表 3　不活化インフルエンザワクチンの有効率[8)]

年齢群	診断基準	有効率(%)	
		日本	欧米
小児　6歳未満	ウイルス学的		58
	ILI	22～25	28
6歳以上	ウイルス学的		65～78
	ILI	24～40	28
成人	ILI		70～90
	入院回避		90
高齢者	ILI	34～55	30～40
	入院回避		50～60
	死亡回避	≧80	80

ILI：インフルエンザ様疾患，ウイルス学的診断：ウイルス分離，血清診断などを用いた実験室診断．

体価であるが，インフルエンザの発症予防抗体価であるHI抗体40倍は50%の成人の発症を予防する抗体価である[6)]．

インフルエンザウイルスは変異が早いウイルスであり，シーズンごとに接種される3種類のワクチン株のいずれかが通常毎年更新されること，一度接種したとしてもインフルエンザワクチン後の抗体陽性率(HI抗体≧40倍)は麻疹と比べて低率であり，しかも抗体価は半年で1/2に低下することから，毎年流行前に1回の接種が勧められている．

インフルエンザワクチンの効果(表3)

インフルエンザワクチンの効果は主として，流行時のインフルエンザ発症予防で評価される．インフルエンザ流行中にILIを発症したとしても，すべての原因はインフルエンザウイルスとは限らないため，インフルエンザの診断を臨床診断で行うと，ウイルス学的に診断したときと比べインフルエンザワクチンの有効率が低下する[7)]．また，流行株とワクチン株の抗原性が一致すると有効率が高く，抗原性が変異すると有効率は低下する．HAの抗原性が4倍以上変異したとき，ワクチン株と流行株に変異があったと診断する．ILIを指標としたときの成人のインフルエンザワクチンの有効率は70～90%であり，ワクチン株と流行株の抗原性が異なると成人の有効率は60%程度に低下する[3)]．

インフルエンザワクチンの有効率が低いのは，乳幼児と高齢者である．高齢者では加齢により免疫応答が低下するためである．高齢者の免疫応答を高めるためには，3～4週間隔で2回接種するよりも1回に多量接種する方が優れている．アメリカでは高齢者用に成人に接種する4倍量のHAが含まれたインフルエンザワクチンが使用されている[2)]．

乳幼児でインフルエンザワクチンの効果が劣る原因として，1歳未満の乳児は1歳以上の子どもよりも抗体反応が低いことや，細胞性免疫が弱いために発症予防には成人よりも高い抗体価が必要なことが示唆されている[8,9)]．2011/12シーズンから小児の抗体反応を高めるために，わが国小児のインフルエンザワクチン接種量がWHO推奨量に増量された．WHO推奨量で接種したときの抗体反応を表4に示した[10)]．1歳未満児ではいずれの型に対しても抗体陽性率が低かったが(ヨーロッパ医薬品庁(EMA)基準≧70%)，A/H1N1およびA/H3N2に対しては抗体陽転率(EMA基準≧40%)および幾何平均抗体価(GMT)上昇率(EMA基準≧2.5倍)ともにEMAの基準を満たしていた．

小児におけるインフルエンザワクチン接種回数に関しては，B型インフルエンザウイルスに対する抗体反応を期待するならば，13歳未満は2回接種が勧められる．なお，今回接種量を増量させたのは2回接種によるプライミング時の抗体反応，または1回追加接種によるブースティング効果を高めるためであり，接種量が増加してもプライミングが必要な人は2回接種が必要である．

インフルエンザワクチンの集団免疫効果

インフルエンザはヒトからヒトに感染する感染症であり，基本再生産数は1.5～2.4，集団免疫率50%程度である(「サイドメモ4」参照)．インフルエンザワクチンを高齢者施設や障害者施設の職員に接種すると，接種率が低い施設と比べて接種率が高い施設では入所者のインフルエンザ発症率やインフルエンザ流行期間中の死亡率が低下する[11)]．また，小児にインフルエンザワクチンを接種すると同居している高齢者のインフルエンザ発症が61%低下するなど[12)]，インフルエンザワクチンの集団免疫効果が示されている．

表 4　小児におけるインフルエンザワクチンの免疫原性(阪大微生物病研究会)[10]

年齢(人数)	ワクチン	A/H1N1			A/H3N2			B		
		陽転率	GMT増加率	陽性率	陽転率	GMT増加率	陽性率	陽転率	GMT増加率	陽性率
6カ月<1歳	1回後	5.9	1.6	5.9	11.8	2.1	11.8	0.0	1.0	0.0
(17)	2回後	41.2	3.8	41.2	58.8	6.0	58.8	23.5	2.4	23.5
1歳<3歳	1回後	47.1	5.3	52.9	64.7	9.4	64.7	52.9	4.3	52.9
(17)	2回後	76.5	7.7	76.5	94.1	13.6	94.1	64.7	6.5	64.7
3歳<6歳	1回後	61.1	6.6	66.7	88.9	6.3	94.4	66.7	5.2	77.8
(18)	2回後	72.2	7.1	72.2	94.4	7.4	94.4	77.8	5.9	83.3
6歳<13歳	1回後	87.5	9.1	87.5	81.3	7.3	100	18.8	5.5	37.5
(16)	2回後	87.5	9.1	87.5	81.3	7.3	100	31.3	3.4	50.0

EMA の基準：抗体陽転率(≧40%)，GMT 増加率(≧2.5 倍)，抗体陽性(HI 抗体≧40 倍)率(≧70%)．

基礎疾患のある人への接種

糖尿病，肝硬変，慢性腎不全などの慢性基礎疾患をもつ人は，インフルエンザに罹患すると重症化するリスクが高い人である．基礎疾患のある人は高齢者と同様にインフルエンザワクチンに対する免疫応答が低下した人である．免疫低下者の抗体反応を高めるためには，理論上高齢者と同様に接種する抗原量を高める必要がある．

妊婦がインフルエンザを発症すると肺炎を合併する頻度が高いため，妊娠期間中がインフルエンザ流行と重なる妊婦にはインフルエンザワクチン接種が勧められている．インフルエンザワクチンは妊娠時期にかかわらず接種が推奨されている．母乳を与えている母親へのインフルエンザワクチン接種も安全性が確認されている．

第三三半期の妊婦にインフルエンザワクチンを接種すると，妊婦が発熱性呼吸器疾患に罹患する率が29%減少し，生まれた生後6カ月未満の子どもも発熱性呼吸器疾患を発症する率が36%減少する[13]．インフルエンザ抗体は3種類ともほぼ同じ濃度で児に移行する[14]．生後6カ月未満の子どもをインフルエンザから予防するために，妊婦にインフルエンザワクチンを接種する対策が検討されている．

サイドメモ4　基本再生産数(R_0)と集団免疫率(H_0)

基本再生産数(R_0)とはひとりの感染者が周囲の免疫のない人に感染させる数で，この数字が高いほど感染力が強いことを示している．R_0は感染症ごとに異なっており，一番感染力が強い感染症は麻疹と百日咳で16～21である．

集団免疫率(H_0)とは，ある集団でヒトからヒトに感染する感染症の流行を阻止するために必要な免疫率のことである．$H_0=(1-1/R_0)\times100$の関係がある．この率も感染症ごとに異なっており，麻疹の集団免疫率は90～95%である．

インフルエンザワクチンの副反応と卵アレルギー児への接種

インフルエンザワクチン接種後約30%に注射部位の紅斑や疼痛が認められる．発熱はまれである．1976年のブタインフルエンザ騒動時に用いられたインフルエンザワクチンではGuillain-Barr症候群(GBS)の出現率が高かったが，近年用いられているインフルエンザワクチンではGBSの有意な増加は認められていない[3]．GBS既往者はインフルエンザワクチンの接種不適当者である．

現行のインフルエンザワクチンは発育鶏卵を用いて製造されるため，欧米では卵を食べてアナフィラキシーを起こす人は接種不適当者とされている．インフルエンザワクチン接種によりアナフィラキシーを引き起こすオボアルブミン量は600～700 ng/dose以上である[2,15]．一方，わが国のインフルエンザワクチンに含まれるオボアルブミン濃度は1 ng/mL程度である[16]．わが国のワクチンを卵アレルギー児に接種したとしても，理論上オボアルブミンによるアナフィラキシーは起こらないと判断されている．

プロトタイプワクチンの製造と接種計画

WHOは2009年のパンデミック後も多くの人が免疫をもたないインフルエンザウイルス(新型インフルエンザウイルス)の流行を危惧している．新型インフルエンザウイルスの出現を予測して準備するワクチンがプロトタイプワクチンであり，新型インフルエンザウイルスがパンデミックを起こしたとき，パンデミック株を用いて製造するのがパンデミックワクチンである．パンデミックワクチンの剤型は出現した亜型により異なってくる．2009年のパンデミックではスプリットワクチンが用いられた．H1，H2，H3以外の亜型が出現した場合はプロトタイプワクチンの剤型が用いられる．

わが国ではA/H5N1亜型の出現を危惧して毎年1,000万人分のプロトタイプワクチンの備蓄を行っている．現在インフルエンザウイルスの増殖に発育鶏卵を用いているが，高病原性A/H5N1がパンデミックを起こしたときは発育鶏卵でのインフルエンザウイルス増殖が不可能であること，発育鶏卵の数に制限があり，急いでワクチンを製造することが困難であることなどの理由で，培養細胞を用いたインフルエンザワクチンの開発が進んでいる[1)]．

現在のところ，新型インフルエンザウイルスとして予測されているのは，①現在流行しているA/H3N2香港型およびA/H1N1 pdm 09と大きく抗原性が異なるA/H3N2亜型またはA/H1N1亜型の出現②，A/H2N2亜型の再燃，③H1，H2，H3以外のHA亜型をもつインフルエンザウイルスの出現の3パターンである．2009年パンデミック時の経験から，現在流行しているA(H3N2)やA(H1N1)と免疫原性が大きく異なる同じ亜型のインフルエンザウイルスが出現した場合，乳幼児を除く多くの人は抗体が陰性でもこれらのウイルスに対して免疫記憶をもっているので，1回の接種で十分である．A(H2N2)が出現した場合は，1968年以前に生まれた人は免疫記憶を有しているので1回接種，それ以降の人は2回接種が必要である．これらの亜型以外のHA亜型が出現した場合は，全員2回接種が必要である．

おわりに

インフルエンザワクチンの剤型および有効性について解説した．現在流行しているインフルエンザウイルスと抗原性が異なるインフルエンザウイルスが出現したとしてもインフルエンザウイルスがヒトに感染して発症する臨床像はILIである．しかし，WHOは高病原性A/H5N1によるパンデミックのリスクをいぜん考えている．このためわが国では，季節性インフルエンザ対策と新型インフルエンザウイルスによるパンデミック対策を考えたインフルエンザワクチン製造および開発を行っている．

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Allergy

ORIGINAL ARTICLE EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Implications of nasopharynx-associated lymphoid tissue (NALT) in the development of allergic responses in an allergic rhinitis mouse model

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Keywords
allergic rhinitis; Id2 protein; IgE; mouse; NALT.

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Abstract

Background: Nasopharynx-associated lymphoid tissue (NALT) serves as an important inductive site for mucosal immunity in the upper respiratory tract. Despite its importance in the mucosal immune system, little is known regarding the role of NALT in airway allergic immune responses. We aimed to elucidate the role of NALT in the induction of upper airway allergic responses in a mouse model.

