has proven to be a challenge and only a limited number of mucosal vaccines are licensed in humans [3]. It is widely established that induction of memory responses is the fundamental basis of vaccination and the development of mucosal vaccines against intracellular pathogens that rely on protective memory T cell responses requires greater understanding about molecular and cellular mechanisms in controlling vaccine-induced mucosal T cell responses.

According to CCR7 and CD62L (L-selectin) expression, memory CD4 and CD8 T cells are divided into two major subsets, the CCR7 $^{+}$CD62L^{high} CD44^{high}CD45RB^{low} central memory T (T_{CM}) and CCR7^{-}CD62L^{low}CD44^{high}CD45RB^{low} effector memory T (T_{EM}) cells [4]. In comparison, naive T cells have the phenotype of CCR7^{+}CD62L^{high}CD44^{low} CD45RB^{high} [5,6]. T_{CM} cells predominantly circulate within secondary lymphoid organs, whereas T_{EM} cells mainly circulate into the peripheral tissues where some

Abbreviations: rAd, recombinant adenovirus-based vaccines; MΦ, respiratory macrophage; i.n., intranasal; i.p., intraperitoneal; i.m., intramuscular; MedLN, mediastinal lymph node; IngLN, inguinal lymph node; DLN, draining lymph node.

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are permanently retained in the tissue and become tissue-resident memory T (T_{RM}) cells [7,8]. Furthermore, T_{CM} cells produce low levels of effector molecules such as IFN-γ and/or cytotoxic granules, but they have high proliferative capacity and are able to convert to T_{EM} upon antigen restimulation. In contrast, T_{EM} and T_{RM} cells produce immediate effector molecules upon stimulation but have very poor proliferative potential [4,9]. Compared to T_{CM} cells that are preferentially localized in the systemic lymphoid organs, T_{EM} and T_{RM} cells located at mucosal sites confer immediate frontline immunity against mucosal pathogens [7,8,10]. Numerous factors such as the strength and duration of the T cell receptor (TCR) signal, the local inflammatory signals at the time of T cell priming, and other microenvironmental signals associated with the route and/or the immunization schedule all play a role in shaping memory T cell responses in terms of their quality, quantity and anatomic distributions [1,4,7–11]. However, most of our understanding has been obtained by studying CD8 memory T cells. CD4 and CD8 memory T cells are different in many aspects; they have different patterns of peripheral migration [12], different proliferation potential and different longevities [13]. Therefore, additional studies are required to understand the regulation of CD4 T memory responses.

Recombinant adenovirus-based (rAd) vaccines have strong abilities to stimulate CD4 and CD8 T cell responses and have been widely explored for developing vaccines against intracellular pathogens including, *Mycobacterium tuberculosis* (*Mtb*), human immunodeficiency virus, malaria and *Chlamydia trachomatis* [14–19]. Using a rAd-based tuberculosis vaccine, we demonstrated previously that intranasal (i.n.) immunization with rAd induces accumulation of CD4 and CD8 T_{RM} cells in the respiratory tract for a prolonged period of time and confers protection against pulmonary *Mtb* challenge via both CD4- and CD8-dependent immune mechanisms [15,20]. In contrast, intramuscular (i.m.) immunization with rAd results in a predominant CD8 T_{CM} cell response in the systemic lymphoid organs, which only translates into a transient partial protection from secondary *Mtb* challenge in the lung [15,20]. While these studies highlight the importance of mucosal immunity in mediating vaccine efficacy against mucosal pathogens, the immune mechanisms that control mucosal CD4 memory T cell responses upon i.n. immunization with rAd are still unclear.

Antigen presenting cells (APCs) play key roles in the induction and regulation of pulmonary immune responses. In particular, respiratory macrophages (MΦs) are demonstrated to modulate respiratory immune responses via various modes of action [21–23]. For instance, respiratory MΦ can modulate immune responses via suppressing migration of dendritic cells (DCs) into the secondary lymphoid organs [21,22,24] or by promoting induction of FoxP3 regulatory T cells [25,26]. Alternatively, respiratory MΦs are demonstrated to participate in respiratory immune responses through directly transporting pathogen/antigen into the draining lymph modes (DLNs) [27,28]. Although respiratory MΦs are known to play essential roles during respiratory viral infections [29–31], it is unclear whether respiratory MΦs may modulate T cell memory responses upon rAd mucosal immunization. In this study, we specifically characterized OVA-specific CD4 T cell responses following i.n. immunization of rAd expressing OVA (AdOVA) and examined the role of respiratory MΦs in controlling CD4 memory T cell responses by depleting respiratory MΦs using clodronate-containing liposome. Our results indicate that respiratory MΦ populations have stage-dependent functional roles in shaping CD4 T memory responses. While respiratory MΦs limit the early stage of CD4 T cell activation and subsequent size of mucosal memory responses, they are critically required for maintaining long-term CD4 T memory responses at both mucosal and systemic compartments.