Methods: Inhibitor of DNA binding/differentiation 2 (Id2)$^{-/-}$ and Id2$^{+/-}$ mice was exposed to the ovalbumin (OVA)-induced allergic rhinitis model, because the former resulted in the NALT deficiency. The allergic parameters, such as allergic symptoms, serum OVA-specific immunoglobulin E (IgE) levels, eosinophil infiltration, and cytokine profiles in the nasal mucosa, were compared between Id2$^{-/-}$ and Id2$^{+/-}$ groups.

Results: NALT-null, Id2$^{-/-}$ mice displayed significantly lower allergic responses compared with Id2$^{+/-}$ mice, as demonstrated by lower levels of allergic symptoms, serum OVA-specific IgE, eosinophilic infiltration, and local Th2 cytokine transcriptions. To determine which of two factors, that is, the absence of NALT or the alteration of immunocompetent cell populations caused by the Id2 deficiency, has a larger effect on the attenuated allergic immune responses in Id2$^{-/-}$ mice, lethally irradiated Id2$^{-/-}$ mice were engrafted with C57BL/6 wild-type bone marrow cells and showed still significantly lower allergic immune responses compared with equally treated Id2$^{+/-}$ mice. In addition, IgE class switch recombination-associated molecules, such as ε immunoglobulin heavy-chain germline gene transcript, ε mRNA, and activation-induced cytidine deaminase mRNA, were detected in NALT from OVA-sensitized wild-type mice.

Conclusion: These results show the critical role of NALT for the induction of allergic responses in the upper airway at least in part by means of class switching to IgE *in situ*.

Abbreviations:
AID, activation-induced cytidine deaminase; BM, bone marrow; CLN, cervical lymph node; CSR, class switch recombination; GFP, green fluorescence protein; i.n., intranasal; i.p., intraperitoneal; Id2, inhibitor of DNA binding/differentiation 2; IgE, immunoglobulin E; NALT, nasopharynx-associated lymphoid tissues; OVA, ovalbumin; ε mRNA, mRNA encoding the heavy chain of IgE; ε GLT, ε immunoglobulin heavy-chain germline gene transcript.

Nasopharynx-associated lymphoid tissue (NALT) is an organized lymphoid structure that is present on both sides of the nasopharyngeal duct, dorsal to the cartilaginous soft palate of mice, which is considered to be equivalent to Waldeyer's ring in humans (1). NALT is an important inductive site for the induction and regulation of mucosal immunity in the upper respiratory tract, similar to Peyer's patch (PP) in the intestinal immune system (2). Nasal immunization with an antigen and cholera toxin as a mucosal adjuvant has been shown to generate antigen-specific IgA-producing cells in the nasal passages and distal mucosal sites, including the genitourinary and intestinal tracts (3, 4).

Despite our knowledge on the importance of NALT in the mucosal immune system, little is known regarding its role in upper airway allergic immune responses. There has been only one study to date on the kinetics of antigen presentation in the lymphoid structures of the upper respiratory tract. Following intranasal application, auto-fluorescent dye was detected in the NALT and cervical lymph nodes (CLN) within 4 and 8 h, respectively. The uptake of dye in the NALT was more rapid and persisted for a longer period of time, compared with the uptake in the CLN, suggesting a prominent role for NALT in allergen processing of the upper airways (5). However, no direct relationship between NALT and airway allergic immune responses has been uncovered.

The major goal of our study was to elucidate the role of NALT in allergic immune responses in the upper airway, using inhibitor of DNA binding/differentiation 2 $(Id2)^{-/-}$ mice, which lack NALT, as an allergic rhinitis model, and directly demonstrating class switch recombination (CSR) to immunoglobulin E (IgE) and IgE synthesis in NALT of allergen-sensitized mice by RT-PCR.

Methods

Animals

57BL/6 mice were purchased from SLC (Shizuoka, Japan). The $Id2^{-/-}$ mice (129/Sv) were generated previously by Yoshifumi Yokota (6). $Id2^{-/-}$ mice lack lymph nodes, PPs, and NALT and show a greatly reduced population of natural killer (NK) cells, which is because of an intrinsic defect in NK cell precursors. By contrast, $Id2^{+/-}$ mice have NALT and normal population of immune cells.

Allergen sensitization and nasal challenge

On days 0, 7, and 14, the mice were sensitized by intraperitoneal (i.p.) injection of 25 μg of ovalbumin (OVA) and 1 mg of alum in 300 μl of phosphate-buffered saline (PBS). One week after the last OVA sensitization (Day 21), the mice received a series of seven daily intranasal (i.n.) OVA (500 μg) challenges (Fig. 1).

Allergic symptoms

For 20 min after the final nasal challenge with OVA, sneezing frequencies and nasal rubbing events were recorded for each mouse by blinded observers, over a 5-min interval. The mice were then killed 24 h after the last nasal challenge for further analyses (7).

Nasal histology

Twenty-four hours after the final OVA nasal challenge, nasal passage tissues were surgically removed from the mice, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Under high magnification (×400), infiltrating eosinophils were counted in the nasal septal mucosa.

OVA-specific immune parameters

To measure total and OVA-specific IgE, serum samples collected from the mice at the time of killing were serially diluted and added to 96-well plates coated with purified anti-mouse IgE mAb (clone R35–72; BD Pharmingen™, San Jose, CA, USA). A purified mouse IgE isotype (27–74; BD Pharmingen™) was used as a standard. To detect total IgE, HRP-conjugated anti-mouse IgE (23G3; Southern Biotechnology, Birmingham, AL, USA) was added to the plate. To detect OVA-specific IgE, biotin-labeled OVA was added, followed by HRP-labeled anti-biotin (Vector Laboratories, Burlingame, CA, USA). The reactions were developed using 3,3′,5,5′-tetramethylbenzidine (Moss Inc., Belfast, ME, USA)

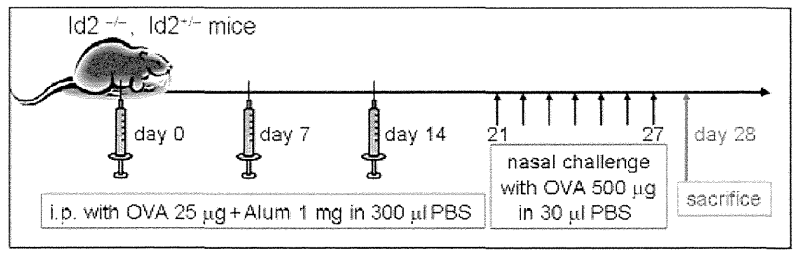

Figure 1 Allergic rhinitis model. On days 0, 7, and 14, inhibitor of DNA binding/differentiation 2 mice received an intraperitoneal (i.p.) injection of 25 μg of ovalbumin (OVA) and 1 mg of alum in 300 μl of phosphate-buffered saline. One week after the last OVA sensitization (day 21), a series of seven daily intranasal (i.n.) OVA (500 μg) challenges were administered. The mice were killed 24 h after the last nasal challenge for further analyses.

and were terminated by adding 2 N H_2SO_4. The optical density (OD) was recorded by a luminometer (iEMS Reader; Labsystems, Helsinki, Finland) set at 450 nm. The end-point titers of OVA-specific IgE are expressed as the reciprocal $\log_2$ of the last dilution of a sample that resulted in an OD value 0.1 higher than background.

Real-time RT-PCR for IL-4, IL-5, IL-13, and IFN-γ in the nasal mucosa

Total RNA was prepared from the nasal mucosa with TriZol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized using superscript reverse transcriptase (Invitrogen) and oligo(dT) primers (Fermentas, Burlington, ON, Canada). For analysis of IL-4 (Mm00445258_g1), IL-5 (Mm00439646-m1), IL-13 (Mm4331182-m1), IFN-γ (Mm99999071_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mm03302249_g1), PDAR (Pre-Developed Assay Reagent) kits of primers and probes were purchased from Applied Biosystems (Foster City, CA, USA). Amplification of IL-4, IL-5, IL-13, IFN-γ, and GAPDH cDNA was carried out in MicroAmp optical 96-well reaction plates (Applied Biosystems). The reaction was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The average transcript levels of genes were then normalized to GAPDH.

Irradiation and bone marrow (BM) transfer

For radiation chimera experiments, a previously reported protocol was adopted (8). Recipient mice (Id2 or C57BL/6) were lethally irradiated with 1100 rad (Gamma cell; Nordion International Inc., Kanata, ON, Canada). The recipient mice were anesthetized immediately following irradiation and were transplanted with 2×10^6 BM cells derived from donor mice (C57BL/6 or Id2). Specifically, the BM cells in 100 μl of medium were transplanted by retro-orbital intravenous injection with a 26-gauge needle. At 6 week after transplantation, flow cytometric analysis from C57BL/6 mice, which were irradiated and engrafted with BM cells derived from green fluorescence protein (GFP)-transgenic mice, revealed that 90–95% of the immune system of the recipient mice had been reconstituted with GFP-positive donor hematopoietic cells (data not shown).

Allergic sensitization began 6 week after transplantation. Allergic symptoms and nasal histology were assessed, and total IgE and OVA-specific IgE were measured as described above.

RNA isolation and RT-PCR

Total RNA was isolated from NALT of OVA-sensitized and PBS-administered mice using the Trizol Reagent (Invitrogen) following the manufacturer's instructions. RNA was suspended in RNase-free water and quantified by spectrophotometry at 260 nm. RNA was reverse transcribed to cDNA, and ε immunoglobulin heavy-chain germline gene transcript (εGLT), mRNA encoding the heavy chain of IgE (ε mRNA), and activation-induced cytidine deaminase (AID) were PCR-amplified as described elsewhere (9). The primers used for PCR were as follows: the oligonucleotide primers specific for εGLT (sense, 5′-TGGGATCAGACGATGGAGAATAG-3′; antisense, 5′-CCAGGGTCATGGAAGCAGTG-3′); the oligonucleotide primers specific for ε mRNA (sense, 5′-TACGACGAGAACGGGTTTGCTTAC-3′; antisense, 5′-AGTTCACAGTGCTCATGTTCAG-3′); the oligonucleotide primers specific for AID (sense, 5′-GGCTGAGGTTAGGGTTCCATCTCAG-3′; antisense, 5′-GAGGGAGTCAAGAAAGTCACGCTGGA-3′); the oligonucleotide primers specific for GAPDH (sense, 5′-ACCACAGTCCATGCCATCAC-3′; antisense, 5′-TCCACCACCCTGTTGCTGTA-3′).

Statistical analysis

Data are expressed as means ± SD. All data were analyzed by a Mann–Whitney *U*-test. P values <0.05 were considered to be statistically significant.

Results

Decreased antigen-specific allergic immune responses in Id2-deficient mice

After the nasal challenge of sensitized mice, $Id2^{-/-}$ mice sneezed and rubbed their noses significantly less frequently than $Id2^{+/-}$ mice did (Fig. 2A,B). Moreover, the serum level of OVA-specific IgE was significantly lower in $Id2^{-/-}$ mice than in $Id2^{+/-}$ mice (Fig. 2D), while $Id2^{-/-}$ mice had a significantly higher total IgE levels as described previously (6) (Fig. 2C). The numbers of eosinophils infiltrating the nasal mucosa per high-magnification field were significantly lower in $Id2^{-/-}$ mice than in $Id2^{+/-}$ mice (Fig. 2E–G).

mRNA expressions of the Th2 cytokines, IL-4 and IL-13, significantly decreased in the nasal mucosa of $Id2^{-/-}$ mice as compared to $Id2^{+/-}$ mice ($P < 0.05$, Fig. 2H,J). On the contrary, mRNA expressions of IL-5 and the Th1 cytokine, IFN-γ, did not differ significantly between $Id2^{-/-}$ and $Id2^{+/-}$ mice (Fig. 2I,K).

Critical role of NALT for the induction of upper airway allergic responses

Adoptive transfer of C57BL/6 wild-type BM cells into lethally irradiated 129/Sv $Id2^{-/-}$ mice did not lead to the formation of NALT, although the immunocompetent cells were replaced by wild type (WT) cells (data not shown). When the engrafted Id2 mice were sensitized with OVA, clinical symptoms of sneezing and nose rubbing were significantly less frequent in the reconstituted $Id2^{-/-}$ mice than similarly treated $Id2^{+/-}$ mice (Fig. 3A,B). The reconstituted $Id2^{-/-}$ mice also had a significantly lower serum OVA-specific IgE level (Fig. 3D) and fewer eosinophils infiltration in the nasal mucosa than similarly treated $Id2^{+/-}$ mice (Fig. 3E). By contrast, the reconstituted $Id2^{-/-}$ mice still had a significantly higher total serum IgE level than $Id2^{+/-}$ mice (Fig. 3C).

Lethally irradiated C57BL/6 wild-type mice that were transplanted with BM cells from $Id2^{-/-}$ or $Id2^{+/-}$ mice did not show any significant differences in the frequencies of

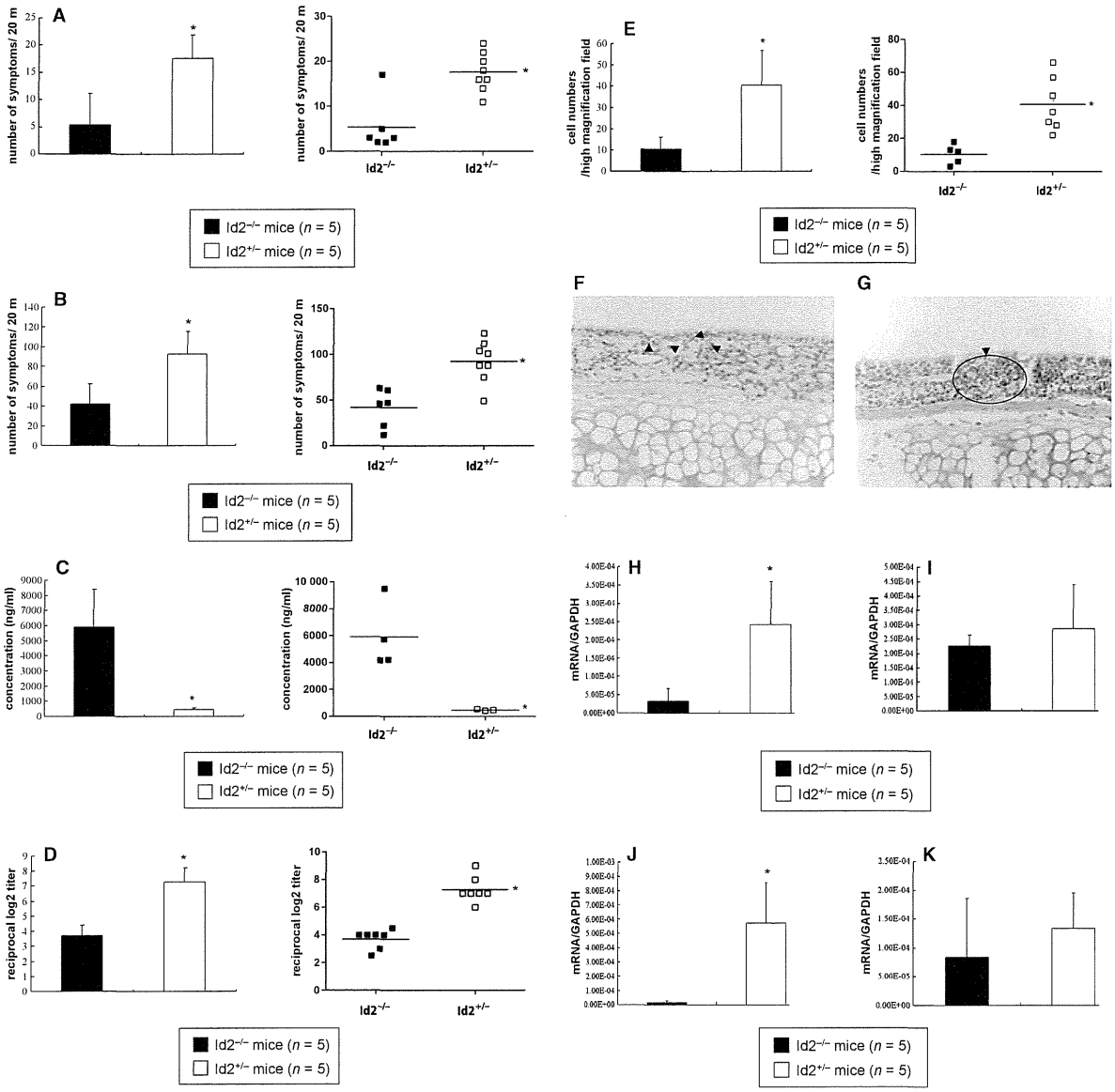

Figure 2 Comparison of nasal symptoms, serum immunoglobulin E (IgE) levels, eosinophilic infiltration, and local cytokine expression levels between $Id2^{-/-}$ and $Id2^{+/-}$ mice. A, sneezing; B, nasal rubbing; C, total serum IgE level; D, serum ovalbumin-specific IgE level; E, eosinophilic infiltration (A–E, the graph on the left represents the mean values with error bars. The graph on the right represents individual data with median values); F,G, The representative H & E staining pictures under 400-fold magnification field demonstrate that a few eosinophils (arrow heads) are seen in the nasal submucosal tissue of $Id2^{-/-}$ mice (F), whereas a lot of eosinophils (in the circle) congregate in $Id2^{+/-}$ mice submucosal tissue (G); H–K, IL-4 (H), IL-5 (I), IL-13 (J), and IFN-γ (K) mRNA expression levels in the nasal mucosa. $*P < 0.05$. Id2, inhibitor of DNA binding/differentiation 2.

sneezing and nasal rubbing (Fig. 4A,B), and in the levels of serum OVA-specific IgE antibodies (Fig. 4D). The total serum IgE levels were significantly higher in the WT mice transplanted with $Id2^{-/-}$ mice marrow cells than those from $Id2^{+/-}$ mice (Fig. 4C), while the wild-type mice transplanted with $Id2^{+/-}$ mice marrow cells had a significantly more severe eosinophilic infiltration (Fig. 4E).

Expression of class switch recombination molecules to IgE in NALT

ε Germline transcription is shown to be the first step for the commitment of B cells to become IgE-producing cells (10). When the εGLT expression was examined in NALT, its expression was detected in the tissue from OVA-sensitized

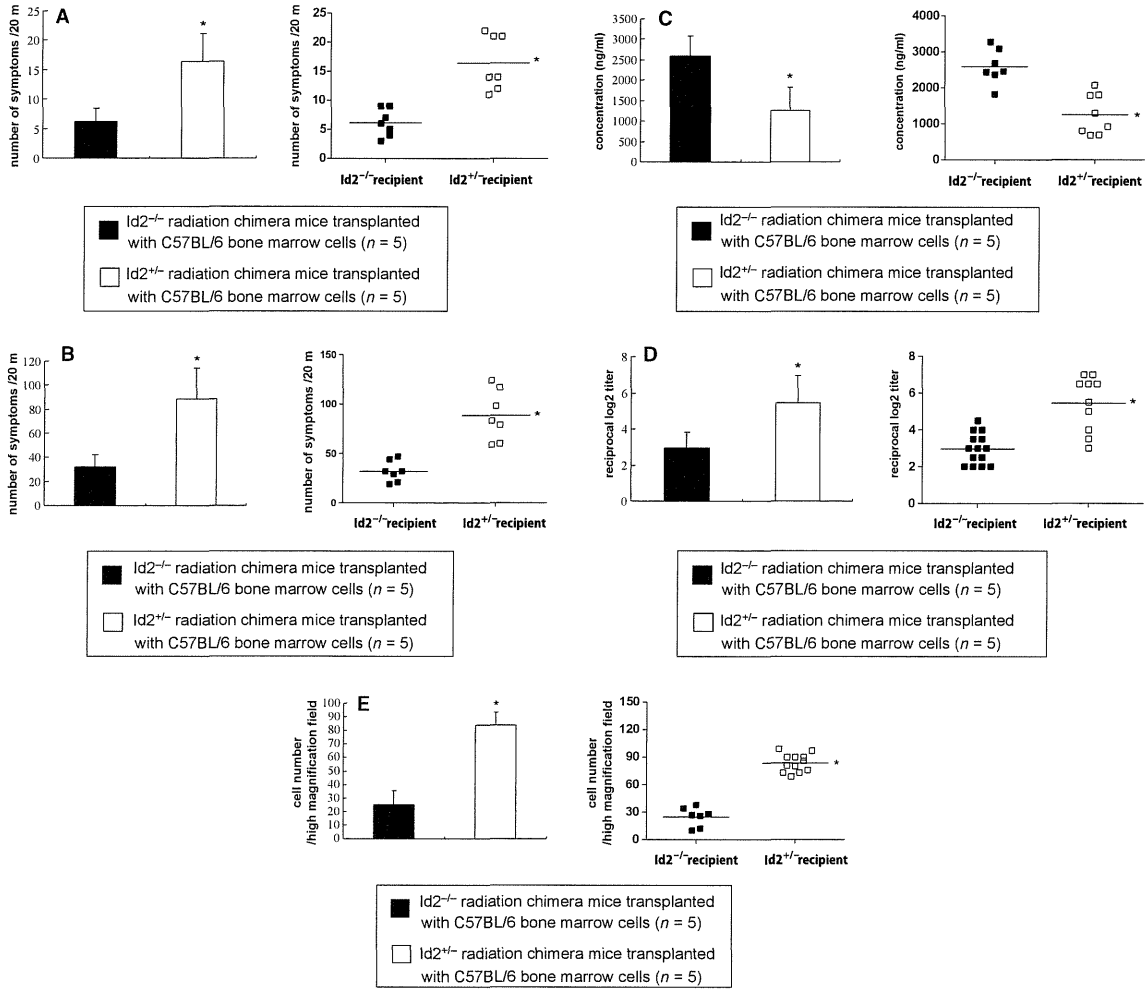

Figure 3 Comparison of nasal symptoms, serum immunoglobulin E (IgE) level, and eosinophilic infiltration between lethally irradiated Id2$^{-/-}$ and Id2$^{+/-}$ mice, transplanted with bone marrow cells from C57BL/6 wild-type mice. A, sneezing; B, nasal rubbing; C, total serum IgE level; D, serum ovalbumin-specific IgE level; E, eosinophilic infiltration (A–E, the graph on the left represents the mean values with error bars. The graph on the right represents individual data with median values). *$P < 0.05$. Id2, inhibitor of DNA binding/differentiation 2.

WT mice (Fig. 5A). IgE synthesis by B cells is the final step in the production of IgE. When NALT of WT mice with allergic rhinitis was examined, the presence of ε heavy-chain-specific mRNA was detected in OVA-sensitized mice, but not those from negative control (Fig. 5B). To further support for the induction of IgE commitment in NALT of OVA-sensitized WT mice, mRNA expression of isotype CSR molecule of AID was markedly increased in mice with allergic rhinitis when compared with control mice (Fig. 5C).