2. Materials and methods

2.1. Animals

Six to eight week-old female BALB/c mice (H-2^d) were ordered from Charles River Laboratories (Senneville, Quebec, Canada). DO11.10 (H-2^d) mice were originally from Jackson laboratory (Bar Harbor, ME, USA) and bred at the IWK Health Centre animal facility. All mice were housed under pathogen-free conditions and used according to the Canadian Council for Animal Care guidelines. Food and water were supplied *ad libitum*.

2.2. Recombinant replication-deficient adenoviral vectors (rAd vectors)

The recombinant human type 5 adenoviral vectors encoding chicken ovalbumin (AdOVA) and empty control vector Addl70-3 have been described before [14,32]. Viral vectors were amplified, purified and titrated according to the protocols previously described [15].

2.3. Animal model of DO11.10:BALB/c mice and sample preparations

DO11.10:BALB/c mice were used for monitoring OVA-specific CD4 T cell responses. To do this, naïve $CD4^+CD62L^+$ T lymphocytes were isolated from pooled peripheral lymph nodes (LN) of DO11.10 mice using mouse naïve CD4 T cell isolation kit (Order No. 130-104-453, Miltenyi Biotec Inc, Auburn, CA, USA) and adoptively transferred into BALB/c mice via tail vein injection ($\sim 2 \times 10^6$ cells/mouse) 24 h prior to immunization.

To monitor CD4 T cell proliferation, purified naïve DO11.10 CD4 T cells were first labeled with carboxyfluorescein succinimidyl ester (CFSE) dye (Life Science, Oakville, ON, Canada) prior to transfer. Naïve $CD4^+CD62L^+$ T cells were resuspended in PBS/0.1% BSA (pre-warmed at 37 °C), mixed with CFSE at final 10 μM concentration and incubated 10 min at 37 °C. Five volumes of ice-cold PBS/10% FBS were used to stop the reaction. Cells were then washed twice with RPMI/5% FBS medium. Twenty-four hours following cell transfer, DO11.10:BALB/c mice were i.n. or i.m. immunized with 5×10^8 or 1×10^9 plaque-forming units (PFU) of AdOVA or empty control virus Addl70-3 as described previously [14,15,33]. At days 0, 3, 5 and 7, single cell suspensions were prepared from peripheral LNs including mediastinal (MedLN), inguinal (IngLN) and axillary LN, bronchoalveolar lavage (BAL), and spleen of each mouse, and stained with DO11.10 TCR-specific monoclonal antibody KJ1-26 (eBioscience, San Diego, CA, USA). OVA-specific CD4 T cell proliferation was analyzed by flow cytometry.

In some experiments, cells isolated from BAL, LN, or spleen were seeded in 96-well plates (1×10^6/well) and restimulated with or without $OVA_{323-339}$ peptide (5 μg/mL) for 6, 12, 24 or 48 h at 37 °C with 5% CO_2. At the end of each time point, culture supernatants were collected and the concentrations of IFN-γ, IL-13, IL-17A, TNF-α, IL-6 and IL-10 were analyzed by ELISA. OVA-specific cytokine measurements in Addl-70-3-immunized mice were only detected at the background levels. Samples without $OVA_{323-339}$ peptide stimulation had no detectable cytokine production. For the purpose of simplicity, these control groups are not included in data presentation.

2.4. In vivo respiratory macrophage migration assay

To examine whether respiratory MΦs migrate into the MedLN following i.n. immunization with rAd, mice were first instilled with 50 μl of PBS containing 2 mM CFSE via i.n. route and then inoculated with 50 μl of PBS containing AdOVA (1×10^9 PFU/mouse)