Discussion

NALT contains all the necessary lymphoid cells, including T cells, B cells, and antigen-presenting cells, for the induction and regulation of inhaled antigen-specific mucosal immune responses (11, 12). Additionally, these tissues are rich in Th0-type CD4$^+$ T cells, which can develop into either Th1- or Th2-type cells (13). NALT also provides a molecular and cellular environment that allows for a CSR of μ to α genes, leading to the generation of IgA-committed B cells and the induction of memory B cells (14, 15). It is thus widely accepted that NALT is a key player in the uptake of nasally delivered antigens and leads to the subsequent induction of antigen-specific IgA immune responses (11).

It is reasonable to hypothesize that allergic immune responses to inhaled allergens can be induced by NALT in a similar manner to the induction of mucosal immune responses. Our group reported that Th2-inducer-type CD8α$^-$ CD11b$^+$ myeloid dendritic cells (m-DCs) increased in NALT after nasal challenge with OVA. We also showed that lymphoid

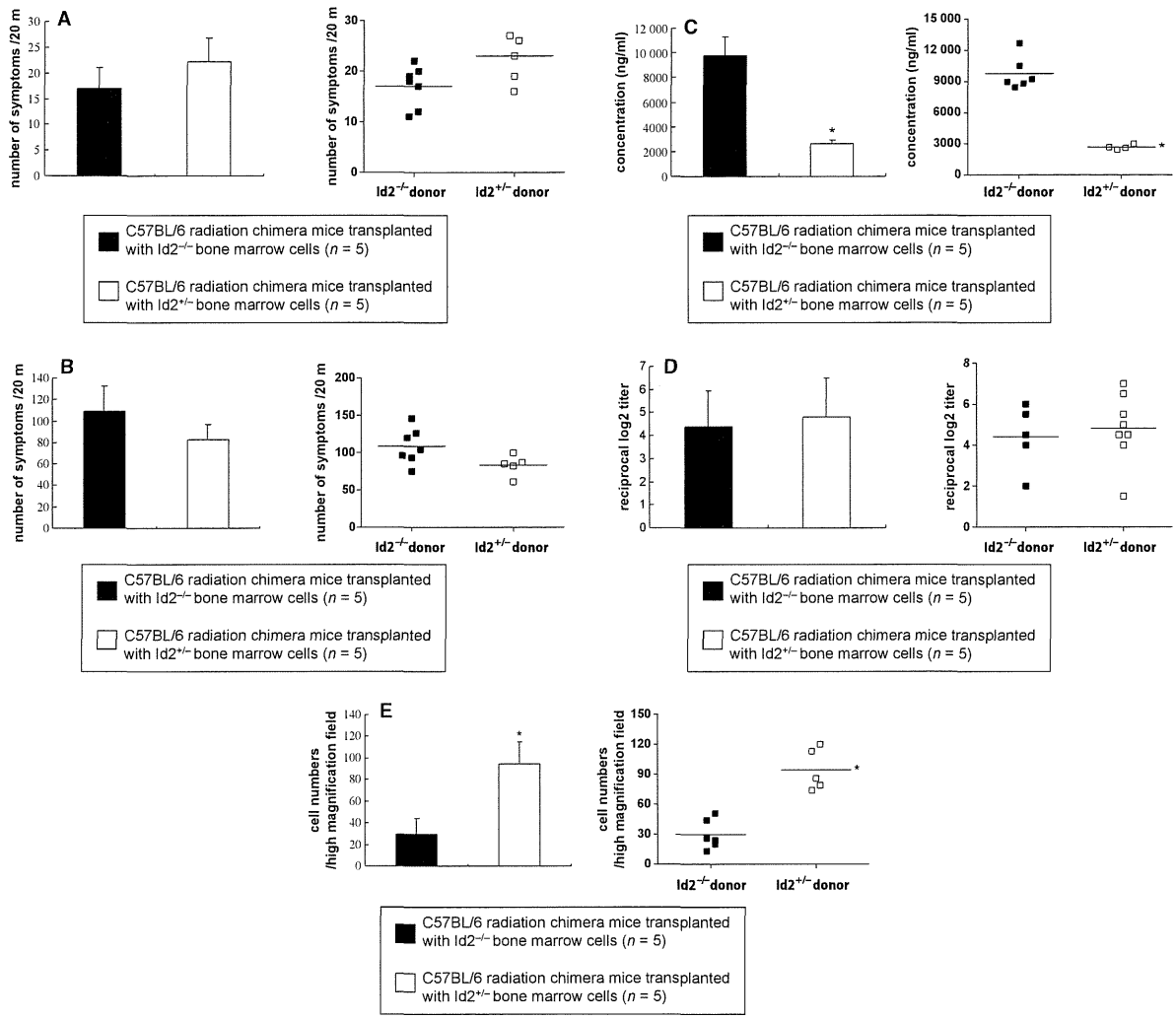

Figure 4 Comparison of nasal symptoms, serum immunoglobulin E (IgE) level, and eosinophilic infiltration between lethally irradiated C57BL/6 wild-type mice, transplanted with bone marrow cells from $Id2^{-/-}$ and $Id2^{+/-}$ mice. A, sneezing; B, nasal rubbing; C, total serum IgE level; D, serum ovalbumin-specific IgE level; E, eosinophilic infiltration (A–E, the graph on the left represents the mean values with error bars. The graph on the right represents individual data with median values). $*P < 0.05$. Id2, inhibitor of DNA binding/differentiation 2.

chemokines CCL19 and CCL21 are involved in the recruitment of CCR7 expressing naturally occurring Tregs in NALT and help suppress the pathological Th2 environment induced by m-DCs during the development of allergic rhinitis (16). However, the direct role of NALT in the induction of upper airway allergic immune responses has not been elucidated.

Inhibitor of DNA binding/differentiation 2 is a transcription factor inhibitor with a basic helix-loop-helix motif that has been shown to be crucial for various cell differentiation processes. As a result, $Id2^{-/-}$ mice lack secondary lymphoid organs such as peripheral lymph nodes, PPs, and NALT. The $Id2^{-/-}$ mice are known to be the only organism that lacks NALT (6). It has also been reported that $Id2^{-/-}$ mice are in a Th2-dominant state and have a selective defect in the $CD8\alpha^+$ dendritic cell (DC) subset (17). Several studies lead us to consider that the Th2 dominance observed in $Id2^{-/-}$ mice is caused, at least in part, by the defect in $CD8\alpha^+$ DCs. It has been demonstrated that distinct DC subsets participate in Th1 and Th2 cell development (18–22). In particular, $CD8\alpha^+$ DCs produce a large amount of IL-12, a crucial cytokine for Th1 development, and prime naïve $CD4^+$ T cells, leading to Th1 differentiation (20–22). In addition, $Id2^{-/-}$ mice exhibit a greatly reduced number of NK cells (14), which produce a large amount of IFN-γ in an IL-12-dependent manner. Furthermore, IFN-γ is a key factor directing the differentiation of naïve $CD4^+$ T cells into Th1

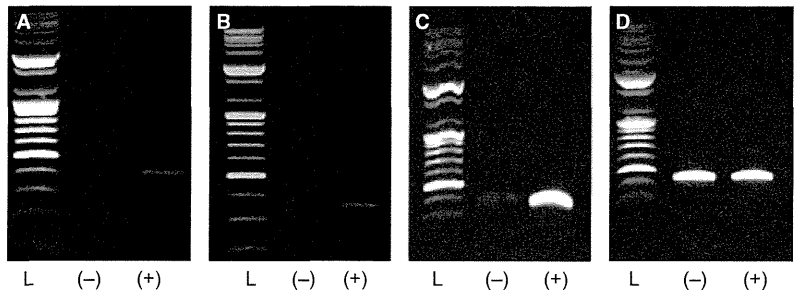

Figure 5 Expressions of ε immunoglobulin heavy-chain germline gene transcript (εGLT), mRNA encoding the heavy chain of immunoglobulin E (ε mRNA), and activation-induced cytidine deaminase (AID) mRNA in nasopharynx-associated lymphoid tissues (NALT). εGLT (A), ε mRNA (B), and AID mRNA (C) were PCR-amplified from NALT of mice with and without ovalbumin (OVA) sensitization. Glyceraldehyde-3-phosphate dehydrogenase (D) was amplified to control cDNA loading. Lanes marked L (–) and (+) indicate DNA ladder, NALT without OVA sensitization, and NALT with OVA sensitization, respectively. The results presented are representative of at least three reproducible independent PCR amplifications.

cells (23). Thus, a decreased NK cell population is one potential cause of skewed Th2 development in $Id2^{-/-}$ mice. Upon B cell activation, Id2 acts as a negative regulator to prevent potentially harmful effects brought about by excessive immunological reactions. One specific role is to maintain a low serum concentration of IgE (24). Taken together with Th2 dominance, this can adequately explain the higher levels of total IgE in $Id2^{-/-}$ mice compared with $Id2^{+/-}$ mice.

Interestingly, despite higher levels of total IgE, $Id2^{-/-}$ mice produced significantly lower levels of antigen-specific IgE compared with $Id2^{+/-}$ mice in the allergic rhinitis model. Furthermore, allergic symptoms, eosinophilic infiltration, and Th2 cytokine production in the nasal mucosa were significantly lower in $Id2^{-/-}$ mice than in $Id2^{+/-}$ mice. As a whole, the major points of difference between $Id2^{-/-}$ and $Id2^{+/-}$ mice are the existence of NALT and defects in immune cells. To determine which of these two factors has a larger effect on attenuated allergic immune responses in $Id2^{-/-}$ mice, we created radiation chimera mice with transplanted donor BM cells. Lethally irradiated 129/Sv $Id2^{-/-}$ or $Id2^{+/-}$ mice and C57BL/6 mice were engrafted with C57BL/6 wild-type and $Id2^{-/-}$ or $Id2^{+/-}$ BM cells, respectively. The $Id2^{-/-}$ radiation chimera mice transplanted with C57BL/6 wild-type BM cells lacked NALT but had normal immune cell populations. On the contrary, C57BL/6 radiation chimera mice transplanted with $Id2^{-/-}$ BM cells had normal NALT but defective immune cell populations. Lethally irradiated $Id2^{-/-}$ mice engrafted with C57BL/6 wild-type BM cells had significantly lower allergic immune responses compared with equally treated $Id2^{+/-}$ mice. However, there were no statistically significant differences in allergic immune responses between C57BL/6 radiation chimera mice transplanted with $Id2^{-/-}$ and $Id2^{+/-}$ BM cells, except for infiltrating eosinophil counts that were significantly higher in C57BL/6 radiation chimera mice transplanted with $Id2^{+/-}$ BM cells than those with $Id2^{-/-}$ BM cells. This finding might be attributable to the fact that Id2 protein is important for the differentiation of eosinophils (25). On the other hand, $Id2^{-/-}$ radiation chimera mice transplanted with C57BL/6 BM cells still showed significantly higher total serum IgE levels than equally treated $Id2^{+/-}$ mice (Fig. 3C). It is speculated that there might be possibilities of another mechanisms involved in Th2 dominance and hyper-IgE in $Id2^{-/-}$ mice, except for immune cell defects as mentioned above.

Activation-induced cytidine deaminase expression is required for CSR (10), thus occurs in lymphoid tissue (26), and has been observed in the nasal mucosa of patients with allergic rhinitis (9, 27). We have demonstrated expression of the enzyme AID as well as εGLT expression in NALT B cells of OVA-sensitized mice. The expression of εGLT and AID demonstrates that NALT is primed for class switching to IgE. Our data also show the evidence of mature ε heavy-chain mRNA production in NALT by OVA sensitization. It is reasonable to assume that this reflects ongoing, elevated synthesis of mature IgE in allergen-sensitized NALT by local B cells.

Taken altogether, these results suggest that NALT plays a critical role in the induction of allergic immune responses in the upper respiratory tract at least in part by means of class switching to IgE *in situ*.

Author contributions

D.Y.K. participated in experimental design, data analysis, and manuscript writing. S.F., T.N., K.T., and I.G.K. assisted with data acquisition and analysis. Y.Y. provided Id2 mice. C.H.L. provided intellectual guidance. H.K. established the initial scientific questions, provided continuing intellectual guidance, and participated in experimental design and manuscript writing.

Declaration

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, see: http://www.textcheck.com/cgi-bin/certificate.cgi?id=SMP32A.

Conflict of interest

No conflicts of interest declared.

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Review

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EXPERT REVIEWS

Novel vaccine development strategies for inducing mucosal immunity

Expert Rev. Vaccines 11(3), 367–379 (2012)

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To develop protective immune responses against mucosal pathogens, the delivery route and adjuvants for vaccination are important. The host, however, strives to maintain mucosal homeostasis by responding to mucosal antigens with tolerance, instead of immune activation. Thus, induction of mucosal immunity through vaccination is a rather difficult task, and potent mucosal adjuvants, vectors or other special delivery systems are often used, especially in the elderly. By taking advantage of the common mucosal immune system, the targeting of mucosal dendritic cells and microfold epithelial cells may facilitate the induction of effective mucosal immunity. Thus, novel routes of immunization and antigen delivery systems also show great potential for the development of effective and safe mucosal vaccines against various pathogens. The purpose of this review is to introduce several recent approaches to induce mucosal immunity to vaccines, with an emphasis on mucosal tissue targeting, new immunization routes and delivery systems. Defining the mechanisms of mucosal vaccines is as important as their efficacy and safety, and in this article, examples of recent approaches, which will likely accelerate progress in mucosal vaccine development, are discussed.

KEYWORDS: delivery system • mucosal adjuvant • secretory IgA

Mucosal immune system

The mucosal immune system can be separated into inductive and effector sites based on the anatomical and functional properties. The migration of immune cells from mucosal inductive to effector tissues is the cellular basis for the common mucosal immune system (CMIS) (FIGURE 1). Thus, mucosal vaccination elicits immune responses in distant, multiple mucosal effector sites [1–5]. Mucosal inductive sites, including gut-associated lymphoreticular tissue (GALT) and nasopharyngeal-associated lymphoreticular tissue (NALT), collectively comprise a mucosa-associated lymphoreticular tissue (MALT) network for provision of a continuous source of memory B and T cells to mucosal effector sites [1,3–5]. The MALT contains T-cell zones, B cell-enriched areas containing a high frequency of surface IgA-positive ($sIgA^+$) B cells and a subepithelial area with APCs for the initiation of specific immune responses. The MALT is covered by a follicle-associated epithelium that consists of a subset of differentiated microfold (M) epithelial cells, columnar epithelial cells and lymphoid cells, which play a central role in the initiation of mucosal immune responses. M cells take up antigens (Ags) from the lumen of the intestinal and nasal mucosa and transport them to the underlying APCs, including dendritic cells (DCs). In addition, recent studies have now identified isolated lymphoid follicles (ILFs) in the mouse small intestine. The ILFs have been identified as a part of GALT and as such are a mucosal inductive tissue [6,7]. These ILFs mainly contain B cells, DCs and M cells in the overlying epithelium. In addition, most recent studies showed that tear duct-associated lymphoreticular tissue (TALT) and conjunctiva-associated lymphoreticular tissue (CALT) play a role as mucosal inductive tissues [8,9]. Mucosal effector sites, including the lamina propria regions of the GI, the upper respiratory (UR), and reproductive tracts, secretory glandular tissues and intestinal intraepithelial lymphocytes, contain Ag-specific mucosal effector cells such as IgA-producing plasma cells and B and T cells.

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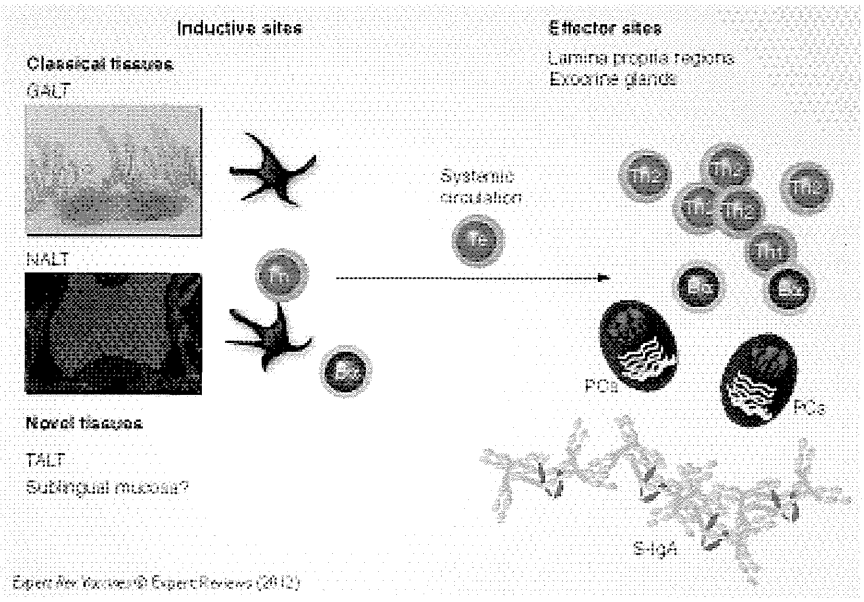

Figure 1. Concept of mucosal inductive and effector sites: when mucosal immunization is initiated, Ags are taken up by mucosal inductive tissues (GALT, NALT and TALT). This is an initial step for eliciting Ag-specific S-IgA Ab responses in mucosal effector tissues. DCs in mucosal inductive tissues play a major role as APCs for the activation of naive CD4+ T cells. In addition, ingested Ags activate IgA-committed B cells. Activated CD4+ T cells and IgA-committed B cells dispatch from mucosal inductive tissues and migrate into the mucosal effector tissues and subsequently interact for the terminal differentiation of IgA-committed B cells into IgA-producing plasma cells. In addition to the classical mucosal inductive tissues, the SL mucosa can initiate mucosal immune responses.
Bα: IgA-committed B cell; GALT: Gut-associated lymphoreticular tissue; MALT: Mucosa-associated lymphoreticular tissue; NALT: Nasopharyngeal-associated lymphoreticular tissue; PC: Plasma cell; Th1: Type 1 helper CD4+ T cell; Th2: Type 2 helper CD4+ T cell; Te: Effector CD4+ T cell; Tn: Naive CD4+ T cell.

Secretory (S)-IgA antibody (Ab) is a major player in the mucosal immune system and is locally produced in effector tissues [1,2,5,10–12]. The presence of Ag-specific S-IgA Abs at mucosal effector sites other than the inductive sites where initial Ag sampling occurred is definitive evidence for the CMIS. To this end, immunization of GALT or NALT effectively elicits Ag-specific mucosal IgA Ab responses in diverse mucosal effector tissues with some notable differences. Indeed, activated T cells in Peyer's patches (PPs) preferentially express α4β7 and CCR9 as gut-homing receptors for their migration into the intestinal lamina propria [13–16]. In this regard, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), the ligand for α4β7, mediates T-cell recruitment into the intestinal endothelium [17]. Furthermore, small intestinal epithelial cells express the CCR9 ligand, thymus-expressed chemokine. Recent studies demonstrated that retinoic acid-producing DCs in PPs and the mesenteric lymph nodes (MLNs) are key players in the enhancement of α4β7 and CCR9 expression by Ag-specific effector CD4+ T cells, which in turn guides their migration into the intestinal lamina propria [18]. In addition to mucosal T-cell homing, retinoic acid-producing DCs in PPs regulate T cell-independent IgA class switching and gut-homing receptor expression on B cells [19,20]. These findings clearly show that the CMIS exhibits distinct sites for induction and regulation of S-IgA Ab responses in mucosal effector tissues.

Although it has been shown that GALT and NALT share common features, it is also clear that a compartmentalization occurs between the oral and nasal immune systems [21–23]. Thus, oral immunization mainly elicits Ag-specific immune responses in the small intestine, in the proximal part of the large intestine, mammary and salivary glands, whereas nasal immunization induces mucosal immunity in the UR tract, nasal and oral cavities, and the cervicovaginal mucosa [21–23]. Furthermore, the organogenesis, lymphocyte trafficking and progression of immunosenescence in PPs and NALT are distinctly regulated [11,13,15,24–35]. Thus, the PPs develop between embryonic days 14 and 17 in an IL-7-IL-7Rα and LTα1β2-LTβR signaling cascade-dependent manner, whereas NALT organogenesis occurs postnatally in the absence of these cytokine cascades [28,32,34,35]. Furthermore, both Id2 and retinoic acid receptor-related orphan receptor-γt transcripts are essential for PP inducer cell development; however, NALT inducer cells require only Id2 [28,36–38]. In addition, activated T and B cells in PPs preferentially express α4β7 and CCR9 as gut-homing receptors, which help guide their migration back to the intestinal lamina propria [13,15]. In contrast, CD62L, α4β1 and CCR10 preferentially control the migration of T and B cells from NALT into the UR tract effector tissues [24,25,32,33]. The compartmentalization of GI and UR tract immune systems is also evident because distinct differences in mucosal aging occurred between the GI and UR tract immune systems [26,27,29–31]. Thus, age-associated alterations, including a reduction in number of PPs and the level of intestinal Ag-specific S-IgA Abs, occur in mice during aging [26,27]. Furthermore, mice lose oral tolerance, which represents another important mucosal immune regulatory function for maintaining systemic homeostasis to orally administered Ags during the aging process (6–12 months) [26,27,30,31]. In contrast, NALT shows a more intact immune response during aging (1-year-old mice), with signs of immunosenescence noted only in mice older than 2 years [26,27,29].

Because mucosal immunization induces not only Ag-specific mucosal S-IgA Abs but also systemic IgG Abs, developing mucosal vaccines could be used in much the same way as currently available licensed parenteral vaccines. Thus, mucosal vaccine delivery can induce systemic T-cell and Ab responses in peripheral lymphoid tissue, as is seen after parenteral vaccine delivery. However, simultaneous induction of mucosal immunity provides a dual protection

against pathogens. Furthermore, mucosal adjuvants and delivery systems are essential to induce Ag-specific immune responses in both mucosal and systemic compartments by avoiding induction of systemic unresponsiveness. This review focuses on several recent approaches to induce mucosal immunity to vaccines, with emphasis on mucosal tissue targeting, new immunization routes and delivery systems that are both effective and safe. As a mucosal targeting strategy, DCs and M cells are discussed as the two major targeting cell types. Although a large number of DC-targeting components have been studied as mucosal adjuvants, CpG oligodeoxynucleotides (CpG ODN) and Flt3 ligand (FL) are selected based on their effectiveness and safety. Importantly, the cellular and molecular mechanisms for these two DC-targeting mucosal adjuvants and an M cell-targeting vaccine delivery system have been well described. In contrast, the precise mechanisms for sublingual (SL) immunization, eye drops, and rice-based and nanogel delivery systems remain to be elucidated; however, the early results are promising. In summary, these novel strategies are attractive and exhibit high potential from a practical point of view. More extensive reviews, which include additional targeting strategies, adjuvants, and delivery systems, are provided. Some specific details are essential to understand the cellular and molecular mechanisms involved in using these novel vaccine strategies.

Targeting vaccines

Mucosal DCs

DCs play a central role in bridging the innate immune system with the adaptive immune system [39–42]. Thus, DCs are found throughout the body and are especially prominent at mucosal surfaces. Immature type DCs are enriched underneath the epithelium of mucosal inductive sites and are poised to capture Ags. When Ag uptake occurs, these DCs change their phenotype by expressing higher levels of MHC class II and costimulatory molecules and move to T-cell areas of inductive sites for Ag presentation. Thus, DCs and their derived cytokines play key roles in the induction of Ag-specific effector Th-cell responses. In this regard, targeting mucosal DCs is not only an effective strategy to induce mucosal immunity but also a safe approach, especially for nasal application, because vaccines mainly initiate immune responses through DCs in the absence of central nervous system toxicity.

Because of the recent progress in the understanding of innate immunity-associated molecules, toll-like receptor (TLR) ligands are now considered to be candidates as potent mucosal adjuvants. Among these, the TLR9 ligand CpG ODN is known to target professional plasmacytoid DCs for their activation, maturation and subsequent induction of Ag-specific Th1-type responses, including cytotoxic T lymphocytes (CTLs) [43,44]. It has been demonstrated that synthetic CpG ODNs can induce innate immune responses [45–48]. In this regard, CpG ODNs as effective immunomodulators, could target malignant tumors, and reduce allergic responses [49,50]. Furthermore, CpG ODNs have been used as potent adjuvants to elicit Ag-specific Ab and cell-mediated immune responses in mice and rats against both bacterial and viral Ags [51–58]. To this end, mucosal administration of CpG ODN exhibits potent adjuvant activity (Figure 2). Mucosal immunization with CpG ODN plus formalin-inactivated influenza virus, hepatitis B virus surface Ag, or tetanus toxoid effectively elicited vaccine-specific immunity in the mucosal compartment of mice [57–59]. CpG ODN as adjuvant mainly induces Th1-type responses. In this regard, CpG ODN could even switch a predominant Th2 into a Th1-type immune response pathway [60]. Although the detailed mechanisms of adjuvant activity of CpG ODN are still unclear, it has been demonstrated that CpG ODN enhanced MAPK-mediated IL-12 production by APCs [61]. Others also clearly showed that nasal immunization with the recombinant protective Ag of the anthrax lethal toxin and CpG ODN induced protective Ag-specific plasma IgG2a and mucosal S-IgA Ab responses with *in vitro* neutralizing activities [62].

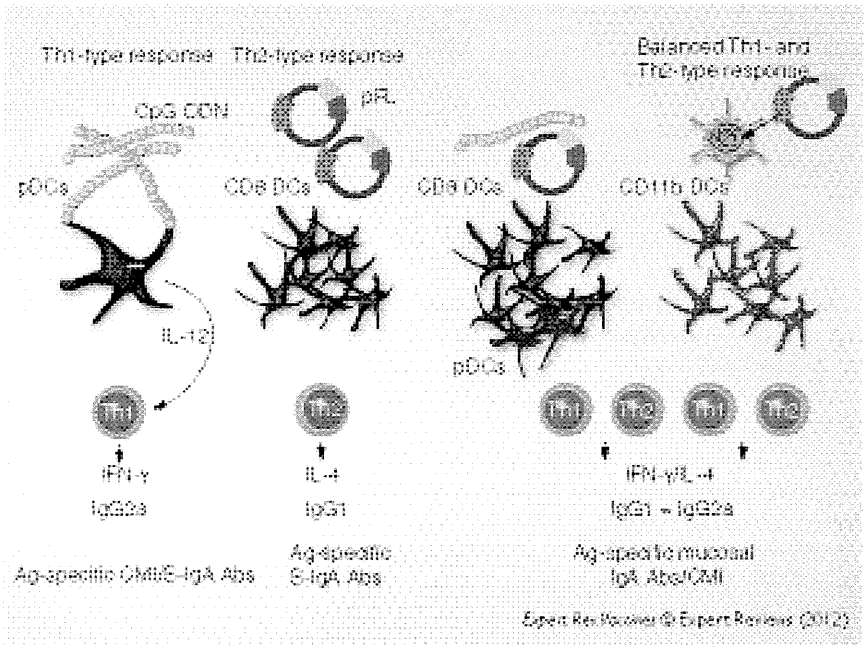

Figure 2. Nasal DC-targeting mucosal vaccines: nasal application of CpG ODN activates plasmacytoid DCs (pDC, B220$^+$ DCs) for the induction of Th1-type cytokine responses. Thus, CMI and cytotoxic T lymphocyte (CTL) activity can be elicited in addition to Ag-specific S-IgA Ab responses. In contrast, pFL as nasal adjuvant preferentially expands the CD8$^+$ DC subset and subsequently elicits Th2-type cytokine-mediated Ag-specific S-IgA Ab responses. Adenovirus expressing FL (Ad-FL) or a combination of CpG ODN and pFL induces a more balanced Th1- and Th2-type immune response. Ad-FL activates CD11b$^+$CD11c$^+$ DCs, whereas a combined nasal CpG ODN and pFL stimulates both CD8$^+$ DCs and pDCs for the induction of CMI and S-IgA Ab responses. Abs: Antibodies; Ag: Antigen; CMI: Common mucosal immune; CPG ODN: CpG oligodeoxynucleotides; DC: Dendritic cell; pDC: Plasmacytoid dendritic cell; pFL: Plasmid-expressing Flt3 ligand; S-IgA: Surface IgA.

FL is a growth factor that binds to the fms-like tyrosine kinase receptor Flt3/Flk2. *In vivo* FL treatment markedly upregulates the number of DCs but not their activation [63,64]. Mouse FL has been cloned and shown to be a key player in the proliferation and differentiation of early hematopoietic precursor stem cells [63,65–68]. Furthermore, it has been reported that FL could mobilize and stimulate not only DCs [64] but also natural killer cells and B cells [69]. Of interest, it was first reported that systemic FL injection facilitated oral tolerance induction because of its ability to result in significant increases in the number of DCs in several lymphoid tissues, including the intestinal lamina propria, PPs, MLNs, and spleen [70,71]. In contrast to tolerance induction, others showed that FL treatment also upregulated immune responses when delivered via mucosal [71], systemic [72], or cutaneous [73] routes. It has also been reported that when plasmid DNA encoding FL (pFL) was coadministered with plasmids encoding protein Ags or linked to the Ag itself, effective immune responses were induced [74,75]. In this regard, it has been suggested that FL possesses adjuvanticity for both humoral and cell-mediated immune responses and that the FL cDNA system may be a potential alternative approach to using the FL protein system [76–79]. To this end, pFL has been used as a mucosal DC-targeting adjuvant for the induction of Ag-specific protective mucosal immune responses (Figure 2). Nasal administration of pFL as mucosal adjuvant facilitated expansion of $CD8^+$ DCs, which subsequently elicited IL-4-producing $CD4^+$ T-cell- and Ag-specific S-IgA Ab responses [80]. NALT has been the major site for sampling pFL and for producing the FL protein locally, which subsequently induced the expansion and activation of DCs [80]. In this regard, pFL did not show any potential to migrate into the CNS.

Other types of FL-based NALT-DC-targeting immune modulators, including an adenovirus serotype 5 vector expressing FL (Ad-FL), were found to elicit Th1- and Th2-type responses, thereby providing both Ag-specific S-IgA Ab and cell-mediated immune responses [81]. When mice were nasally immunized with ovalbumin (OVA) and Ad-FL, high levels of Ag-specific Ab responses were elicited in both mucosal and systemic compartments. Furthermore, significantly increased levels of Ag-specific IFN-γ and IL-4 production were noted in cervical lymph nodes and spleen [81]. Because of OVA-specific Th1-type cytokine responses, Ag-specific CTL responses were upregulated in mice administered with nasal OVA and Ad-FL. Interestingly, the number of $CD11b^+$ $CD11c^+$ DCs was preferentially increased. This DC subset expressed high levels of costimulatory molecules and migrated from the NALT to mucosal effector tissues [81]. These findings show that nasal administration of Ad-FL facilitated the induction of mature-type $CD11b^+$ $CD11c^+$ DCs and Th1- and Th2-type $CD4^+$ T cells in the NALT for Ag-specific Ab and CTL responses (Figure 2). Balanced Th1- and Th2-type responses have become key issues in mucosal vaccine development because this type of cytokine response would not only provide Ag-specific S-IgA Ab and CTL responses against viral and bacterial infections but also avoid induction of allergic (IgE) and inflammatory-type responses.

CpG ODN has been shown to induce polarized Th1-type cytokine responses in mice [62]. In contrast, pFL preferentially elicits coadministered Ag-specific Th2-type cytokine immunity [80]. To this end, one could hypothesize that an ideal but balanced Th1- and Th2-type cytokine response would be elicited by using a combination of pFL and CpG ODN as DC-targeting nasal adjuvants. Indeed, recent studies clearly showed that pFL and CpG ODN as a combined nasal adjuvant induced the activation and expansion of plasmacytoid DCs and $CD8^+$ DCs in the nasal cavity for the development of Th1- and Th2-type cytokine-producing $CD4^+$ T cells. Thus, these Ag-specific $CD4^+$ T cells successfully upregulated coadministered Ag-specific immunity in both the mucosal and systemic immune compartments (Figure 2) [82,83]. Increased frequencies of mature-type DCs in NALT correlated well with induction of Ag-specific immune responses. Of significance, nasal delivery of pFL and CpG ODN successfully elicited significant levels of Ag-specific S-IgA Ab responses in 2-year-old mice [82,83]. To this end, aged mice given nasal pneumococcal surface protein A and a combination of pFL and CpG ODN showed protective immunity against nasal *Streptococcus pneumoniae* colonization [83]. These results suggest that nasal administration of pFL and CpG ODN as mucosal adjuvants provides an attractive possibility for the development of a vaccine against *S. pneumoniae* in the elderly.

M cells

As discussed earlier, GALT, including PPs, is covered by a specialized follicle-associated epithelium, 10–20% of which is composed of M cells that show a unique topical morphology (microfold/membranous) and form pockets for the inclusion of lymphoid cells, including B and T cells, DCs, and macrophages [84–89]. M cells show significantly different features compared with intestinal epithelial cells. M cells possess relatively short microvilli, small cytoplasmic vesicles and few lysosomes. Thus, M cells are able to capture and transport lumenal Ags, including viruses, bacteria, small parasites, and microspheres [86,87,89,90]. It has been suggested that M cells may also play a role as APCs because M cells express MHC class II molecules and acidic endosomal–lysosomal compartments [91]. In this regard, activation and potential MHC class II expression by M cells may depend on the nature of endocytosed Ag. M cells serve not only for transport of lumenal Ags but also for provision of an entry way for pathogens to invade the host. In particular, it has been shown that invasive but not noninvasive strains of *Salmonella typhimurium* enter the host through PP M cells [92]. In addition to PPs, the ILFs and NALT also contain a lymphoepithelium with M cells. Thus, *Mycobacterium tuberculosis* uses NALT M cells for host entry [93]. In addition, it was reported that M cells are also detected in nonlymphoid follicle-associated epithelium that covers small intestinal villi [94]. Thus, villous M cells in the small intestine were present in several PP-deficient mouse strains, including *in utero* LT-βR-Ig-treated, LT-$\alpha^{-/-}$, TNF/LT-$\alpha^{-/-}$ and inhibition of differentiation 2 $(Id2)^{-/-}$ mice [94]. Importantly, these villous M cells functionally take up bacteria and induce bacterial Ag-specific immune responses [94]. Indeed,

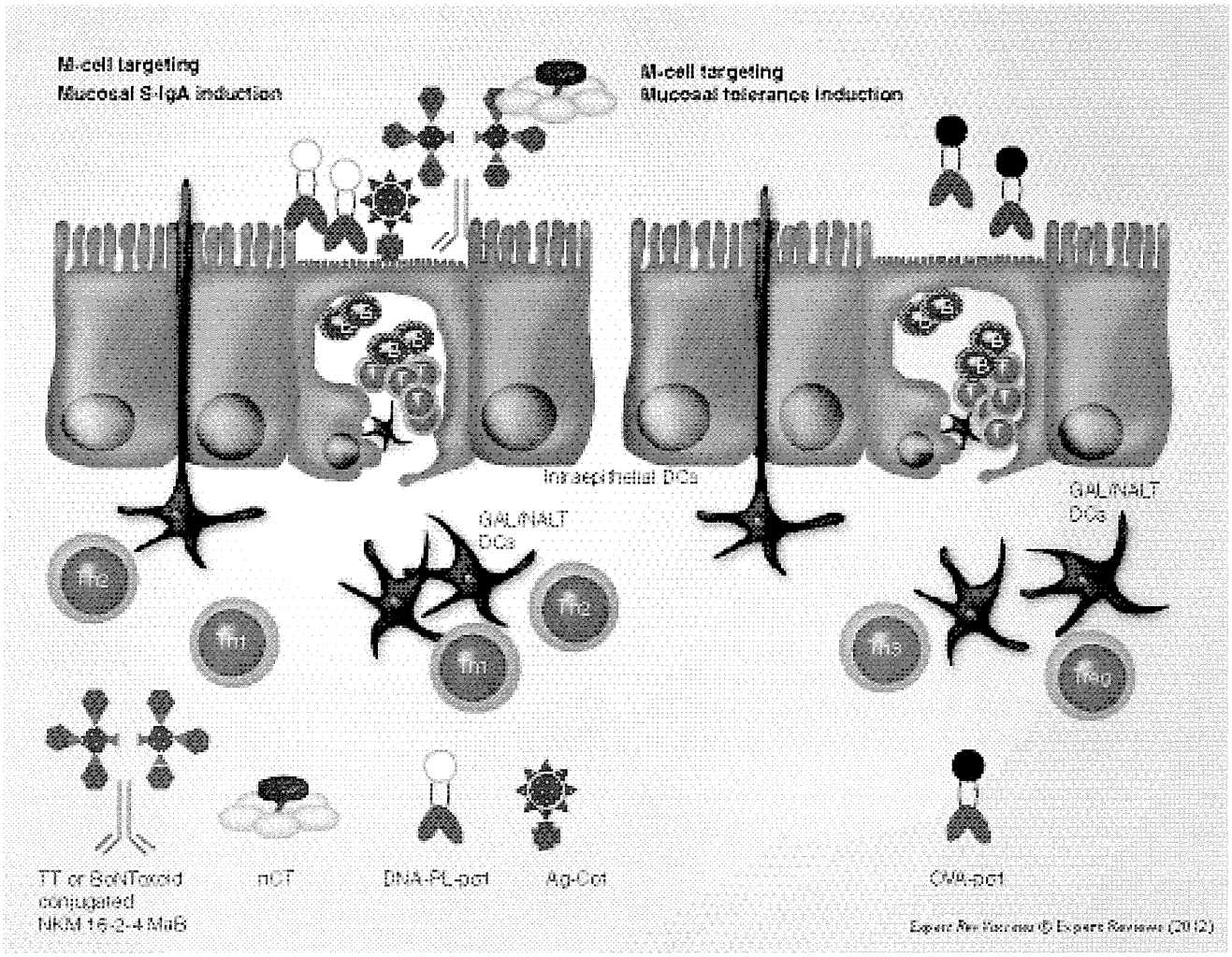

Figure 3. Potential for an M cell-targeting strategy: mucosal M-cell targeting by M cell-specific monoclonal antibody or surface proteins can facilitate Ag delivery for the induction of Ag-specific S-IgA antibody responses to provide effective immunity at the entry site of pathogens. M-cell targeting is achieved by using the protein sigma-1 (pσ1) from reovirus, the ligand for M cell-specific peptide (Co1) or M cell-specific mAb. However, mucosal administration of genetically conjugated OVA protein with pσ1 in the absence of an adjuvant elicits mucosal tolerance.
Ag: Antigen; DC: Dendritic cell; GAL: Gut-associated lymphoreticular; mAb: Monoclonal antibody; NALT: Nasopharyngeal-associated lymphoreticular tissue; S-IgA: Surface IgA; Th1: Type 1 helper CD4+ T cell; Th2: Type 2 helper CD4+ T cell; Th3: Type 3 helper CD4+ T cell; Treg: T regulatory cell.

the MLNs from PP-deficient mice play a key backup role as a mucosal inductive tissue [95]. It has been suggested that MHC class II+ sIgA+ B cells and lamina propria macrophages may be able to capture Ag through endocytic pathways and process and present peptides to CD4+ Th cells. These findings clearly suggest that the intestinal lamina propria–MLN axis performs a potent mucosal inductive function in addition to the PPs.

If one could identify the key molecules expressed by bacteria and viruses that are needed for their invasion or infection of M cells, it would be a great advantage for designing and constructing effective delivery systems for M-cell targeting of vaccines. Reoviruses initially infect the mouse through M cells [96], by using their surface protein sigma-1 (pσ1) [97,98]. In this regard, an M cell-targeting DNA vaccine complex consisting of plasmid DNA and the reovirus pσ1 covalently attached to poly-L-lysine induced significant mucosal S-IgA Ab responses and systemic immunity (Figure 3) [99]. Furthermore, a newly developed M cell-specific monoclonal Ab (NKM 16-2-4) was used as an M cell-targeting carrier for mucosal vaccines. Thus, oral administration of a chimeric vaccine consisting of NKM 16-2-4 and tetanus toxoid or botulinum neurotoxin type A toxoid (BoNToxoid/A), together with native cholera toxin, elicited increased levels of Ag-specific S-IgA and plasma IgG Ab responses (Figure 3) [100]. Importantly, oral immunization with BoNToxoid/A-NKM 16-2-4 provided protective immunity against lethal challenge with botulinum neurotoxin [100]. In addition, oral immunization of Ag fused with M cell-targeting peptide ligand (Co1) resulted in enhanced Ag-specific immune responses [101]. These studies show that an

M cell-targeting delivery system may be of central importance in developing effective mucosal vaccines. Furthermore, it is likely that M cells are also involved in the induction of oral tolerance. In this latter regard, one must carefully consider the nature of formulation of vaccine (or inclusion of adjuvant) because both nasal and oral administration of pσ1 of reovirus genetically conjugated with OVA (OVA-pσ1) alone induced systemic unresponsiveness instead of mucosal IgA immunity (Figure 3) [102,103]. Thus, mucosally induced tolerance was achieved with doses as low as 10–50 μg of OVA-pσ1 when given by the nasal or oral routes [102,103].

Mucosal delivery systems

MucoRice

In 1997, Curtiss and Cardineau successfully filed for and received a US patent (5686079) describing tobacco leaves expressing *Streptococcus mutans* surface protein Ag as an initial indication of a potential plant-based mucosal vaccine. Furthermore, others have developed edible plant-based vaccines by expressing Ags from enterotoxins, hepatitis B, Norwalk virus and respiratory syncytial virus expressed in tobacco leaves or potato tubers [104–111]. Although these plant-based vaccines exhibited some functional properties in experimental systems, their practical application still remains to be elucidated. To develop practical oral vaccines for global immunization, one should consider that the vaccine must maintain effectiveness despite *in vivo* and *ex vivo* environmental changes. In this regard, several practical merits can be found in a rice-based oral vaccine compared with most traditional and other plant-based vaccines. For example, a rice-based vaccine is a rather safe approach. Because this vaccine can be given in a powder form, one could avoid potential problems by using a food-based delivery system. Although the lot-to-lot quality control of a rice-based vaccine may be challenging, stable vaccine Ag expression could be achieved by the third generation of rice-based vaccine. Furthermore, a rice-based vaccine showed stability at room temperature for 2–3 years [112,113]. Oral administration of this rice-based vaccine did not lose activity when exposed to digestive enzymes and subsequently induced protective, Ag-specific Ab responses in mice and non-human primates [112–115]. Recent studies have provided direct evidence that oral MucoRice-cholera toxin B-subunit (CT-B) induced Ag-specific S-IgA Abs that played a critical role in protection against CT-induced diarrhea (Figure 4) [113]. Importantly, cold chain-free oral MucoRice-CT-B induced long-lasting cross-protective immunity against heat-labile enterotoxin-producing enterotoxigenic *Escherichia coli* in addition to CT-producing *Vibrio cholerae* [113]. These results demonstrate that oral administration of a rice-based vaccine provides a potent practical global strategy for the development of cold chain- and needle-free vaccines that protect from gastrointestinal infection.

Nanogels

The application of biomaterials, such as encapsulating Ags in polymer nanoparticles, microparticles, virosomes and liposomes, shows significant potential in the development of vaccines and immunotherapy [116–123]. Although use of liposomes can enhance Ag delivery across mucosal surfaces, they are rapidly cleared and do not allow for long-term Ag release at the mucosal surface [124–126]. In this regard, it is possible that using a bioadhesive gel one could upregulate the residence time and enhance Ag release and retention onto the epithelial cells themselves. Indeed, it has been shown that surface modifications or coadministration with bioadhesive materials, that is, chitosan, resulted in influenza-specific S-IgA Ab responses in nasal washes [127]. A nanometer-sized (<100 nm) bioadhesive polymer hydrogel (nanogel) system has been developed and used as an attractive drug delivery system [128]. Cholesteryl group-bearing pullulan (CHP) form self-assembly of associating polymers as physically crosslinked nanogels in water [129,130]. In general, hydrophobic interactions between CHP and various proteins revealed a CHP nanogel containing the protein inside. When CHP nanogels capture the proteins inside, they form a hydrated nanogel polymer network (nanomatrix) without aggregation. In this regard, trapped proteins maintained their native form and were slowly released [130]. On the basis of these advantages, a CHP nanogel strategy has been used for the development of adjuvant-free nasal vaccines. It was recently shown that nasal administration of a cationic type of CHP nanogel (cCHP nanogel) containing the C-terminus of the H chain (Hc) of botulinum neurotoxin-type A (BoNT/A; nanogel-Hc-BoNT/A) allowed adherence to the nasal epithelium for a longer period compared with naked Hc-BoNT/A (Figure 4) [131]. In this regard, gradually released Hc-BoNT/A was effectively taken up by mucosal APCs and subsequently elicited protective Ag-specific S-IgA Ab responses against BoNT/A intoxication [131]. In summary, this cCHP nanogel system could represent an ideal and effective mucosal delivery system to enhance pathogen-specific mucosal immune responses at the mucosal surface. Because vaccine Ags are retained for a longer period at mucosal surfaces, it is essential to consider the potential side effects of this delivery system in future applications.

Mucosal immunization routes

SL immunization

Oral and nasal routes have been the preferred ones to induce protective immunity in different mucosal compartments [1,5,21]. However, it has been demonstrated that rectal, vaginal or paramucosal (iliac and inguinal lymph nodes) immunization are also effective strategies for the induction of protective immunity against sexually transmitted infectious diseases, including HIV [132–135]. In addition to these mucosal immunization routes, SL administration of Ags has been used to treat allergic, autoimmune or infection-induced pathologic reactions [21,136], by taking advantage of the induction of oral tolerance [137–140]. It is well known that nasal immunization effectively elicits Ag-specific immunity in both mucosal and systemic compartments; however, one must consider that some nasal immunization strategies risk Ag trafficking into olfactory tissues and the CNS [141–145]. To obviate this potential problem, SL immunization may be an ideal mucosal Ag delivery system that avoids CNS involvement. SL administration is also a noninvasive route that has the advantage of requiring lower doses of Ag than the oral route because of the reduced exposure to proteolytic enzymes and lower pH of the stomach encountered

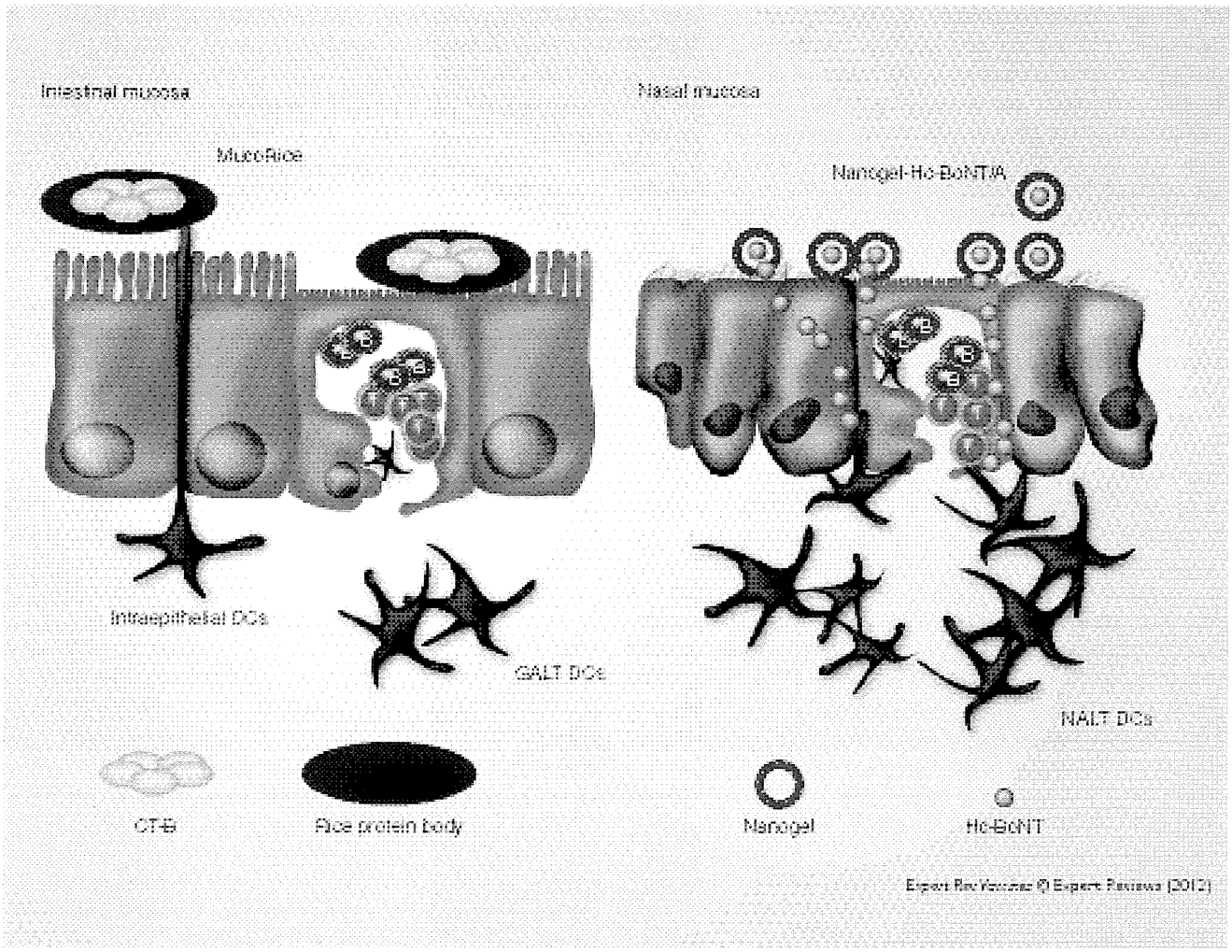

Figure 4. Novel mucosal delivery systems: a plant-based MucoRice-CT-B vaccine effectively induces CT-B-specific protective immunity when orally administered. Because CT-B can be delivered to the small intestine in the rice protein body, MucoRice-CT-B effectively induced CT-B-specific Ab responses in the absence of the CT-A subunit or other potential adjuvants. The cationic nanogel-Hc-BoNT/A is retained for a longer period at the nasal epithelium for slow release of Ag when nasally administered. Thus, nasal APCs, including DCs, can more effectively take up Hc-BoNT/A to initiate Ag-specific immune responses.
DC: Dendritic cell; GALT: Gut-associated lymphoreticular tissue; NALT: Nasopharyngeal-associated lymphoreticular tissue.

after oral immunization. Furthermore, vaccine uptake may be more efficient based on the number of APCs present at the SL site [138]. Recently, studies have used the SL route for vaccine delivery [146–151]. When plasmid DNA encoding hepatitis B surface Ag was sublingually administered to mice, comparable levels of Ag-specific humoral and $CD8^+$ CTL responses were induced as seen after intradermal injection [147]. The SL delivery of a soluble Ag 2,4-dinitrophenyl bovine serum albumin in starch microparticles in combination with a penetration enhancer resulted in good salivary IgA Ab responses [148]. Finally, SL delivery of lipopeptides induced increased serum Abs and T-cell responses in the spleen and inguinal lymph nodes of mice [146]. Compared with subcutaneous administration of the same vaccine preparation, SL application preferentially induced IFN-γ-producing T cells and IgG2a Ab responses, whereas subcutaneous injection elicited IL-4 and IgG1 Ab responses [146]. More recently, SL immunization with influenza virus successfully elicited influenza-specific immunity and provided protection against lethal viral infection [150]. Furthermore, SL immunization with the outer membrane protein of *Porphyromonas gingivalis* plus the plasmid expressing FL cDNA (pFL) elicited increased frequencies of DCs in submandibular lymph nodes and protective immunity in the oral cavity [151]. In addition, CCR7-expressing DCs in cervical lymph nodes were the key players in the induction of Ag-specific immune responses [149]. These findings show that by using the appropriate quantity and form of Ag with a targeted delivery system, the SL route could be the preferred one for inducing both mucosal and systemic immunity, without induction of T-cell unresponsiveness.

Eye drops

The ocular surface leading to the lacrimal sac and nasolacrimal duct also forms an interface with the outside environment.

In fact, it has been proposed that CALT, together with TALT, organizes eye-associated lymphoreticular tissue to create mucosal surveillance and a barrier in the eye region of humans [152,153]. Although TALT develops in human tear ducts, little information was available on mouse TALT until recently. Thus, it was reported that TALT is located in the murine lacrimal sac covered by an epithelium with M cells for Ag uptake [8]. The administration of Ags using eye drops induced Ag-specific S-IgA Ab responses in both ocular and nasal cavities in addition to serum IgG Abs because of the presence of TALT in the conjunctival sac, located in the tear duct, which bridges the ocular and nasal cavities [8,152–154]. Ocular administered Ags migrate to tear ducts and then to the nasal cavity and thus are taken up by TALT and NALT M cells for the induction of Ag-specific immune responses. Past investigations tended to emphasize the identification and characterization of CALT [153,155–158]. Unlike other mammals (e.g., cat, dog, and human), mice and rats do not possess CALT [155]. However, recent findings showed that eye drop administration of Ag induced CALT development in mice with increased numbers of M cell-like cells [9]. Although it remains unclear whether eye drop immunization induces potential adverse effects, including inflammatory responses, it was reported that the administered Ag did not migrate into the CNS [9]. Taken together, these findings clearly showed that eye drop administration of vaccine would be a novel strategy for the induction of Ag-specific mucosal immune responses, if inflammatory responses could be avoided.

Expert commentary

The CMIS provides both an essential concept and a practical means for the development of mucosal vaccines. Thus, it is essential to effectively activate mucosal inductive tissues or MALT for effective mucosal immunity. For targeting MALT, different routes of mucosal immunization have been developed and shown to successfully elicit protective mucosal immunity against several pathogens. However, one could easily fail to elicit protective mucosal immunity without a better understanding of the cellular and molecular mechanisms that regulate the mucosal immune system. Thus, one must carefully consider the route of immunization, the adjuvant and method of Ag delivery to elicit appropriate and desired mucosal immune responses to a particular pathogen. For example, oral vaccination may have fewer side effects and be the most preferred immunization route from a practical point of view; however, oral vaccines require that one maintain their original quality and efficacy until they reach the GALT, because the GI tract represents a harsh environment. In this regard, the MucoRice delivery system could be potentially beneficial for oral vaccine development. Thus, it is important to test whether this system can be easily adapted to other types of vaccine Ags. Nasal vaccines must be safe and not be taken up by the CNS because the nasal immunization route has an advantage for the induction of Ag-specific S-IgA Ab responses in the elderly. Indeed, targeting DCs or M cells in the MALT not only facilitates Ag uptake but also avoids potential CNS toxicity. Furthermore, SL and eye drop immunization successfully elicit mucosal immunity without serious toxicity or side effects so far. Novel delivery systems significantly enhance Ag uptake by MALT for the induction of Ag- or pathogen-specific mucosal immunity. However, the precise cellular and molecular mechanisms for these immunization systems in the induction of mucosal immunity still remain to be elucidated. Nevertheless, it is possible that a strategy that uses the appropriate combination of mucosal adjuvants and delivery systems and optimizes the immunization schedule by repeating and combining different routes of mucosal immunization as a primary and boosting strategy could lead to development of a new generation of safe and effective mucosal vaccines.

Five-year view

Mucosal vaccination is a needle- and medical waste-free vaccine strategy that provides protective immunity against pathogenic bacteria and viruses in both mucosal and systemic compartments. However, mucosal vaccines must overcome two major hurdles (effectiveness and safety), which are both relatively difficult tasks compared with systemic vaccine development because of the uniqueness of the mucosal environment. Future global warming could introduce unexpected pathogens, such as the malaria parasite, into new areas where they have never been seen causing pandemic infectious diseases. Furthermore, some of the currently available vaccines, including nasal FLuMist, are less effective in the immunocompromized population such as young children and the elderly. These facts indicate that the development of novel mucosal vaccines have the potential to provide a better quality of life. According to current knowledge of mucosal vaccines, an appropriate combination of several mucosal vaccine strategies could facilitate the development of practical vaccines over the next 5 years. However, one must realize that developing licensed products is a time-consuming and difficult task from the point of view of a promising outcome. Furthermore, more intensive vaccine development studies need to be performed using novel approaches such as SL immunization, eye drop delivery, nanomatrix and plant-based delivery systems because recent evidence supports both their effectiveness and safety.

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Key issues

- The concept of a common mucosal immune system with specialized compartments is required for the development of effective mucosal vaccines.
- Mucosal vaccines elicit immune defense in both mucosal and systemic tissue compartments.
- Mucosal adjuvants and delivery systems are needed for the induction of more effective mucosal immune responses.
- Targeting mucosal dendritic cells is an effective and safe strategy for inducing antigen-specific immunity.
- New routes of mucosal immunization and antigen delivery systems should facilitate mucosal vaccine development.
- A combination of appropriate mucosal vaccine strategies is essential for future mucosal vaccine development.

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